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Acetone-Butanol Fermentation of Rice Straw by Simultaneous Saccharification and Fermentation

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동시당화 발효법에 의한 볏짚의 Acetone-Butanol 발효

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ABSTRACT: Rice straw was used in the acetone-butanol fermentation by simultaneous saccharification and fermentation (SSF) using *Clostridium acetobutylicum* and cellulolytic enzyme. Over 230 mM of solvent was produced from alkali treated rice straw or from ball-milled microcrystalline cellulose whilst only acidic fermentation products were formed from ball-milled rice straw. From the results it is concluded that rice straw used in the study contained an inhibitor for the solventogenesis by the organism which is insoluble in water and some organic solvent and destroyed by alkaline treatment.

KEY WORDS□*Clostridium acetobutylicum*, Simultaneous Saccharification and Fermentation, Solventogenesis, Acetone-Butanol Fermentation

Acetone-butanol fermentation by *Clostridium acetobutylicum* is one of the oldest industrially practiced processes based on starch or molasses. Recently a renewed effort has been placed on this process to produce alternative fuel from the renewable biomass (Zeikus 1980, Volesky and Szczesny 1983).

Butanol producing clostridia utilize a wide variety of sugars ranging from pentoses and hexoses to some of their polymers. Cellulose is the most abundant biomass, and the most promising candidate for the starting material for the alternative fuel production. However, most of the solvent producing microorganisms including *Clostridium acetobutylicum* cannot ferment cellulose.

Acids were used to hydrolyse cellulose before fermentation (Leonard *et al.* 1947) but the enzymic hydrolysis was found better process (Deverell 1983). Cellulose can be fermented to ethanol (Kim *et al.* 1980) or to butanol (Marchal *et al.* 1984) in a one step process, the simultaneous saccharification and fermentation (SSF) using cellulolytic enzyme and sugar fermenting microorganism. Cellulosic biomass has been reported to contain inhibitor hindering the fermentation (Soni *et al.* 1982, Maddox and Murray 1983). Marchal *et al.* (1986) claimed that washing with water can remove the inhibitor from steam exploded wood.

MATERIALS AND METHODS

Bacterial Strain and Its Maintenance

Clostridium acetobutylicum KCTC 1037 (ATCC 4259) was used throughout the study. Spore suspension in distilled water was kept at 4 °C and used to develop inoculum (Kim *et al.* 1984).

Substrates

Microcrystalline cellulose, Avicel PH-101 (FMC Co., Philadelphia, PA, USA) and locally produced rice straw were used as cellulosic sub-

strates. The cellulosic substrates were treated to increase its susceptibility to enzyme by ball-mill or by 1 N NaOH according to Kim *et al.*(1980). The amorphous Avicel was prepared using phosphoric acid according to Walseth (1952).

Cellulase

The cellulase T.v. was obtained from Pacific Chemical Co. (Suwon, Korea) as crude extract powder (3,000 IU/g, produced by *Trichoderma viride*). The enzyme was used at the concentration of 6.6 IU/g of substrate.

The enzyme was dissolved in 0.1 M sodium citrate buffer (pH 4.8). The enzyme solution was membrane filtered into an anaerobic sterile serum vial and evacuation and N_2 gassing were repeated to remove oxygen through sterile cotton filter. This enzyme solution was stored at 4 °C before use.

Cultures Conditions

Cultures were made at 35 °C in anaerobic pressure tubes (Bellco Glass Inc.) containing 10 m*l* of complex CAB medium (Kim *et al.* 1984) with 45 g/*l* glucose or 100 g/*l* cellulosic substrates.

Inocula were prepared using CAB medium with 45 g/*l* glucose in a pressure tube inoculated by 0.1 m*l* spore suspension. The inoculated tube was subjected to the heat shock at 85 °C for 5 min before incubated at 35 °C for 24 hrs. A five percent inoculum was used to initiate the main fermentation. The anaerobic sterile cellulase solution was added to the autoclaved CAB medium with cellulosic substrate before inoculation. All cultures were conducted under a stringent anaerobic conditions (Kim *et al.* 1984) using N_2 gas.

Analytical Methods

Soluble fermentation products were analyzed by chromatographic methods with a Varian 3700 gas chromatograph, equipped with a flame ionization detector. Conditions for the analyses were the same as described previously (Kim *et al.* 1984). Reducing sugar was determined by Somogyi-Nelson's method described in elsewhere (Kim *et al.* 1980).

RESULTS

Fermentation of Ball-Milled and Alkali-Treated Rice Straw

C. acetobutylicum was inoculated to CAB medium containing varying concentration of cellulosic substrates and incubated for 4 days at 35 °C before the products (Fig. 1) and the soluble reducing sugar (Table 1) were analysed.

In the fermentations with alkali-treated rice straw (Fig. 1-A) and ball-milled Avicel (Fig. 1-C), solvent production was increased as the concentration of the substrate increased. On the other hand, the ball-milled rice straw produced mainly acetate and butyrate and the amounts of fermentation products did not increase with the increase of

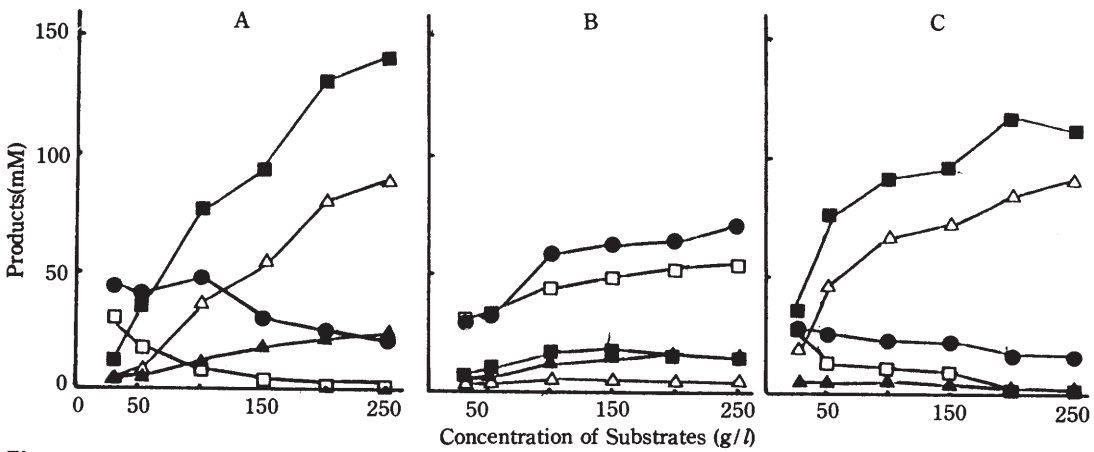

Fig. 1. *Fermentation profiles of Rice Straw and Avicel by SSF.*
▲—▲; Ethanol △—△; Acetone ■—■; Butanol ●—●; Acetate □—□; Butyrate
A; Alkali-treated Rice Straw B; Ball-milled Rice Straw C; Ball-milled Avicel

Table 1. *Residual Reducing Sugar Contents in Filterates of SSF of Cellulosic Substrate*

Substrates	Concentration(mM)
Ball-milled Avicel	12.5
Alkali-treated Rice Straw	8.3
Ball-milled Rice Straw	97.1

CAB medium containing 100 g/*l* substrate was added by cellulase solution and incubated at 35 °C for 4 days. See Fig. 1 for the fermentation products analyses.

substrate used. SSF of the ball-milled rice straw left about 100 mM of reducing sugar whilst about 10 mM of reducing sugar was found in the alkali-treated rice straw and ball-milled Avicel fermentation (Table 1).

Successful solventogenic fermentations were obtained in the SSF of alkali-treated and ballmilled rice straw (Data not shown).

During the SSF process polysaccharides are hydrolysed by enzyme and the hydrolysis products are fermented by the bacterium. Since the SSF of ball-milled rice straw produced less fermentation products with the high residual soluble sugar, it is concluded that the ball-milled rice straw contains a compound which inhibits the fermentation step but not the hydrolysis.

Glucose Fermentation Added by Alkaline-Extractables of Rice Straw

Alkaline solution used in the rice straw treatment was neutralized by 1.0 M H_2SO_4 and the precipitate was recovered. In order to investigate if the inhibitory compound is soluble in the alkaline solution, the CAB medium with glucose was fermented with or without the precipitates (Table 2).

Table 2. *Glucose Fermentation with the Acid Precipitates of Alkaline Solution used in Rice Straw Treatment*

Medium	Products (mM)				
	Acetate	Butyrate	Ethanol	Acetone	Butanol
4.5% Glucose CAB Medium	10.2	3.1	18.2	66.3	94.3
4.5% Glucose CAB Medium with Precipitates	27.3	6.5	16.2	54.0	90.6

The precipitates showed little effects on the glucose fermentation. From this result it is concluded that the inhibitory compound is either destroyed by alkaline treatment, or soluble in Na_2SO_4 solution.

Fermentation of Washed Rice Straw

Ball-milled rice straw was washed by selected organic solvents such as benzene, toluene, hexane and acetone, water or Na_2SO_4 solution before used in SSF to test if the inhibitory compound is soluble in solvent or in neutral salt solution (Table 3). As shown in the Table a good solvent fermentation was achieved in the SSF of the alkaline treated rice straw, but the ball-milled substrate gave poor solvent productions regardless the washings. These results show that the inhibitory compound is not soluble in organic solvents, water or neutral salt solution.

Electron Flow Modulators

Table 3. *Rice Straw Fermentation by SSF*

Substrates	Products (mM)				
	Acetate	Butyrate	Ethanol	Acetone	Butanol
Alkali-treated Rice Straw	47.9	7.6	23.1	26.3	109.5
Ball-milled Rice Straw	58.3	48.8	9.7	4.8	17.7
Ball-milled Rice Straw washed by					
Benzene	59.9	45.1	4.6	8.8	13.9
Toluene	49.5	41.1	4.5	10.1	21.2
Hexane	46.0	32.7	4.1	10.6	22.9
Acetone	46.7	34.7	6.5	10.9	22.1
Water	54.3	45.9	4.5	5.2	19.8
1 N Na_2SO_4	33.6	25.6	4.8	6.5	17.6

Table 4. *SSF of Ball-milled Rice Straw with Carbon Monoxide and Neutral Red*

	Products (mM)				
	Acetate	Butyrate	Ethanol	Acetone	Butanol
—	58.8	44.6	9.7	4.8	17.4
5% CO	50.9	33.8	9.2	10.1	36.2
5mM NR	52.4	50.6	10.2	6.7	19.3

Various electron flow modulators have been reported to enhance the solvent production by *C. acetobutylicum*. Carbon monoxide (Kim *et al.* 1984) and neutral red (Hongo 1957) were used as the electron flow modulators if they can alter the fermentation pattern of ball-milled rice straw (Table 4). As shown in the table carbon monoxide or neutral red did not removed the inhibitory effects on the solventogenesis.

DISCUSSION

The hydrolysis of cellulosic materials by enzyme is retarded by the crystalline nature of the substrate and lignin which block the enzyme molecules to form complex with the substrate. Various methods have been developed to increase the digestibility of cellulosic materials by enzyme. Alkaline treatment and ball-milling methods were employed to use rice straw as the substrate for the solvent production by SSF using fungal cellulolytic enzyme and *C. acetobutylicum*.

A satisfactory solventogenic fermentations were achieved in SSF of the ball-milled microcrystalline cellulose or of the alkali-treated rice straw, but ball-milled rice straw was fermented to produce less solvents with the accumulation of the acidic fermentation products and the reducing sugar. This poor fermentation of the ball-milled rice straw seems due to the presence of an inhibitory compound in the substrate. Because the acidic fermentation products were accumulated to the level at which the growth of the butanol producer is inhibited (Kim and Shin, unpublished data), the inhibitory effect seems to be specific for the solventogenic step.

A successful solventogenesis requires the solventogenic enzymes and the reducing equivalent. The solventogenic enzymes were reported to be induced during the sporulation processes of *C. acetobutylicum* (Andersch *et al.* 1984, Long *et al.* 1984). The electron flow modulators are known to increase the available electron for the reduction of metabolic intermediates and acids into alcohols (Kim *et al.* 1984). The inhibitory effect seems to be related to the solventogenic enzyme synthesis since electron flow modulators such as carbon monoxide and neutral red were not effective to enhance the solventogenesis of ball-milled rice straw.

The nature of the inhibitory compound is different from those of steam exploded cellulosic material which is removed by washing with water (Marchal *et al.* 1986). The inhibitory effect cannot be removed by organic solvents, water or neutral salt, but removed by alkaline treatment. The inhibitory compound might be lignin derivatives as assumed by Jeffries (1983) and Detroy *et al.* (1982). Another possibility is that the inhibitory compound is the residual agricultural chemicals which are widely used in rice cultivation.

적 요

벗짚을 이용하여 acetone-butanol을 생산하기 위해 전처리한 벗짚을 *C. acetobutylicum* KCTC 1037(ATCC 4259)과 *Trichoderma viride*로부터 얻은 섬유소 분해효소를 이용하여 동시당화 발효법(SSF)으로 발효하였다. Ball-mill로 처리한 벗짚을 SSF로 발효한 결과 acetate와 butyrate만을 생산하였으나, alkali로 전처리한 기질은 230 mM 이상의 solvent를 생산하였다. 이와 같은 발효의 차이는 벗짚에는 alkali 처리로 분해되는 물질이 있으며, 이 물질이 solvent 생산을 저해하기 때문인 것으로 판단된다. 이러한 solvent 생산 저해물질은 물이나 유기용매에 불용성으로 lignin 유도체 혹은 잔류농약으로 추측된다.

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