Appl Biochem Biotechnol (2010) 160:477–485
DOI 10.1007/s12010-008-8367-0

Succinic Acid Production from Acid Hydrolysate of Corn Fiber by *Actinobacillus succinogenes*

Kequan Chen · Min Jiang · Ping Wei · Jiaming Yao · Hao Wu

Received: 14 March 2008 / Accepted: 11 September 2008 /
Published online: 2 October 2008

Abstract Dilute acid hydrolysate of corn fiber was used as carbon source for the production of succinic acid by *Actinobacillus succinogenes* NJ113. The optimized hydrolysis conditions were obtained by orthogonal experiments. When corn fiber particles were of 20 mesh in size and treated with 1.0% sulfuric acid at 121 °C for 2 h, the total sugar yield could reach 63.3%. It was found that $CaCO_3$ neutralization combined with activated carbon adsorption was an effective method to remove fermentation inhibitors especially furfural that presented in the acid hydrolysate of corn fiber. Only 5.2% of the total sugar was lost, while 91.9% of furfural was removed. The yield of succinic acid was higher than 72.0% with the detoxified corn fiber hydrolysate as the carbon source in anaerobic bottles or 7.5 L fermentor cultures. It was proved that the corn fiber hydrolysate could be an alternative to glucose for the production of succinic acid by *A. succinogenes* NJ113.

Keywords Succinic acid · Corn fiber · Acid hydrolysate · Detoxification · *Actinobacillus succinogenes* NJ113

Introduction

Succinic acid, a common metabolite in plants, animals, and microorganisms, is valued for its derivative chemicals, which can be used directly or as a precursor for many industrial chemicals production including 1,4-butanediol, tetrahydrofuran, *N*-methyl pyrrolidinone, 2-pyrrolidinone, gamma-butyrolactone, and biodegradable polymers such as polybutyrate succinate, and polyamides [1]. Currently, most of the commercialized production of succinic acid has been successfully chemical synthesized from butane derived from petroleum. However, because of concerns about future scarcity, cost, and environmental impact of fossil fuel, much attention has recently been focused on the production of succinic acid by microorganisms as an alternative to the petroleum-based processes [2, 3].

K. Chen · M. Jiang (✉) · P. Wei · J. Yao · H. Wu
State Key Laboratory of Materials-Oriented Chemical Engineering,
College of Life Science and Pharmacy, Nanjing University of Technology, Nanjing 210009,
People's Republic of China
e-mail: Jiangmin@njut.edu.cn

Fermentation process for succinic acid production is desirable because renewable resources and CO_2 can be used as feedstock. Glucose, whey, and wood hydrolysate have been reported as raw materials for the production of succinic acid [4–7].

Corn fiber, a by-product of corn wet milling facilities, is marketed as a low-cost animal feed ingredient currently. It contains 60–70% carbohydrates which include starch, cellulose, and hemicellulose [8]. Low cost and high carbohydrate content of corn fiber make it an attractive potential substrate for bioconversion to chemicals. Several saccharification procedures for conversion of corn fiber carbohydrate to fermentable sugars have been reported [9–13]. Among the reported hydrolysis methods, dilute acid catalyzed hydrolysis could be performed easily at low cost. Unfortunately, non-fermentable or toxic compounds, especially furfural which is an inhibitor for cell growth by lowering cells membrane permeability [14], are formed in side reactions during acid hydrolysis process [15–17]. Thus, the hydrolysate must be detoxified to enable the microorganisms to grow and ferment. Several treatment approaches have been used for detoxification including overliming [18, 19], fungal treatment [20], ion-exchange resin [21], and activated charcoal [22–24].Nevertheless, the effectivity of each method varies when different microorganism is used.

In this paper, dilute sulfuric acid was used for corn fiber hydrolysis, followed by treatment processes to remove the inhibitors in the acid hydrolysate of corn fiber. Then the effects of treatment methods were evaluated on fermentability of the corn fiber hydrolysate for the production of succinic acid by *Actinobacillus succinogenes* NJ113.

Materials and Methods

Chemicals and Materials

All the chemicals were reagent grade and were received from Sinochem (Shanghai, P.R. China) and from Fluka Chemical (Buchs, Switzerland). N_2 and CO_2 were obtained from Nanjing Special Gases Factory (Nanjing, P.R. China). Dry corn fiber was obtained from Shandong Zhengde Foods, P.R. China.

Preparation of Corn Fiber Hydrolysate

Prior to hydrolysis, corn fiber was ground using a commercial plant grinder (Tianjing Taisite Apparatus, P.R. China) and passed through different mesh screens. Dry corn fiber particles were mixed with dilute sulfuric acid aqueous solution at a concentration of 18% (*w*/*v*). The suspensions were placed in 250 mL flasks and hydrolysed in an autoclave at 110 °C, 121 °C, or in water bath at 90 °C and 100 °C, respectively. Effects of the corn fiber particle size, sulfuric acid concentration, hydrolysis temperature, and hydrolysis time on the total sugar yields were investigated by orthogonal experimental design (Table 1).

Treatment Methods of Corn Fiber Hydrolysate

Two methods were applied to treat the hydrolysate.

NaOH Neutralization

The hydrolysate was neutralized to pH 6.0 by NaOH, and the resultant precipitates were filtered with a Busher funnel. One drop of antifoaming agent per 200 mL of hydrolysate

Table 1 Levels and factors of orthogonal experiments.

Factors	Levels			
Sulfuric acid concentration (%, *v*/*v*)	0.30	0.50	0.75	1.00
Particle size (mesh)	20	40	60	80
Hydrolysis temperature (°C)	90	100	110	121
Hydrolysis time (min)	50	75	100	120

was added into the filtrate, and then the filtrate was concentrated from 200 to 80 mL using a rotary vacuum evaporator at 60 °C, 100 rpm and 80 kPa. This is designated as the corn fiber hydrolysate treated by sodium hydroxide neutralization (SCFH).

$CaCO_3$ Neutralization Combined with Activated Carbon Adsorption

$CaCO_3$ was added to the hydrolysate to adjust the pH at 6.0, and the subsequent treatment process was the same as in "NaOH Neutralization." After concentration, 1% (*w*/*v*) of activated carbon was added to the concentrated hydrolysate, and the suspension was agitated at 150 rpm and 30 °C for 30 min. The activated carbon was filtered with a Busher funnel, and the filtrate was designated as the corn fiber hydrolysate (CCFH) treated by $CaCO_3$ neutralization combined with activated carbon adsorption (CNAA).

Microorganism and Cultivation Conditions

Microorganism

A. succinogenes NJ113 (isolated from rumen by our laboratory and stored at China General Microbiological Culture Collection Center, CGMCC NO.1716) was used in all experiments.

Culture Media

The medium for inoculum preparation contained the following (in g/L): 10.0 glucose, 5.0 yeast extract, 10.0 $NaHCO_3$, 8.5 $NaH_2PO_4{\cdot}H_2O$, and 15.5 K_2HPO_4.

The production medium contained the following (in g/L): 3.0 KH_2PO_4, 0.2 $MgCl_2{\cdot}6H_2O$, 0.2 $CaCl_2$, 1.0 NaCl, 5.0 corn liquor steep, and 10.0 yeast extract. Carbon sources (glucose, SCFH, and CCFH) were autoclaved separately and added aseptically.

Cultivation Conditions

Inoculum was prepared in 100 mL sealed anaerobic bottles with polytetrafluoroethylene on open top caps containing 50 mL medium with CO_2 as the gas phase. Vials were inoculated with a syringe from the headspace to ensure anaerobic conditions. The cultures were grown in a rotary shaker at 37 °C and 180 rpm for 12 h.

For anaerobic bottle fermentation, the cultures were performed using 100 mL anaerobic bottles containing 50 mL medium with CO_2 as the gas phase, and 50 g/L $MgCO_3$ was added to maintain the pH of medium. The cultures were grown in a rotary shaker at 37 °C and 180 rpm for 36 h.

Batch fermentation was carried out in a 7.5 L fermentor (Bioflo 110, USA) with an initial broth volume of 4.5 L, and nitrogen was bubbled through the medium for 30 min to

remove oxygen before inoculation. All fermentations were performed at 37 °C with the agitation speed of 200 rpm and CO_2 flow rate of 0.5 L/min. The pH was set at 6.8 and maintained by addition of a concentrated base solution (10 N NaOH).

Analytical Methods

For evaluation of the corn fiber samples, Chinese standard method GB/T 5009.3-2003 and GB/T 5009.9-2003 were used to determine moisture and starch content, respectively [25]. Cellulose was determined by nitric acid-ethanol method, and hemicellulose was determined by phloroglucinol method [26]. All determinations were carried out in triplicate.

Glucose was analyzed by a SBA-40C biosensor analyzer (Institute of Biology, Shandong Province Academy of Sciences, P.R. China); after acid hydrolysis, the total sugar concentration in the corn fiber hydrolysate was determined by dinitrosalicyclic acid method [27], and the gross concentration of other sugars was calculated by the total sugar minus glucose concentration.

Furfural and fermentation products were analyzed by high-performance liquid chromatography (Chromeleon server monitor, UVD 170U detector, P680 pump, Dionex, USA). For determining furfural, a reversed-phase chromatographic column (Prevail C18 column, Grace, USA) was used, and the mobile phase was acetonitrile and water (50:50, *v*/*v*) with the flow rate of 1 mL/min. To determine the fermentation products, an ion exchange chromatographic column (Prevail organic acid column, Grace, USA) was used, and 25 mM KH_2PO_4 (adjusted to pH 2.5 by H_3PO_4) was used as the mobile phase with the flow rate of 1 mL/min.

Dry cell weight (DCW) was computed from a curve relating optical density at 660 nm (OD_{660}) to dry weight. An OD_{660} of 1.0 represented 520 mg dry weight per liter.

Results and Discussion

Feedstock Composition

The chemical composition of the original untreated corn fiber was determined. The moisture content of the corn fiber was 4.6%. The total polysaccharide content was proved to be very high (68.7%), which included 15.4% starch in addition to 21.7% cellulose and 31.6% hemicellulose. These results conform to a previous literature report [28]. This agricultural residue was an attractive material for saccharification and fermentation processes for its high carbohydrate content. The remaining components (26.7%) of the original untreated corn fiber, which may include protein, fat, lignin, and so on, were not quantified.

Hydrolysis of Corn Fiber Based on Orthogonal Experimental Design

L16 (4^4) orthogonal experiments were designed to estimate the effects of particle size, sulfuric acid concentration, hydrolysis temperature, and hydrolysis time on total sugar yields. The results were shown in Table 2. The total sugar yield was calculated as a percentage of the maximum theoretical yield based on the monosaccharides in starch, cellulose, and hemicellulose.

It was shown that hydrolysis temperature had the most significant influence on the total sugar yield; sulfuric acid concentration and hydrolysis time also had strong influence, while

Table 2 Arrangement and results of L16 (4^4) orthogonal experiments.

No.	Levels				Total sugar yield (%)
	Sulfuric acid concentration (%)	Particle size (mesh)	Hydrolysis temperature (°C)	Hydrolysis time (min)	
1	0.30	20	90	50	6.3
2	0.30	40	100	75	13.1
3	0.30	60	110	100	25.7
4	0.30	80	121	120	56.9
5	0.50	20	100	100	15.9
6	0.50	40	90	120	11.6
7	0.50	60	121	50	54.9
8	0.50	80	110	75	27.3
9	0.75	20	110	120	45.7
10	0.75	40	121	100	62.8
11	0.75	60	90	75	11.2
12	0.75	80	100	50	14.8
13	1.00	20	121	75	62.7
14	1.00	40	110	50	38.5
15	1.00	60	100	120	30.0
16	1.00	80	90	100	17.9
K1[a]	25.5	32.6	11.7	28.6	
K2	27.4	31.5	18.5	28.6	
K3	33.6	30.5	34.3	30.6	
K4	37.2	29.2	59.3	36.1	
R[b]	11.8	3.4	47.6	7.5	
Optimized design	1	20	121	120	

[a] The average of the sum of experimental results

[b] $R=Ki,\max-Ki,\min$

particle size had least influence among the four factors. The optimized hydrolysis conditions were determined as follows: particle size was 20 mesh with 1.0% sulfuric acid at 121 °C for 120 min. Under the optimized conditions, the total sugar yield could reach 63.3%. However, there were toxic compounds in the hydrolysate such as furfural which would inhibit the growth of bacteria. The solution also presented chocolate, which complicated the downstream process.

Treatment of Corn Fiber Hydrolysate

The effects of treatment methods of the corn fiber hydrolysate on the removal of furfural and the loss of sugar were shown in Table 3.

Table 3 The effects of treatment methods on the removal of furfural and the loss of sugar.

Hydrolysate	Total sugar concentration (g/L)	Furfural (g/L)
Untreated hydrolysate[a]	217.5	2.98
SCFH	214.6	2.64
CCFH	206.1	0.24

[a] The hydrolysate was not neutralized by NaOH or $CaCO_3$ before filtration and concentration

It could be seen that the method treating with CNAA was more effective which caused 91.9% reduction of furfural with only 5.2% loss of the total sugar. Although NaOH neutralization caused less sugar loss too, the removal efficiency of furfural was low.

Both calcic compounds neutralization and activated carbon absorption were effective ways to remove furfural from the hydrolysates [16, 29]. In this work, it was proved that $CaCO_3$ neutralization combined with activated carbon absorption was a more effective method to remove furfural in the corn fiber hydrolysate.

Succinic Acid Production from Different Carbon Sources

To investigate the fermentability of the corn fiber hydrolysate, succinic acid fermentations by *A. succinogenes* NJ113 from glucose, SCFH, and CCFH at the concentration from 10 to 50 g/L in medium were carried out, and the results were shown in Fig. 1.

As shown in Fig. 1, the succinic acid yield decreased 1.41-fold with the increase of SCFH from 10 to 50 g/L. However, when CCFH was used as carbon source, even at a high concentration of 50 g/L, the production yield reached above 72.0%, which was 23.2% higher than that of SCFH. The poor fermentability of NaOH neutralized hydrolysate was primarily due to the presence of high level of furfural which could decrease fermentation efficiency of *A. succinogenes* NJ113. Therefore, it was proved again that CNAA was an effective method to remove inhibitors especially furfural from acid hydrolysate of corn fiber for succinic acid production by *A. succinogenes* NJ113.

As reported, the use of the more-reduced sugars resulted in the formation of higher amounts of the more-reduced end products succinate [30]. Since saccharides in the corn fiber hydrolysate are composed of xylose and arabinose in addition to glucose [10], reducing power of the hydrolysate is lower than that of glucose [30]. So for the production of succinic acid, if corn fiber hydrolysate was used as carbon source, the production yield from CCFH was about 8% lower than that with glucose (data shown in Fig. 1).

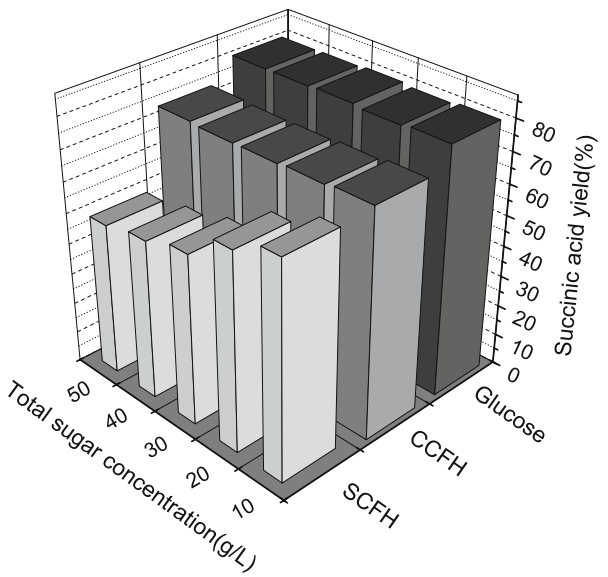

Fig. 1 Effects of carbon sources on succinic acid yield

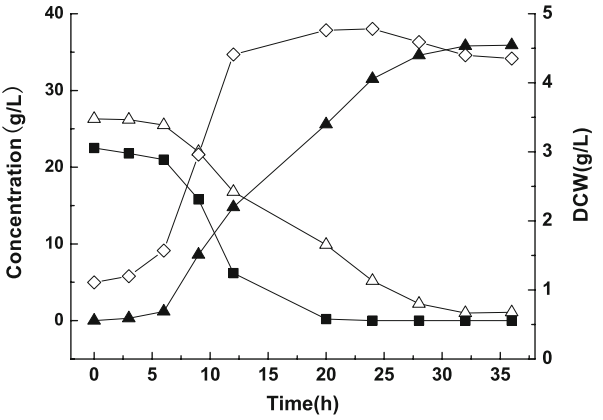

Fig. 2 Succinic acid production from CCFH in 7.5 L fermentor. *Open triangles* other sugars, *closed squares* glucose, *open diamonds* DCW, *closed triangles* succinic acid

Succinic Acid Production from CCFH in Batch Fermentation

The results of succinic acid production from CCFH in a 7.5 L fermentor were shown in Fig. 2.

There existed some residual inhibition in the CCFH since the growth of *A. succinogenes* NJ113 had a longer lag phase (6 h) than that with glucose as sole carbon source (Fig. 3). As shown in Fig. 2, when CCFH was used as carbon source, glucose was consumed rapidly and could be exhausted within 24 h. In the meanwhile, other sugars were also utilized but with much lower utilization rate in comparison with that of glucose. It appeared that there was no catabolite repression from glucose present for *A. succinogenes* NJ113.

At the end of 36 h fermentation, the concentration of succinic acid reached 35.4 g/L with the yield of 72.5%, while 1.0 g/L of other sugars still remained.

Concluding remarks

Cost of raw materials is one of the major factors in economics of production media for succinic acid fermentation. $CaCO_3$ neutralization combined with activated carbon absorption was an effective method to remove the inhibitory compound especially furfural from acid hydrolysate of corn fiber, and the fermentability of hydrolysate for the production

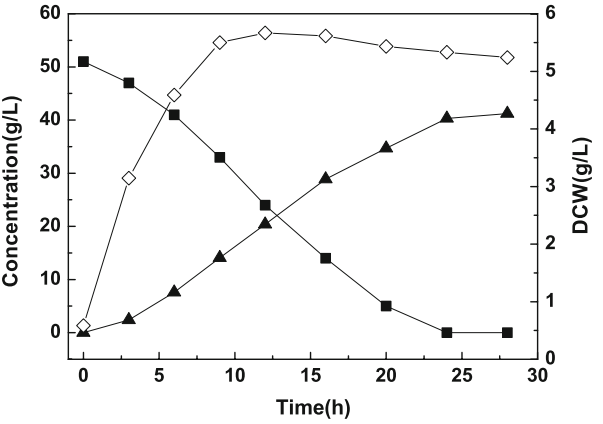

Fig. 3 Succinic acid production from glucose in 7.5 L fermentor. *Closed squares* glucose, *open diamonds* DCW, *closed triangles* succinic acid

of succinic acid by *A. succinogenes* NJ113 was improved. Therefore, corn fiber could be an alternative carbon source for the economic production of succinic acid by *A. succinogenes* NJ113.

Acknowledgements This work was supported by grant no. 2006AA02Z235 from "863" Program of China, grant no. 20606017 from National Natural Science Foundation of China, and grant no. BSCX200608 from Doctoral Dissertations' innovation Foundation of Nanjing University of Technology.

References

1. Willke, T., & Vorlop, K. D. (2004). *Applied Microbiology and Biotechnology*, *66*, 131–142. doi:10.1007/s00253-004-1733-0.
2. Song, H., & Lee, S. Y. (2006). *Enzyme and Microbial Technology*, *39*, 352–361. doi:10.1016/j.enzmictec.2005.11.043.
3. Zeikus, J. G., Jain, M. K., & Elankovan, P. (1999). *Applied Microbiology and Biotechnology*, *51*, 525–545. doi:10.1007/s002530051431.
4. Lee, P. C., Lee, W. G., Kwon, S., & Lee, S. Y. (2000). *Applied Microbiology and Biotechnology*, *54*, 23–27. doi:10.1007/s002530000331.
5. Guettler, M. V., Jain, M. K., & Rumler, D. (1996). US Patent 5,573,931.
6. Kim, D. Y., Yim, S. C., Lee, P. C., & Lee, W. G. (2004). *Enzyme and Microbial Technology*, *35*, 648–653. doi:10.1016/j.enzmictec.2004.08.018.
7. Mckinlay, J. B., Zeikus, J. G., & Vieille, C. (2005). *Applied and Environmental Microbiology*, *71*, 6651–6656. doi:10.1128/AEM.71.11.6651-6656.2005.
8. Hespell, R. B., O'Bryan, P. J., Moniruzzaman, M., & Bothast, R. J. (1997). *Applied Biochemistry and Biotechnology*, *62*, 87–97. doi:10.1007/BF02787986.
9. Saha, B. C., & Bothast, R. J. (1999). *Applied Biochemistry and Biotechnology*, *76*, 65–77. doi:10.1385/ABAB:76:2:65.
10. Zhu, Y., Wu, Z., & Yang, S. (2002). *Process Biochemistry*, *38*, 657–666. doi:10.1016/S0032-9592(02)00162-0.
11. Karel, G., & Rodney, J. B. (1997). *Process Biochemistry*, *32*, 405–415. doi:10.1016/S0032-9592(96)00095-7.
12. Gáspár, M., Kálmán, G., & Réczey, K. (2007). *Process Biochemistry*, *42*, 1135–1139. doi:10.1016/j.procbio.2007.04.003.
13. Timothy, D. L., & Bruce, S. D. (2000). *Process Biochemistry*, *35*, 765–769. doi:10.1016/S0032-9592(99)00137-5.
14. Larsson, S., Palmqvist, E., Tengborg, C., Stenberg, K., & Zacchi, G. (1999). *Enzyme and Microbial Technology*, *24*, 151–159. doi:10.1016/S0141-0229(98)00101-X.
15. Buhner, J., & Agblevor, F. A. (2004). *Applied Biochemistry and Biotechnology*, *119*, 13–30. doi:10.1385/ABAB:119:1:13.
16. Palmqvist, E., & Hahn-Hagerdal, B. (2000). *Bioresource Technology*, *74*, 17–24. doi:10.1016/S0960-8524(99)00160-1.
17. Luo, C., Brink, D., & Blanch, H. (2002). *Biomass and Bioenergy*, *22*, 125–138. doi:10.1016/S0961-9534(01)00061-7.
18. Roberto, I. C., Lacis, L. C., Barbosa, M. F. S., & Mancilha, I. M. (1991). *Process Biochemistry*, *26*, 15–21. doi:10.1016/0032-9592(91)80003-8.
19. Martinez, A., Rodriguez, M. E., Wells, M. L., & York, S. W. (2001). *Biotechnology Progress*, *17*, 287–293. doi:10.1021/bp0001720.
20. Jonsson, L. (1998). *Applied Microbiology and Biotechnology*, *49*, 691–697. doi:10.1007/s002530051233.
21. Nilvebrant, N. O., Reinmann, A., & Larsson, S. (2001). *Applied Biochemistry and Biotechnology*, *91–93*, 35–49. doi:10.1385/ABAB:91-93:1-9:35.
22. Villarreal, M. L. M., Prata, A. M. R., Felipe, M. G. A., Almeida, E., & Silva, J. B. (2006). *Enzyme and Microbial Technology*, *40*, 17–24. doi:10.1016/j.enzmictec.2005.10.032.
23. Parajo, J. C., Domınguez, H., & Domınguez, J. M. (1997). *Enzyme and Microbial Technology*, *21*, 18–24. doi:10.1016/S0141-0229(96)00210-4.
24. Alves, L. A., Felipe, M. G. A., Almeida e Silva, J. B., Silva, S. S., & Prata, A. M. R. (1998). *Applied Biochemistry and Biotechnology*, *70–72*, 89–98. doi:10.1007/BF02920126.
25. Press editors (2003), *National standard assembly of China*, Standard Press of China, Beijing.
26. Ouyang, P. K., & Chen, Y. R. (1995). *Theory and application for hydrolysis of plant biomass*, China Science and Technology Press, pp. 21–24.

27. Miller, G. L. (1959). *Analytical Chemistry*, *31*, 426–428. doi:10.1021/ac60147a030.
28. Saha, B. C., Dien, B. S., & Bothast, R. J. (1998). *Applied Biochemistry and Biotechnology*, *70–72*, 115–125. doi:10.1007/BF02920129.
29. Chandel, A. K., Kapoor, R. K., Singh, A., & Kuhad, R. C. (2007). *Bioresource Technology*, *98*, 1947–1950. doi:10.1016/j.biortech.2006.07.047.
30. Mariët, J., Werf, V. D., Guettler, M. V., Jain, M. K., & Zeikus, J. G. (1997). *Archives of Microbiology*, *167*, 332–342. doi:10.1007/s002030050452.