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Butanol production by *Clostridium beijerinckii* ATCC 55025 from wheat bran

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Abstract Wheat bran, a by-product of the wheat milling industry, consists mainly of hemicellulose, starch and protein. In this study, the hydrolysate of wheat bran pretreated with dilute sulfuric acid was used as a substrate to produce ABE (acetone, butanol and ethanol) using *Clostridium beijerinckii* ATCC 55025. The wheat bran hydrolysate contained 53.1 g/l total reducing sugars, including 21.3 g/l of glucose, 17.4 g/l of xylose and 10.6 g/l of arabinose. *C. beijerinckii* ATCC 55025 can utilize hexose and pentose simultaneously in the hydrolysate to produce ABE. After 72 h of fermentation, the total ABE in the system was 11.8 g/l, of which acetone, butanol and ethanol were 2.2, 8.8 and 0.8 g/l, respectively. The fermentation resulted in an ABE yield of 0.32 and productivity of 0.16 g l^{-1} h^{-1}. This study suggests that wheat bran can be a potential renewable resource for ABE fermentation.

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Introduction

Butanol fermentation once ranked second to ethanol in its importance and scale of production, but declined because of increasing feedstock costs and the availability of much cheaper, petrochemically derived butanol [9, 13]. However, recently butanol and ethanol fermentation has been attracting more and more attention because of the unsustainable supply of fossil fuels and the fluctuating prices of petroleum [1, 2, 27, 38]. Butanol, as a kind of biofuel, is superior to ethanol, considering its more hydrophobic property and higher energy density, and that it allows the use of existing pipeline infrastructures for transportation and can be mixed with gasoline at any ratio. The best known strains for butanol fermentation are the mesophiles *Clostridium acetobutylicum* and *C. beijerinckii* [17, 18]. Like other bioprocesses, high substrate cost, low product yield and high recovery cost limit the production of butanol fermentation [35]. Metabolic engineering and advanced fermentation techniques have been used in *Clostridia* to overcome these problems [12, 14]. Seeking potential substrates for butanol fermentation is also an active field [8].

So far, a variety of renewable resources, such as wheat straw, corn stover and corn fiber, has been employed for the production of butanol [19, 23]. Since no microorganism currently can efficiently hydrolyze lignocellulosic biomass to produce butanol, both physical-chemical pretreatment [28, 36] and enzymatic hydrolysis [31, 33, 34] are required. During pretreatment, the biomass is subjected to severe conditions such as high temperature, followed by reactions with various chemicals. However, fermentation inhibitors

are produced in the process of pretreatment [11, 25, 32]. Removal of these inhibitors from pretreated hydrolysate increases the cost. With enzymatical pretreatment, fewer fermentation inhibitors in the hydrolysate are produced, whereas the low efficiency of the enzymes, especially cellulase, increases the cost of fermentation and prolongs the experiment cycle [8]. Therefore, the fermentation substrate is an important factor influencing the cost of butanol production [3, 30]. Thus, it is necessary to find substrates that are constantly supplied, contain highly fermentable sugars and can be hydrolyzed with low-cost simple pretreatment [22].

It is well known that wheat bran represents a renewable resource that is available in significant quantities from the flour industries in East Asia. Approximately 10 million tons of wheat bran are produced annually in China. Wheat bran in general consists mainly of non-starch polysaccharides (NSP, 41–60%), starch (10–20%) and protein (15–20%) [4, 24, 37]. Currently, wheat bran is sold as animal feed stock in China. It is also a perfect feed for microorganisms. Through dilute sulfuric acid pretreatment, NSP, which consists mainly of hemicellulose, was easily turned into a monosaccharide; simultaneously starch was hydrolyzed to glucose. Moreover, the trace elements and amino acids from the wheat bran hydrolysate were suitable nutrients for the organism.

In this study, sugars were released from wheat bran by dilute sulfuric acid pretreatment. The inhibitors reported in wheat bran hydrolysate formed during pretreatment were monitored, and their effects on butanol fermentation were studied. Butanol productions using glucose or wheat bran hydrolysate pretreated with dilute sulfuric acid were compared, and wheat bran was shown to have good potential as an alternative substrate for butanol production.

Materials and methods

Chemicals and materials

Resazurin sodium salt was purchased from Sigma-Aldrich (St. Louis, MO). Yeast extract was obtained from Oxoid Ltd. (Thermo Fisher Biochemical, Beijing, China). The laboratory media components of analytical grade were purchased from Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The wheat bran was collected from the MinTian flour mill factory (Jinan, China) and maintained at room temperature. The moisture content of wheat bran was determined by using a convection oven. One hundred grams of wheat bran was put into the oven at 105°C for 8 h until the mass varied less than 0.1 g. The reduced weight by 100 (g/g) was the moisture content of the wheat bran [20].

Microorganism and culture conditions

Clostridium beijerinckii ATCC 55025 [15], an asporogenic mutant of *C. beijerinckii* ATCC 4259, was purchased from American Type Culture Collection (Manassas, VA). A stock culture of *C. beijerinckii* 55025 was maintained as a cell suspension in 30% (v/v) sterile glycerol at −20°C in screw-capped bottles.

In 100-ml screw-capped bottles, 50 ml medium containing a carbon source, yeast extract (1 g/l) and 0.0001% (w/v) resazurine was put into the anaerobic chamber (Coy, Ann Arbor, MI) filled with 95% N_2-5% H_2 and containing a palladium catalyst for O_2 reduction with H_2. After being covered with butyl rubber stoppers, the bottles were sterilized at 115°C for 15 min. On cooling to room temperature, 1 ml each of filter-sterilized P2 stock solutions (buffer: KH_2PO_4, 50 g/l; K_2HPO_4, 50 g/l; ammonium acetate, 220 g/l; mineral: $MgSO_4$•$7H_2O$, 20 g/l; $MnSO_4$•H_2O, 1 g/l; $FeSO_4$•$7H_2O$, 1 g/l; NaCl, 1 g/l; and vitamin: para-aminobenzoic acid, 0.1 g/l; thiamin, 0.1 g/l; biotin, 0.001 g/l)] [29] was added. Two milliliters of cell suspension was inoculated into 50 ml P2 medium with 20 g/l glucose as carbon source in a 100-ml screw capped bottle and incubated anaerobically for 14–16 h at 37°C before fermentation studies. Sodium hydrosulfite was added at a final concentration of 50 mg/l to the medium to reduce the redox potential.

Wheat bran hydrolysis

One hundred grams of wheat bran (moisture content 10%, w/w) was soaked in 1 l of 0.75% (v/v) sulfuric acid in a 2-l beaker for 15 min with agitation at 150 rpm followed by autoclaving at 121°C for 45 min. Then the pH of the solution was adjusted to 6–6.5 with 12 g $Ca(OH)_2$ at 50°C for 2 h with agitation at 150 rpm. The $Ca(OH)_2$ was used for neutralization and detoxification of wheat bran hydrolysate [25]. The mixture was then centrifuged at 10,000*g* for 10 min. The clear supernatant, sulfuric acid treated wheat bran hydrolysate (SAWBH), was used as carbon source in the following fermentation studies. The mixture without centrifugation was also tested as carbon source directly for strain *C. beijerinckii* 55025.

Fermentation studies

The fermentation was conducted in 100-ml screw-capped bottles containing 50 ml P2 medium with 50 g/l glucose or SAWBH as carbon source. Then 2.5 ml of highly motile cells (3.6×10^7 CFU/ml) of *C. beijerinckii* 55025 was inoculated and incubated at 37°C. Unless otherwise stated, the fermentation time was 96 h. At 24-h intervals samples were withdrawn for ABE and sugar analysis. Before analysis,

the samples were centrifuged at 10,000*g* for 3 min. ABEs were extracted using ethyl acetate, and isomylol was used as the internal standard. All experiments were conducted in duplicate in this study.

Analysis

The fermentation products ABE were analyzed by using gas chromatography (GC-2010, Shimadazu Scientific Instruments, Japan) equipped with a flame ionization detector (FID) and an InterCap WAX column (0.25 mm by 30 m, GL Sciences Inc., Japan). Yield was defined as grams of ABE produced per gram of glucose or sugar utilized. ABE productivity was calculated as total ABE produced in g l^{-1} divided by the fermentation time and is expressed as g l^{-1} h^{-1}.

Sugars (glucose, xylose and arabinose); acetic acid and butyric acid were measured using Agilent 1100 HPLC (Agilent Technologies Co., Ltd., Beijing, China) equipped with a differential refractive index detector. The HPLC column (Aminex HPX-87H, 7.8 mm by 30 cm) was obtained from BioRad Laboratories (Beijing, China) and maintained at 55°C with 10 mmol/l of H_2SO_4 as a mobile phase and at a flow rate of 0.4 ml/min. Furfural and hydroxymethyl furfural (HMF) were determined on HPLC equipped with the same column and a UV detector at 286 nm. Total reducing sugar concentration was measured according to the 3,5-dinitrosalicyclic acid method [5, 16].

Results and discussion

Wheat bran hydrolysis and sugars analysis

After the hydrolysis of 100 g/l wheat bran under the above-mentioned conditions, the SAWBH contained 53.1 g/l total reducing sugars including mainly 21.3 g/l glucose, 17.4 g/l xylose and 10.6 g/l arabinose, which accounted for 93% of the total sugars.

Various by-products were formed in the process of pretreatment because of high temperature and acids. Among them, the most abundant substances are furfural and 5-hydroxymethyl-2-furaldehyde (HMF) [21]. Hexoses and pentose degradation under more severe pretreatment conditions resulted in an increased yield of HMF and furfural, respectively. The concentrations of HMF and furfural in the hydrolysate are highly dependent on the temperature, residence time and acid concentration in the hydrolysis, and more severe conditions give higher yields of inhibitors [7]. In the hydrolysate of wheat bran, the concentration of furfural and HMF were 0.1 and 0.13 g/l, respectively. When 0.1 g/l furfural and 0.13 g/l HMF were added in the P2 medium with 50 g/l glucose as the sole carbon source, the growth and ABE fermentation of the *C. beijerinckii* 55025 were not affected (data not shown). The fermentation results were similar to those shown in Fig. 1. In another report, the growth and ABE production of *C. beijerinckii* BA101 were found to be stimulated by low concentration of furfural and HMF. In the presence of 2 g/l furfural and HMF, the ABE concentration was even 6 and 15% higher than the concentration achieved in the control [11].

In this study, the pretreatment didn't involve the enzymatic hydrolysis of cellulose and detoxification; the main components of wheat bran were starch and hemicellulose, which were easily pretreated by dilute sulfuric acid into soluble sugars. Corn fiber and rice bran, which were also by-products of food factories in the USA and Korea, were also used as substrates for butanol fermentation [22, 33]. When 84 g/l corn fiber and 100 g/l rice bran were pretreated by dilute sulfuric acid, 30 and 25 g/l soluble sugars were obtained in the hydrolysate, respectively. In this study, 53.1 g/l total reducing sugars were released from 100 g/l wheat bran by using dilute sulfuric acid, which can meet *C. beijerinckii* 55025 butanol fermentation.

ABE fermentation using SAWBH-based medium and glucose-based medium

The hydrolysate of wheat bran contained 53.1 g/l reducing sugars, and the total concentration of glucose, xylose and arabinose was 49.3 g/l. Prior to carrying out ABE fermentation using SAWBH-based medium, control batch fermentation was conducted using 50 g/l glucose as sole carbon source (Fig. 1). In 72 h, ABE productivity and yield of 0.15 g l^{-1} h^{-1} and 0.28 were obtained. At the end of fermentation, concentrations of acetone, ethanol and butanol were 2.6 ± 0.62, 1.5 ± 0.05 and 7.1 ± 0.07 g/l, respectively. Acetic and butyric acids were also detected, the concentrations of which were both below 0.6 g/l.

ABEs were produced using SAWBH as carbon source in P2 medium by *C. beijerinckii* 55025. It was shown that at 72 h total ABE in the system was 11.8 g/l, of which

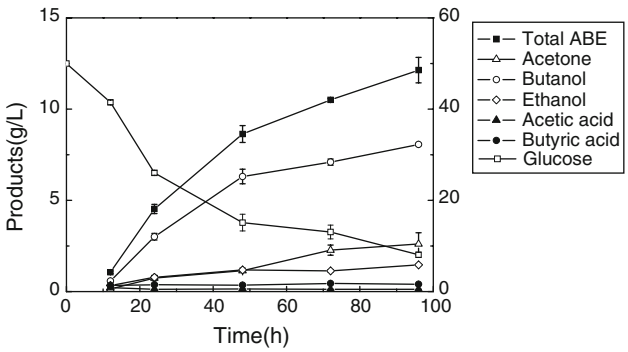

Fig. 1 Production of ABE from glucose in a batch reactor by *Clostridium beijerinckii* 55025

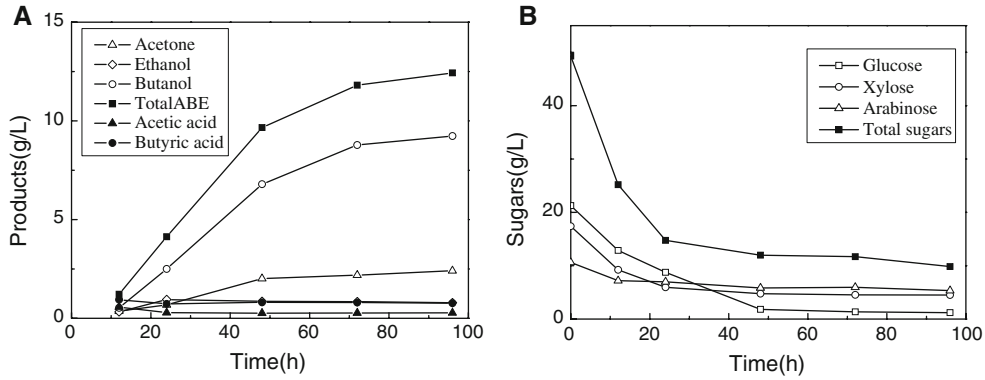

Fig. 2 Production of ABE from SAWBH in a batch reactor by *Clostridium beijerinckii* 55025. ABE production versus fermentation time (**a**). Residual sugars in fermentation broth versus fermentation time (**b**)

acetone, butanol and ethanol were 2.2, 8.8 and 0.8 g/l, respectively (Fig. 2a). Based on the fermentation time of 72 h, 20.1 g/l glucose, 12.9 g/l xylose and 5.3 g/l arabinose were utilized, and an ABE yield of 0.32 and productivity of 0.16 g l^{-1} h^{-1} were obtained, which was almost equivalent to the productivity when using glucose as carbon source. This was similar to the results reported by using other agricultural by-products (Table 2) [10, 28, 32, 33, 36].

The glucose in the SAWBH was used quickly and almost consumed in 48 h by *C. beijerinckii* 55025 (Fig. 2b). Glucose and xylose were utilized efficiently in the first day, whereas during the second day the xylose utilization rate was much slower than that of glucose. After 48 h the residual sugars were basically unchanged in accordance with the growth of the strain. In a study with *C. acetobutylicum* ATCC 824, butanol was found to be a potent inhibitor of the growth of the organism and of the rate of sugar uptake as well as of sugar incorporation into cell materials [26]. Moreover, butanol completely inhibited growth at a concentration of 14 g/l for cultures growing on glucose and 8 g/l for cultures growing on xylose. In this study, the uptake rate of xylose decreased during the second day when 2.5–7 g/l of butanol was produced. This implied that microorganisms showing high tolerance to butanol must be found in order to improve the process. It was reported that the genus *Lactobacillus* bacteria could grow under 2.5% (w/v) of butanol on agar plates [6]. In addition, we also found the uptake rate of arabinose was even less than that of xylose.

When SAWBH was used as carbon source, only 1.2 g of glucose was left in the medium at the end of fermentation, while 26% of xylose and 50% of arabinose were not utilized. To examine whether glucose was sufficient in the SAWBH for batch fermentation by *C. beijerinckii*, it was added to the medium at a concentration of 10 and 20 g/l. The results suggested that only 35–40 g/l of sugars (glucose, xylose, arabinose or mixture) could be utilized by *C. beijerinckii* during the batch fermentation process, and supplemented glucose did not increase the butanol production (Table 1). The fermentation ceased because of the end product inhibition and butanol toxicity [12].

Till now, several renewable resources including wheat straw, corn stover and corn fiber have been reported for the production of butanol, but fermentation of the renewable lignocellulose requires complicated pretreatments [11, 31, 33]. The inhibitors that affected microbial growth and ABE production needed to be removed. However, the acid treatment has been proven to be a cost-effective method [22], and in our experiment it was suggested that wheat bran had advantages over other natural biomasses as the fermentation substrate (Table 2). In SAWBH there were enough sugars for *C. beijerinckii* 55025, and the inhibitors reported in the hydrolysate after the dilute sulfuric acid pretreatment didn't affect ABE production.

Study of fermentation products using SAWBH as medium without adding P2 medium

Wheat bran is a biomass with a complex chemical composition including carbohydrates, proteins, minerals and vitamins. Therefore, we carried out an experiment using SAWBH as medium without adding P2 medium for ABE fermentation (Fig. 3). In the experiment, 9.5 ± 0.9 g/l ABE and 6.3 ± 0.5 g/l butanol were produced in 72 h, and approximately 35 g/l of sugars was consumed at the end of fermentation. Compared with the fermentation when using SAWBH supplemented with P2 medium, the total sugars consumed changed little in the fermentation process, whereas the production of ABE decreased. This suggested that the supplementation of P2 into SAWBH is required, which is consistent with the finding when pretreated rice bran was used as the carbon source [22].

From an industrial point of view, the removal of suspended solids by centrifugation would bring additional costs. Therefore, SAWBH, as the fermentation substrate

Table 1 Butanol production and sugar utilization by *C. beijerinckii* 55025 with different substrates

Experiment	Substrate (g/l)	Residual sugars (g/l)	Consumed sugars (g/l)	Butanol (g/l)
Control	50[a]	15.5[a]	34.5	7.2
SAWBH (53.1 g/l)	21.3[a]	1.2[a]	38.2	8.5
	17.4[b]	4.5[b]		
	10.6[c]	5.3[c]		
Glu + SAWBH (10 + 53.1 g/l)	31.3[a]	12.2[a]	39.8	8.9
	17.4[b]	3.3[b]		
	10.6[c]	3.9[c]		
Glu + SAWBH (20 + 53.1 g/l)	41.3[a]	21.4[a]	38.2	8.4
	17.4[b]	4.3[b]		
	10.6[c]	5.4[c]		

[a] Glucose

[b] Xylose

[c] Arabinose

Table 2 Butanol production from agriculture residue hydrolysate

Substrate	Hydrolysis	Inhibitors removal	Culture	ABE (g/l)	Yield (g ABE/g sugar)	Productivity (g/l h)	Reference
Corn stover[a]	SO_2-catalysed prehydrolysis + emzyme hydrolysed	None	*C. acetobutylicum* P262	25.8	0.34	1.08	[28]
Wheat straw[b]	Dilute sulfuric acid prehydrolysis and emzyme hydrolysed	None	*C. beijerinckii* P260	25.0	0.42	0.60	[36]
Wheat straw	Alkaline peroxide +emzyme hydrolysed	Electrodialysis	*C. beijerinckii* P260	22.17	0.30	0.55	[32]
Distillers dried grains and solubles	Ammonium fiber expansion + emzyme hydrolysed	None	*C. beijerinckii* BA101	10.4	0.34	0.14	[10]
Corn fiber	Dilute sulfuric acid	XAD-4 resin	*C. beijerinckii* BA101	9.3	0.39	0.10	[33]
Wheat bran	Dilute sulfuric acid	None	*C. beijerinckii* ATCC 55025	11.8	0.32	0.16	This work

[a,b] The batch fermentation was integrated with cell recycling and product recovery, which is as expected in high ABE concentration and productivity

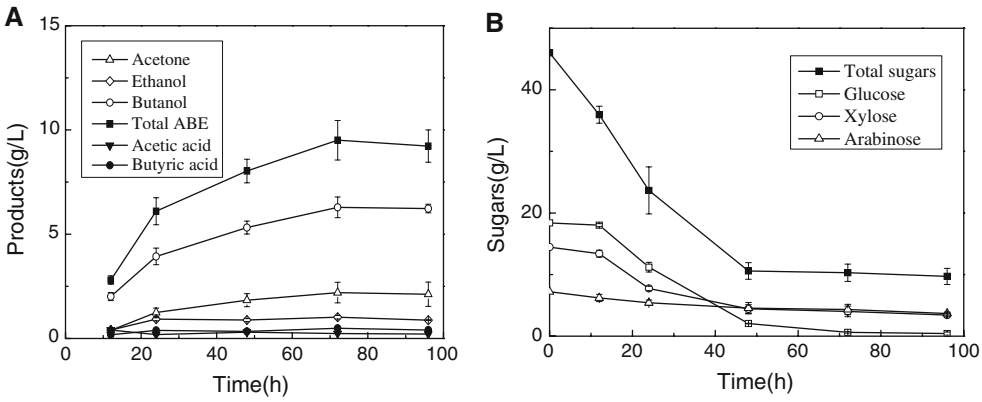

Fig. 3 Production of ABE from SAWBH without adding P2 medium in a batch reactor by *Clostridium beijerinckii* 55025. ABE production versus fermentation time (**a**) and residual sugars in fermentation broth versus fermentation time (**b**)

that did not need centrifugation or the addition of P2 medium was also investigated (Fig. 4). In the experiment using SAWBH without centrifugation or adding P2 medium, 8.6 ± 0.6 g/l ABE and 5.9 ± 0.5 g/l butanol were produced in 72 h, and approximately 40 g/l of sugars was consumed at the end of fermentation.

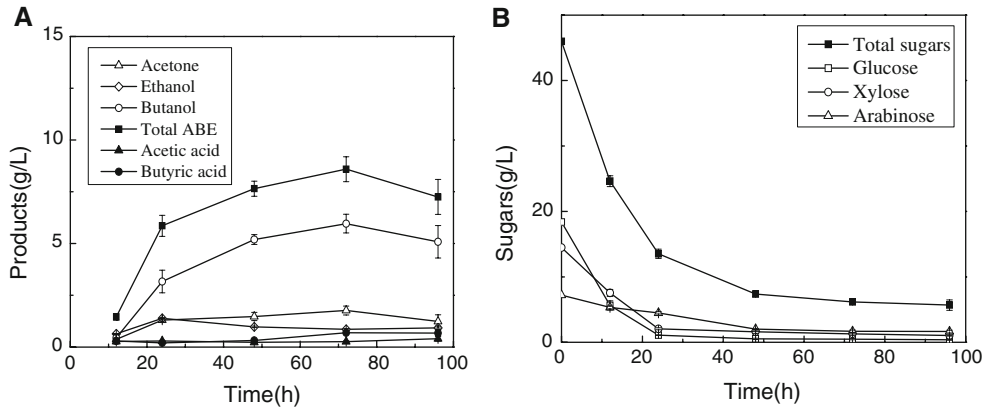

Fig. 4 Production of ABE from SAWBH without centrifugation or adding P2 medium in a batch reactor by *Clostridium beijerinckii* 55025. ABE production versus fermentation time (**a**) and residual sugars in fermentation broth versus fermentation time (**b**)

Conclusions

Wheat bran, a renewable biomass, is the byproduct of flour production. Starch and hemicellulose, the main components of wheat bran, could be easily hydrolyzed to monosaccharides using dilute sulfuric acid pretreatment. In the hydrolysate, the concentrations of glucose, xylose and arabinose were 21.3, 17.4 and 10.6 g/l, respectively. SAWBH could supply enough sugars to *C. beijerinckii* 55025 for growth and ABE fermentation. When SAWBH was used as the carbon source, the fermentation resulted in the production of 11.8 g/l ABE and a yield of 0.32 in 72 h. This study suggested that wheat bran is a potential substrate for ABE production.

C. beijerinckii ATCC 55025 was a mutant strain and could produce a mixture of solvents including acetone, butanol and ethanol. During batch fermentation, only 35–40 g/l of total sugars was utilized by strain 55025, but additional sugars didn't help to increase the ABE production. These results suggested that the strain needs to be further modified in order to get a high sugar utilization ratio and production of ABE.

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