한국생물공학회지 제 11권 제 1호
Korean J. Biotechnol. Bioeng.
Vol. 11. No. 1. 58-64(1996)

*Zymomonas mobilis*에 의한 Packed Bed Reactor를 이용한 연속적인 sorbitol의 형성

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Continuous Production of Sorbitol with *Zymomonas mobilis* in a Packed Bed Reactor

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ABSTRACT

κ-carrageenan에 고정화시킨 *Zymomonas mobilis*를 이용한 연속적인 sorbitol 생산에 대하여 연구하였다. Toluene과 glutaraldehyde로 처리하여 투과성을 증대시킨 세포를 alginate나 chitin 고정화 공정에서 어느 정도의 효소활성을 보였으나 210시간 이상의 공정반응에서는 sorbitol 생성량이 감소되었다. 독성이 적고 투과성을 증가시키는 물질로서 toluene 대신 CTAB(Cetyltrimethylammoniumbromide)를 사용하였다. CTAB로 투과성을 증가시킨 세포를 κ-carrageenan에 고정화하여 CSTR과 packed bed reactor를 이용하여 sorbitol 생산을 시도하였으며 CSTR보다도 (25일) packed bed reactor에서 (30일) 더 긴 시간 효소활성도가 유지되었다. Two-stage 연속공정에서는 희석비율이 (dilution rate, h^{-1}) 증가함에 따라서 sorbitol 생성률이 증가되었으며 희석비율 0.32 h^{-1}에서 첫번째와 두번째 반응조에서 각각 15g/ℓ-h, 22g/ℓ-h의 sorbitol 생성률을 얻었다. 0.32h^{-1}보다 높은 희석비율에서는 생성률이 감소되었다.

SUMMARY

The purpose of this study is to develop a continuous process for sorbitol production using *Zymomonas mobilis* immobilized in κ-carra-geenan. The glutaraldehyde cross-linking of toluene-treated cells immobilized in alginate or chitin showed high enzyme stability for long period. However, loss of enzyme activity was observed at 23% during 210h. In order to investigate the stability of glucose-fructose oxidoreductase of cethyltrimethylammoniumbromide (CTAB) treated cells, the long term continuous process was carried out with *Z. mobilis* immobilized in κ-carrageenan in the continuous stirred tank reactor(CSTR) and the packed bed reactor. The continuous production of sorbitol with the immobilized CTAB permeabilized cells in packed bed reactor was

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more stable than in CSTR. Two stage continuous process with CTAB treated cells of *Z. mobilis* immobilized in κ-carrageenan was carried out at various dilution rates. At the first stage, the productivity was increased up to 15 g/ℓ-h as dilution rate increased and decreased over $0.32h^{-1}$ of dilution rate. Similarly, maximum productivity obtained at the second stage was 22g/ℓ-h at $0.32h^{-1}$.

INTRODUCTION

The bacterium, *Zymomonas mobilis*, is able to convert glucose and fructose to gluconic acid and sorbitol simultaneously(1, 2). The enzyme complex, which is responsible for glucose oxidation and fructose reduction, has been isolated(3, 4) and described as glucose fructose oxidoreductase (GFOR) that is strongly bound to its cofactor, NADP(5, 6). This enzyme shows the highest activity in *Z. mobilis* when equimolar concentrations of glucose and fructose were used as substrates, and gluconic acid is produced. The gluconic acid is phosphorylated by gluconate kinase to produce ethanol via the Entner Doudoroff pathway(7). GFOR constitutes up to 1% of the soluble protein in *Z. mobilis* and is located in the periplasmic space(4). According to earlier observation, only permeabilized cells were to able to produce gluconate and sorbitol(8). Cells with unaltered membrane permeability would convert glucose and fructose to ethanol, CO_2 and some sorbitol via the Entner Doudoroff pathway. However, it was established that when permeabilizing cell membranes with proper concentration of toluene, cofactors like ATP, Mg^{++} or phosphate which are key compounds for fermentation of glucose and fructose could be removed through the cell membrane by washing while an enzyme such as GFOR required for conversion of glucose and fructose remains inside the cell. In the absence of these cofactors, it is unlikely that gluconate accumulated via 6-phosphogluconolactone would be further metabolized to ethanol via the Entner Doudoroff pathway. When cells of *Z. mobilis* were treated with toluene or cetyltrimethylammon-iumbromide(CTAB), glucose and fructose were converted to gluconic acid and sorbitol instead of undergoing further conversion to ethanol(8, 9, 10, 11). The use of whole cells containing an interesting alternate enzyme for the production of sorbitol and gluconic acid alleviates the problems involved in the isolation of enzyme and also reduces the cost. However, a major drawback in the use of whole cells is the poor permeability of the cell membrane to substrate and its stability. Thus, we have investigated the immobilization process with various materials such as alginate and κ-carrageenan for the long term process in view of commercial production of sorbitol. In this paper, we also show the effect of the several parameters such as dilution rate, recycle speed, and substrate concentration on the production of sorbitol using immobilized *Z. mobilis*.

MATERIALS AND METHODS

Chemicals

The Cetyltrimethylammoniumbromide(CTAB) was purchased from Fluka, Switzerland. The κ-carrageenan was purchased from the Korean Carrageenan Company. All other chemicals used were reagent grade.

Microorganisms and growth conditions

All experiments were performed with *Z. mobilis* ZM4(ATCC 31821). They were maintained and grown as described previously(8).

Preparation of permeabilized cells and glutaraldehyde cross linking

Cells were harvested in the late exponential phase after 20h after inoculation by centrifugation (4,000 rpm) prior to treatment with CTAB (0.2% v/v) at 4℃, pH 6.0~6.2. After gently stirring with CTAB for 10 min, cells were washed twice with saline buffer as reported previously(8). The yield of cells from the fermenter

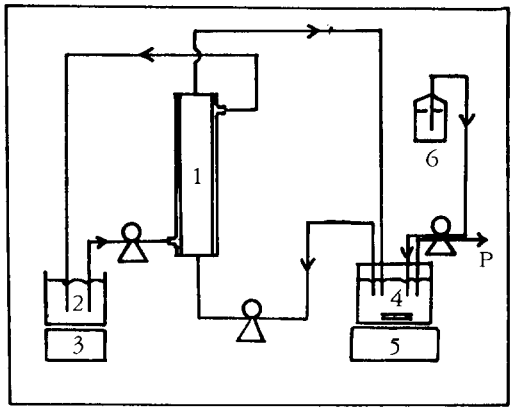

Fig. 1. Diagram of the experimental packed bed bioreactor for continuous production of sorbitol and gluconic acid.
1: packed bed column; 2: water reservoir for temperature control; 3: hot plate; 4: reservoir for pH control; 5: magnetic stirrer; 6: feed reservoir; p: product stream.

was approximately 2.0~3.0g(dry weight)/ℓ. Cross linking with glutaraldehyde involved suspending the permeabilized and washed cells in 0.3 % (v/v) glutaraldehyde in 0.1 M Kpi buffer(pH 6.2) and stirring at 4℃ for 10 min. The suspensions were centrifuged(4000 rpm) for 5 min and then washed twice with same buffer.

Procedure for cell immobilization

To evaluate sorbitol production, the immobilized cells with κ-carrageenan were prepared as follows. For immobilization with κ-carrageenan, CTAB permeabilized cells (12~13g, wet weight), which were also treated with glutaraldehyde before immobilization, were mixed with 100 mL of κ-carrageenan solution(3.2%, w/v). The κ-carrageenan beads were produced in 0.1 M Kpi buffer(pH 6.2) solution containing 20g/ℓ KCl and 0.15g/ℓ $CaCl_2$. Bead size ranged between 2.5 ~3.0 mm 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, the liquid volume was maintained at 102ml. The continuous process for sorbitol production was performed in a packed bed column reactor as shown in Fig. 1. The total working volume was 136ml : 41ml for the main column reactor and 95ml for the pH adjusting unit including connecting tubes. Dilution rates were determined by using the total working volume unless otherwise stated. The temperature and pH during the operation was maintained at 39℃ and 6.2, respectively. For the packed bed column, pH and temperature was controlled in the recycle vessel. Various concentrations of sugar solution (equimolar glucose and fructose) were used for the continuous reaction under the controlled recycle rate and dilution rate.

Analytical methods

Glucose, fructose, sorbitol and ethanol concentrations were determined by using a Waters Model HPLC at 85℃ with a BioRad(Richmond, Calif, U. S. A.) Aminex HPX-87C column(with flow rate of 0.6ml/min). The biomass concentrations for the free cells were determined as dry weights following oven drying at 104℃ for 24 hr. For the experiments with immobilized cells a known mass of cells was used.

RESULTS AND DISCUSSION

In the results discussed below, we examine the effect of κ-carrageenan immobilized *Z. mobilis* in both batch and continuous process and its effect on the activity and stability of Glucose-fructose oxidoreductase(GFOR). All runs were performed at 39℃ and the pH of reaction substrate was maintained between 6.0~6.2 unless stated otherwise.

Cell permeabilization

Various agents and methods for cell permeabilization have been investigated in order to de-

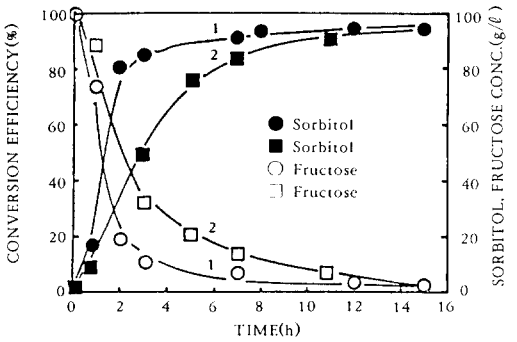

Fig. 2. Comparison of sorbitol conversion with free cells(1) and CTAB-treated immobilized cells in κ-carrageenan(2). The reaction was performed on 300ml sugar solution (100g/ℓ glucose+100 g/ℓ fructose) at 39℃, pH 6.2. The conversion efficiency was calculated from product formation by enzymatic conversion from fructose to sorbitol.

velop an adequate procedure for efficient sorbitol production. Earlier studies(8) show that CTAB appeared to be the best permeabilizing agent among the methods and agents investigated. This procedure obtained 60% conversion of fructose to sorbitol. Whereas untreated cells only convert 6% of fructose to sorbitol. The optimal concentration of CTAB for sorbitol production using *Z. mobilis* was 0.3%(w/v). Extended CTAB treatment led to decrease in conversion efficiency(8). The reduction in conversion efficiency was probably due to enzyme leakage through the permeabilized membrane of cells. In view of preventing enzyme loss from the permeabilized cell, cells were treated with glutaraldehyde prior to immobilization in κ-carrageenan, since it was reported that glutaraldehyde treated cells of *Saccharomyces carlsbergensis* were repeatedly used for the phosphorylation of glucose to fructose-1,6-diphosphate over 10 days(12).

Various concentrations of glutaraldehyde were investigated as a cross linking agent. The optimum concentration of glutaraldehyde as a cross linking agent was 0.2%. At this concentration, sorbitol conversion was 22.5g/ℓ after 14 h at 39℃ (Table 1). The pH for this experiment was not controlled. Due to glutaraldehyde toxicity, the glutaraldehyde treated cells were washed with saline buffer(pH 6.2) prior to immobilization with κ-carrageenan.

Table 1. The effect of glutaraldehyde concentrations on sorbitol formation. The productivity of the sorbitol production was calculated based on time of 14 h at 39℃, without pH control.

Kinetic parameters	Glutaraldehyde concentrations(%)				
	0.15	0.20	0.25	0.30	0.35
Sorbitol concentration (g/ℓ)	18.5	22.5	20.5	22.2	16.5
Conversion efficiency (%)	37	45	41	44	33

Fig. 2 shows sorbitol production with CTAB treated cells containing 100g/ℓ of each glucose and fructose. After 10 h of fermentation, both free and immobilized cells show almost 90% conversion efficiency of fructose to sorbitol indicating that κ-carrageenan is a suitable matrix to immobilize permeabilized cells. The specific rate for sorbitol production with immobilized cells was shown to be slightly lower than free cells. This indicated the presence of a mass transfer limitation in the κ−carrageenan bead. However, the maximum concentration of sorbitol was reached at 14h, as obtained for free cells. Other studies have also reported that κ-carrageenan is an adequate material for immobilization of microbial cells(11, 13).

Continuous process

Previous studies showed that the cross-linking of glutaraldehyde to toluene treated cells immobilized in alginate and chitin showed a higher enzyme stability for longer periods(8). However, the loss of enzyme activity was observed at 23% during 210h. This was probably due to the effect of residual toluene in cell lysis. In order to investigate the stability of GFOR in *Z. mobilis*, the long term continuous process was carried out with *Z. mobilis* immobilized in κ-carrageenan in the continuous stirred tank reactor(CSTR) and the

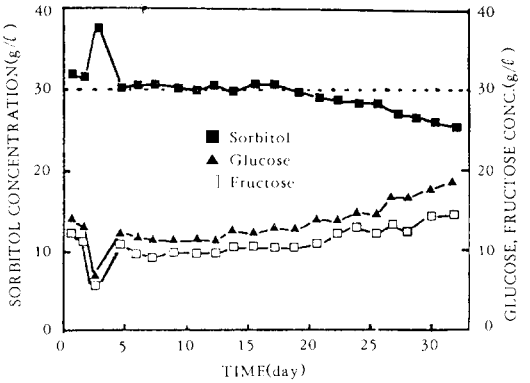

Fig. 3. Sorbitol production by immobilized CTAB-permeabilized cells of *Z. mobilis* in κ-carrageenan. Permeabilized cells were treated with glutaraldehyde prior to immobilization. Condition for continuous CSTR were T =39℃, pH 6.2 and dilution rate=0.2h^{-1}.

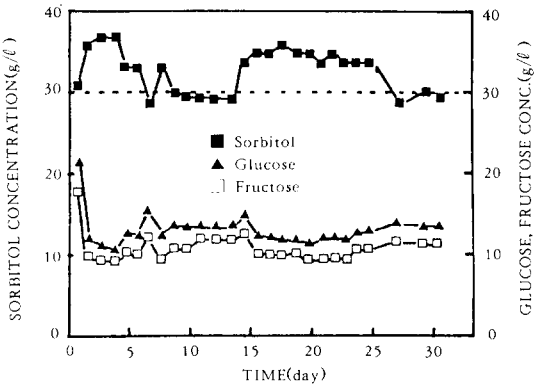

Fig. 4. Continuous production of sorbitol by κ-carrageenan immobilized CTAB-treated cells in the packed bed reactor at dilution rate of 0.2h^{-1}.

packed bed reactor. The conformation of the beads was maintained by the addition of 2N $CaCl_2$ to the input substrate solution. $CaCl_2$ has been commonly used by other authors to stabilize the alginate support(14). However, it is unknown whether $CaCl_2$ is likely to cause any inhibition of the activity of GFOR, although Ca^{2+} is reported to be important in maintaining activity of the enzyme(3). Fig. 3 and Fig. 4 represent a very stable operational activity that was maintained over a period of 30 days. Due to the mechanical shear impeller in the CSTR should affect in the beads rigidity as negative results. Therefore, reduction in enzyme activity was investigated in CSTR after 25 days(Fig. 3). The continuous process for sorbitol production using immobilized *Z. mobilis* showed the possibility of extended sorbitol production using κ-carrageenan gel and glutaraldehyde as the support material. The productivity of the continuous process at a fixed dilution rate of 0.2 h^{-1} was 6.51g/ℓ-h of sorbitol which was calculated on the based of total working volume (beads+liquid in both reactor and connector). Gluconic acid showed similar profiles to sorbitol in all experiments and are not shown here. Glucose and fructose exhibited a similar inverse trend to the production of sorbitol and Gluconic acid. The sorbitol conversion efficiency was usually slightly higher than that for gluconic acid due to the formation of a small amount of ethanol(3~4g/ℓ) via the Entner Doudoroff pathway as reported earlier(15).

Increasing sorbitol productivity in a continuous process by varying conditions

Sorbitol production in packed bed reactor using immobilized CTAB-permeabilized cells showed some of glucose and fructose remained unutilized, indicating that the conditions had been unfavorable to achieve full conversion(Fig. 3 and Fig. 4).

In order to increase the conversion efficiency in the packed bed reactor, the effect of recycle rate, initial substrate concentration, and two stage reactor were studied.

Effect of dilution rate on the productivity of sorbitol

The high conversion of fructose to sorbitol can be obtained at lower dilution rates. To improve productivity in the immobilized reactor system, various dilution rates were used in the two-stage continuous packed bed reactor. This involved collecting substrate and product the reactor. The productivity was increased with an increase dilu-

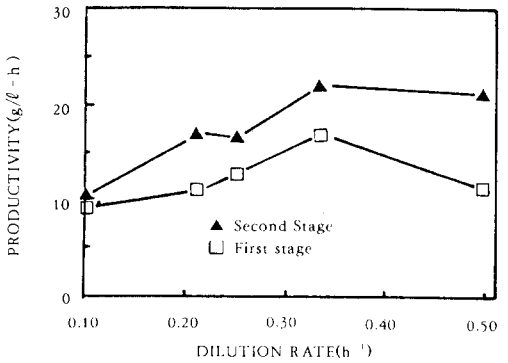

Fig. 5. Effect of dilution rate for production of sorbitol in a two stage continuous packed bed reactor(working volume: 260ml) at 39℃, pH 6.2 and recycle rate 1560ml/h. Cell loading was 8.2g for first stage and 5.8g for second stage, respectively.

tion rate until $0.32h^{-1}$ for both the first and second stages. Any further increase in dilution rate reduced the productivity of sorbitol(Fig. 5). It appeared that slightly higher productivity was obtained with two-stage process when compared to the single reactor process. The maximal productivities obtained were 17g/ℓ-h (at first stage) and 22g/ℓ-h (second stage) at a recycle speed of 1950ml/h, although trace of unutilized substrate remained in the reactor. Cell loading was 6g.

Effect of recycle speed on the productivity

As summarized in Table 2, the conversion efficiency for sorbitol with immobilized *Z. mobilis* was increased as the recycle rates increase. When recycle speed was increased from 432 to 1950ml/h, the productivity was double. The higher recycle speed led to a higher conversion efficiency. This is due to the increase in the supply of reactants and the removal of product from the reactor. The difference in pH between input and output in the column was caused byproduction of gluconic acid from glucose, and was more than pH unit of 0.7 at the 432ml/h of recycle speed, and difference was reduced as increase recycle speed(data not shown).

Table 2. Production of sorbitol with various recycle speed in packed bed continuous process. Continuous process was performed using κ-carrageenan beads on 100g/ℓ each of glucose and fructose at 39℃, pH 6.2 and dilution rate of 0.1 h^{-1}.

Parameters	Recycle speed (ml/h)			
	432	1000	1200	1950
Productivity(g/ℓ-h)	4.46	7.6	7.4	8.05
Conversion efficiency(%)	44.6	76	74	80.5
$q_{p\,sorbitol}$(g/g-h)	0.124	0.211	0.206	0.237
Biomass concentration (g/L)	36	36	36	36

[a] $q_{p\,sorbitol}$ = dilution rate × ($dP_{sorbitol}/dX_{cell}$)

Table 3. Conversion of glucose and fructose to gluconic acid and sorbitol in a two-stage continuous packed bed reactor. Cell loading in the first was 16.4 and second stage was 9.4g/ℓ. Recycle speed was 1500ml/h.

Substrate conc.(g/ℓ) (glucose+fructose)	Dilution rate(h^{-1})	Stage 1		Stage 2	
		Conversion(%)	Productivity(g/ℓ-h)	Conversion(%)	Productivity(g/ℓ-h)
100+100	0.11	85.4	9.4	87.6	9.6
200+200	0.13	78.2	20.3	82.6	21.3
300+300	0.10	73.0	21.5	78.5	23.1

Effect of substrate concentration on the productivity of sorbitol

In order to improve the production of sorbitol, varing of sorbitol concentrations of fructose were analysed. At the lower fructose concentration (100g/ℓ), higher conversion efficiency was obtained. However, the conversion efficiency was reduced by 12% when the fructose concentration was increased from 100 to 300g/ℓ (Table 3). It

has been reported that a conversion approaching 100% would be difficult to obtain from a continuous process because the K_m fructose value for the GFOR is quite high (250g/ℓ)(9). Zachariou and Scopes(3) also showed that at a sorbitol concentration of 0.8 M (144g/ℓ) the GFOR was inhibited by 27%.

From this data, it is evident that κ-carrageenan was successfully applied to produce sorbitol and gluconic acid by using CTAB-permeabilized *Z. mobilis*, since little mass limitation was observed during the reaction. Further experiments revealed that the enzyme activity was maintained for 30 days without decrease of activity in the continuous process. Recently, we have observed that further stability of GFOR in the continuous system can be achieved by drying the κ-carrageenan bead or with modifying the κ-carrageenan bead by using agents such as polyols (in preparation).

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