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Studies on Whole Cell Immobilized Glucose Isomerase

II. Operational Studies on the Batchwise and Continuous Isomerization of *D*-Glucose

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포도당 이성화 효소의 세포 고정화에 관한 연구

제 2 보 : 회분식 및 연속 반응조를 사용한 포도당의 이성화

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Abstract

Using the whole cell immobilized glucose isomerase which was prepared in the previous work (Korean J. Food Sci. & Technol., 11(3), 192 (1979), the specific activity of the immobilized enzyme was 48.1 units in the batch reaction system and 114 units in the continuous reaction system per *g* of matrix, respectively.

In the continuous reactor the voidity was 0.36, which was suitable for the packed bed reactor. This immobilized enzyme showed a good operational stability of 115 days of half life which was sufficient for the continuous operation. The experimental result showed that 55 % of the substrate was converted to the product in the packed bed reactor. The productivity was dependent on the flow rate, column geometry, enzyme loading, and substrate concentration.

An intrapaticle diffusion was observed by the effectiveness factor of 0.75 and interparticle diffusion by the decrease of Km' with increasing the superficial velocity.

Introduction

In the previous work[1], we have obtained a mechanically stable form of whole cell glucose isomerase (hereafter called GI) of *Streptomyces K*-45 by a series of treatments : heat treatment, carefully manipulated drying, extrusion with a thickening agent, and glutaraldehyde-induced crosslinking.

Using this whole cell immobilized GI, two types of reactor are adapted for the laboratory scale performance of *D*-glucose isomerization to examine the kinetic behaviors of the immobilized enzyme. A continuous stirred tank and plug flow type are used as the batchwise and continuous reaction systems, respectively.

Various kinds of reactor configurations were used by many investigators for the enzymatic isomeriza-

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tion of *D*-glucose[2~5]. Basically, the configurations included the batchwise or continuous stirring tank reactors and continuous packed bed reactors. Lilly and Dunnill classified the types of enzyme reactor according to the mode of operation, catalyst retention and flow characteristics[6] and compared the reaction properties of the batchwise and continuous reactors[7]. Havewala and Pitcher expressed their opinions that the continuous stirring tank reactor was less efficient than the plug flow reactor for the pseudo-first order reversible kinetics and the labor costs were higher for the batch reactor[8]. Although the packed beds suffer from some operational disadvantages, it has been most frequently used as an enzyme reactor with immobilized enzymes[9,10] or immobilized whole cells[11,12], especially for the particulated forms[13].

In this communication, therefore, we chose two typical reactors and we reported the operational stability, the space time dependency of substrate conversion and productivity, and apparent Michaelis-Menten constants at different superficial velocity of the whole cell immobilized GI which we prepared in the previous work.

Materials and Methods

Materials

The whole cell immobilized enzyme was prepared by the method described in the previous work[1]. Other chemicals used were also presented in the previous work. The amount of fructose produced was determined by the cysteine-carbazole method [14]. One unit of glucose isomerase activity was defined as the amount of enzyme which catalyzes the production of 1 *μmole* of fructose for 1 min under the condition described below.

Batch reactor

The exact amount of the immobilized GI beween 20 to 50 mg was swollen for 3 hr and centrifuged to remove the supernatant. The enzyme was incubated in 10 *ml* of incubation mixture containing 5 *ml* of 0.1 *M* phosphate buffer, pH 7.2, 1.0 *ml* of 0.05 *M* $MgSO_4$, 1.0 *ml* of various concentrations of *D*-glucose, and 3.0 *ml* of distilled water. Before the enzyme added, the mixture was preincubated at 65°C for 10 min and the whole reaction mixture was incubated for 1 hr. During the reaction, the mixture was well stirred with a submerged magnetic stirrer to minimize the diffusional limitation. After the incubation was completed, the reaction was stopped by adding 10 *ml* of 0.5 *N* perchloric acid solution and the enzyme was removed by centrifugation at 3,000 r.p.m. for 15 min. The concentration of fructose in the supernatant was determined by the cysteine-carbazole method and the glucose was determined with Glucostat[15]. To determine the effect of internal diffusional limitation, the pellet form of the enzyme was carefully ground in a cold mortar into powder. With 100 mg of this powder the enzyme activity was assayed at the above condition, except of relatively short time for 10 min in 100 *ml* of the reaction mixture with vigorous agitation to eliminate the external diffusional resistance. As comparison, the same procedure was carried out with a commercial enzyme of Novo Corp. (Sweetzyme, S. type). For large scale production of fructose, 4 *g* of the immobilized GI was used in 100 *ml* of reaction mixture.

Continuous reactor

The enzyme was swollen for 3 hr and packed in a column attached a preheating and cooling unit.

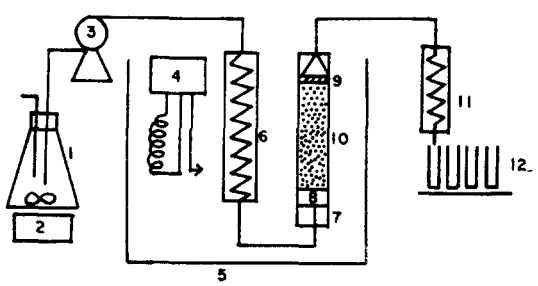

Fig. 1. Packed bed reactor containing the whole cell immobilized glucose isomerase

1. Substrate reservoir
2. Magnetic stirrer
3. Poystaltic pump
4. Thermostat
5. Water bath
6. Preheating unit
7. Rubber stopper
8. Sponge support
9. Glass filter
10. Immobilized GI
11. Cooling unit
12. Product collecting device

The temperature was maintained at 65±0.5°C and substrate solution was pumped upward with Büchler polystaltic pump. All the substrate solutions contained 5 *mM* Mg^{+} as an activactor and pH was adjusted to 8.0. A fraction 10 *ml* was collected with an ISCO fraction collector and each fraction was analyzed. The detailed configuration of the continuous reactor is shown in Fig. 1.

Flow characteristics of packed bed reactor

To examine the flow characteristics and the voidage of the reactor, residence time distribution was measured by an impulse concentration technique[16] which characterizes dead space, by-passing, mixing and channelling phenomena in the packed bed reactor. Ten *μl* of 0.3 % of *D,L*-phenylalanine, as a tracer, was suddenly injected at the inlet of the column and the absorbancy of the effluent at the outlet was read at 280 nm, while distilled H_2O was continuously pumped through the column at a flow rate of 2 *ml*/min and 0.84 *ml*/min for the columns of 1.53×12.2 and 1.53×5.8 cm, respectively.

Specific activity

The specific activity was determined with the column reactor as the amount of steady state conversion. One *M* of substrate was passed throuh a short column of 1.53×2 cm, packed with 1 *g* of the immobilized GI. The amount of fructose produced in the effluent was measured by the cysteine-carbazole method.

Operational stability

Ten *g* of the preswollen enzyme was packed in 1.53×12.6 cm column and 2.5 *M* of *D*-glucose was pumped upward at a flow rate of 2.1 *ml*/hr (τ=5.3hr) for 220 hr. The enzyme activity was determined at every 10 hr to see the deactivation during the operation of the continuous reactor.

Apparent K_m

Four columns of different ratio of column length to diameter (L/D) were prepared and the flow rate was adjusted to have the same space time of 2 min which results in the different superficial velocity. To each column, the substrate solution used were 0.1, 0.5, 1, and 2.5 *M*. The reaction was operated at the condition of the continuous reactor described. The apparent Michaelis-Menten constants (K'_m) were determined by plotting S_0X *vs ln* X from the equation ; $S_0X=K'_m\ ln\ (1-X)+V'_m\ L/V_s$, where S_0 is the initial substrate concentration, X is the mole fraction of conversion, $(S_0-S)/S_0$, K'_m is the apparent Michaelis-Menten constant, V'_m is the apparent maximum reaction rate, V_s is the superficial velocity in column reactor, and L is the length of column reactor, according to the method of Havewala and Pitcher[8].

Effect of the enzyme and substrate concentrations on the productivity

At a constant space time the effects of different enzyme loading at the various substrate concentrations on the productivity and the steady state conversion were observed. The immobilized GI of 10, 7.2, and 4.7 *g* was packed in the column of which the length was 12.2, 8.9, and 5.8 cm with the same diameter of 1.53 cm, respectively. The flow rate of each column was adjusted to have the same space time of 2 min. The substrate concentrations used were 0.05 to 1 *M*.

Results

Batch reactor

The whole cell immobilized GI which was prepared by the method reported in the previous work[1] showed an equilibrium conversion of *D*-glucose to fructose at various substrate con centrations in the batch reaction system. The time to reach the equilibrium conversion was dependent on the substrate concentration and the observed one was 0.92 (48 % conversion). Above 60 % of substrate concentration there was no increase in the conversion. The result was illustrated in Fig. 2. In this batch reactor the specific activity of the whole cell immobilized GI was 48 units/g.

It was suggested that the internal diffusional limitation might be more significant than the external one in batch reactor and in continuous stirred tank reactor. This can be expressed quantatively by the effectiveness factor (η), which is defined as the ratio of actual reaction rate to one of no internal diffusion limitation[17]. Effectiveness factor was obtained in this reaction system for the

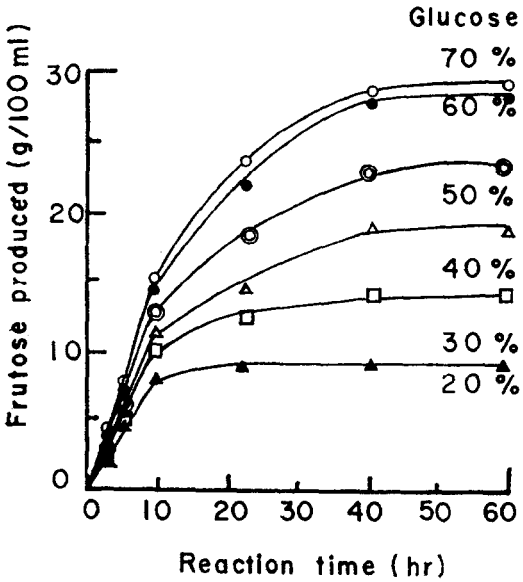

Fig. 2. Equilibrium conversion of substrate by the whole cell immobilized GI in the batch reactor
E_o. Amount of the immobilized enzyme: 4 g

Table 1. Effect of internal diffusional limitation of the whole cell immobilized glucose isomerase

	Enzyme activity (Unit/g of immobilized GI)	
	Immobilized GI[1]	Sweetzyme of Novo[2]
Pellet from (E_1)[3]	41.9	72.9
Powderized from (E_2)[4]	55.9	75.9
Effectiveness factor (E_1/E_2)	0.75	0.96

[1] Glucose isomerase
[2] Novo Corp. product
[3] The whole cell immobilized GI preparation which was prepared in the previous work(Ref. 1)
[4] The pellet form of GI was powderized as described in the text

internal diffusion-limited reaction by eliminating the external one with vigorous stirring. For this, the immobilized whole cell pellets were powderized in cooled mortar and the enzyme activities of the original pellet form and the powderized form were determined in relatively short time of about 10min. The effectiveness factor was obtained from $\eta = E_1/E_2$, where E_2 was the activity of the powderized form and E_1 was one of the pellet forms.

Table 1 shows the effect of internal diffusional limitation. For comparison, same procedure was carried out with a commercial enzyme preparation from Novo Corp. (Sweetzyme, S type). This result indicated that the whole cell immobilized GI showed some internal diffusional drawback ($\eta = 0.75$) to compare with Sweetzyme ($\eta = 0.96$).

Continuous reactor

Continuous reactor for glucose isomerization was designed with column reactor packed with the preswollen whole cell pellet as shown in Fig. 1. To examine packing state and flow of the packed bed reactor the distribution mode of residence time was observed by impulse concentration technique to characterize presence of dead space, by-passing and channeling phenomena. This was very similar to the typical "E-curve" with a rather short tail, which suggested a good packing property with little mixing but no by-passing or channeling phenomena[18]. When packing density was 450 g/l, voidity of the packed bed reactor was calculated to 0.36 from $\varepsilon = V_o/V_b$, $V_o = Q \cdot \bar{t}$, where ε is voidity of the packed bed, V_o void volume, V_b bed volum, Q volume flow rate, and $\bar{t}$ mean residence time which was obtained in this experiment.

Specific activity of the immobilized enzyme in column reactor is defined as amount of product formed per unit time with unit amount of immobilized enzyme. However, this varies as the flow rate, substrate concentration, packing density and geometry of reactor. The experimental conditions

Table 2. Specific activity of the whole cell immobilized glucose isomerase in the packed bed reactor

Flow rate (ml/min)	Space time (min)	Specific activity (unit/g)
5	0.150	108.2
6	0.110	109.3
7	0.094	121.3
10	0.066	116.2

Average specific activity : 114.0
Colum size : 1.53×2 cm
Amount of the immobilized GI : 1 g

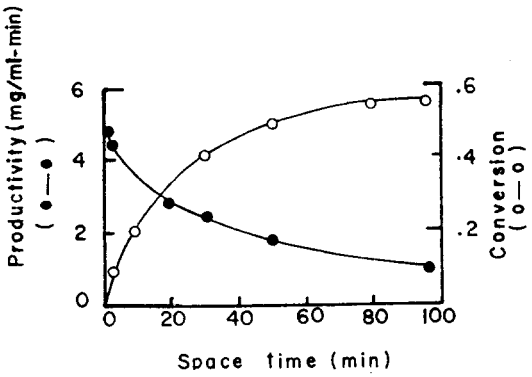

Fig. 3. Effect of space time on productivity and steady state conversion in the packed bd reactor

E_o: Amount of the immobilized GI, 10 *g*
S_o: Substrate concentration, 1 *M*
Columtn size: 1.53×12.59 cm

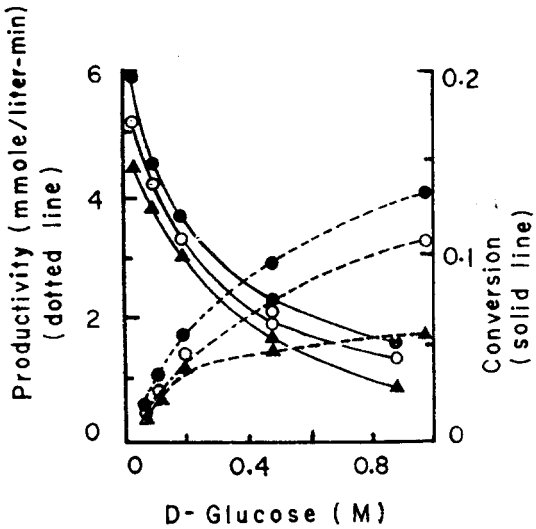

Fig. 4. Effect of substrate concentration and enzyme loading on seady state conversion and productivity

E_o, Amount of the immobilized GI
● ● ● : 10 *g*
○ ○ ○ : 7.2 *g*
▲ ▲ ▲ : 4.7 *g*
τ, Space time : 2 min

are specified in Table 2 and specific activity was calculated from PQ/E_0 to yield average value of 114 units/*g*, where P was the concentration of product, Q flow rate and E_0 amount of the immobilized GI.

Space time dependent conversion

Steady state conversion in the continuous reactor was observed in a 1.53×12.19 cm column which was packed with 10 *g* of the whole cell immobilized GI with 1 *M* *D*-glucose solution. Productivity was calculated by an equation[16], $\pi=S_0 \cdot X/\tau$, where π is productivity, S_0 initial substrate concentration, X fraction of substrate conversion (P/S_0), and τ the space time. Fig. 3 shows effect of space time on productivity and steady state conversion by the immobilized GI. As results, equilibrium conversion was observed with space time of above 60 min and 55 % of the initial *D*-glucose solution was converted to fructose when holding time was over 80 min.

Enzyme loading effect

To know effects of enzyme loading on productivity, 3 reactors packed with different amounts of enzyme were operated at various substrate concentrations. As shown in Fig. 4, productivity was more dependent on enzyme loading at higher substrate concentration.

Table 3. Flow conditions of different column reactors

Column	Length / diameter	Enzyme amount (*g*)	Superficial velocty (cm/min)	K'_m (*M*)
1	8.0	10.0	6.74	0.234
2	3.8	4.8	3.20	0.254
3	2.8	3.5	2.14	0.261
4	1.5	1.9	1.14	0.281

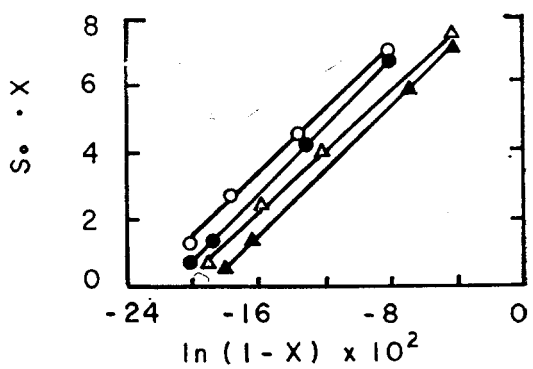

Fig. 5. Determination of K'_m of various column reactors

□□□ : Column 1 ○○○ : Column 2
▲▲▲ : Column 3 ●●● : Column 4
X: Conversion of *D*-glucose to fructose
S_o: *D*-glucose concentration (*M*)

Effect of superficial velocity on apparent *Km'*

The whole cell immobilized GI preparation showed internal diffusion controlled reaction rate as in Table 1. It was interesting, therefore, to examine effects of external diffusion resistance on overall reaction rate in the packed bed reactor. The column geometry and other flow conditions are described in Table 3. Apparent Michaelis constants (*Km'*) for the forward reaction rate were obtained from slope of plotting integrated from of Michaelis equation as was in Fig. 5.

Different superficial velocity was given by changing the ratio of length to diameter (*L/D*) of the column reactor. As shown in Fig. 6, reaction rate was depended on the external diffusion. Apparent *Km'* value decreased as superficial velocity increased.

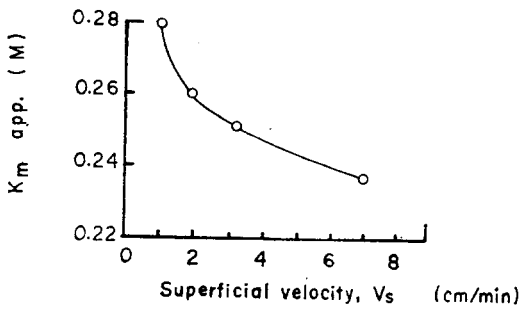

Fig. 6. **Effect of superficial velocity on the apparent K_m**

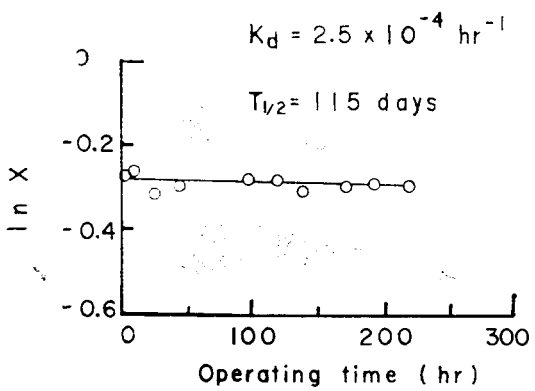

Fig. 7. **Operational stability of the whole cell immobilized GI in the packed bed reactor**
E_o: Amount of the immobilized GI, 10 *g*
S_o: Substrate concentration, 2.5 *M*
X: Conversion of *D*-glucose to fructose
τ: Space time, 5.3 hr
Column size : 1.53×12.59 cm

In column 1 which had a relatively large superficial velocity of 6.74 cm/min, apparent *Km'* value was contiguous to that of batch reactor, which indicates that there is minimum external diffusional limitation.

Operational stability

Deactivation of the whole cell immobilized GI during the reaction is unavoidable and operational stability of the whole cell immobilized GI is one of the most critical factors in continuous reactor in view point of cost of enzyme and productivity. In this study time course of inactivation of the whole cell immobliized GI showed a straight line, which obeyed first order decay mode as shown in Fig. 7. In the first order kinetics time course decrease of enzyme concentration or residual enzyme activity is represented as : $-d(E)/dt=K_d(E)$. The decay constant, K_d was determined to be 2.5×10^{-4} hr from the plot *ln X vs* operating time, *t*.

Half life of the enzyme activity was about 115 days at the given condition and this suggested availability of the whole cell immobilized GI in continuous operation.

Discussion

Use of immobilized enzymes in industrial processes depends on many factors. Ability to produce, by simple means, a relatively low cost immobilized enzyme product with high activity per weight of support, an adequate operational stability, and efficient use of that product, are important technical considerations. In the previous work[1], we prepared a mechanically stable form of whole cell GI. Uuing this one, we have examined the laboratory scale performance of *D*-glucose isomerization with two types of reactors, batchwise and plug flow type reactors.

In batch reactor, the specific activity was 48 units per *g* and the observed equilibrium conversion was about 48 %. The equilibrium conversion reported by Takasaki[2], was 53.5 % and 56.5 % at 60°C and 70°C, respectively. Takasaki's ones were determined using dilute sugar solution (about 0.55 *mM*). Havewala and Pitcher[8] also reported it 51 %. Their

experiment was carried out using relatively concentrated glucose solution (2.0 *M*). It is important to improve the degree of equilibrium conversion which is dependent upon the microbial enzyme source. But time to reach the equilibrium conversion should be considered together in the practical application. Many works have improved the equilibrium conversion of glucose isomerization. Takasaki[19] has demonstrated that 88 % of glucose present at an initial concentration of 1.0 *M* glucose was converted to fructose at around pH 7.5 in the presence of 0.3 *M* sodium tetraborate.

In the continuous reactor, specific activity was depended on flow rate, amount of enzyme loaded, substrate concentration, and column geometry. Those must be specified in determination of specific activity of immobilized enzyme in column reactor. Novo Corp. [20] uses IGIC unit(Immobilized Glucose Isomerase Column) as amounts of enzyme converting glucose to fructose with initial rate of 1 *μmole* per minute in a 2.5×40 cm column reactor at 65°C with 40 %(w/w) of glucose solution passed through at a flow rate of 80 *g* per hour. The experimental conditions were in Table 2 and specific activity was the average value of 114 units/*g*. Specific activity of immobilized enzyme is important in view point of immobilization cost and efficiency of reactor operation, which may be largely dependent on immobilization process itself and enzymatic property of microbial source. Many works have studied to improve activity of immobilized enzyme. Lloyd, *et al.*[21] reported 110 units per gram of dried cell-fixed isomerase of *S. spp.* (ATCC 21175) at 60°C in continuous packed bed reactor and Dworschack *et al.* reported the activity of 200 units per gram of the same microbial species[22]. Vieth *et al.* demonstrated the continuous packed bed reactor with spiral wound collagen membrane which entrapped the whole cell to have 12.1 units per gram of complex at 70°C[4].

Table 4 shows compared results of the batch and packed bed reactor performance with the whole cell immobilized GI. As results the continuous packed bed reactor was preferred to yield higher productivity and substrate conversion.

Table 4. Comparison of the batch and backed bed reactor performance

Reactor conditions	Batch	Packed bed
Reactor volume, V_o (cm^3)	100	8.3
Amount of enzyme, E_o (*g*)	4	10
Substrate concentration, S_o (%)	20	18
Reaction parameters		
Specific activity (unit/*g*)	48	114
Equilibrium conversion (%)	48	55
Space time to reach the equilibrium conversion, τ (hr)	10.5	1.3
Productivity,* π (mg/*ml*-min)	0.15	1.40

* Productivity was calculated for the minimum space time to reach the equilibrium conversion state

In the practical process, the catalyst packing density (enzyme loading), diffusional restriction and operational stability are also important factors affecting productivity. The immobilized GI prepared in this work showed good voidity of 0.36 which is suitable for packed bed reactor[18], in spite of relatively high packing density of 450 *g* per liter. As possible as high enzyme loading is recommended in case of low cost immobilized enzyme, if problems of mass transfer and pressure drop across the packed bed can be avoided. As shown in Fig. 4, productivity increased as the enzyme loading increased in high substrate concentration which is necessary in practical operation.

In application of immobilized enzyme, mass transfer should be considered, especially in case of whole cell immobilization. Mass transfer effects on oberved kinetic behavior for the immobilized enzyme may be divided into two categories : (a) intraparticle diffusive effects and, (b) influences of external mass transfer. The observed kinetics of immobilized enzyme can depend significantly on reactor operating conditions such as flow velocity or agitation rate. As shown in Table 1, we still observed the intraparticle one. This may be caused by tight cross linking by glutaraldehyde. To solve this problem, further studies are required to improve spinning apparatus for immobilization, as suggested in the previous work[1].

We also examined the external mass transfer barrier between the surface of whole cell particulate and liquid layer of substrate from the dependency of apparent *Km* value on the superficial velocity.

Deactivation of enzyme activity during the operation may result from the physical loss of enzyme molecules from the system, pore blockage or channeling, inactivation of the functional group by oxidation, and mainly from the thermal deactivation of enzymes retained in the system. The thermal deactivation of glucose isomerase was known to show two stage decrease. Havewala and Pitcher[8] reported half life of GI immobilized to zircornia coated glass to be about 24 days and 120 days for first stage and second stage activity decrease, respectively. Operational stability can be varied by reaction conditions, especially operating temperature and space time. The immobilized GI prepared by us showed good operational stability of half life 115 days with the space time of 5.3 hr at 65°C which is sufficient for the continuous operation.

In this communication, we studied the basic properties and the possibility of practical application of the whole cell immobilized GI which was prepared in the previous work[1]. More detailed and scaled-up studies are recommended to set up the optimal operating conditions for the practical application of this product. These include the optimal flow condition, length to diameter ratio and the period of operation with respect to the price of enzyme and cost of operation.

요 약

전보에서 저자들을 물리적 견고성이 우수한 포도당 이성화 효소를 세포 고정화 시킨 제품을 얻었다.(한국 식품 과학 회지, **11** (No. 3), 192 (1979). 이 효소 제품을 사용하여 실험실 조건에서 회분식 반응조와 충진식 반응조를 운영하여 효소의 반응 특성과 생산성, 활성 감소 현상을 비교 하였다.

본 고정화 효소의 비활성 역가는 회분식 반응조와 충진식 반응조에서 1 *g*당 각각 48 및 114 units였으며 연속적인 공정에서 더 높은 생산성과 이성화율을 보였다. 효소의 생산성은 체장 시간, 기질의 농도, 효소 부하율(附荷率) 및 반응조의 의형에 영향을 받았으며 충진 밀도가 450 *g/l*일 때 실제 공간율은 0.36이었으며 비교적 좋은 충진 현상을 보였다.

연속 공정중 효소의 활성 감퇴 현상을 고찰하기 위하여 2.5 *M* 포도당 용액을 체장 시간이 5.3시간이 되도록 약 220시간 동안 반응시켜 본 결과, 효소 활성 감퇴 곡선은 일차 반응을 따르며 활성 반감기는 115일로 연속 공정에 이용 가능함을 알았다.

이 고정화 효소 제품은 물리적 안정성이 높은 반면 물질 전달 계수가 반응 속도에 큰 영향을 미치는 것으로 나타났다.

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