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Continuous Production of Sorbitol with Permeabilized *Zymomonas mobilis* Immobilized in Alginate and Chitin

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알저네이트 및 카이틴 고정화 *Zymomonas mobilis* 에 의한 쏠비톨의 연속생산

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ABSTRACT

This study describes the sorbitol production with permeabilized cells of *Zymomonas mobilis* immobilized in Ca-alginate. Toluene treated cells lose glucose-fructose oxidoreductase activity due to leaking of enzyme from the cells. In order to prevent this leakage, the permeabilized cells were immobilized in alginate and chitin. No significant loss of enzyme activity was apparent during 210h operation in a continuous process. The productivity of the continuous process was estimated to be about 3.5 g/*l* -h for sorbitol at dilution rate $0.2h^{-1}$.

INTRODUCTION

The bacterium *Zymomonas mobilis* has been shown to be a very fast, efficient producer of ethanol(1). However, Viikari(2) and Barrow et al.,(3) demonstrated that quite high levels of sorbitol accumulated when either sucrose of a mixture of glucose and fructose was used as the carbon source during growth. The new enzyme complex, which is responsible for glucose oxidation and fructose reduction, has been described as glucose-fructose oxidoreductase(4) . This enzyme is present at significant level in *Z. mobilis*, especially when the organism is grown on glucose as a carbon source(5) .

The enzyme glucose-fructose oxidoreductase which converts fructose to sorbitol has several biotechnological applications in food industries. Sorbitol has been used as a low calorie sweetner in foods for diabetics. Sorbitol can be converted also to L-sorbose, an intermediate in the production of Vitamin C. A three stage continuous fermentation which involves the biodegradation of the sorbitol by *Acetobacter suboxydans* or *A. xylinum* is involved in the conversion(6).

The purified enzymes have been used as a soluble or immobilized form. However, enzyme purification is time consuming and tedious. The use of whole cells as a source of enzyme is an interesting alternative for specific bioconversion. It was reported that formation of fructose 1,6-diphosphate during glucose fermentation by permeabilized yeast cells *Saccharomyces carlsbergensis* depended on the degree of permeabilization(7) . Joshi et al.,(8) also reported that permeabilized cells of yeast *Kluyveromyces fragilis* increased β-galactosidase activity 480 fold.

In view of the interesting properties of the oxidoreductase identified in *Z. mobilis*, the aim of present study is to investigate the efficiency of conversion by the permeabilized cells with various methods. Results are also

presented showing the stability of the biocatalyst immobilized with alginate and glutaraldehyde.

MATERIALS AND METHODS

Microorganism and growth conditions

All experiments were performed with *Zymomonas mobilis* ZM4(ATCC31821) grown in a medium containing 100 g / l glucose, 1 g / l $(NH_4)_2SO_4$, 1 g / l $MgSO_4$ $7H_2O$ and 5 g / l yeast extract(Sigma) under controlled environmental conditions of 30℃ and pH 5.0. No phosphate salts were added to the final growth medium (although they were present in the medium for inoculum growth) in order to minimize the levels of phosphorylated intermediates in the cells.

Cell permeabilization

Approximately 10 g cells taken from the late exponential phase of growth were separated by centrifugation and suspended in citrate buffer (pH 6.2) to give a total volume of 100ml. In order to select the most practical methods of permeabilization a preliminary series of experiments was carried out using solvent, surfactant or heat shock methods.

Procedure for cell immobilization

A concentrated suspension of cells was mixed with a solution of sodium alginate (2% w / v) in the volume ratio of 1:10. In order to evaluate the enzyme stability for long term processes, cross-linking was carried out by adding 0.25% glutaraldehyde with stirring for 1h at room temperature before immobilization. Also, permeabilized cells were suspended in 50ml of 2% Na-alginate solution. For immobilization with chitin, 6 N HCl was used for treatment of chitin(from crab shells, Sigma). Then, chitin solution was neutralized by washing with H_2O and dried in a vacuum oven. Glutaraldehyde treated cells were mixed with chitin(10 g in 50ml physiological saline solution) and then the mixture was allowed to stand for 1h at room temperature and followed by standing overnight at 4℃(9).

Spherical beads of immobilized cells were then produced by adding the above mixture dropwise into a 40 g / l calcium chloride solution. The procedure was automated by using a syringe and peristaltic pump to give beads of 1.0 to 1.5 mm diameter. With chitin bead, sizes were at ranges of 3-4mm 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 processes, liquid volume was 102ml and the productivities were calculated based on the total volume(liquid + void volume).

The temperature during operation was maintained at 39℃ by heating plate, and the liquid and alginate beads in the reactor were mixed by a magnetic stirrer. A 10% sugar solution(5% glucose + 5% fructose) was pumped into the reactor at various dilution rates for the stude of enzyme stability.

Analytical methods

Samples were taken from the reactor and immediately frozen. Analysis of glucose, fructose, ethanol and sorbitol was performed using a Waters model HPLC with a BioRad Aminex HPX-87C column.

The biomass concentrations for the free cells were determined as dry weights following oven drying at 104 ℃ for 24h.

For the immobilized cell experiments, a known mass of cells was immobilized. The kinetic parameters were estimated based on the total volume of liquid plus alginate beads.

RESULTS AND DISCUSSION

Cell permeabilization

Table 1 shows the effect of cell wall permeability of the different agents or methods tested in the preliminary study of the most adequate procedure to be adopted. Toluene treated cells appeared to produce the highest amount of sorbitol. Previously, it was reported that a 10 % v / v toluene concentration with 5 min of vortexing was sufficient to minimize ethanol production and achieve high conversion efficiency for sorbitol formation(10). It is interesting to note that permeabilized *Zymomonas* cells are able to accumulate sorbitol at a high level. Incompletely permeabilized cells will mostly convert sugars to ethanol and CO_2 in the presence of phosphorylated intermediates.

Enzymatic activity

Fed-batch experiments were carried out with toluene treated cells of *Z. mobilis*. After 10h, the supernatant in the bioreactor was removed by centrifugation and fresh

Table 1. Sorbitol formed by permeabilized *Zymomonas mobilis*

Permeabilization procedure	Max. Sorbitol concentration(g/l)	Conversion (%)
Toluene[a]	28	56
Glycerol[b]	20	40
Tween 20[c]	11	22
Heat shock[d]	20	40

(a): Vortexing with 10% v/v toluene for 5 min.
(b): Glycerol added at late stationary phase to give 10 ml/l and stirring for 1h.
(c): Tween 20 added after 2h of inoculation. Final concentration: 0.4% (w/v).
(d): Stirring harvested cells in 10ml of 10% ethanol for 1h.

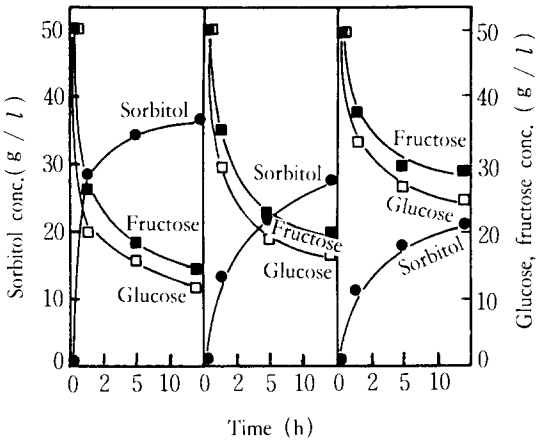

Fig. 1. Production of sorbitol in fed-batch process with toluene-treated cells of *Z. mobilis*. Three cycles of fed-batch culture were carried out(T=39℃).

sugar solution (50 g/l each glucose and fructose) added. The procedure was repeated 5 times. Similar experiments to those were performed with toluene treated cells immobilized in calcium alginate beads. The gel particle sizes were designed to be 1.00−1.15 mm diameter in order to overcome mass transfer limitation because such sizes were recommended to give optimal values of the kinetic constants, maximum reaction rate(Vm) and apparent saturation constant(Ks)(11).

As can be seen from Fig. 1, a conversion efficiency for sorbitol reduced after lst run of batch cycle since enzyme leakage occurred due to permeabilization of cell wall.

At the 3rd stage of the cycle(48h) with immobilized cells, almost a loss of 71% of the enzyme activity occurred with immobilized cells(see Table 2). Leakage of enzyme activity was also reported to be serious with permeabilized cells of *Saccharomyces calsbergensis*(7). About 71% of enzyme activity was leaked from the toluene treated cells at the end of second run of batch cycle.

Enzyme stability in a continuous process

Using immobilized toluene treated cells of *Z.mobilis* in a continuous stired tank reactor(CSTR), the sorbitol level was determined for a 50 g/l glucose + 50 g/l fructose solution. Conditions of temperature = 39℃ and pH6.2 were maintained. The calcium alginate gel beads were found to be very stable in a continuous process over 210h. Hardness of the beads was maintained by addition of 2 g/l $CaCl_2$ to the input substrate solution. $CaCl_2$ has been used by other aut hors to stabilize the alginate

Table 2. Kinetic parameters in fed-batch process with toluene treated cells of Z. *mobilis* immobiliz ed in Na-alginate

Kinetic parameters	Batch cycle		
	lst	2nd	3rd
Max. sorbitol concentration(g/l)	33.5	16.0	9.5
Conversion efficience(%)	66	32	19
Enzyme activity loss at 3rd cycle (%)		71	

support (12−13). It is not known whether $CaCl_2$ i likely to cause any inhibition of the activity of glucose-fructose oxidoreductase responsible for formation of sorbitol, although Ca^{2+} was reported to be important in maintaining activity of this enzyme(5). Fig. 2 shows the sorbitol, fructose, glucose concentrations at dilution rate $0.18h^{-1}$. Sorbitol concentration decreased with time and corresponding glucose and fructose concentrations increased. This indicates that some enzyme loss occurred in the immobilized cells and alginate matrix.

In view of preventing enzyme loss from immobilized system, cells were treated with glutaraldehyde prior to immobilization with alginate. As can be seen in Fig. 3, during 210h operation, sorbitol concentrations of 40−41.5 g / l were sustained throughout the steady state period. The productivities were estimated to be about 7.2−7.5 g / l-h for sorbitol at dilution rate $0.18h^{-1}$.

The enzyme stability with permeabilized cells immobilized in alginate+chitin was also examined in the continuous process using 10% sugar solution(5% glucose+5% fructose). The profiles of sorbitol, glucose and fructose concentrations for CSTR at dilution rate of $0.2h^{-1}$ are shown in Figure 4. Very little enzyme deactivation occurred over 210h, which is comparable with the immobilized process using glutaraldehyde and alginate. Some glucose and fructose remained unutilized indicating that the dilution rate had been set at too high a value to achieve full conversion. A continuous process for sorbitol production using immobilized *Z. mobilis* showed the possibility of extended production using Ca-alginate gel and glutarald

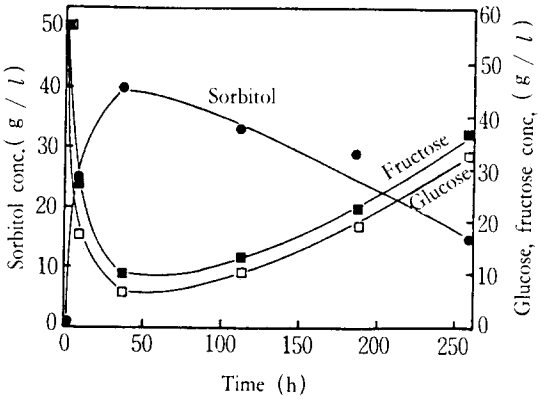

Fig. 3. Sorbitol production by permeabilized cells of *Z. mobilis* immobilized in alginate plus glutaraldehyde at dilution rate 0.18^{-1} in continuous process(T=39℃, pH 6.2).

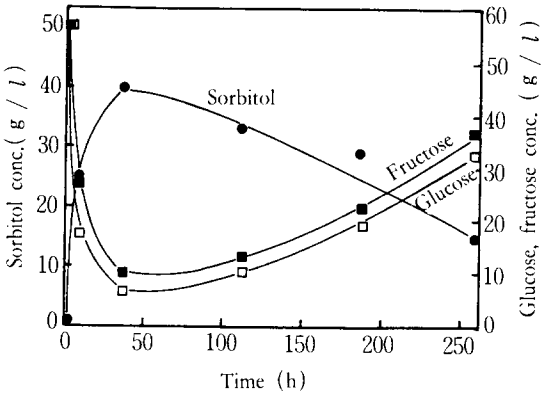

Fig. 2. Sorbitol producing capability of immobilized cells of *Z. mobilis*(ZM4) on 50 g / l each of glucose and fructose at dilution rate $0.18h^{-1}$ in continuous processes(T=39℃, pH6.2).

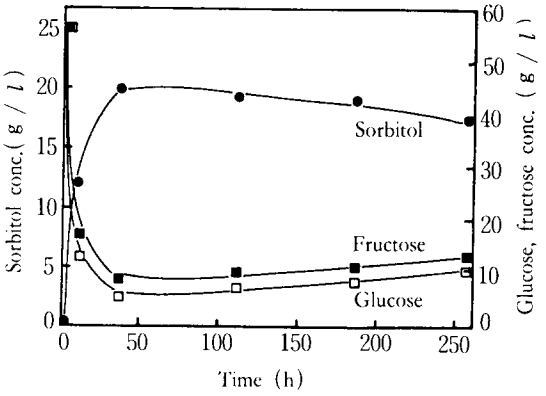

Fig. 4. Sorbitol production by permeabilized cells of *Z. mobilis* immobilized in alginate, glutaraldehyde and chitin in continuous process(T=39℃, pH6.2, dilution rate= $0.2h^{-1}$).

ehyde as support materials. No cell leakage was evident from the beads in both processes indicating that the calcium alginate beads themselves were quite stable. When treating cells with glutaraldehyde, the stable enzyme activity was observed for considerably long period of process indcating that enzyme loss could be prevented due to a cross-linking between enzymes by glutaraldehyde inside cells(14). It was reported that glutaraldehyde treated cells of *Saccharomyces carlsbergensis* was repeatedly used for the phosphorylation of glucose to fructose-1,6-diphosphate over 100 days(7). Furthermore, immobilization of permeabilized cells of *Z. mobilis* in alginate and chitin provides higher enzyme stability than those in previous report(8).

요 약

Ethanol을 주로 생산하는 균주인 *Z. mobilis*의 cell wall 투과성을 높인 후 고정화하여 sorbitol 생산에 이용하였다. 그러나, toluene으로 투과성을 높인 cell은 oxidoreductase의 유출, 손실로 인하여 sorbitol conversion efficiency가 급격히 저하되었다. 따라서, 이와같은 enzyme의 유출을 방지 하기 위하여, 투과성을 향상시킨 cell을 0.25% glutaraldehyde로 처리한 후 alginate와 chitin에 고정화하여 희석율 $0.2h^{-1}$에서 연속배양을 한 결과 210시간동안 효소활성도의 저하는 거의 일어나지 않았다. 이와같은 연속배양에서 얻어진 sorbitol productivity는 3.5 g /l·h로 측정 되었다.

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