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Improvement of Process for Sorbitol Production with Cetyltrimethylammoniumbromide Permeabilized Cells of *Zymomonas mobilis* through Glutaraldehyde Crosslinking

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Cetyltrimethylammoniumbromide로 투과성을 높힌 *Zymomonas mobilis*의 Glutaraldehyde Crosslinking에 의한 Sorbitol 생산 안정성의 향상

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ABSTRACT

Permeabilization of *Zymomonas mobilis* with CTAB(Cetyltrimethylammoniumbromide) was investigated in order to obtain stable process for sorbitol production in the immobilized system. The optimum conditions for sorbitol formation were obtained in the case of using cells treated with 0.2% CTAB at 4℃ for 10 min. Permeabilized cells were treated with glutaraldehyde to cross-link the internal enzyme for the improvement of the enzyme stability. In this way, no significant loss of enzyme activity was apparent during 30 day operation in a continuous process. The productivity of the continuous process at dilution rate $0.2h^{-1}$ was 6.51 g / l / h for sorbitol. The CTAB permeabilized cells could be used to produce sorbitol in the long term continuous process.

INTRODUCTION

Zymomonas mobilis has been known to produce ethanol at fast rate(1). The formation of sorbitol by this bacteria has been evaluated over the last few years when *Zymomonas mobilis* was cultured on sucrose or a mixture of glucose and fructose(2, 3). The mechanism of sorbitol formation with *Zymomonas mobilis* was found to be that the reduction of fructose to sorbitol is coupled with the dehydrogenation of glucose to gluconolatone(4). Gluconolactone is then further hydrolyzed to gluconate by a gluconolactonase(5).

The newly found enzyme complex that is responsible for conversion of fructose to sorbitol has been described as glucose-fructose oxidoreductase with a tightly coupled cofactor, NADP(5). This cofactor is nondialyzable. Only permeabilized cells produce sorbitol and gluconate simultaniously due to removal of soluble cofactors necessary for phosphorylation of gluconate by the gluconate kinase in the Entner-Doudoroff pathway(6).

The enzymatic process for the production of sorbitol by oxidoreductase is important in food industry because of the mild operating conditions and the specific activity of the enzymes which minimize by-product formation.

Currently, permeabilized cells of *Zymomonas mobilis* treated with toluene were used either in the free or in the immobilized form for the production of both sorbitol and gluconic acid(6). The process using permeabilized whole cells as a source of enzyme is an interesting alternative for the specific bioconversion of fructose to sorbitol which alleviates problems of isolations and also reduces the production cost.

A continuous process employing cells treated with toluene and immobilized in alginate has been developed and some loss of enzyme activity was observed after 120 h due to leakage of oxidoreductase from the permeabilized cells or further proteolysis by toluene during the process (7).

In order to enhance the stability of the sorbitol production system, we attempted to improve the *K*-carrageenan method. Treatment of the cells with a new permeabilizing agent, CTAB(Cetyltrimethylamoniumbromide) was found to be effective in increasing the stability of oxidoreductase activity in the long term process.

MATERIALS AND METHODS

Microorganism and growth conditions

All experiments were performed with *Zymomonas mobilis* ZM4(ATCC 31821) grown anaerobically in the medium containing 100 g / L glucose, 1 g / L $(NH_4)_2SO_4$, 1 g / L $MgSO_4 \cdot 7H_2O$, 2 g / L KH_2PO_4 and 5 g / L yeast extract(Sigma). For cell-production, batch culture was performed in 2L fermentor under controlled enviro nmental conditions at 30℃ and pH5.0. No phosphate was included in the growth medium, although it was added to the medium for inoculum in order to minimize the levels of phosphorylated intermediates in the cells.

Preparation of permeabilized cells and glutaraldehyde cross-linking

Cells were harvested in the late exponential phase after 20 h growth by centrifugation (4000 rpm) prior to treatment with toluene(10% v / v in 0.1N saline buffer at pH6.2) or CTAB(0.2% v / v at 4℃ and pH 6.0−6.2). After vortexing(toluene) or gently stirring(CTAB) for 10 min, cells were washed twice with saline buffer as reported by Chun and Rogers(6). The yield of cells from the fermentor was approximately 2.5−3.0 g (dry weight) / L. For cross-linking with glutaraldehyde, the permeabilized and washed cells were suspended in 0.3%(v / v) glutaraldehyde in 0.1M Kpi buffer(6.2) and stirred at 4 ℃ for 10 min. The suspension was centrifuged(4000 rpm) for 5 min and then washed twice with same buffer.

Procedure for cell immobilization.

Both alginate and *K*-carrageenan immobilized cells were used for their ability to produce sorbitol and gluconic acid from fructose and glucose respectively.

For cell immobilization with alginate, a concentrated suspension of cells was mixed with a solution of Na-alginate(2% w / v) in the volume ratio of 1:10. Cross-linking was carried out by adding 0.25% glutaraldehyde with stirring for 10 min before immobilization. Chitin was also used for alginate immobilization. The chitin(from crab shells, Sigma) treated with 6N HCl was neutralized by washing with distilled water and dried in a vacuum oven. To a chitin solution(10 g in 50mL physiological saline solution), the glutaraldehyde treated cells were added and the mixture was allowed to stand for 1 h at room temperature and followed by standing overnight at 4℃(8). Spherical beads of immobilized cells were then produced by adding the above mixture dropwise into a 40 g / L $CaCl_2$ solution. For immobilization with *K*-carrageenan, CTAB permeabilized cells(12−13 g, wet weight) which were also treated with glutaraldehyde before immobilization were mixed with 100mL of *K*-carrageenan solution(3.2% w / v). And then the *K*-carragenan beads were produced in a solution containing 20 g / L KCl and 0.15 g / L $CaCl_2$. Bead sizes were at ranges of 2.5−3.0mm in diameter.

Reactor operation.

The reactor used for free and immobilized cells was a continuous stirred tank reactor(CSTR) with working volume of 186mL. For the immobilized process, liquid volume was 102mL.

The continuous process for sorbitol production was carried out also in a packed bead column reactor as shown in Fig. 1. The total working volume was 136mL:95mL for the main column reactor and 41mL for the pH adjusting unit including connecting tubes. Dilution rates were determined using the total working volume unless otherwise stated. The temperature and pH during the operation were maintained at 39℃ and 6.2 respectively. For the packed

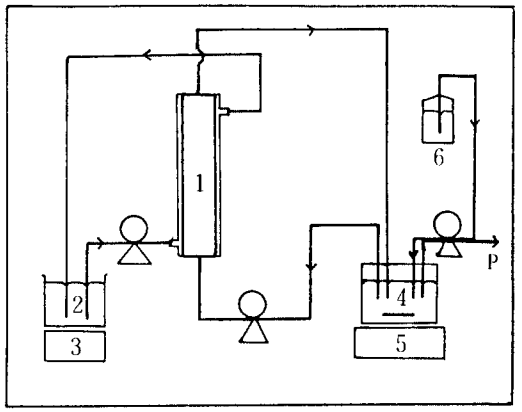

Fig. 1. Diagram of the experimental packed bead bioreactor for continuous production of sorbitol and gluconic acid.
1: packed beads column; 2: water reservoir for temperature control; 3: hot plate; 4: reservoir for pH control; 5: magnetic stirrer; 6: feed reservoir; p: product stream.

bead column, pH was controlled in the recycle vessel.

Analytical methods.

Glucose, fructose, sorbitol and ethanol concentrations were analyzed by using a Waters model HPLC with a BioRad(Richmond, Calf, USA) Aminex HPX-87C column. The biomass concentrations for the free cells were determined as dry weight following oven drying at 104℃ for 24hr. For the immobilized cell experiments, a known mass of cells was immobilized.

RESULTS AND DISCUSSION

Sorbitol formation with toluene treated cells.

Repeated fed-batch experiments were carried out with toluene permeabilized cells of *Zymomonas mobilis*. After 10 h, the supernatant in the reactor was removed by centrifugation and fresh sugar solution(50 g / L equimolar mixtures of glucose and fructose) added to the cell concentrate. The procedure was repeated 3 times. Similar experiments to those were performed with toluene treated cells immobilized in Ca-alginate beads. As summarized in Table 1, the conversion efficiency for sorbitol with free cells reduced after first run of batch cycle, and in the third cycle there was significant decrease in conversion of fructose to sorbitol. Similar decreases in the sorbitol formation capacity of cells upon recycling were observed with the toluene treated cells immobilized in Ca-alginate. The reduction in conversion efficiency is probably due to enzyme leakage through permeabilized membrane of cells. The leakage of enzyme was reported to be serious with permeabilized cells of *Saccharomyces carlsbergensis*(9). It was found that more than 50% of the total hexokinase activity at the first stage of the cycle and 85% at the final stage leaked off the toluene treated permeabilized cell.

Cells immobilized in Ca-alginate beads showed slightly lower conversion efficiencies compared with free cells. It might be that some mass transfer limitations occurred with immobilized cells. Using immobilized toluene treated cells of *Zymomonas mobilis* in a continuous stirred tank reactor (CSTR), the sorbitol level was determined for a 50 g / L fructose solution. The conditions at 39℃ and pH6.2 were maintained.

In view of preventing enzyme loss from immobilized system, cells were treated with glutaraldehyde prior to immobilization in plus alginate chitin. The profile of sorbitol, glucose and fructose concentractions for CSTR at dilution rate of 0.2 h^{-1} are shown in Fig. 2. The relatively stable enzyme activity was observed over 210 h.

Table 1. Kinetic parameters in the repeated batch process with toluene treated cells in both free and immobilized form. The pH was not controlled for these experiments

Parameters	Batch cycle					
	Free cell			Immobilized cells		
	1st	2nd	3rd	1st	2nd	3rd
Sorbitol concentration(g / L)	35.4	26.7	21.2	33.5	16.0	9.5
Conversion efficiency(%)	70.8	53.4	42.4	67.0	32.0	19.0

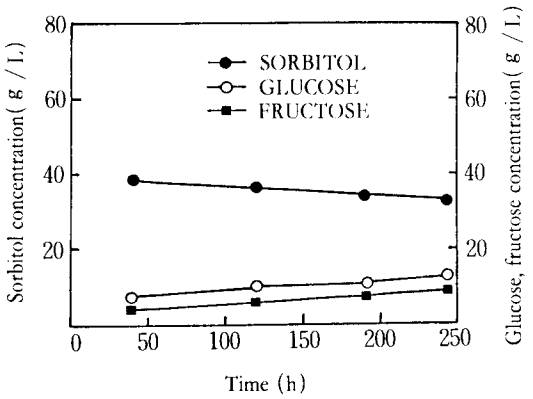

Fig. 2. Sorbitol production by permeabilized cells of *Z. mobilis* immobilized in Ca-alginate, glutaraldehyde and chitin in a continuous process(T=39℃, pH6.2, D=0.2h^{-1}).

This indicates that enzyme loss could be prevented due to a cross-linking between enzymes by glutaraldehyde inside cells. It was reported that glutaraldehyde treated cells of *Saccharomyces carlsbergensis* were repeatedly used for the phosphorylation of glucose to fructose-1.6-diphosphate over 100 days(Bisso and Melleli, 1986).

Sorbitol production with CTAB treated cells.

Although the glutaraldehyde cross-linking of toluene treated cells immobilized in alginate and chitin showed enzyme stability for 210 h of process, some enzyme deactivation (about 23%) was observed during such period. This was probably due to the effect of residual tolune on the cell lysis. Therefore, a cationic detergent, CTAB, was used to permeablize cells. As compared in Table 2, the similar conversion efficiency to toluene permeabilized cells was obtained from the cells treated with 0.2% CTAB. As expected with untreated cells, very low concentration of sorbitol (3 g / L) formed since both glucose and fructose were metabolized to ethanol and byproducts via normal ED pathway.

The maximum conversion efficiency(60%) was obtained from the cells stirred in 0.2% CTAB solution at 4℃ for 10min (see Table 3). The conversion efficiency of 95-97% for sorbitol resulted from the pH controlled reaction by cells treated with 0.2% CTAB solution (data not shown).

Table 3. The effect of CTAB concentrations on sorbitol formation. The reaction was performed on 50 g / L glucose and 50 g / L fructose at 39℃ without pH control for 15 h

Parameters	CTAB concentration (%)			
	0.05	0.1	0.2	0.3
Sorbitol concentration(g / L)	7.5	25.6	30.0	29.1
Ethanol concentration(g / l)	42.0	16.6	6.2	9.1
Conversion efficiency(%)	15.0	51.2	60.0	58.2

Stability of continuous process

Table 2. Sorbitol formation by *Zymomonas mobilis* treated with various permeabilizing agents. Experiments were performed on 50 g / L each of glucose and fructose at 39℃ without pH control for 15 h

Methods	Max. sorbitol concentration (g / L)	Conversion (%)
CTAB[a]	30.0	60
Toluene[b]	28.1	56
Tween 20[c]	11.2	22
Untreated cells	3.0	6

a) Stirring with 0.2%(v / v) CTAB for 10min at 4℃

b) Vortexing with 10%(v / v) toluene for 5 min at room temperature

c) Tween 20 added to the reactor at 2 h after inoculation. Final concentration=0.4%(w / v)

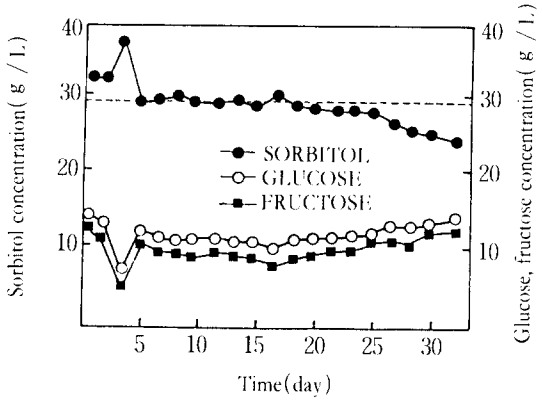

Fig. 3. Sorbitol production by immobilized CTAB permeabilized cells of *Z. mobilis* in *K*-carrageenan. Permeabilized cells were treated with glutaraldehyde prior to immobilization. Continuous process was performed on 50 g / l each of glucose and fructose at 39℃, pH6.2 and dilution rate of 0.2 h^{-1} in the CSTR.

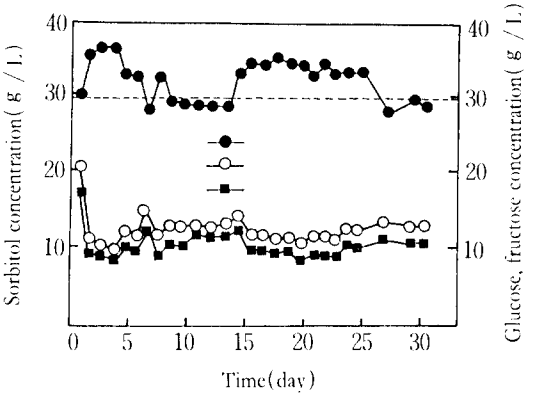

Fig. 4. Continuous production of sorbitol by *K*-carrageenan immobilized CTAB treated cells in the packed bead reactor at a dilution rate of 0.2h^{-1}, 39℃ and pH 6.2.

The sorbitol was produced using CTAB treated cells of *Zymomonas mobilis* immobilized in *K*-carrageenan in the CSTR. Hardness of the beads was maintained by addition of 2N $CaCl_2$ to the input substrate solution. Also, cells were treated with glutaraldehyde prior to immobiliz ation with *K*-carrageenan. As can be seen in Fig. 3, a stable production of sorbitol was sustained over 24 days.

This is comparable with the stability of process for 210 h with immobilized toluene treated cells. During 20 day operation, sorbitol concentrations of 32−35 g / L were sustained thoughout the steady state period and the half-life of enzyme process was estimated to be about 35 days. The improvement of the process stability may be due to that CTAB was not effective for further proteolysis after washing and a NH_2 group in enzyme was cross-linked by glutaraldehyde. Treatment with hardening agent has been investigated and Bajpai and Margaritis(10) reported that inulinase stability of glutaraldehyde hardened immobilized cells increased two folds.

The continuous production of sorbitol with the immobilized CTAB permeabilized cells in packed bead reactor was evaluated and the process was more stable than that of CSTR. As represented in Fig. 4, very stable operational activity was maintained over a period of 30 days. The productivity of the continuous process at a fixed dilution rate of 0.2 h^{-1} was 6.51 g / L / h for sorbitol which was calculated on the total working volume liquid in both reactor and connector). Some glucose and fructose remained unutilized indicating that the dilution rate had been set at too high a value to achieve full conversion. For full conversion in packed bead reactor, the outlet stream from the reactor was recycled at various rates(see Fig. 1), and an increase in conversion efficiency was observed indicating the possibility of full conversion at high dilution rate (data not shown). Gluconic acid showed similar profiles to sorbitol in all experiments and are not reproduced here.

요 약

고정화 세포 공정에서 안정적으로 sorbitol을 생산하기 위하여 CTAB에 의한 *Zymomonas mobilis*의 투과성에 관하여 연구하였다. Sorbitol생성의 최적조건은 세포를 0.2%의CTAB로 4℃에서 10분간 처리 하였을때이며, 투과성이 증가된 세포를 고정화 시키기 전에 system의 안정성을 향상시키기 위하여 glutaraldehyde로 처리하였다. 이와 같이 했을때 연속 공정에서 약 30일간 enzyme activity의 감소가 거의 없었으며, 희석비율 0.2h^{-1}에서 6.51 g / l / h 의 productivity를 보였다. CTAB로 처리된 *Z. mobilis*

를 장시간 연속공정으로 sorbitol 생산에 이용할 수 있다.

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