A Combined Process for Production of Fumaric Acid and Xylooligosaccharides from Corncob

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Production of fumaric acid and xylooligosaccharides from corncob was investigated using a combined process. Corncob was fractionated into a cellulose-rich fraction and a hemicellulose-rich fraction by an alkali pretreatment. The cellulose-rich fraction was converted into fumaric acid by *Rhizopus oryzae* in fed-batch simultaneous saccharification and fermentation (SSF). Maximal fumaric acid concentration reached 35.22 g/L at a final 15% (w/v) solid loading in the fed-batch SSF. The hemicellulose-rich fraction was converted into xylooligosaccharides (XOSs) by endo-*β*-1,4-xylanase. The yield of XOSs was 62.35% after 24 h of xylanase hydrolysis. Xylobiose, xylotriose, and xylotetraose were the three major components in the XOSs. A mass balance analysis demonstrated that 100.6 g of fumaric acid and 148.1 g of XOSs were produced from 1000 g of dry corncob matter. The production of fumaric acid and XOSs by the combined process could make the utilization of corncob more efficient and more promising.

Keywords: Corncob; Fumaric acid; Xylooligosaccharides; Fed-batch simultaneous saccharification; Fermentation; Rhizopus oryzae; Enzymatic hydrolysis; Xylanase

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INTRODUCTION

Lignocellulose, the most abundant renewable biomass on Earth, is composed of cellulose, hemicellulose, and lignin. Cellulose and hemicellulose can be further converted into oligosaccharides (such as cellooligosaccharides and xylooligosaccharides), fermentable sugars (such as glucose and xylose), or bio-based products (such as ethanol, L-lactic acid, and fumaric acid). Therefore, lignocellulosic materials might be a cost-effective alternative feedstock for producing value-added products (FitzPatrick *et al*. 2010; Vejdovszky *et al*. 2015; Zhou *et al*. 2017).

Agricultural wastes (such as corncob, corn stover, and wheat straw) are major lignocellulosic resources in China. For example, corncob waste is estimated to be at nearly 20 million tons per year (Chang *et al*. 2017). However, most of the available agricultural wastes are not effectively used, or are simply burned off. Fortunately, environment-friendly bioconversion processes could convert agricultural wastes into value-added products.

Fumaric acid is a four-carbon unsaturated dicarboxylic acid that is widely used as a food additive in the food industry. The dicarboxylic nature of fumaric acid, as well as the unsaturated property of fumaric acid, makes it an important intermediate for polymer

production. Therefore, fumaric acid was classified as a top 10 chemical by the United States of America Department of Energy in 2004 (Bozell and Petersen 2010). Fermentative production of fumaric acid is regarded as a promising approach using renewable biomass as the feedstock, such as lignocellulosic materials (Xu *et al*. 2012). The mycelia fungi *Rhizopus oryzae* is an excellent fumaric acid producer in nature. *R. oryzae* has a limited nutrient requirement for fumaric acid production. It has been reported that *R. oryzae* can grow on carbohydrates in lignocellulose (Xu *et al*. 2012). Wheat straw, corn stover, corncob, and pulp and paper solid waste are just some of the materials that have been used as raw materials for the production of bio-based chemicals by *R. oryzae* (Xu *et al*. 2010; Saito *et al*. 2012; Li *et al*. 2016; Das *et al*. 2016). All these works show that *R. oryzae* is suitable for the production of fumaric acid from lignocellulosic materials.

Xylooligosaccharides (XOSs), as a prebiotic, are a nondigestible food ingredient and can selectively promote the growth of gut microflora, possess antioxidant properties, and enhance the immune system (Samanta *et al*. 2015). XOSs also have other healthy properties, such as low caloric content and noncariogenicity (Vázquez *et al*. 2000). Because of XOSs' healthy properties, they are widely used as functional foods and feed additives. Producing XOSs with endo-xylanase is a promising approach for the conversion of xylan into value-added products using agricultural residues (Samanta *et al*. 2015).

Although lignocellulose is rich in cellulose and hemicellulose, the complex structure of lignocellulose is an obstacle that limits its application for the production of value-added products. Generally, three steps are taken to convert lignocellulose into value-added products: (1) pretreatment, (2) saccharification or enzymatic hydrolysis, and (3) fermentation (Yu *et al*. 2014). Corncob is rich in cellulose and hemicellulose, representing a potential source of carbohydrates (Jiang *et al*. 2017).

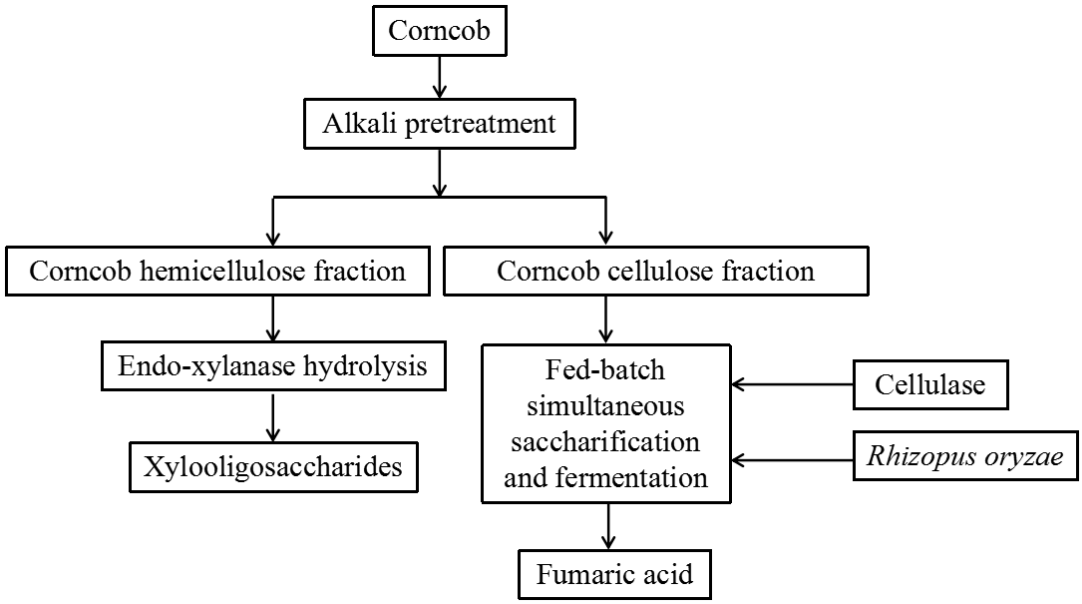

Fig. 1. Flowchart of production of fumaric acid and xylooligosaccharides from corncob

Some studies have focused on the production of XOSs from lignocellulosic xylan using various methods (Akpinar *et al*. 2010; Chapla *et al*. 2012; Zhang *et al*. 2017). Other

studies have paid more attention to the production of fumaric acid from different raw materials (Xu *et al*. 2010; Das *et al*. 2015; Wang *et al*. 2015). However, little research has been reported on a process for producing fumaric acid and XOSs from lignocellulosic materials. The present work reports a combined process to produce fumaric acid and XOSs using corncob as the feedstock. The corncob was fractionated into the cellulose-rich solid (CRS) fraction and the hemicellulose-rich fraction by alkali pretreatment. Fumaric acid was produced from CRS by fed-batch simultaneous saccharification and fermentation (SSF) with cellulase and *Rhizopus oryzae* at a high solid loading, and XOSs were produced from the hemicellulose-rich fraction by endo-β-1,4-xylanase hydrolysis. This is the combined process for the production of fumaric acid and XOSs from corncob, as presented in Fig. 1.

EXPERIMENTAL

Materials

Strain

Rhizopus oryzae CICC 40351 was obtained from the China Center of Industrial Culture Collection (CICC, Beijing, China).

Enzymes

Cellulase (Cellic® CTec 2) was obtained from Novozymes (Bagsvaerd, Denmark) and used without further purification. Cellulase activity was 257.57 FPIU per gram of liquor.

Endo-β-1,4-xylanase was expressed using a recombinant *Pichia pastoris* strain, as reported by Ouyang *et al*. (2007). Xylanase activity was 1241 IU per milliliter.

Xylooligosaccharide standards

Xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose were purchased from Megazyme (Bray, Ireland).

Corncob

Corncob was a gift from Jiangsu Kangwei Biologic Co., Ltd., Dongtai, Jiangsu Province, China. The corncob was air-dried, cut into 2- to 3-cm chips, and stored in plastic bags at 4 °C until further use. The chemical composition of the corncob was as follows (% dry wt.): cellulose 36.01%, hemicellulose 36.84%, lignin 17.43%, and others 9.72%.

Agar slant

The agar slant used in this work contained 10.0 g/L glucose, 3.0 g/L yeast extract, 3.0 g/L malt extract, 5.0 g/L peptone, and 20.0 g/L agar. The inoculated slant was cultured in an incubator at 30 °C for one week and then stored at 4 °C until further use.

Spore suspension

The spores of *R. oryzae* were washed from the slant with sterile water, and the spore density was adjusted to 10^7 spores per milliliter.

Seed medium

The seed medium used in this work contained 40 g/L glucose, 4.4 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.6 g/L KH_2PO_4, 17.6 mg/L $ZnSO_4 \cdot 7H_2O$, and 0.498 mg/L $FeSO_4 \cdot 7H_2O$.

Fermentation medium

The SSF medium contained CRS, 0.71 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.6 g/L KH_2PO_4, 0.01 g/L $ZnSO_4 \cdot 7H_2O$, 0.0004 g/L $FeSO_4 \cdot 7H_2O$, and 30 g/L $CaCO_3$ in 0.05 mol/L sodium acetate-acetic acid (NaOAc-AcH) buffer (pH of 4.8).

The separated hydrolysis and fermentation (SHF) medium contained enzymatic hydrolysate of CRS, 0.71 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.6 g/L KH_2PO_4, 0.01 g/L $ZnSO_4 \cdot 7H_2O$, 0.0004 g/L $FeSO_4 \cdot 7H_2O$, and 30 g/L $CaCO_3$.

Methods

Alkali pretreatment

The corncob chips were soaked in 7% (w/v) sodium hydroxide at 85 to 90 °C for 1 h. After solid/liquid separation, the liquid fraction containing hemicellulose was neutralized with 72% (w/w) sulfuric acid to a pH of 6.0 to 7.0. Then, the neutralized liquid fraction was further desalinated and used for producing XOSs by endo-*β*-1,4-xylanase hydrolysis. The solid fraction was mixed with purified water at a solid-liquid ratio of 1:10 (w/v), and then neutralized by 72% (w/w) sulfuric acid to a pH of 4.8 to 5.0. The mixture was centrifuged to remove the supernatant at 5000 rpm for 15 min. The solid fraction was then further washed with purified water three times. After centrifugation, the solid fraction (named CRS) was stored in plastic bags at 4 °C. The chemical composition of the CRS was as follows (% dry wt.): cellulose 66.95%, hemicellulose 21.87%, and lignin 5.92%.

Enzymatic hydrolysis

Enzymatic hydrolysis of the CRS by cellulase was conducted at a pH of 4.8, 50 °C, and 150 rpm in a round-bottom, three-necked flask equipped with a mechanical agitator. The cellulase (Cellic® CTec 2) dosage was 25 FPIU per gram of cellulose. The enzymatic hydrolysate was centrifuged to remove solid residue at 5000 rpm for 15 min, and the supernatant was used for fermentation by *R. oryzae*.

The corncob hemicellulose fraction was adjusted to a pH of 5.0 with diluted HCl. Enzymatic hydrolysis of the corncob hemicellulose fraction by endo-*β*-1,4-xylanase was conducted at a pH of 5.0, 60 °C, and 150 rpm in a round-bottom, three-necked flask equipped with a mechanical agitator. The endo-*β*-1,4-xylanase dosage was 50 IU per gram of hemicellulose.

Seed culture

The spore suspension was transferred to the seed medium and cultured in an incubator (New Brunswick Scientific, INNOVA 40R, USA) for 24 h at 35 °C and 200 rpm.

Simultaneous saccharification and fermentation (SSF)

The SSF was conducted using cellulase (Cellic® CTec 2) at 38 °C and 220 rpm in a SSF medium with a 10% (v/v) inoculum. The conversion yield was calculated as follows:

$$\text{Conversion yield} = \text{fumaric acid (g)}/\text{substrate (g)} \quad (1)$$

Separated hydrolysis and fermentation (SHF)

The enzymatic hydrolysate from the CRS was centrifuged to obtain a supernatant solution, and the supernatant was used as the sole carbon source for *R. oryzae* growth. Fermentation by *R. oryzae* was carried out in an incubator (New Brunswick Scientific, INNOVA 40R, USA) at 38 °C and 220 rpm in the SHF medium with a 10% (v/v) inoculum.

Analytical methods

The fumaric acid, ethanol, and glucose concentrations were determined by high-performance liquid chromatography (HPLC, Agilent Technologies, 1260 Infinity, USA) equipped with a Bio-Rad Aminex HPX-87H column and a refractive index detector. The mobile phase was 5 mM H_2SO_4, and the flow rate was maintained at 0.6 mL/min.

The XOSs were determined using an ion chromatography system (Dionex, ICS-3000, USA) equipped with PAD and CarboPac PA200 column (3×250 mm) at 30 °C. The eluent included 100 mM NaOH (A) and 500 mM sodium acetate (B). The XOSs were analyzed using a linear gradient (B: 0 to 24% for 40 min), followed by elution with 100 mM NaOH (15 min). The flow rate was 0.3 mL/min.

The chemical composition of the lignocellulosic material was analyzed according to the National Renewable Energy Laboratory standard method for the determination of structural carbohydrates and lignin in biomass (Sluiter *et al*. 2008). Filter paper activity of the cellulase was determined according to the International Union of Pure and Applied Chemistry procedures (Ghose 1987). One FPIU was defined as the amount of enzyme needed to release 1 μmol of glucose equivalent from Whatman No. 1 filter paper per min.

The alcohol dehydrogenase (ADH) activities were determined using the method presented by Zhang *et al*. (2016). ADH activities include ADH (forward, f) activities and ADH (backward, b) activities. ADH (f) converts acetaldehyde into ethanol, and ADH (b) converts ethanol into acetaldehyde.

Both the ADH (f) and ADH (b) activities were measured changes in the absorbance at 340 nm. The solution contained 5 mM of the reduced form of nicotinamide adenine dinucleotide (NADH) and 1% (v/v) acetaldehyde in 0.1 M phosphate buffer for the forward reaction.

For the backward reaction, the solution contained 10% (v/v) ethanol and 6 mM of the oxidized form of nicotinamide adenine dinucleotide (NAD^+) in 0.1 M phosphate buffer. One unit of ADH activity was defined as the amount of enzyme needed to oxidize or reduce 1 μmol of NADH per min.

The fumarase activities were determined using the method of Song *et al*. (2011) with some modifications. Fumarase converts L-malate into fumarate. The fumarase activities were measured changes in the absorbance at 250 nm using sodium L-malate (50 mM) as the substrate. One unit of fumarase activity was defined as the amount of enzyme needed to produce 1 μmol of fumaric acid or fumarate per min.

The protein was determined by the Bradford method using bovine serum albumin as the standard. The specific activities of enzyme were defined as enzyme activities per mg of protein.

RESULTS AND DISCUSSION

Production of Xylooligosaccharides from Corncob Hemicellulose Fraction by Endo-*β*-1,4-xylanase

In enzymatic production of XOSs, endo-*β*-1,4-xylanase randomly cleaves the xylan chain to produce xylose-based oligomers with different degrees of polymerization (DP) (Conejo-Saucedo *et al*. 2017). In this work, endo-*β*-1,4-xylanase gene from *Trichoderma reesei* was expressed by *Pichia pastoris*. Figure 2 shows that the endo-*β*-1,4-xylanase hydrolyzed the corncob hemicellulose into XOSs. The concentration and yield of XOSs at 24 h were 6.49 g/L and 62.35%, respectively. Endo-*β*-1,4-xylanase derived from *T. reesei* demonstrated good enzymatic hydrolysis for the production of XOSs. Mao *et al*. (2008) reported a 54.22% XOSs yield obtain by endo-*β*-1,4-xylanase from *T. reesei.* In the current work, the distribution of XOSs was as follows: xylose 12.3%, xylobiose 41.07%, xylotriose 30%, xylotetraose 11.21%, xylopentaose 4.72%, and xylohexaose 0.7%. Therefore, the XOSs were rich in xylobiose, xylotriose and xylotetraose. Nieto-Dominguez *et al*. (2017) reported that xylobiose, xylotriose, and xylotetraose promoted the proliferation of *Bifidobacteria*.

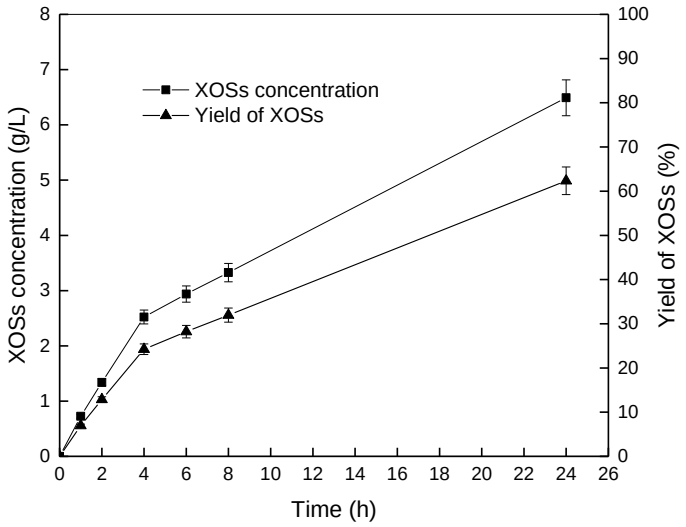

Fig. 2. Enzymatic hydrolysis of corncob hemicellulose for production of xylooligosaccharides (XOSs) by endo-*β*-1,4-xylanase (Yield of XOSs = XOSs (g/L)×Volume (L)/corncob hemicellulose (g)×100%; XOSs = Xylobiose+Xylotriose+Xylotetraose+Xylopentaose+Xylohexaose)

Production of Fumaric Acid from Corncob Cellulose Fraction by *Rhizopus oryzae*

Production of fumaric acid from glucose by batch fermentation

Figure 3 shows the batch fermentation profiles for *R. oryzae* using glucose as the sole carbon source. The glucose (37 g/L) was fully consumed within 24 h along with the production of 11.55 g/L fumaric acid and 2.15 g/L ethanol. A little fumaric acid and 2.65 g/L ethanol were produced in the first 12 h. The specific activities of fumarase, ADH (f), and ADH (b) after 12 h were 1.75 U/(mg protein), 9.23 U/(mg protein), and 0.79 U/(mg protein), respectively. From 12 to 60 h of fermentation, the fumarase specific activity

reached a maximum of 2.28 U/(mg protein) at 36 h and then decreased afterwards, whereas both ADH (f) and ADH (b) reached a maximum at 12 h and then decreased afterwards. In addition, the ratio of ADH (f) to ADH (b) indicated ethanol production *vs* ethanol consumption. *R. oryzae* accumulated ethanol when the ratio of ADH (f) to ADH (b) was higher than 11. On the contrary, ethanol was consumed by *R. oryzae* when the ratio of ADH (f) to ADH (b) was less than 11.

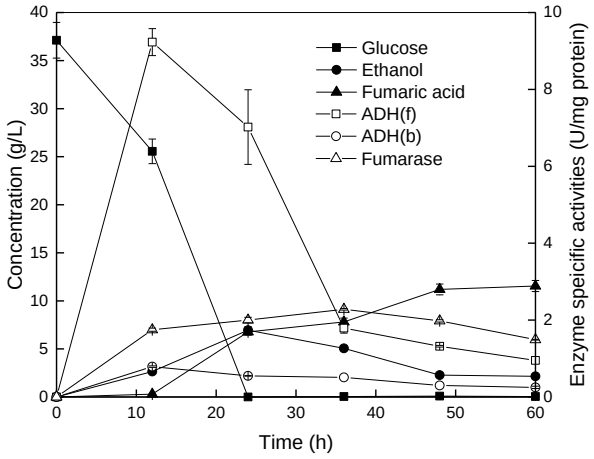

Fig. 3. Profiles of fermentation by *R. oryzae* using glucose as the sole carbon source. ADH (f): alcohol dehydrogenase (forward); and ADH (b): alcohol dehydrogenase (backward)

Production of fumaric acid by simultaneous saccharification and fermentation (SSF) and separated hydrolysis and fermentation (SHF) from corncob cellulose

Figure 4 shows the fermentation profiles of simultaneous saccharification and fermentation (SSF) (Fig. 4A) and separated hydrolysis and fermentation (SHF) (Fig. 4B) by *R. oryzae*. No fumaric acid and little ethanol were detected in the first 12 h of both SSF and SHF. Comparably, little fumaric acid and 2.65 g/L ethanol were found in the first 12 h of pure glucose fermentation (Fig. 3). Correspondingly, the highest ADH specific activity of pure glucose fermentation was higher than those in SSF and SHF. As shown in Fig. 4, both the ADH and fumarase activities increased with time in both SSF and SHF, coupled with increased production of fumaric acid and ethanol. The fumaric acid concentrations in SSF and SHF at 48 h were 14.72 and 13.11 g/L, respectively. The ethanol concentration decreased after the glucose was completely consumed, which occurred at 36 h in SHF. No carbon source was available after 36 h in SHF, and ethanol was consumed by *R. oryzae* while the ratio of ADH (f) to ADH (b) was less than 11. Interestingly, fumarase specific activities after 24 h were higher than 2 U/(mg protein) in SSF compared to those in both SHF and pure glucose fermentation. The maximal fumaric acid concentration in SSF was higher than those in both SHF and pure glucose fermentation. These results indicate that the higher fumarase specific activity resulted in higher fumaric acid concentration. Ding *et al*. (2011) reported that the increased fumarase activity in *R. oryzae* notably enhanced fumaric acid production. Profiles of the fumarase specific activity suggest that the SSF process was more favorable for fumaric acid production than SHF using lignocellulose as the carbon source.

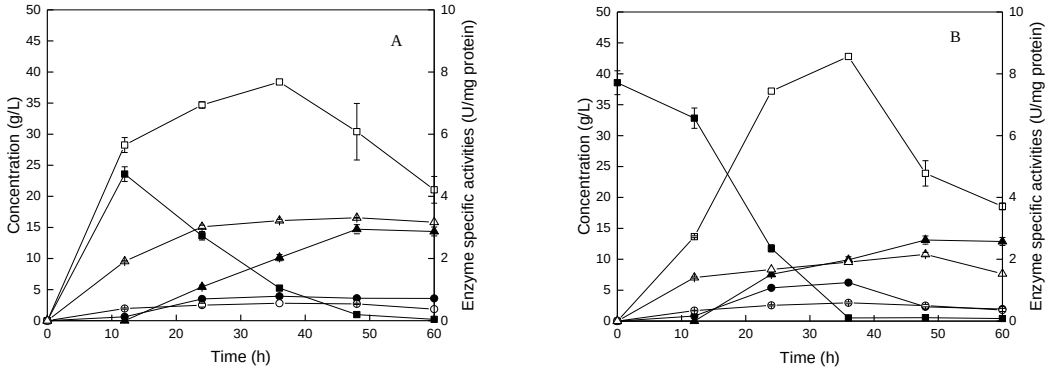

Fig. 4. Profiles of simultaneous saccharification and fermentation at 5% (w/v) CRS (A) and separated hydrolysis and fermentation using enzymatic hydrolysate of 5% (w/v) CRS as the sole carbon source (B). CRS: the cellulose-rich solid; ADH (f): alcohol dehydrogenase (forward); ADH (b): alcohol dehydrogenase (backward). (■) Glucose; (●) Ethanol; (▲) Fumaric acid; (□) ADH(f); (○) ADH(b); and (△) Fumarase.

Simultaneous saccharification and fermentation at different solid loadings

High solid loading is critical for improving process economics using lignocellulosic materials (Jørgensen *et al*. 2007). Although higher solid loading in SSF resulted in higher concentration of the final product, but in practically, it will be met with mass transfer limitation and higher viscosity (Romaní *et al*. 2012). As shown in Fig. 5, a maximal fumaric acid concentration was observed at 5% (w/v) CRS with a conversion yield of 0.29 g/(g substrate).

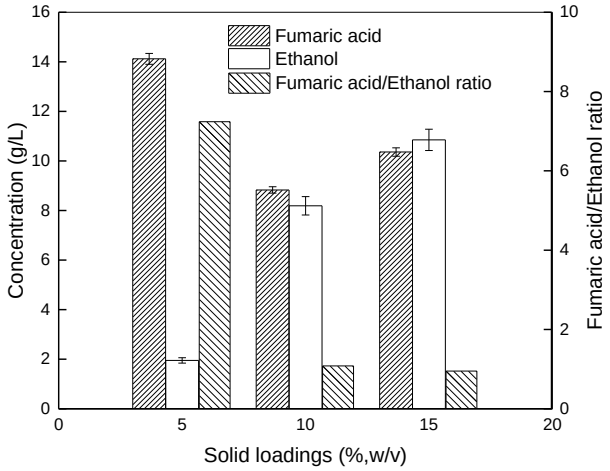

Fig. 5. Batch process of simultaneous saccharification and fermentation at different solid loadings

Increasing the solid loading from 5% (w/v) to 15% (w/v) reduced the production of fumaric acid in the batch SSF process. On the contrary, the ethanol concentration increased with the solid loading increase from 5% (w/v) to 15% (w/v). *R. oryzae* in an aerobic condition is favorable for fumaric acid production (Xu *et al*. 2012), and *R. oryzae* in an anaerobic condition tends to produce much more ethanol (Abedinifar *et al*. 2009).

Increased ethanol production suggests a limited oxygen supply in SSF at a higher solid loading. It is noted that the fumaric acid/ethanol ratio was higher than 6 at 5% (w/v) CRS and around 1 at 10 to 15% (w/v) CRS. Higher solid loading redirected carbon flux toward ethanol production. Apparently, higher solid loading was more favorable for ethanol production, and lower solid loading was beneficial for fumaric acid production. These results imply that a low level of solid loading is favorable for fumaric acid production by *R. oryzae* in a SSF process. For improving mass transfer in SSF at a higher solid loading, Zhang *et al*. (2010) reported that fed-batch SSF improved production of ethanol at a final dry matter content of 25% (w/v) and alleviated high viscosity, which usually results from high dry matter concentration. In the following experiments, a fed-batch SSF process was applied for production of fumaric acid by *R. oryzae*.

Fed-batch simultaneous saccharification and fermentation for fumaric acid production

Figure 6 shows the time course of fed-batch SSF by *R. oryzae* at a final 15% (w/v) solid loading with two feeding strategies. No fumaric acid and a little ethanol were found in the first 12 h. Afterward, the fumaric acid and ethanol concentrations increased with time in the fed-batch SSF process. Maximal fumaric acid concentration reached 35.22 g/L at 132 h with a conversion yield of 0.23 g/(g substrate) while the ethanol concentration was 1.92 g/L. The conversion yield of 0.23 g/(g substrate) was near to 0.29 g/(g substrate) in the batch process of SSF at a 5% (w/v) solid loading. The higher fumaric acid/ethanol ratio (18.34) suggests that carbon flux was redirected toward fumaric acid production by the fed-batch SSF compared to a 0.95 fumaric acid/ethanol ratio in the batch SSF (Fig. 5). The fed-batch SSF improved mass transfer, alleviated higher viscosity caused by higher solid loading, and resulted in a higher end-product concentration (Zhang *et al*. 2010). For a common batch SSF process, solid loading is usually limited to 10% (w/v) dry matter of lignocellulosic material due to its high viscosity (Tomás-Pejó *et al*. 2009; Zhang *et al*. 2015). In the present work, the fed-batch SSF process notably enhanced fumaric acid production by *R. oryzae* at a final 15% (w/v) solid loading.

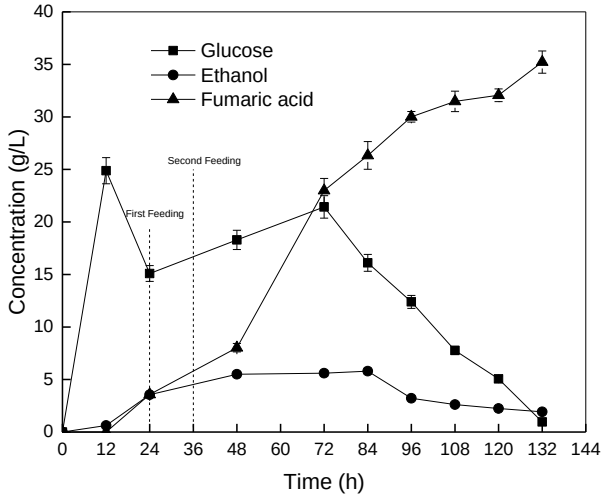

Fig. 6. Fermentation time course of the fed-batch simultaneous saccharification and fermentation (SSF) at a final 15% (w/v) solid loading. The fed-batch SSF was started with a batch SSF process at a 5% (w/v) solid loading. Two feeding strategies (feeding 5% (w/v) solid loading every time) were set at 24 h and 36 h, respectively.

Mass Balance for Production of Fumaric Acid and Xylooligosaccharides

Figure 7 shows the mass balance of the combined process for the production of fumaric acid and xylooligosaccharides. Corncob dry matter (1000 g) contained 360.1 g of cellulose, 368.4 g of hemicellulose, and 174.3 g of lignin. After alkali pretreatment at 85 to 90 °C for 1 h, the recovery of the solid fraction was 43.75%. The solid fraction was composed of 292.9 g of cellulose and 95.7 g of hemicellulose. After that, fed-batch SSF at a final 15% (w/v) solid loading obtained 100.6 g of fumaric acid. At the same time, the liquid fraction contained 283.2 g of hemicellulose, which means a 76.9% recovery of hemicellulose. Afterwards, enzymatic hydrolysis of hemicellulose in the liquid fraction by endo-*β*-1,4-xylanase was carried out to produce 148.1 g of xylooligosaccharides. These lab-scale results reveal that on a larger scale the combined process could produce 1000 kg of fumaric acid from 9940.4 kg of corncob dry matter, coupled with 1472.2 kg of xylooligosaccharides. The results indicate that production of fumaric acid and xylooligosaccharides by the combined process present an alternative for maximizing the utilization of corncob.

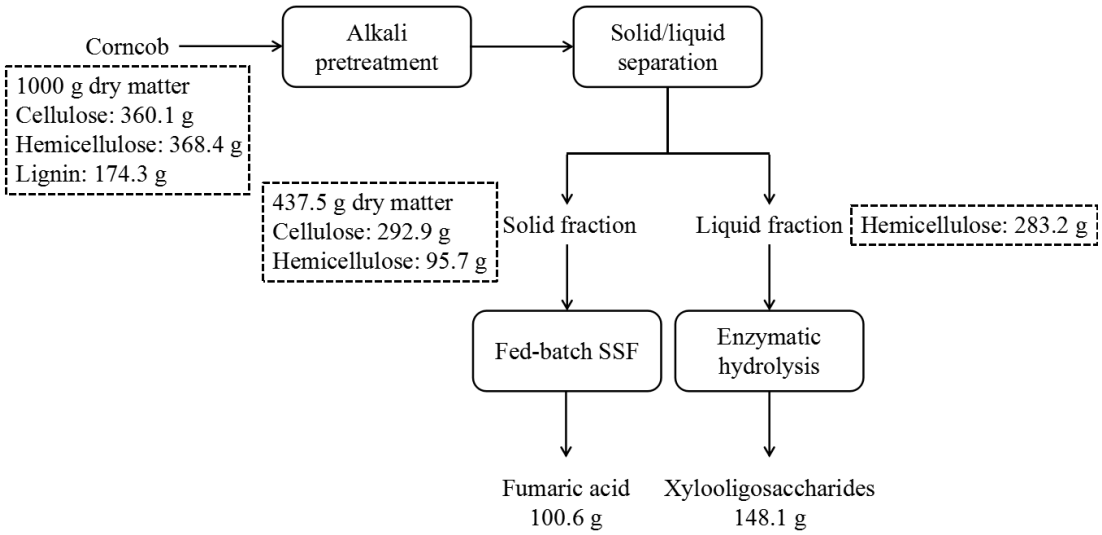

Fig. 7. Mass balance for the production of fumaric acid and xylooligosaccharides from corncob by the combined process

CONCLUSIONS

1. A combined process was developed for the production of fumaric acid and XOSs from corncob.
2. The corncob hemicellulose fraction was efficiently converted into XOSs with a XOSs yield of 62.35%.
3. A higher fumarase specific activity in the SSF process showed that SSF was more favorable for fumaric acid production than SHF.
4. The fed-batch SSF process notably enhanced fumaric acid production by *R. oryzae* at a final 15% (w/v) solid loading.

5. Mass balance analysis of the combine process demonstrated that 100.6 g of fumaric acid and 148.1 g of xylooligosaccharides were produced from 1000 g of corncob dry matter.

ACKNOWLEDGMENTS

The authors are grateful for the support from the Key Research and Development Program of Jiangsu Province (BF2015007), the Key Research and Development Program of Qinghai Province (2016-HZ-819), and the Priority Academic Program Development of the Jiangsu Higher Education Institutions (PAPD).

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Article submitted: October 5, 2017; Peer review completed: November 5, 2017; Revised version received and accepted: November 14, 2017; Published: November 22, 2017.
DOI: 10.15376/biores.13.1.399-411