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Optimization of Simultaneous Saccharification and Fermentation of Rice Straw to Produce Butanol

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Butanol 생산을 위한 동시 당화 발효법의 최적화

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Studies were made to optimize the simultaneous saccharification and fermentation (SSF) of rice straw to produce butanol using *Clostridium acetobutylicum* KCTC 1037 and a cellulolytic enzyme preparation from *Trichoderma viride*. The fermentation was inhibited when the liquid enzyme preparation from Novo was used, whilst a successful fermentation was achieved in the SSF using the enzyme manufactured by Pacific Chemical Co. The minimum cellulase concentration for the successful fermentation of pure cellulose was found to be 4 IU/g of substrate used. Alkaline treatment was better method for the fermentation of rice straw by the system. SSF using 25% alkaline treated rice straw produced 150 mM butanol, 90 mM acetone. On the other hand, fermentation of ball milled rice straw was mainly acidogenic producing 98 mM acetate and 64 mM butyrate with less than 20 mM butanol. These results show that rice straw contains (a) specific inhibitor(s) for solventogenesis which is destroyed or soluble in alkali.

The utilization of biomass is the most promising route among the various alternative energy technologies, since the biomass is replenished every year. Cellulosic materials such as agricultural materials are one of the most abundant biomass and studied as fermentation substrate for alcohol production (1, 2). Acetone-butanol fermentation also has been tried to utilize cellulosic substrate for the same purpose (3, 4). Butanol has several advantages over ethanol as an alternative energy form. They are; (1) Butanol is more expensive than ethanol, 2) Butanol is better to blend with gasoline, 3) Butanol with 4 carbons can be used as different chemical feedstock, 4) Butanol fermentation can utilize starch, amorphous celluloses and hemicellulose without hydrolysis, 5) Butanol fermenters can utilize both pentoses and hexoses found in hemicellulose (5).

It is *Clostridium acetobutylicum* that carries out butanol fermentation. This bacterium is strict anaerobe, forms a spore and produces three major classes of fermentation end products: acids; acetate and butyrate, solvents; butanol, acetone and ethanol, and gases; CO_2 and H_2 (6). In a batch fermentation acidic products are produced at early stage (acidogenic phase) which reduces the culture pH. Solvents are produced at low pH with the accumulation of acids (solventogenic phase). The factor(s) which induces or effects on this metabolic shift from acidogenic phase to solventogenic phase is not certain but the availability of coenzyme A in the cell (7), the internal pH of the cell (8), the specific activity of hydrogenase (6), and the intracellular undissociated acid concentration (9) were suggested as

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important physiological changes of shift during the growth of *C. acetobutylicum*.

C. acetobutylicum can not hydrolyze and utilize the cellulosic substrates because this organism is unable to attack the native cellulose. Simultaneous saccharification and fermentation (SSF) have several advantages over other processes; 1) It is possible to achieve all processes by one-step, 2) It can remove the end product inhibition by glucose and substrate resistance, 3) It can reduce the amount of enzyme. From the ethanol fermentation, SSF was studied very intensively by some workers (1,2) and it was also applied to butanol fermentation (3). Other studies on the one step conversion from cellulosic substrate to butanol resulted in the major acid production (4) and required the pretreatment of cellulosic materials (10). Studies were made to optimize SSF using rice straw and cellulolytic enzyme preparation.

Materials and Methods

Microorganisms and their maintenance

Clostridium acetobutylicum KCTC 1037 (ATCC 4259) was used throughout the study. *C. acetobutylicum* KCTC 1788 (NRRL B527), KCTC 1789 (DSM 1731), KCTC 1790 (NCIB 8052), and KCTC 1669 (ATCC 10132) were also used in the study. All strains were grown under the strict anaerobic conditions (11) using a complex medium (CAB) and stored as spore suspensions at 4 °C.

CAB medium contained, in grams per liter of distilled water (11): yeast extract (Difco Laboratories), 4; tryptone (Difco), 1; KH_2PO_4, 0.7; K_2HPO_4, 0.7; DL-asparazine, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; $MnSO_4 \cdot H_2O$, 0.1; $FeSO_4$, 0.015; and NaCl, 0.1; the medium was added by 1 m*l* of 0.2% resazurin as redox indicator. As carbon source 45 g/*l* glucose or cellulosic substrate (amount described in the text) was used. The medium was prepared under an N_2 headspace and the pH was adjusted to 5.4 before autoclaving. After dispensing 10 m*l* or more CAB medium into anaerobic pressure tube (Bellco Glass Inc.) or serum vial (Wheaton Glass), the tube and vial were sealed with butyl rubber bung and aluminium seal. All anaerobic media were autoclaved at 121 C for 20-30 min.

Spore suspension (0.1 m*l*) was inoculated to 10 m*l* of sterile CAB (with glucose) medium in an anaerobic pressure tube using sterile syringe. The tube was heated at 85 C for 5 min. The heat-shocked tube was incubated at 35 C water bath for 24 hours and the culture was transferred to fresh medium at the size of 5%. The second 24 hour-old culture was used as inoculum for SSF. Always fresh inoculum was prepared from spore suspension for each experiment.

Cellulase

The Cellulase T.v. was a generous gift from Pacific Chemical Co. (Seoul, Korea) as crude extract powder (3 000 IU/g of powder, from *Trichoderma viride*). Celluclast (Novo Industries) was also used in form of brown liquid. The activity of cellulases was measured using the standard method (12) and expressed in filter paper unit (FPU).

Cellulosic substrates and their pretreatments

The rice straw and Avicel PH-101 (microcrystalline cellulose, FMC Co., Philadelphia, USA) were used as cellulosic substrates. The rice straw was cut to 2-3 cm using a laboratory cutting mill and dried overnight at 60 C before ball milled for 24 hours. The alkaline treatment of rice straw was done by the method as described earlier (1) and used without drying. The amorphous Avicel was prepared using 85% phosphoric acid according to the method of Walseth (13). The wet alkaline treated rice straw and the amorphous Avicel were stored at 4 °C before use. The amount of wet substrates is expressed as the weight prior to the treatment.

Simultaneous saccharification and fermentation

CAB medium was used with either 45 g/*l* of glucose or cellulosic substrates. For the preparation of cellulosic CAB medium, 1g of ball milled rice straw or other cellulosic substrate was placed into the each anaerobic pressure tube or serum vial before dispensing the CAB (without glucose) medum. The cellulase was dissolved in 0.1M sodium citrate buffer (pH 4.8), and centrifuged at 12000 rpm for 20 min. The supernatant was membrane-filtered to ste-

rile serum vial and made anaerobic condition. This anaerobic sterile cellulase preparation was added to the autoclaved CAB medium with cellulosic substrates using sterile syringe. After mixing the solution throughly, the 5% of fresh *C. acetobutylicum* culture grown on CAB (with glucose) medium were inoculated. The SSF was conducted in a water bath at 35 C without shaking.

Assays

Soluble fermentation products were analyzed by chromatographic methods (11) using a varian 3700 gas chromatograph, equipped with a flame ionization detector. Fermentation broth was centrifuged and directly analyzed for acids and solvents after acidification using 1/10 volume of 10 M phosphoric acid. Total fermentation product was calculated as the amount of glucose (C_6) equivalent, i.e. 1/2 for acetate (C_2) and ethanol (C_2) and 1 for acetone (C_3), butanol (C_4) and butyrate (C_4).

Results and Discussion

Strain selection

C. acetobutylicum cultures maintained by Korea Collection for Type Cultures (KCTC) were used in butanol fermentation using CAB medium containing 45 g/*l* glucose (Table 1). Among the cultures used KCTC 1037 and 1788 produced butanol at the concentration over 120 mM. The repeated fermentation showed that butanol yields are similar in both strains. Since *C. acetobutylicum* KCTC 1037 produced less acidic fermentation products than 1788, 1037 was used in latter experiments. This strain produced solvents at ratio of 1:3.8:5.5 for ethanol, acetone and butanol. This figure shows that the strain produces higher acetone in the system we employed.

Use of cellulolytic enzyme preparation

C. acetobutylicum KCTC 1037 was cultured using CAB medium containing 20% ball milled rice straw added by varying concentration of cellulase preparations, Cellulase T.v. and Celluclast (Fig. 1). SSF using Celluclast showed gradual decrease in the total fermentation products with the increasing enzyme concentration. On the other hand, about 125 mM of fermentation products, mainly acidic products were formed in the SSF added by cellulase T.v. regardless with the enzyme concentration us-

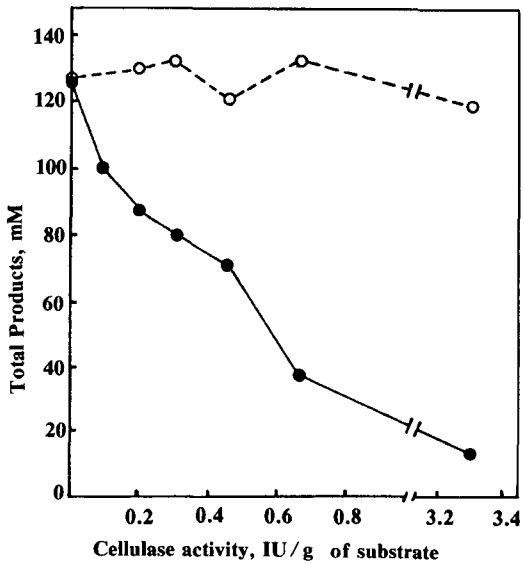

Fig. 1. SSF of ball milled rice straw using Cellulase T.v. and Celluclast.
○---○, Cellulase T.v.: ● - ●, Celluclast

Table 1. Glucose fermentation by *Clostridium acetobutylicum* cultures from KCTC.

Strain	Products, mM					
	Acetate	Butyrate	Ethanol	Acetone	Butanol	Total as glucose
KCTC 1037	10.1	8.3	23.4	89.1	128.1	243.1
KCTC 1669	9.5	22.9	0.5	10.9	17.1	55.9
KCTC 1788	15.1	21.1	32.7	64.0	146.7	255.7
KCTC 1789	30.3	18.7	6.7	16.1	47.2	100.5
KCTC 1790	5.8	7.6	0.6	19.7	28.4	58.9

Fermentation was made using CAB medum with 4.5% glucose at 35 C for 4 days.

ed. In addition to the fermentation products high concentration of reducing sugar were accumulated in the cultures especially cultures added by over 0.8 IU/g substrate regardless the enzyme preparation used (data not shown).

The cultures without enzyme produced over 125 mM of fermentation products. *C. acetobutylicum* is known to be able to ferment xylan (5). Cultures without added enzyme could ferment hemicellulose in rice straw to produce 125 mM of products.

In spite of high reducing sugar accumulation in the culture added by Celluclast, the organism could not utilize the sugar, probably due to the bacteriostatic agent contained in the liquid enzyme preparation. This argument is further substantiated by the fact that the inhibition was more significant at high enzyme concentration.

It seems to be abnormal that cultures added by high Cellulase T.v. produced similar fermentation products (mainly acidic) as the control. The concentration of the acids at pH value below 4.5 is known to dissipate the protonmotive force (Shin and Kim, paper presented at the International Union of Microbiological Societies, Sept. 1986, Manchester, England), In normal fermentation, solventogenesis initiated before the concentration of the acidic products reaches over 120 mM. From this discussion it concluded that the substrate contains some kind of inhibitor(s) for the initiation of solventogenesis.

Avicel fermentation by SSF

In the previous experiment using ball milled rice straw it was not possible to determine the optimum cellulase concentration of the substrate and the presence of hemicellulose in the substrate. Avicel PH-101 was used at the concentration of 5% in a similar experiment using varying concentration of enzyme Cellulase T.v. (Fig. 2). Butanol production was increased linearly up to the enzyme concentration of 4 IU/g of substrate, after which the increase was lower. Similar tendencies were observed in acetone and ethanol productions. The fermentation products found in control tube without enzyme addition stayed at about the amount carried over by the inoculum. From these results it is concluded

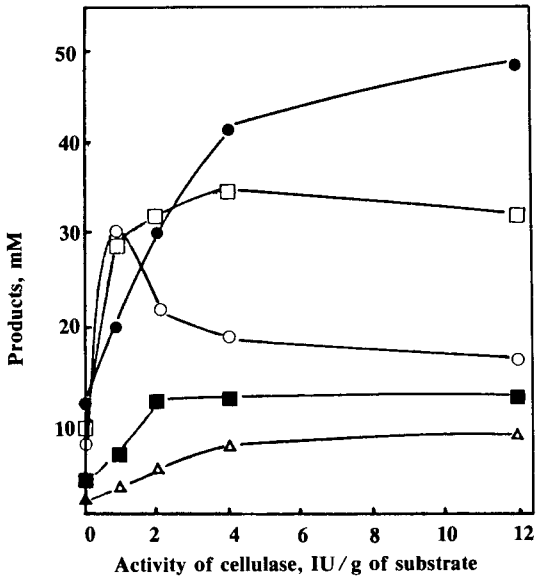

Fig. 2. Optimum Cellulase T.v. concentration for SSF of 50 g/l Avicel.

●-●, Butanol: □-□, Acetate: ○-○, Butyrate: ■-■, Acetone: △-△, Ethanol

that Avicel is not very good substrate for SSF and that the optimum enzyme concentration is 4 IU/g substrate for successful SSF.

The effects of pretreatment of rice straw

It was found that Avicel PH-101 and ball milled rice straw are not very good substrate for SSF to produce butanol. SSF were made using CAB medium containing 10% of Avicel PH-101, ball milled Avicel, ball milled rice straw or alkaline treated rice straw. Cellulase T.v. was used at the concentration of 4.4 IU/g of substrate used. Table 2 shows the fermentation results. As shown in the table crystalline and amorphous Avicel gave similar fermentation results in total fermentation products as well as in solvents. On the other hand, solvents yield was much higher in the alkaline treated rice straw than in ball milled rice straw.

SSF of rice straw with varying concentration

In the previous experiment 70 mM of butanol was produced from 100 g/l of the alkaline treated rice straw which is lower than butanol concentration found in starch fermentation, 120-130 mM (14). To establish the optimum substrate concentra-

Table 2. Simultaneous saccharification and fermentation of cellulosic substrate.

substrate used	Products, mM					
	Acetate	Butyrate	Ethanol	Acetone	Butanol	Total as glucose
Avicel	28.1	41.4	3.9	27.1	56.6	141.2
Ball milled Avicel	23.4	29.0	5.7	40.3	71.4	155.3
Ball milled rice straw	58.8	44.6	9.7	4.8	17.4	101.1
alkaline treated rice straw	48.5	11.7	12.2	37.5	76.2	155.8

CAB medium with 10% substrate was autoclaved and added by 4.4 IU/ml of cellulase and fresh culture of *C. acetobutylicum* before incubates for 4 days at 35 C.

tion for SSF alkaline treated and ball milled rice straw were used at various concentrations up to the maximum amount to be suspended in the liquid. The fermentation profiles are shown in Fig. 3. In the case of the alkaline treated substrate the solvent production was increased gradually with increasing substrate concentration. The cultures with 2.5% alkaline treated rice straw produced only acidic fermentation products. 150 mM of butanol was produced from 25% substrate, which is a good yield in terms of final concentration though only one fifth of the substrate originally provided was recovered in the fermentation products.

On the other hand, the culture with 8% ball milled rice straw produced the highest butanol yield. By increasing the substrate concentration more than 8% solvent production was decreased with the increase in acid production. In other words

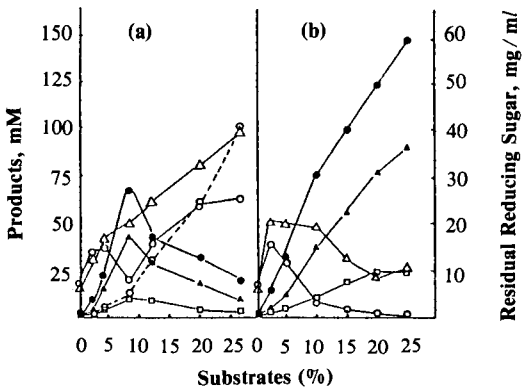

Fig. 3. Fermentation profiles of SSF used different concentration of rice straw. (a) ball milled rice straw (b) alkaline treated rice straw

●–●, Butanol: △–△, Acetate: ○–○, Butyrate: ▲–▲, Acetone: □–□, Ethanol ○---○, Sugar

the solventogenesis is repressed when substrate concentration was higher than 8%. From these results it is hypothesized that the rice straw contains (an) inhibitor(s) for the solventogenesis. These results are consistent with the results shown in Fig. 1. The rice straw is converted to solvents in three steps during the SSF. First the polysaccharides are hydrolyzed to the reducing sugars by cellulase and the reducing sugars are transported into the microorganism, which ferments the reducing sugars to butyrate and acetate, and the acidic products are reduced to butanol, acetone and ethanol. The scheme of SSF using rice straw is as follows:

Polysaccharides (Rice straw)
↓ hydrolysis
Reducing sugars
↓ acidogenesis
Butyrate and acetate
↓ solventogenesis
Butanol, acetone and ethanol

The ball milled rice straw fermentation resulted in the accumulation of reducing sugars and acidic fermentation products with the increase in the substrate concentrations. Since the acidic fermentation products function as uncouplers dissipating the protonmotive force, the organism cannot ferments the reducing sugars when the solventogenesis is inhibited. The accumulations of acids and reducing sugars in the ball milled rice straw are due to the inhibition of solventogenesis caused by the inhibitor found in the rice straw. The inhibitor seems not to hinder the hydrolysis of the polysaccharide or the acidogenesis.

The inhibition was not significant at low concentration since the fermentation patterns of ball milled and alkaline treated rice straw were similar when the substrates were used at the concentration lower than 8%.

요 약

섬유소 폐기물인 볏짚으로부터 butanol를 생산하기 위하여 *Clostridium acetobutylicum* KCTC 1037와 cellulase(from *Trichoderma viride*)을 발효액에 동시에 첨가하여 발효시키는 동시당화 발효법(Simultaneous saccharification and fermentation, SSF)을 수행하였다. Alkali 처리한 볏짚을 발효기질로 사용한 결과 그 농도를 25%로 사용하였을 때 최고 150 mM의 butanol이 생산되었고, 15% 볏짚을 사용하였을 때는 97 mM의 butanol이 생산되었다. 그러나 ball milled 볏짚의 경우 발효산물 중 대부분이 acetate와 butyrate로 주로 산이 생산되었으며 따라서 solventogenesis는 거의 일어나지 않았다. 또한 그 농도별 실험에서 보면 8%의 ball milled 볏짚 사용시 66 mM의 butanol이 생산된 반면 그 이상의 농도에서는 butanol 생산량이 점차 감소하는 추세를 보였으며 acetate, butyrate 같은 산은 계속 증가 추세를 보였다. 이것으로 보아 ball milled 볏짚에는 butanol 발효 과정중 acidogenesis에서 solventogenesis로의 전이(shift)를 방해하는 어떤 인자가 있으리라고 추측되었으며 alkali 처리방법에 의해서 이 방해자는 제거될 수 있는 것으로 관찰되었다.

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