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Production of fermentable sugars from corn fiber using soaking in aqueous ammonia (SAA) pretreatment and fermentation to succinic acid using *Escherichia coli* AFP184

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Abstract−Conversion of corn fiber (CF), a by-product from the corn-to-ethanol conversion process, into fermentable sugar and succinic acid was investigated using soaking in aqueous ammonia (SAA) pretreatment followed by biological conversions, including enzymatic hydrolysis and fermentation using genetically engineered *E. coli* (AFP184). The SAA pretreatment (using a 15% w/w NH_4OH solution at a solid-to-liquid ratio of 1 : 10 at 60 °C for 24 h) removed 20-38% of lignin and significantly improved the digestibility of the treated solid (85-99% of glucan digestibility). Following the enzymatic hydrolysis, the sugar-rich hydrolysate was subjected to dilute sulfuric acid treatment (1 wt% sulfuric acid and 120 °C for 1 h), which hydrolyzed the oligosaccharides in the hydrolysate into fermentable monomeric sugars. The mixed sugar hydrolysates containing hexose and pentose obtained from the two-step hydrolysis and SAA pretreatment were fermented to succinic acid using a genetically engineered microorganism, *Escherichia coli* AFP184, for evaluating the fermentability. Engineered *E. coli* AFP184 effectively converted soluble sugars in the hydrolysate to succinic acid (20.7 g/L), and the production rate and yield were further enhanced with additional nutrients; the highest concentration of succinic acid was 26.3 g/L for 48 h of fermentation.

Keywords: Corn Fiber, Ammonia Pretreatment, Succinic Acid, Fermentable Sugars

INTRODUCTION

There has been strong interest in fuel ethanol, which is a biodegradable, high-octane alternative fuel. It can be produced from lignocellulosic biomass, such as forest and agricultural residues, as well as from grains like corn and sugarcane. Ethanol can replace some fossil fuels and thus reduce dependency on petroleum and thus lower gasoline costs. Moreover, it is expected to create domestic jobs and decrease carbon dioxide emissions [1]. According to the most recent RFA's report [2], the annual ethanol production in the U.S. was 3.9 billion gal/year in 2005 and increased to 14.3 billion gal/year in 2014. This ethanol production accounts for 10% of the U.S. gasoline supply [3]. Although different sources of fermentable sugars, including corn, sugarcane, woody biomass, grasses and algae, have been investigated, corn is still the largest feedstock for commercial fuel ethanol production in the U.S. (98% in 2014) [3]. Unfortunately, profiting from corn ethanol still relies on government support. Therefore, more efficient conversion processes and/or value-added co-products are necessary.

Corn fiber (CF) is one of the major byproducts from the corn wet-milling process. It is cellulosic material that is mainly composed of carbohydrates, including starch, cellulose and hemicellulose (~65%) with small fractions of lignin and protein [4]. It has been used as a low-value animal feed. However, a high content of polysaccharides in CF allows it to be a potential feedstock for producing additional ethanol or value-added products. This promising feature has attracted strong interest in the utilization of carbohydrates in CF for production of value-added products such as succinic acid.

Succinic acid is a useful 4-carbon intermediate for many applications. It is a platform chemical in pharmaceuticals, foods, cosmetics and biodegradable polymers [5-7]. Several routes including hydrogenation of maleic acid, oxidation of 1,4-butanediol, carbonylation of ethylene glycol and fermentation of carbohydrates have been introduced for succinic acid production [6,8,9]. Thus far, most succinic acid is petroleum-based, but a bio-based route via fermentation has been getting more attention in recent years. It has been shown that bio-based succinic acid obtained by fermentation of renewable carbohydrates has many advantages because it is environmentally friendly and cost-competitive over chemical processes [5]. For these reasons, succinic acid fermentation could be one of the possible applications for CF utilization.

For economically-feasible CF utilization, effective pretreatment methods for overcoming the native recalcitrance of CF and well-developed organisms for fermenting carbohydrates to the target product are essential. Since CF contains a large fraction of hemicellulose compared to other lignocellulose and is relatively easy to remove and decompose during pretreatment, minimizing this loss is

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as important as reducing the structural barriers of the solid matrix to improve enzymatic hydrolysis for production of fermentable sugars. Soaking in aqueous ammonia (SAA) has been demonstrated as a pretreatment method that can be used to effectively remove lignin without significant losses of cellulose and hemicellulose under moderate reaction conditions compared to other pretreatment methods [10,11]. In this study, we investigated the effects of SAA pretreatment on chemical composition and enzymatic digestibility of CF and production of succinic acid from the fermentable sugars obtained in sequential enzymatic and mild dilute acid hydrolysis of the pretreated material. *Escherichia coli* strain AFP184, which is capable of metabolizing both glucose and xylose [12], was used in the production of the succinic acid.

MATERIALS AND METHODS

1. Materials

1-1. Chemicals and Substrates

Dried CF was obtained from the Cargill corn wet mill in Dayton, Ohio, USA. Corn steep liquor, sugars used for analytical standards including glucose, xylose, arabinose, galactose and mannose, and chemicals used in the formulation of fermentation media were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium hydroxide (ACS grade, Fisher catalog no. A669) and sulfuric acid (ACS grade, Fisher catalog no. A300) for the CF pretreatment and hydrolysis of hemicellulose oligomers were purchased from Fisher Scientific (Pittsburgh, PA, USA).

1-2. Microbial Strain

E. coli AFP184 was obtained from the American Type Culture Collection (ATCC PTA 5132, Manassas, Virginia, USA). The freeze-dried culture was reconstituted in an LB medium [13]. The reconstituted culture was grown in 50-mL medium in a 250-mL shake flask. Following incubation at 37 °C and 250 rpm for 16 h, two volumes of the broth were mixed with one volume of sterile glycerol and then the stock cultures were stored at −70 °C.

1-3. Corn Fiber Destarching

Corn fiber (CF) was processed in batches of 500 g (dry basis – db) to make the hydrolysate for use in succinic acid fermentation experiments. Fig. 1 presents the overall process flow diagram from corn fiber to succinic acid. The first step in the hydrolysate making process was destarching. In each batch, 500 g (db) CF was placed in a 4-L beaker with 2,500 g deionized (DI) water. The pH was adjusted to 5, and then 45.5 μL Spezyme XTRA was added (0.1 g enzyme per kg of CF on db). The beaker was maintained at 95 °C in a water bath for 2 h. The contents of the beaker were mixed by a mechanical agitator. DI water was added as needed to replace the water loss by evaporation. At the end of the 2-h period, the beaker content was allowed to cool to about 25 °C. The pH was readjusted to 5 and 91 μL Fermenzyme L-400 was added (0.2 g/kg CF on db). The beaker content was split into two approximately equal portions and placed in two Fernbach flasks. The flasks were then incubated at 55 °C and shaken at 250 rpm for 16 h to complete the hydrolysis. The destarching water and the residual solids were separated by centrifugation at 2,000 rpm for 15 min. The supernatant (destarching water) was collected and stored frozen until further use. The wet cake was spread out on aluminum foil and dried in an oven at 55 °C with periodic stirring for 2 to 3 days. The dried solids (with about 5.4% moisture) were ground in a small tabletop coffee grinder (Krups, model F203) and used in the SAA pretreatment.

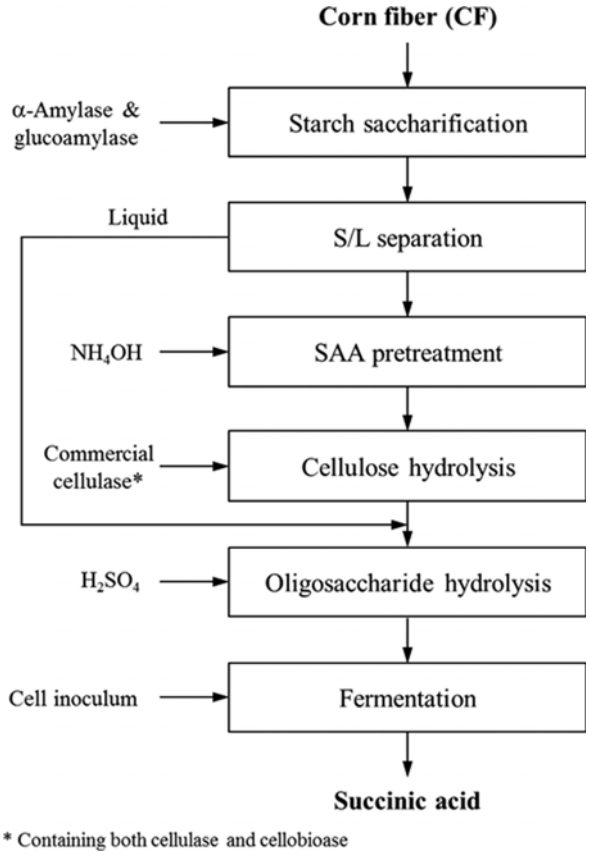

Fig. 1. Overall process diagram for succinic acid fermentation with corn fiber.

1-4. SAA Pretreatment and Hydrolysate Preparation

Pretreatment of the destarched CF was performed in Pyrex glass medium bottles with plastic caps with a soft plastic lining inside. Each bottle contained 70 g (db) of destarched CF and 700 g of 15 wt% NH_4OH (a solid-to-liquid ratio of 1 : 10). The bottles with the caps tightly squeezed in place were incubated in an oven at 60 °C for 24 h. The pretreated solids then were collected by vacuum filtration and washed repeatedly with DI water until the optical density at 465 nm (OD 465) of the wash water (diluted 10 times with DI water) measured by a Spectronic® 20 D+ spectrophotometer (Thermo Electron Corporation, Waltham, MA, USA) reached a constant value (about 0.03-0.05). The recovered solids were stored refrigerated at about 4 °C until they were used in the subsequent enzymatic hydrolysis.

Enzymatic hydrolysis of the SAA-pretreated CF (at 60 °C for 24 h) was performed in destarching water at 5 wt% solid and pH 5 in a 2-L flask. Accellerase® 1500 was added at a dosage of 0.25 mL/g solid (db). The flask was incubated at 50 °C and 250 rpm for 45 h. The liquid and residual solids were separated by centrifugation at 2,000 rpm for 15 min. Sulfuric acid was added to the recovered supernatant to a final concentration of 1 wt% H_2SO_4 in the hydrolysate. The acidified hydrolysate then was placed in an auto-

clave at 121 °C for 1 h to hydrolyze the solubilized arabinoxylan to free xylose and arabinose.

1-5. Succinic Acid Fermentation

The final hydrolysate containing glucose, xylose and arabinose was used for succinic acid fermentation in 500-ml fermentors (Moubio Knowledge, Taipei, Taiwan). Two experiments were performed. In the first experiment, the final hydrolysate was used without any modification. In the second experiment, the final hydrolysate was enriched with the following ingredients (in g/L): K_2HPO_4 1.4, KH_2PO_4 0.6, $(NH4)_2SO_4$ 3.3, $MgSO_4 \cdot 7H_2O$ 0.4, and corn steep liquor (Sigma-Aldrich, St. Louis, MO, USA) 15. In each fermentor, 350 mL of fermentation media was prepared, and these fermentors were then sterilized by autoclaving at 121 °C for 20 min. The inoculum medium was prepared in DI water, which contained the same components as in the nutrient-enriched final hydrolysate used for the second experiment plus 10 g/L glucose and was also adjusted to pH 6.5. The inoculum medium was placed in 250-ml flasks (25 mL/flask). The flasks were capped with foam plugs and sterilized as described previously. Upon cooling, each flask was inoculated with 0.1 mL thawed glycerol stock culture of *E. coli* AFP184. The flasks were incubated at 37 °C and 250 rpm for 16 h before they were used to inoculate the fermentors. Each fermentor received the entire content of one inoculum flask. Prior to inoculation, the fermentors were placed in an incubator maintained at 37 °C for about 1 h, and then kept under the same conditions for the rest of the experiments. During the first 6 h, the fermentors were aerated with 0.2-micron filter-sterilized air at 1 vvm (volume per volume per min). Aeration was then stopped to establish an anaerobic environment, which triggered succinic acid synthesis. During the entire course of the fermentation, the pH was maintained at 6.5 by automatic addition of 1.5 M Na_2CO_3 via a pH controller (model 270002, Aquatic Life, Burbank, CA). Samples (1 mL) were taken at intervals, centrifuged at 12,000 rpm on an Eppendorf microcentrifuge (Eppendorf, Hauppauge, NY, USA) and syringe-filtered through a 0.2-micron membrane for subsequent analysis.

1-6. Analysis

Lignin and carbohydrate content in the solid samples were analyzed by the NREL Procedure [13]. Quantification of monosaccharides including glucose, xylose, and arabinose after hydrolysis was conducted using a high performance liquid chromatography system (HPLC, Varian 356-LC, Agilent, CA, USA) using a refractive index (RI) detector and Bio-Rad Aminex HPX-87P column (Bio-Rad Laboratories Inc., Hercules, CA, USA). HPLC analysis conditions were 85 °C of column temperature and 0.6 mL of DI water/min.

The fermentation samples were analyzed for sugars (glucose, xylose, and arabinose) and organic acids (succinic and acetic acid) by HPLC using an Agilent Technologies system (series 1200) (Santa Clara, CA, USA) equipped with a refractive index detector. The sugar concentration was determined using a Bio-Rad Aminex HPX-87P column maintained at 80 °C with DI water as the mobile phase at a flow rate of 0.6 mL/min. For organic acid analysis, a Bio-Rad Aminex HPX-87H column maintained at 65 °C was used with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min.

RESULTS AND DISCUSSION

1. SAA Pretreatment on Chemical Composition and Enzymatic Hydrolysis of CF

The compositional data of untreated (but destarched) and SAA-pretreated CF are shown in Table 1. The carbohydrates and lignin compositions of CF under different pretreatment conditions were calculated based on the initial destarched CF weight. Delignification after the pretreatment was also presented. The results in Table 1 indicate that delignification generally increased as the reaction time increased; however, an extended reaction increased the risk of carbohydrate fraction loss, in particular, pentosan such as xylan and arabinan. Delignification of CF was not significantly increased when the temperature was increased from 60 °C to 80 °C, while more carbohydrate fractions in destarched CF were solubilized at a higher reaction temperature.

The effect of SAA pretreatment on enzymatic digestibility of CF was also evaluated. Fig. 2 summarizes the glucan, xylan, and arabinan digestibilities of the SAA-pretreated CF treated with the aforementioned reaction conditions. In Fig. 2(a), the glucan digestibility of CF was notably improved from 19% to 85% by 6 h-SAA pretreatment at 60 °C, and the digestibility further increased to 92% as the pretreatment time was extended to 24 h. With the SAA-pretreated CF at a higher reaction temperature (80 °C) and longer time (up to 48 h), the glucan digestibilities increased to the range of 88-99%. It has been reported that the removal of lignin and hemicellulose enhances enzyme access to cellulose; therefore, the enzymatic digestibility of the cellulose increased. In addition, effective pretreatment can change the cellulose structure to be more amena-

Table 1. Composition of SAA pretreated corn fiber

Temp. [°C]	Time [h]	S.R. [wt%]	Lignin [wt%]	Delignification [%]	Solid composition (based on the initial untreated biomass)				
					Glucan [wt%]	Xylan [wt%]	Galactan [wt%]	Arabinan [wt%]	Mannan [wt%]
Untreated		100	8.8	-	22.1	25.7	4.5	16.1	1.9
60	6	71.7	7.1	20.1	21.5	20.9	3.8	14.6	1.1
	12	62.2	6.2	29.6	20.5	16.4	3.0	11.5	1.0
	24	52.7	6.1	30.6	19.9	12.6	2.3	8.7	1.0
80	12	50.4	7.1	19.6	20.0	11.5	2.0	7.7	0.9
	24	46.1	6.2	29.2	20.0	10.1	1.8	6.3	1.0
	48	40.8	5.5	37.5	19.6	8.6	1.5	5.1	1.0

Note. S.R.: Solid remaining (wt%) after pretreatment; Lignin: Acid insoluble lignin

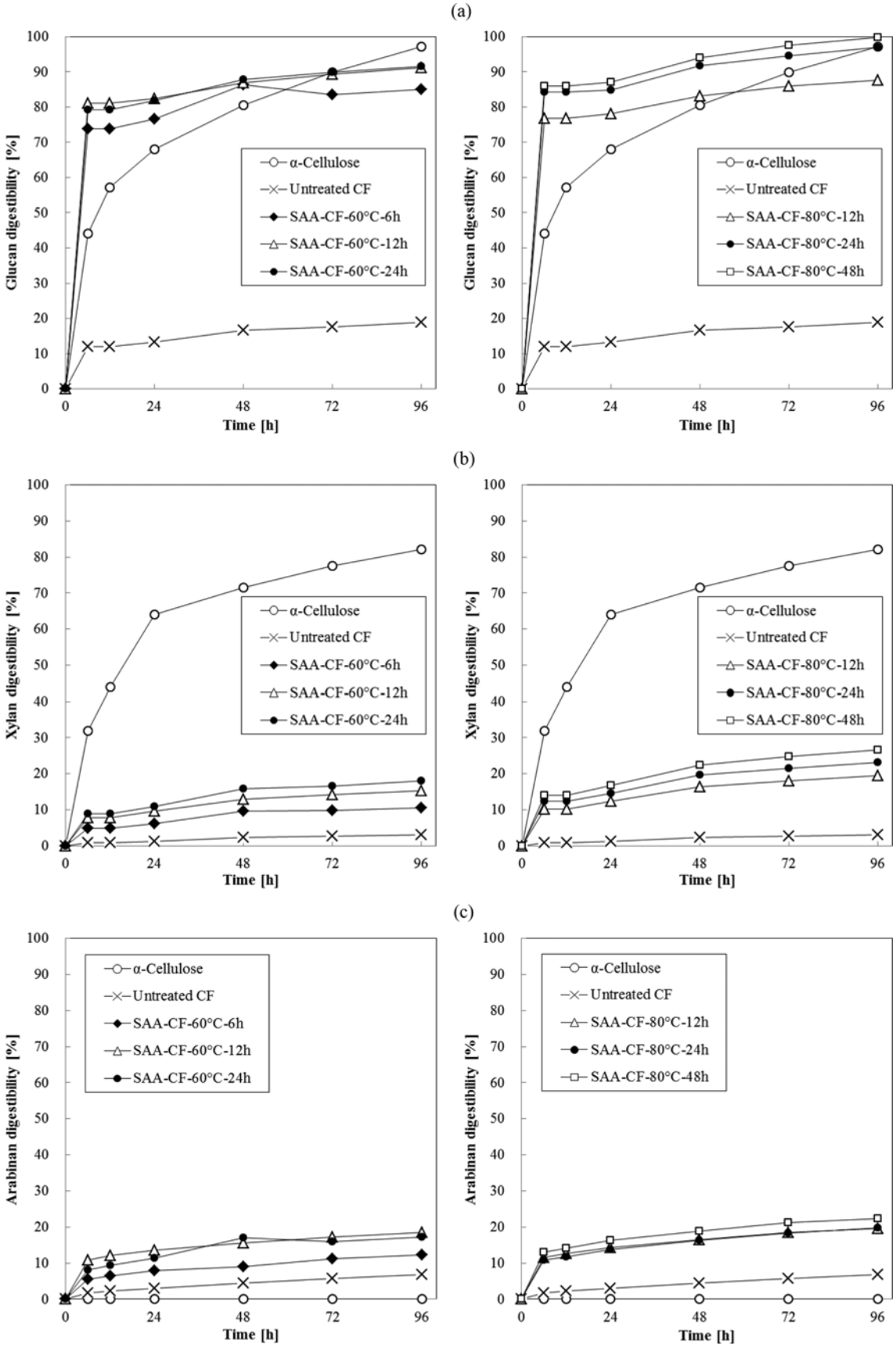

Fig. 2. Enzymatic digestibilities with SAA-pretreated corn fiber: (a) Glucan digestibility; (b) xylan digestibility; (c) arabinan digestibility. Note. Pretreatment reaction conditions: 15 wt% NH_4OH at 60-80 °C for 6-48 h.

ble to enzymatic reaction, as discussed in previous studies [14-16]. Therefore, the initial enzymatic hydrolysis rate of the pretreated CF was even faster than that of α-cellulose, pure cellulose as control, for the first 48 h.

Figs. 2(b) and (c) show the xylan and arabinan digestibilities of SAA pretreated CF treated at various reaction temperatures and times. The xylan and arabinan digestibilities were not significantly improved by SAA pretreatment compared to the increase in glucan digestibility. The xylan digestibility of pretreated CF was measured in the range of 11-27% after the pretreatment, which was increased from that (~3%) of untreated CF. Similarly, the arabinan digestibility also slightly increased from 7% of untreated CF to 22% of pretreated CF. Note that most commercial cellulase products contain both glucanase and hemicellulase activities. Although the digestibilities in Figs. 2(b) and (c) were higher than the xylose and arabinose yields from untreated CF, further hydrolysis with additional hemicellulase or acids is necessary to efficiently utilize the pentose sugars in the CF.

Overall, SAA pretreatment of CF rendered it more digestible by changing its chemical composition and structure as reaction conditions became more severe, while the total available carbohydrate content decreased with an increasing reaction temperature and time. We also propose that further optimization for SAA pretreatment of CF should be able to achieve maximum utilization of carbohydrates in CF for production of fermentable sugars.

2. Fermentation of CF Hydrolysate

We produced the succinic acid with hydrolysate obtained from sequential hydrolysis of pretreated CF (see Fig. 1). The destarching water contained 20.0 g/L glucose. After enzymatic hydrolysis with Accellerase® 1500 and 1 wt% H_2SO_4 treatment, the glucose concentration was increased to 31.2 g/L. The concentration of xylose and arabinose in the final hydrolysate was 15.0 and 7.0 g/L, respectively. In the first succinic acid fermentation experiment, where the final hydrolysate was used with no nutrient addition, the OD_{660} at the end of the growth stage (first 6 h) was 5.8. The concentration profiles of the substrates (glucose, xylose and arabinose) and product (succinic acid) are shown in Fig. 3(a). It has been reported that *E. coli* AFP184 is capable of simultaneously utilizing glucose and xylose [17]. The results obtained in the present study indicate that this strain is also capable of simultaneously utilizing arabinose together with the other two sugars. When the experiment was stopped at 73 h, all of the arabinose was consumed and only about 6% of the initial glucose remained, but xylose utilization was incomplete at 59% consumption of the initial amount. The final succinic acid concentration was 20.7 g/L. Thus, the overall productivity of the succinic acid was 0.31 g/L-h. The final succinic acid yield was 0.35 g succinic acid/g total sugar consumed. Significant improvement of succinic acid fermentation was observed when nutrients were added to the medium. At the end of the 6-h growth stage, the OD_{660} was 10.0. The concentration profiles of the substrates (glucose, xylose and arabinose) and product (succinic acid) in this experiment are shown in Fig. 3(b). Complete consumption of glucose and arabinose was observed and xylose consumption was increased to 80% of the initial amount. It was observed that the rate of succinic acid production was also relatively high. Maximum succinic acid concentration was achieved in less than 48 h. Because no sample was taken between 33.5 h and 48 h where the succinic acid concentration was 25.6 g/L and 26.3 g/L, respectively, it was not possible to determine the exact time when maximum succinic acid concentration was reached. The overall productivity, which was calculated using the end point of 48 h, was 0.49 g/L-h. This value is much higher than the productivity obtained in the experiment in which the additional nutrients were not added to the medium. The final succinic acid yield obtained with addition of nutrients to the medium was 0.53 g succinic acid/g total sugar consumed, which was also higher than the value observed in the experiment without nutrient addition. The results obtained in these two experiments demonstrate the feasibility of using the hydrolysate obtained by sequential enzymatic and dilute sulfuric acid hydrolysis of the SAA-treated corn fiber for production of succinic acid. The results obtained in the second experiment in which nutrients were supplemented to the medium also pointed to the possibility that addition of glucose in this case might increase the final succinic acid concentration.

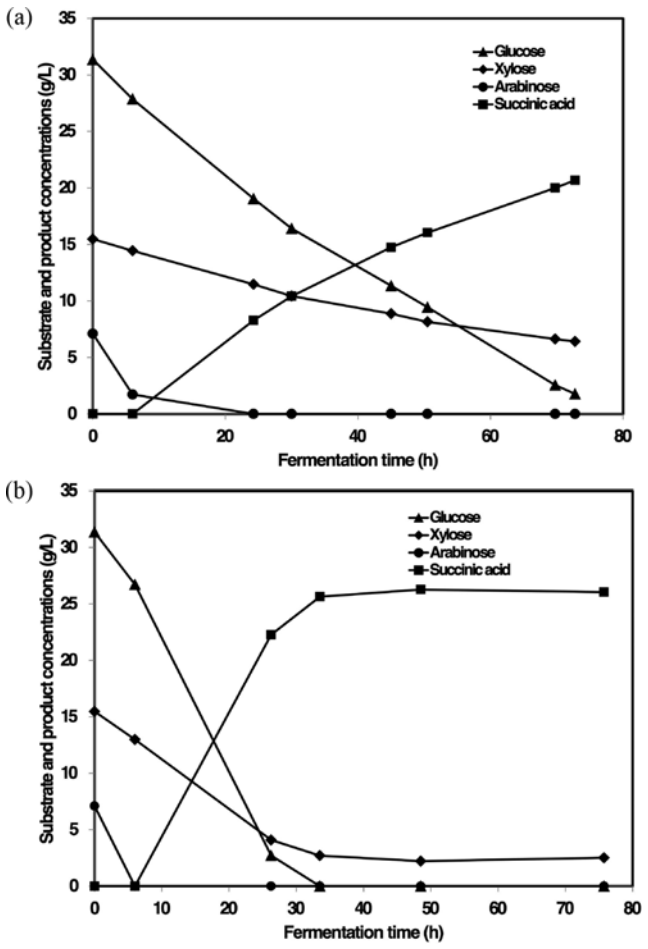

Fig. 3. Succinic acid fermentation with hydrolysate from corn fiber: (a) Without nutrients; (b) with nutrients.

CONCLUSION

Pretreatment of CF, a by-product of the corn wet milling process, can provide an additional carbohydrate source to the existing

corn ethanol process. It was found that the effective delignification and minimal carbohydrate loss along with low cost pretreatment should be factors to consider, as they enhance the enzymatic hydrolysis yield and rate of pretreated solid. Fermentation of succinic acid using hydrolysate obtained from sequential hydrolysis process resulted in 20.7 g-succinic acid/L without additional nutrients in 73 h. Addition of nutrients to the medium resulted in near complete consumption of glucose and arabinose in the hydrolysate, which also improved xylose consumption effectively. Overall it improved not only succinic acid conversion (26.3 g/L), but also the productivity of the fermentation process, which allowed succinic acid to reach the maximum concentration within 48 h.

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REFERENCES

1. A. Demirbas, *Energy Convers. Manage.*, **50**(9), 2239 (2009).
2. Ethanol Industry Outlook, Renewable Fuels Association (RFA, website; http://www.ethanolrfa.org/), January, Washington, DC, USA (2015).
3. Pocket guide to ethanol 2015, Renewable Fuels Association. Available from: http://www.ethanolrfa.org/pages/rfa-pocket-guide-to-ethanol, Accessed August 7, 2015 (2015).
4. N. S. Mosier, R. Hendrickson, M. Brewer, N. Ho, M. Sedlak, R. Dreshel, G. Welch, B. S. Dien, A. Aden and M. R. Ladisch, *Appl. Biochem. Biotechnol.*, **125**(2), 77 (2005).
5. I. J. Oh, H. W. Lee, C. H. Park, S. Y. Lee and J. Lee, *J. Microbiol. Biotechnol.*, **18**(5), 908 (2008).
6. A. M. Sánchez, G. N. Bennett and K. Y. San, *Biotechnol. Prog.*, **21**(2), 358 (2005).
7. J. Zeikus, M. Jain and P. Elankovan, *Appl. Microbiol. Biotechnol.*, **51**(5), 545 (1999).
8. B. Cornils and P. Lappe, "Dicarboxylic acids, aliphatic" in Ullmann's encyclopedia of industrial chemistry, Wiley-VCH, Weinheim, DOI:10.1002/14356007.a08_523 (2006).
9. B. Bai, J. M. Zhou, M. H. Yang, Y. L. Liu, X. H. Xu and J. M. Xing, *Bioresour. Technol.*, **185**, 56 (2015).
10. T. H. Kim, F. Taylor and K. B. Hicks, *Bioresour. Technol.*, **99**(13), 5694 (2008).
11. C. G. Yoo, N. P. Nghiem, K. B. Hicks and T. H. Kim, *Appl. Biochem. Biotechnol.*, **169**(8), 2430 (2013).
12. C. Andersson, D. Hodge, K. A. Berglund and U. Rova, *Biotechnol. Prog.*, **23**(2), 381 (2007).
13. A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton and D. Crocker, *Determination of structural carbohydrates and lignin in biomass*, Vol NREL/TP-510-42618 (2012).
14. J. A. Rollin, Z. Zhu, N. Sathitsuksanoh and Y. H. P. Zhang, *Biotechnol. Bioeng.*, **108**(1), 22 (2011).
15. N. P. Nghiem, T. H. Kim, C. G. Yoo and K. B. Hicks, *Appl. Biochem. Biotechnol.*, **171**, 341 (2013).
16. T. H. Kim, *Korean J. Chem. Eng.*, **28**(11), 2156 (2011).
17. M. I. Donnelly, C. Y. Sanville-Millard and N. P. Nghiem, US Patent, 6,743,610 (2004).