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Sorbitol Production by *Zymomonas mobilis* Immobilized in Calcium Alginate Gels and Glutaraldehyde

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알지네이트 및 글루타르알데하이드 고정화 *Zymomonas mobilis*에 의한 솔비톨 생산

정인호 · 최도진 · 박철진 · 전억한
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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 activity of glucose-fructose oxidoreductase due to the leaking of enzyme from the cells. To prevent this leakage, the permeabilized cells were treated with 0.25% glutaraldehyde by stirring for 1 h at room temperature. A continuous process with glutaraldehyde treated cells was developed and no significant reduction in the degree of conversion occurred during 210 h operation. The productivities were estimated to be about 7.2~7.5 g/*l*-h for sorbitol at dilution rate 0.18 h^{-1}.

Key words : sorbitol, permeabilization, immobilization, glutaraldehyde treatment

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 some sorbitol accumulated when either sucrose or a mixture of glucose and fructose was used as a 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 application in food industries. 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[6]. Joshi *et al.*[7], 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 also showed the stability of the biocatalyst immobilized with alginate and glutaraldehyde.

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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* $MgSO_4 \cdot 7H_2O$ 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. Thus, metabolism of gluconate to 2-keto-3-deoxy-6-phosphogluconate via 6-phosphogluconate would be partly prevented.

Cell permeabilization

Approximately 10g 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 100 m*l*. In order to select the most practical methods of permeabilization instead of toluene treatment due to its toxicity a preliminary series of experiments was carried out using solvent, surfactant or heat shock methods which increase cell permeability[6].

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 1 h at room temperature before immobilization. Also, permeabilized cells were suspended in 50 m*l* of 2% Na-alginate solution. 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.

Analytical methods

5 m*l* of samples were taken from the reactor ,and frozen immediately. Analysis of glucose, fructose, ethanol and sorbitol was performed using a Waters model HPLC(r401) with a BioRad Aminex HPX-87C column. HPLC analysis was carried out at 85℃ with solvent flow rate of 0.6 m*l*/min. The samples were pretreated by diluting to an appropriate sugar concentration(2 Brix), desalted and then filtered through a 0.45 μm pore size filter(Millipore).

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

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 treatment with permeabilizing agents or methods in the preliminary study of the most adequate procedure to be adopted. In the experimental system the temperature was maintained at 39℃ and the pH was not controlled. Toluene treated cells appeared to produce the highest amount of sorbitol, whereas untreated cells were able to accumulate sorbitol at low level. The low conversion of sugars to sorbitol exhibited by untreated cells is probably due to the poor permeability of the cell membrane. 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[8]. The authors also reported that only permeabilized cells(with toluene) were able to accumulate sorbitol and gluconate. Cells with incomplete permeability mostly transformed sugars to ethanol and CO_2. In a normal conversion of the glucose and fructose, sorbitol accumulated in the culture medium while gluconic acid was metabolized via 6-phosphogluconate to 2-keto-3-deoxy-6-phosphogluconate(an intermediate in the Enter-Doudoroff pathway). However, the amount of sorbitol formed was very low[9].

Table 1. Comparison of efficiencies for sorbitol formation for treated and untreated cells of *Zymomonas mobilis* on 50 g/*l* each of glucose and fructose

Cell treatment	Max. Sorbitol concentration (g/*l*)	Conversion (%)	Ethanol concentration (g/*l*)
Toluene [a]	28	56	1.7
Glycerol [b]	20	40	12.4
Tween 20 [c]	11	22	29.5
Heat shock [d]	20	40	13.3
Untreated cells	3	6	36.6

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

From the Table 1, ethanol formed at high concentration with untreated cells compared to toluene treated cells indicating that the gluconate which accumulated originally was finally converted to ethanol. Chun and Rogers[9] confirmed in previous report that no further gluconate accumulated, whereas almost equimolar concentrations of sorbitol and gluconate formed with fully permeabilized cells of *Z. mobilis*. Although the gluco-

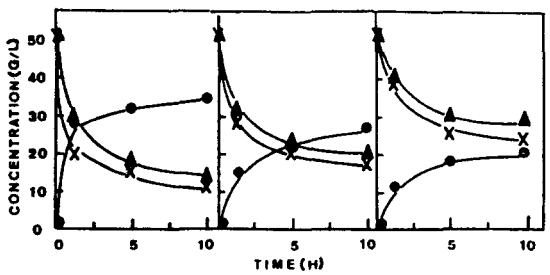

Fig. 1. Production of sorbitol in semi-batch process with toluene-treted cells of *Z. mobilis*. Three cycles of batch culture were carried out (T=39℃, pH 6.2)
—●— ; sorbitol, —▲— ; glucose, —x— ; fructose

Table 2. Kinetic parameters in semi-batch process with toluene treated cells of *Z. mobilis* immobilized in Ca-alginate

Kinetic parameters	Batch cycle		
	1st	2nd	3rd
Max. sorbitol concentration(g/l)	33.5	16.0	9.5
Conversion efficiency(%)	66	32	19

nate would accumulate in the reactor with toluene treated cells, data are not presented here.

Conversion efficiency

In order to evaluate the stability of the oxidoreductase in cells, semi-batch culture experiments were carried out with toluene treated cells of *Z. mobilis*. After 10 h. the supernatant in the bioreactor was removed by centrifugation and fresh sugar solution(50 g/l each of glucose and fructose) added. The procedure was repeated 3 times, and in each case the glucose, fructose and sorbitol concentrations were determined(see Fig. 1). Similar experiments to those were performed with toluene treated cells immobilized in calcium alginate beads and results are summarized in Table 2. The reaction of each cycle was carried out for 16 h. 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 (V_m) and apparent saturation constant(K_s)[10].

As can be seen from Fig. 1, a conversion efficiency for sorbitol reduced after 1st run of batch cycle which is probably due to enzyme leakage from the permeabilized cell wall.

Similar results were obtained with immobilized cells.

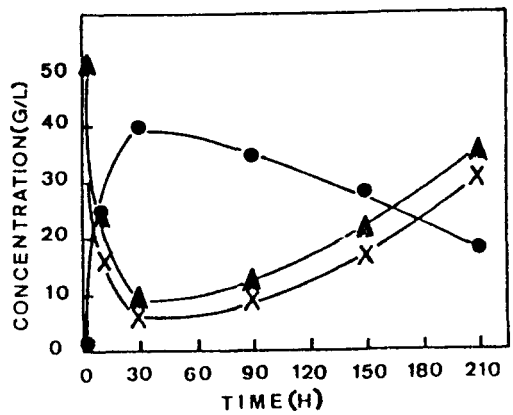

Fig. 2. Sorbitol producing capability of immobilized cells of *Z. mobilis*(ZM4) on 50 g/l each of glucose and fructose at dilution rate 0.18 h^{-1} in continuous processes (T=30℃, pH 6.2)
—●— ; sorbitol, —▲— ; glucose, —x— ; fructose

At the 3rd stage of the cycle(48 h), sugars(e.g. 50 g/l each of glucose and fructose) were converted to sorbitol with conversion efficiency of only 19% with immobilized cells(see Table 2). Leakage of enzyme activity (hexokinase and pyruvatekinase) was also reported to be serious with permeabilized cells of yeast, *Saccharomyces calsbergensis*[6]. More than 85% of the total hexokinase activity at the 3rd stage of the cycle leaked off the permeabilized cells.

Continuous process

Using immobilized toluene treated cells of *Z. mobilis* in a continuous stirred tank reactor(CSTR), the sorbitol level was determined for a 50 g/l glucose+50 g/l fructose solution. Conditions of temperature=39℃ and pH 6.2 were maintained. The calcium alginate gel beads were found to be very stable in a continuous process over 210 h. 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 authors to stabilize the alginate support[11,12]. It is not known whether $CaCl_2$ is 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 0.18 h^{-1}. Sorbitol concentration decreased as time increased and corresponding glucose and fructose concentrations increased. This indicates that some enzyme leakage occurred in the permeabilized

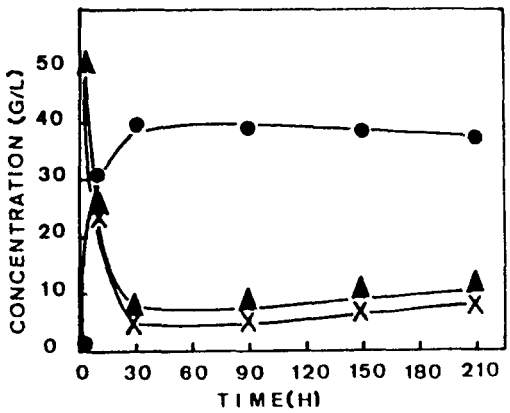

Fig. 3. Sorbitol production by permeabilized cells of *Z. mobilis* immobilized in alginate plus glutaraldehyde at dilution rate 0.18^{-1} in continuous process(T=39℃, pH 6.2)
—●— ; sorbitol, —▲— ; glucose, —x— ; fructose

cells immobilized in alginate matrix.

In view of preventing enzyme leakage from immobilized system, cells were treated with glutaraldehyde prior to immobilization with alginate. As can be seen in Fig. 3, during 210 h 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 $0.18\ h^{-1}$. 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 glutaraldehyde 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 conversion efficiency was observed for considerably long period of process indicating that enzyme leakage could be prevented due to a cross-linking between enzymes by glutaraldehyde inside cells[12]. 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[6].

요 약

쏠비톨 생산을 위해서 *Zymomonas mobilis*의 cell wall 투과성을 높인 후 alginate에 고정화 하였다. 투과성을 높이는데 toluene이 가장 효과적인 것으로 나타났으나, 3회의 회분공정을 행한 바 glucose-fructose oxidoreductase의 유출, 손실로 인하여 쏠비톨 생산이 급격히 저하되었다. 효소의 활성도를 장시간 유지하기 위하여 투과성을 높인 cell을 상온에서 1시간 동안 0.25%(v/v)의 glutaraldehyde로 처리한 후 alginate에 고정화 하여 연속공정을 행하였을 때 210시간 동안 높은 전환효율(82%)을 보였으며, 희석비율 $0.18\ h^{-1}$에서 7.2~7.5 g/*l*-h의 쏠비톨 productivity를 얻었다. 이는 glutaraldehyde로 처리하지 않고 alginate에 cell을 고정화하였을 때에 비해서 효소의 안정성이 아주 높은 것으로 나타났다.

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