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ORIGINAL PAPER

Ultrasonic pretreatment and acid hydrolysis of sugarcane bagasse for succinic acid production using *Actinobacillus succinogenes*

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Abstract Immense interest has been devoted to the production of bulk chemicals from lignocellulose biomass. Diluted sulfuric acid treatment is currently one of the main pretreatment methods. However, the low total sugar concentration obtained via such pretreatment limits industrial fermentation systems that use lignocellulosic hydrolysate. Sugarcane bagasse hemicellulose hydrolysate is used as the carbon and nitrogen sources to achieve a green and economical production of succinic acid in this study. Sugarcane bagasse was ultrasonically pretreated for 40 min, with 43.9 g/L total sugar obtained after dilute acid hydrolysis. The total sugar concentration increased by 29.5 %. In a 3-L fermentor, using 30 g/L non-detoxified total sugar as the carbon source, succinic acid production increased to 23.7 g/L with a succinic acid yield of 79.0 % and a productivity of 0.99 g/L/h, and 60 % yeast extract in the medium could be reduced. Compared with the detoxified sugar preparation method, succinic acid production and yield were improved by 20.9 and 20.2 %, respectively.

Keywords Biomass · Succinic acid · *Actinobacillus succinogenes* · Sugarcane bagasse · Pretreatment

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Introduction

Succinic acid, a common metabolite in plants, animals and microorganisms, is widely used in the agricultural, food, and pharmaceutical industries [1]. Currently, succinic acid has attracted a lot of interest because of its application as a C4 platform chemical to promote polyester degradation for the synthesis of 1,4-butanediol, tetrahydrofuran, gamma-butyrolacetone and other bulk chemicals, and polybutylene succinate (PBS) [2]. Among various succinic acid producers, such as *Actinobacillus succinogenes* [3, 4], *Anaerobiospirillum succiniciproducens* [5, 6], *Mannheimia succiniciproducens* [7–9], and *Escherichia coli* [10, 11], *A. succinogenes* is considered one of the most promising strains for industrial succinic acid production because of its ability to produce comparatively large amounts of succinic acid using a wide range of carbon sources [12, 13].

Considering the abundance of raw materials and low production cost, many studies have focused on producing bulk chemicals from lignocellulosic biomass, with dilute sulfuric acid treatment combined with enzymatic hydrolysis of lignocellulose as the main pretreatment method [14, 15]. However, the high pretreatment cost limits industrial fermentation systems using lignocellulosic hydrolysate to obtain carbon source. As one of the most important lignocellulosic materials from agricultural residues, sugarcane bagasse is mainly composed of cellulose, hemicellulose, and lignin [16]. At present, many studies have reported a fermentation process using cheap raw materials to prepare succinic acid [17–19]. For example, Elcio reported the pretreatment of sugarcane bagasse using diluted acid to gain xylose as the carbon source by optimizing the medium components, and fermentation was carried out in an instrumented bioreactor, with a final succinic acid concentration of 22.5 g/L [20]. These studies

indicate that the cost of succinic acid production is greatly reduced by maximizing the use of sugarcane bagasse hemicellulose as the carbon source for fermentation. The pretreatment of sugarcane bagasse will provide a simpler, more effective method for the utilization of the hydrolysates produced in this study. Besides xylose, the utilization of glucose and arabinose are also investigated.

A higher total sugar concentration was obtained from pretreated sugarcane bagasse under different ultrasonic times before acid hydrolysis in this study. Pretreated sugarcane bagasse acts as the carbon source and replaces a part of the nitrogen source in the culture medium, which reduces the yeast extract required for the growth of *A. succinogenes* NJ113 in succinic acid production. The method is highly important in the usage of renewable resources considering the depletion of fossil resources and the strong demand for environment-friendly energy sources.

Materials and methods

Chemicals and gas

The phenolic compounds *p*-coumaric acid, ferulic acid, 4-hydroxybutyl acrylate (4-HBA), vanillic acid, syringaldehyde, and vanillin were purchased from Sigma Chemicals. Yeast extract was purchased from Oxoid Ltd. (Cambridge, UK). Other chemicals were of reagent grade and either from Sinochen (Shanghai, China) or Fluka Chemical (Buchs, Switzerland). CO_2 and N_2 were obtained from Nanjing Special Gases Factory (Nanjing, China).

Materials and preparation

Sugarcane bagasse was obtained from the Guangzhou Sugarcane Industry Research Institute (Guangzhou, China) and milled to particle sizes less than 1 mm. Acid hydrolysis of sugarcane bagasse was carried out in a medium containing 2 % H_2SO_4 with a liquid to solid ratio of 10:2 (w/w) at 125 °C for 150 min [21]. The concentration of sugars on the basis of per kilogram sugarcane bagasse dry mass was 153 g/L reducing sugars, which comprised about 17.5 g/L glucose, 126 g/L xylose, and 9.5 g/L arabinose. The sugarcane bagasse was ultrasonicated at 150 W (ultrasonic extraction device from Kedao Company, Shanghai, China). The liquid–solid complex was heated at room temperature and filtered to remove the solids [22, 23]. Activated charcoal (2 % w/v) was used for detoxification at 30 °C. The hydrolysates were concentrated by vacuum evaporation at 65 °C to a third of the initial volume. The hydrolysate concentrate was stored at 4 °C before use.

Microorganism and growth conditions

A. succinogenes NJ113 (China General Microbiological Culture Collection Center, CGMCC NO.1716) was used in all experiments. Cells were grown in 50 mL medium sealed in anaerobic bottles with a volume of 100 mL. The medium for inoculum cultures was composed of (per liter): 10.0 g glucose, 5.0 g yeast extract, 10.0 g $NaHCO_3$, 8.5 g $NaH_2PO_4{\cdot}H_2O$, and 15.5 g K_2HPO_4. It was heat sterilized at 121 °C for 15 min. The anaerobic bottles were inoculated with 1 mL of a −70 °C glycerol stock culture and incubated at 37 °C.

For anaerobic bottle cultivation, exponentially growing cells were inoculated into 100 mL sealed anaerobic bottles filled with 30 mL of fermentation medium containing the following (per liter): 3.0 g KH_2PO_4, 0.2 g $MgCl_2{\cdot}6H_2O$, 0.2 g $CaCl_2$, 1.0 g NaCl, and 10 g yeast extract. The carbon source (sugarcane bagasse hydrolysate) was autoclaved separately. The pH of the medium was maintained by the addition of an equal quality carbon source of $MgCO_3$. The anaerobic bottle cultivation was carried out in a rotary shaker at 37 °C and 180 rpm.

Batch fermentation was conducted in a 3-L fermentor (Bioflo 110, USA) with an initial broth volume of 1.5 L, which contained (per liter): 3.0 g KH_2PO_4, 0.2 g $MgCl_2{\cdot}6H_2O$, 0.2 g $CaCl_2$, 1.0 g NaCl, 5.0 g corn steep liquor, and 10.0 g yeast extract. The carbon sources (non-detoxified sugarcane bagasse cellulose hydrolysate) was separately sterilized and added to the medium at a final concentration of 30 g/L. Carbon dioxide (CO_2) was bubbled through the medium for 30 min to remove oxygen before inoculation. All fermentation processes were carried out at an agitation speed of 200 rpm and CO_2 flow rate of 0.5 L/min. The pH was controlled at 6.8 by automatically adding 2.0 mol/L Na_2CO_3. All experiments were carried out in triplicate.

Analytical methods

The 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 of dry weight per liter.

Glucose was analyzed by an SBA-40C biosensor analyzer (Institute of Biology, Shandong Province Academy of Sciences, P.R. China). Xylose, arabinose, hydroxymethylfurfural (HMF), and organic acid were analyzed by high-performance liquid chromatography (Chromeleon server monitor, UVD 170U detector, P680 pump, Dionex, USA). 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 a

flow rate of 1 mL/min. Furfural was estimated by a spectrophotometric method described by Martinez et al. [24]. The total soluble phenolic compounds (TPC) in acid hydrolysate was determined by the Folin–Ciocalteu method [25] with vanillin as calibration standard. The yields of organic acid, including succinic acid and acetic acid, defined as the amount of the final organic acid produced from 1 g glucose consumption, were expressed as percentages.

The total nitrogen/protein content in the samples was measured by a combustion method (AOAC, 2002), using a protein analyzer (K9860 Kjeltec Analyzer Unit, Hanon Instruments, China). The protein content was calculated with a conversion factor of 6.25.

Results and discussion

Effect of ultrasonication time on sugarcane bagasse hydrolysis

Ultrasonication is widely applied as an energy injection method at room temperature in the physical, biological, and chemical fields. Sugarcane bagasse hydrolysis was performed with and without ultrasonication to investigate the effects of ultrasonication time on the total sugar concentration. As shown in Table 1, when 150 W of ultrasonication was used at room temperature, the glucose, xylose, and arabinose concentrations increased as the ultrasonication time increased from 10 to 40 min. Compared with treatment without ultrasonication, the total sugar concentration after 40 min of ultrasonication increased by 29.5 %, and the glucose, xylose, and arabinose concentrations increased by 28.6, 29.1, and 34.9 %, respectively. Ultrasound in aqueous media produces mechanical action, and the cavitation induced by the internal jet promotes macromolecular degradation [22]. The ultrasonic pretreatment of sugarcane bagasse can release the polymers from the biomass, dramatically change the ultrastructure, and increase the total sugar concentration. When the ultrasonication time increased from 40 to 60 min, the total sugar concentration was 40.4 g/L, a 19.2 % increase compared with sugarcane bagasse without ultrasonic pretreatment. However, this figure is about 8.7 % lower than the yield with 40 min of ultrasonication. With the long pretreatment time of ultrasonication, the polymer morphology of the biomass was possibly damaged; thus, the total sugar concentration that was obtained began to decrease at 60 min of ultrasonication.

Succinic acid production using different total sugar concentrations

Diluted acid pretreatment of sugarcane bagasse generates a liquid hemicellulose hydrolysate composed mainly of xylose with moderate amounts of glucose and arabinose. In this study, succinic acid production from hemicellulose hydrolysate with total sugar concentrations ranging from 10 to 50 g/L was investigated through anaerobic bottle cultivation (Table 2). When the total sugar concentration was 30 g/L, the maximum succinic acid concentration, the maximum DCW, and the yield of succinic acid were 19.7, 4.28 g/L, and 65.7 %, respectively. As shown in Fig. 1, the consumption rates for glucose, xylose, and arabinose were 4.18, 18.92, and 4.8 g/L, respectively, as the sugar consumption reached maximum, which are obviously higher than in other succinic acid production methods. With the further increase in total sugar concentration, the succinic acid yield decreased from 65.7 % or 4.6 g/L. The lowest yield was obtained at a total sugar concentration of 50 g/L. At this condition, the growth of the strain was severely inhibited and only a DCW of 1.56 g/L was obtained. When

Table 1 Effect of ultrasonic time on the sugar concentration

Ultrasonic time (min)	Glucose concentration (g/L)	Xylose concentration (g/L)	Arabinose concentration (g/L)	Total sugar concentration (g/L)
0	4.2 ± 0.6[a]	25.4 ± 0.6	4.3 ± 0.3	33.9 ± 0.7
20	4.6 ± 0.6	29.7 ± 0.1	5.2 ± 0.5	39.5 ± 0.6
40	5.4 ± 1.0	32.8 ± 0.9	5.8 ± 0.5	43.9 ± 0.3
60	5.3 ± 0.7	30.5 ± 0.4	4.6 ± 0.6	40.4 ± 1.0

Sugarcane bagasse was treated at different ultrasound times, the ultrasonic power was 150 W and pretreatment temperature was room temperature

[a] Each value is an average of three parallel replicates and is represented as mean ± standard deviation

Table 2 Effect of the total sugar concentration on succinic acid production

Total sugar concentration (g/L)	DCW (g/L)	Succinic acid (g/L)	Formic acid (g/L)	Acetic acid (g/L)	Succinic acid yield (%)
10	2.14 ± 0.21[a]	7.8 ± 0.4	1.0 ± 0.1	2.3 ± 0.3	78.0 ± 0.4
20	3.43 ± 0.16	16.9 ± 1.2	2.9 ± 0.2	7.7 ± 0.8	84.5 ± 0.7
30	4.28 ± 0.27	19.7 ± 1.0	3.1 ± 0.2	9.0 ± 0.5	65.7 ± 1.2
40	2.37 ± 0.13	12.5 ± 0.9	1.2 ± 0.1	4.6 ± 0.2	31.3 ± 0.7
50	1.56 ± 0.19	4.6 ± 0.5	0.7 ± 0.1	8.3 ± 0.3	9.2 ± 0.3

Cells were grown in anaerobic bottles with different total sugar concentrations for 48 h

[a] Each value is an average of three parallel replicates and is represented as mean ± standard deviation

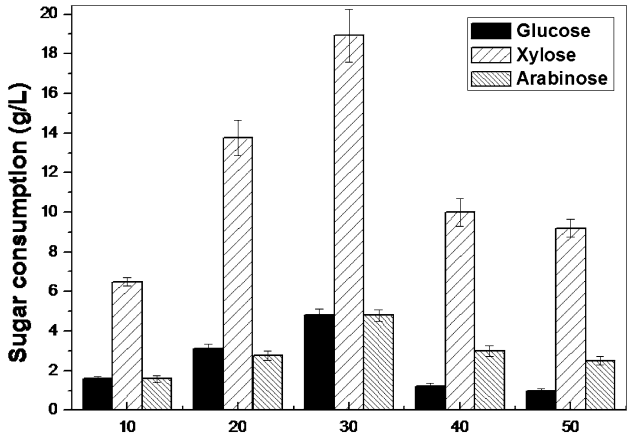

Fig. 1 Sugar consumption at the different total sugar concentration in the succinic acid fermentation. Cells were grown in anaerobic bottles for 40 h

the total sugar concentration was too high, toxic substances harmful to strain growth were probably generated in the medium. Consequently, a total sugar concentration of 30 g/L was found to be the optimum condition for succinic acid production. Subsequent experiments were performed under this optimum condition.

Effect of hydrolysate detoxification on succinic acid production

Acid hydrolysis of lignocellulose, especially concentrated acid hydrolysis, usually generates harmful substrates such as formic acid, phenol, and furfural, which may have inhibitory effects on microorganisms [26]. However, in the experiments, *A. succinogenes* NJ113 resisted a certain amount of inhibitors in the hydrolysates. As shown in Table 3, hydrolysate detoxification during succinic acid production was investigated, and the initial total sugar concentration was 30 g/L. The inhibitors in diluted-acid hemicellulose hydrolysate including TPC, HMF, and furfural were detected. We found 2.84 g/L of TPC, 0.42 g/L of HMF, and 0.71 g/L of furfural in the hydrolysate. The concentration of TPC, HMF, and furfural in detoxified hemicellulose hydrolysate were decreased to 78.5, 83.3, and 81.7 %, respectively, compared with non-detoxified hemicellulose hydrolysate. In contrast, the increase of cell growth was not obvious using non-detoxified hemicellulose hydrolysate as a carbon source, but higher succinic acid concentrations and lower acetic acid concentrations were obtained. The succinic acid concentration obtained was 23.5 g/L, a 17.8 % increase. The acetic acid and formic acid productions were 5.4 and 3.6 g/L, respectively. Acetic acid productivity was reduced by 40.3 %, but formic acid productivity was enhanced by 20 %. The contents of N-compounds and crude protein were 0.03 and 0.19 % in non-detoxified hemicellulose hydrolysate, which were decreased greatly in the detoxified hemicellulose hydrolysate. Perhaps, *A. succinogenes* NJ113 is resistant to the low concentration of inhibitors in the non-detoxified hemicellulose hydrolysate and the contents of N-compounds and crude protein can be beneficial to bacterial growth. In Fig. 2, compared with detoxified hemicellulose hydrolysate, the xylose and glucose consumption using non-detoxified hydrolysate increased by 5.7 and 12.5 %, respectively, whereas arabinose consumption decreased by 4.2 %. However, the total sugar consumption increased. Detoxification during hydrolysis may have destroyed some components beneficial to bacterial growth. Nevertheless, *A. succinogenes* was able to resist toxins in the sugarcane bagasse hemicellulose hydrolysate to a certain extent. Batch fermentation of non-detoxified sugarcane bagasse hemicellulose hydrolysate was conducted to produce succinic acid by *A. succinogenes* NJ113 in a 3-L stirred bioreactor with an initial sugar concentration of 30 g/L containing 22.4 g/L xylose, 3.6 g/L glucose, and 3.9 g/L arabinose. As shown in Fig. 3, 3.6 g/L of glucose and 3.9 g/L of arabinose were consumed completely at the end of this fermentation, along with 22.4 g/L of xylose. The final concentration of succinic acid achieved at the end of the

Table 3 Effect of detoxification on succinic acid production

	TPC[a] (g/L)	HMF[c] (g/L)	Furfural (g/L)	N-compounds (%)[d]	Crude protein (%)[d]	DCW (g/L)	Glucose consumed (g/L)	Xylose consumed (g/L)	Arabinose consumed (g/L)	Succinic acid (g/L)	Formic acid (g/L)	Acetic acid (g/L)	Succinic acid yield (%)
Non-detoxified sugar	2.84 ± 0.13[b]	0.42 ± 0.10	0.71 ± 0.06	0.03 ± 0.01	0.19 ± 0.03	4.41 ± 0.21	5.4 ± 0.27	20 ± 0.46	4.6 ± 0.24	23.2 ± 0.4	3.6 ± 0.1	5.4 ± 0.2	77.3 ± 0.6
Detoxified sugar	0.61 ± 0.09	0.07 ± 0.02	0.13 ± 0.01	0.01 ± 0.01	0.10 ± 0.02	4.33 ± 0.14	4.8 ± 0.33	18.9 ± 0.62	4.8 ± 0.41	19.6 ± 0.3	3.0 ± 0.1	9.2 ± 0.9	65.7 ± 0.4

Total sugar concentration = 30 g/L

Cells were grown in anaerobic bottles with an initial total sugar concentration of 30 g/L for 24 h

[a] Total soluble phenolic compounds

[b] Each value is an average of three parallel replicates and is represented as mean ± standard deviation

[c] Hydroxymethylfurfural

[d] w/w, on wet basis

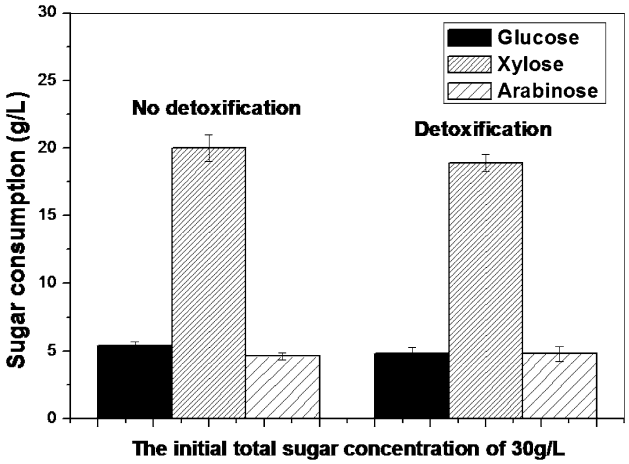

Fig. 2 Effect of hydrolysate detoxification on succinic acid production. Cells were grown in anaerobic bottles with an initial total sugar concentration of 30 g/L for 24 h

fermentation was 23.7 g/L, with a succinic acid yield of 79.0 % and a productivity of 0.99 g/L/h.

Optimization of yeast extract concentrations from the medium for succinic acid fermentation

Yeast extract is recognized as the best nutrient source for succinic acid fermentation using *A. succinogenes* [27]. Given the abundant nutrients in sugarcane bagasse, such as amino acids, vitamins, and minerals, succinic acid fermentation using sugarcane bagasse as a carbon source may reduce the required yeast extract concentration. As shown in Table 4, the DCW and succinic acid concentration decreased when the yeast extract concentration decreased from 10 to 6 g/L. In contrast, the addition of 8 and 6 g/L yeast extract decreased succinic acid concentration from 17.7 to 9.5 %. Perhaps the contents of N-compounds and crude protein in non-detoxified hemicellulose hydrolysate can be beneficial to bacterial growth and succinic acid production. When 4 g/L yeast extract was used in the culture medium, the DCW was 4.12 g/L and the succinic acid concentration was 23.5 g/L, equal to the succinic concentration obtained using 10 g/L yeast extract in the medium. However, when the yeast extract concentration decreased to 2 g/L in the culture medium, a 12.5 % decrease in succinic acid concentration was observed. Cell growth was very slow and no formic acid was produced without yeast extract supplementation. Furthermore, the DCW, succinic acid, and acetic acid concentrations were only 1.77, 1, and 1.4 g/L, respectively. Thus, the nitrogen source in the medium, i.e., the yeast extract supplement, for succinic acid fermentation with sugarcane bagasse hydrolysate could be reduced from 10 g/L yeast extract to just 4 g/L.

Table 4 Effect of yeast extract concentration on succinic acid production

Yeast extract concentration (g/L)	DCW (g/L)	Succinic acid (g/L)	Formic acid (g/L)	Acetic acid (g/L)	Succinic acid yield (%)
10	4.41 ± 0.21[a]	23.2 ± 0.4	3.6 ± 0.1	5.4 ± 0.2	77.3 ± 0.6
8	4.36 ± 0.16	19.1 ± 0.9	2.7 ± 0.3	8.2 ± 0.7	63.7 ± 1.0
6	4.29 ± 0.21	21.0 ± 0.4	3.6 ± 0.6	8.5 ± 0.4	70.0 ± 0.4
4	4.12 ± 0.14	23.5 ± 0.8	3.0 ± 0.1	9.2 ± 0.7	78.3 ± 0.6
2	3.99 ± 0.28	20.3 ± 1.2	3.4 ± 0.3	8.7 ± 0.5	67.7 ± 1.2
0	1.77 ± 0.13	1.0 ± 0.2	0	1.4 ± 0.3	3.3 ± 0.2

Cells were grown in anaerobic bottles with an initial total sugar concentration of 30 g/L for 24 h

[a] Each value is an average of three parallel replicates and is represented as mean ± standard deviation

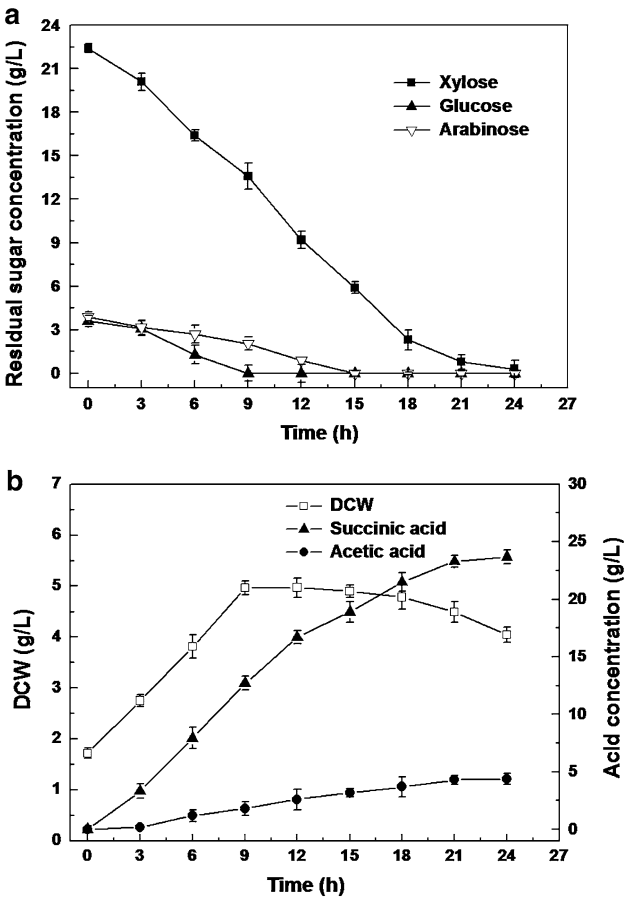

Fig. 3 Time course of cell growth and production of organic acids in batch fermentation from non-detoxified sugarcane bagasse hemicellulose hydrolysate by *A. succinogenes* NJ113 in a 3-L stirred bioreactor. Cells were grown with an initial total sugar concentration of 30 g/L containing 22.4 g/L xylose, 3.6 g/L glucose, and 3.9 g/L arabinose. The plotted data were the averages from parallel experiments. *DCW* dry cell weight, *FA* formic acid, *Ac* acetic acid, *Glu* glucose, *SA* succinic acid

Conclusion

This study investigated a simple method to pretreat sugarcane bagasse. Ultrasonic pretreatment combined with dilute acid hydrolysis demonstrated a time-saving and economical method for hydrolyzing sugarcane bagasse. Through this method, the total sugar concentration was enhanced by 29.5 %. Using non-detoxified 30 g/L total sugar as the carbon source for succinic acid production using *A. succinogenes*, the amount of yeast extract required was reduced by 60 %, and the succinic acid production increased up to 23.7 g/L with a succinic acid yield of 79.0 % and a productivity of 0.99 g/L/h.

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