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Immobilization of Xylose Isomerase and Trial Production of High Fructose Corn Syrup

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Xylose 이성화 효소의 고정화 및 이성화당의 생산

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요 약

본 연구에서는 저자등이 분리한 바 있는 *Streptomyces griseolus*가 분비한 xylose 이성화 효소(D-xylose ketol isomerase, EC 5.3.1.5, 포도당 이성화 효소)의 고정화 및 이 고정화 효소에 의한 파일롯트 규모의 이성화당 생산에의 응용을 시도하였다.

세포내 분비된 Xylose 이성화 효소를 함유한 미생물 균체를 호모 게나이저로 500kg/cm² 압력하에 파쇄한 결과 원 효소 역가의 98.8%가 얻어졌고, lysozyme으로 분해 했을 때는 54.7%가 얻어졌다.

이 효소의 고정화를 위한 담체로서는 Diaion HP 20, Duolite A-7, Amberlite IRA 93 및 94와 같은 포리스형 수지류가 효과적임이 밝혀졌고, Amberlite IRA 93에 대한 재생 형태는 BO_4^{--}가, Diaion HP 20에 대해서는 HCO_3^-가 효과적이었다.

Amberlite IRA 93에 대한 고정화 최적 조건은 pH 8.0에 55°C로서 80.6%의 효소 고정화 수율을 나타내었으며 효소 활성 반감기는 65°C에서 24일 이상이었다. Amberlite IRA 93에 효소를 脫着 실험한 결과, 담체는 재 사용이 가능 하였으며 2, 3, 4, 5회째의 효소의 재 고정화율은 각각 98.2, 93.3, 90.7 및 87.5%를 나타내었다.

고정화 효소의 최적 반응 온도는 60~75°C로 원 효소의 경우에 비하여 다소 낮아지면서 폭이 넓어졌으며, 최적 pH는 8.0~8.3으로 알카리 쪽으로 이동하였다.

파일롯트 규모로 본 고정화 효소 충전탑(내경 30cm, 높이 85cm)에 의한 이성화당의 생산을 시도하였던 바, 고정화 효소(350 IXIU/ml-R) 1리터가 30일 동안에 약 293리터의 이성화당을 생산할 수 있는 것으로 나타났다.

Introduction

The bioreactor column packed with immobilized D-xylose ketol isomerase(glucose isomerase) and/or enzyme inducing cellmass has been widely applied in isomerization process of D-glucose to D-fructose. Increased attention has been directed toward whole cell immobilization[1~5] due to its reported advantages[6], compared to immobilized enzyme process. For example, whole cell immobilization process reguires neither isolation nor

purification so that high yield of enzyme can be obtained; enzyme stability may be generally higher in whole cells; and it is possible to achieve sequential reactions using a whole enzyme system coexisting in the cell, etc.

But dispite the above suggested merits the process still has some limitations. The cellmass may contain other enzymes that catalyze the unwanted side reactions and it is difficult to maintatain the integrity of cell; cell walls and membranes offer permeability and diffusion barriers.

As is well-know, there are three typical immobilization technics such as carrier bonding, cross linking and entrapping. As Okada[7] pointed out, ionic bonding method belonging to carrier bonding has many merits in immobilizing the enzyme onto the carrier on a commerical base, due to easy manufacturing process, resuable carrier, reduced production cost, etc. The carrier should be satisfied to meet the above requirements in terms of particle size, wide surface[8,9] area, high hydrophillic radicals, proper chemical components, etc. According to Yoshimura et al[10], relationship of enzyme adsorption and activity reactions depend on (1) internal pore volume and numbers of internal ion exchanging group, (2) glucose isomerase can be adsorbed into the a certain size of macropore, (3) the correct size of macropore, (4) ionic exchange capacity proportional to that of total space area of the specific macropore mentioned above, and additionally, anion exchange resins have higher ion exchange capacity and are more stable than cation exchange resins.

The following resins can be considered testworthy; Amberlin IRA type(Rohm & Hass 904, 938, 400, 93, 94), phenol-formaldehyde type(Diamond Sharmrock Duolite A-7 etc.), high prorous and/or chelate type(Mitsubishi Kasei PA-308, PA304, WA30 etc.).

In this experiment, some kinds of macro porous ion exchange resins were tested for immobilization of xylose isomerase from *Streptomyces griseolus* isolated by the authors[11].

Materials and Methods

The following glucose syrups for activity decay test were donated by Miwon Co., Ltd.; Glucose syrup(50% dry substance), hydrol(D.E. 93% pH 4~5, colorness 0.15), starch acid hydrolysate D.E. 65). Chemical reagnts were extra pure grade and ion exchange resins were chemical grade(Table 1).

Culture of microorganism

Streptomyces griseolus was inoculated and transferred to 7k*l* fermentor through the seeding

Table 1. Ion exchange resins for immobilization of xylose isomerase.

Resin	Resin form	Surface area (m^2/g-R)	Pore volume (m*l*/g)	I.E.C.* (meg/g)	Kind of resin**	
Diaion SA 20A	Cl^-			>1.3	SB Ⅱ (gel)	Mitsubishi Chemical Co.
Diaion PA 406	Cl^-			>0.7	SB Ⅱ (porous)	Mitsubishi Chemical Co.
Diaion HP 20		718	1.16		HP(MR)	Mitsubishi Chemical Co.
Duolite A-1	OH				SB	Diamond Sharmrock Co.
Doulite A-7	OH^-			2.4	SB(porous)	Diamond Sharmrock Co.
Dowex 2(SAR)					SB	Dow Chemical Co.
Amberlite IRA 400	SO^{4--}			1.4	SB(gel)	Rohm & Hass Co.
Amberlite IRA 904	SO_4^{--}	43.6	0.73	0.7	SB(porous)	Rohm & Hass Co.
Amberlite IRA 93	SO_4^{--}	23.1	0.88	1.25	SB(porous)	Rohm & Hass Co.

* I.E.C.: Ion exchange capacity
** SB: Strong base, HP: High porous

Seeding lines	Media volum	Inoculum(%)	Sterilization conditions	Cultural conditions
STOCK SLANT	—	One platinum wire	120°C, 15min.	30°C, pH 6.8~7.0 7~10 days
↓				
ACTIVE SLANT	—	One platinum wire	120°C, 15min.	30°C, pH 6.8~7.0 7~10 days
↓				
FLASK SEED I	80m*l*/500m*l* F	One platinum wire	115°C, 20min.	30°C, pH 7.0 24 days
↓				
FLASK SEED II	80m*l*/500m*l* F	10	115°C, 20min.	30°C, pH 7.0 24 days
↓				
JAR SEED I	4*l*/10*l*	5	115°C, 20min.	31±2°C, pH 6.6~7.0 0.3kg/cm², 0.5uum 230rpm, 18~20hrs.
↓				
JAR SEED II	20*l*/50*l*	1.5	115°C, 20min.	31±2°C, pH 6.7~7.0 0.3kg/cm², 0.5uum 180rpm, 15~18hrs.
↓				
JAR SEED III	250*l*/500*l*	5.0	115°C, 20min.	31±2°C, pH 6.6~7.0 0.3kg/cm², 0.5uum 130rpm, 133~15hrs.
↓				
MAIN CULTURE	3K*l*/7K*l*	7.5	115°C, 20min.	30±2°C, pH 6.8~8.0 0.3kg/cm², 0.5uum 120rpm.

Fig. 1. Seeding line and cultural conditions for 7k*l* fermentor.

lines shown in Fig. 1.

Preparation of crude enzyme solution

The culture broth was heat-stabilized as reported by Takasaki[12] and then filtered. Wet cell mass(870 XIU/g) were measured, crushed and filtered to remove cell debris.

1) Extraction by mixer: 62 gram of wet cell was suspended with 125m*l* of 0.02M phosphoric acid buffer(pH 7.8) in the mixer for five minutes and filtered.

2) Extraction by lysozyme: 62 gram of wet cell was suspended with 125m*l* of 0.02M-phosphoric acid buffer(pH 7.8) and 0.01% of lysozyme(Taipyungyang Chemical Co.) and incubated at 55°C for 2 hours and filtered.

3) Extraction by glass flake: 62 gram of wet cell was suspended with 5 gram of glass flake on the mortar and crushed vigorously for ten minutes. 125m*l* of 0.02M phosphoric acid buffer (pH 7.8) was added, stirred and filtered.

4) Extraction by homogenizer: 2 kilogram of wet cell was suspended with 8*l* of 0.20M phosphoric acid buffer(pH 7.8) and homogenized by using a Gulin-Menton Homogenizer with two passes under the 300~500kg/cm² pressure.

Immobilization of enzyme

125 of each crude enzyme solutions prepared was mixed with 25m*l* of ion exchange resin precipitation. The reaction conditions were 65°C for 3 hours. The mass was filtered and after cooling to room temperature was washed with 1M NaCl solution, in order to remove the loosely bound enzymes, then renised with distilled water and dried.

Assay of enzyme activity

Native enzyme activity was determined by the same method reported previously. 20m*l* of immobilized glucose isomerase was packed in a glass column(2cm dia, 50cm hight) and heated to 65°C by the warm water circulation through the jacket. Substrate solution(0.8M glucose, 0.1M $MgSO_4 \cdot 7H_2O$, 1×10^{-4}M $CoCl_2 \cdot 6H_2O$ 0.05M $NaHCO_3$, pH 8.2) was passed through this column at an initial space velocity of 10 and elution was sampled and analyzed for fructose content. The activity was calculated by the following equation.

One immobilized xylose isomerase unit(IXIU/

From	Procedure	Flow	Reagent	Remarks
R-	Soaking		D.W	
	Washing	D	D.W	*SV 2
	Back Washing	U	D.W	For 10 Minites
	↓			
RH	Regeneration	D	2N-HCl	SV 2
	Washing	D	D.W	For 5hrs, ph 5.2
	Back Washing	U	D.W	
	↓			
R-	Regeneration	D	2N-NaOH	SV 2
	Washing	D	D.W	For 4.5hrs, pH 8.8
	Back Washing	U	D.W	For 10 Minites
	↓			
RH(HCO_3^-)	Forming	D	0.2M-$NaHCO_3$	SV 2, pH 8.4
	Back Washing	U	D.W	For 10 Minites
	↓			
	Forming	D	0.05M~$NaHCO_3$	SV 2, pH 8.4
	Sieving			80 Mesh
	Weighing			

*One SV(Spice velocity) is defined as the ratio of the volume of solution to that of the packed material in the column per hour.)

Fig. 2. Conditioning flow for ion exchange resin, RH(HCO_3^-) type

m*l*-R) =

$$\frac{(OD_{560}-\text{blank } OD_{560})\times\text{Dilution rate}\times 220}{0.02465\times 10^3\times 20}$$

Therefore, one immobilized xylose isomerase unit(IXIU) can be expressed as glucose isomerizing activity capable of producing one milligam of fructose at 65°C for one hour.

Regeneration of ion exchange resin

Raw resins were regenerated as shown in Fig. 2.

Activity decay test

20m*l* immobilized xylose isomerase(colum activity 250~300 IXIU/m*l*-R) was packed in a glass column(2cm dia, 50cm hight) and substrate solution(glucose 50%, 0.01M $MgSO_4 \cdot 7H_2O$, 0.02M $NaHCO_3$, 5×10^{-4} $CoCl_2$, $5H_2O$, pH 8.2) was passed at an initial velocity of 0.6 while keeping the temperature at 65°C. Fructose content in eluent was analyzed to calculate the enzyme activity. Besides, hydrol(D.E. 93) and

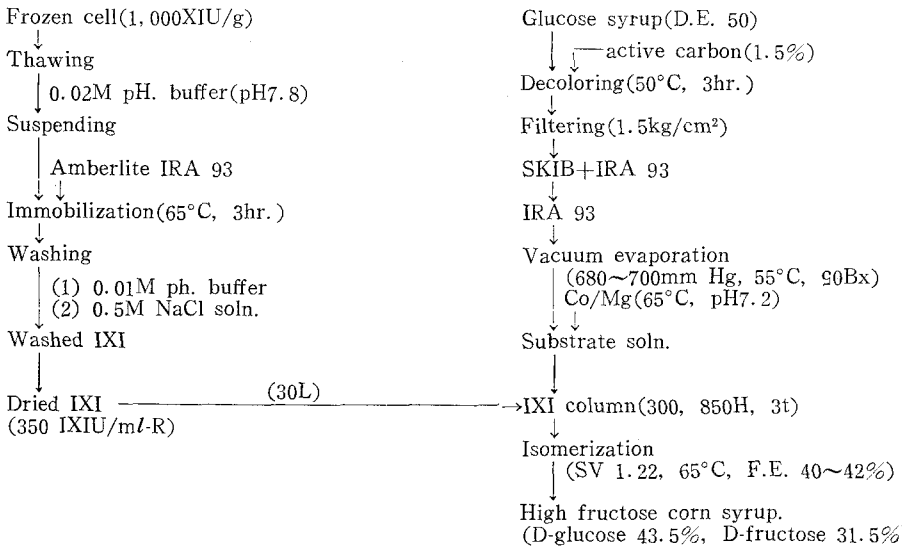

Fig. 3. Production flow diagram of high fructose corn syrup by the immobilized xylose isomerase

starch acid hydrolysate(D.E. 93) was also passed through a different column for comparison.

Reuse of the carrier

To evaluate the resusable possibility of the carrier, 20ml of immobilized xylose isomerase was treated with 7.5M NaCl solution for 30 minutes to remove the fixed enzyme and washed with distilled water three times, regenerated according to Fig. 2 and re-immobilized with new crude enzyme solution as described above.

Production of high fructose corn syrup in pilot scale(Fig. 3).

3k*l*(30kg×1,000XIU/g=3×10^{-7} XIU/30kg) of culture broth in 7k*l* fermentor was heated at 70°C for one hour after adding Na_2CO_3 to 0.3% concentration level of the broth volume, pumped to filter press, airpressed(2.5kg/cm_2) for further dehydration until the moisture content becomes below 75% and was frozen to −20°C. Xylose isomerase unit was 1,000 XIU per gram of wet cell weight.

For immobilization, the frozen cell was thawed and suspended with 0.02M phosphoric acid buffer as described previously and homogenized by two passes under 500kg/cm^2 pressure. After filtration the supernant was mixed with Amberlite IRA 93 while stirring slowly. Washed immobilized enzyme showed 350 IXIU permiliter of dry form.

30*l* of immobilized xylose isomerase was packed in the epoxy coated stainless steel column (30cm dia, 85cm hight) fitted with a jacket for warm water circulation. Through this column, preheated substrate solution(50 Brix purified glucose syrup, 0.02% $MgSO_4 \cdot 7H_2O$) was passed at an initial space velocity of 1.22 for isomerization of D-glucose to D-fructose and concentrated in vaccum evaporator at 680~700mmHg(55°C) to 75 Brix. Activity decay test was conducted for checking its half life for a 24 day period.

Result and Discussion

Enzyme extraction

Table 2. showed homogenizing treatment as effective enzyme extraction method with 52.7% yield. Maximum enzyme extraction was obtained at 500kg/cm^2 pressure in homogenizing as indicated in Table 3.

Table 2. Enzyme extraction.

Method \ Activity	Extracted (XIU/ml)	Total (10^{-4}XIU/ml)	Yield (%)
Mixter (5,000rpm, 5min.)	192	2.40	35.6
Lysozyme (0.01%, 55°C, 2hr.)	208	2.60	38.6
Glassbead (Hand mix, 10min.)	232	2.90	43.0
Homogenizing (300kg/cm^2)	284	3.55	52.7

Table 3. Effect of homogenizing pressure on enzyme extraction

Pressure (kg/cm^2)	Enz. Activity (XIU/ml)	Total Activity (10^{-6} XIU/ml)	Yield (%)
300	159	1.27	73.0
400	181	1.45	83.3
500	197	1.58	90.8
600	173	1.38	79.3

1. Raw enzyme activity used: 1.74×10^6 XIU
2. Each biomass suspension was homogenated by two passage

Table 4. Slection of immobilization yield of ion exchange resin for xylose isomerase.

Resin	Column activity (IXIU/ml)	Total Activity Retained (IXIU)	Immobilization Yield (%)
Diaion SA 20A	120	3,000	55.6
Diaion PA 406	132	3,300	61.2
Diaion HP 20	171	4,725	79.3
Duolite A-1	124	3,100	57.5
Duolite A-7	158	3,950	73.2
Dowex 2(SAR)	127	3,175	58.9
Amberlite IRA 400	150	3,750	69.5
Amberlite IRA 904	139	3,475	64.4
Amberlite IRA 93	174	4,356	80.6

Total raw enzyme activity: 53,940XIU

Table 5. Effect of resion types(IRA 93) and temperature on immobilization of xylose isomerase.

Resin Type (IRA 93)	Temp (°C)	Column IXIU/ml	Activity IXIU/ Total	Immobilization Yield (%)
Cl^-	40	182	4,550	60.7
	50	175	4,375	58.3
	60	176	4,400	58.7
	70	175	4,375	58.3
HCO_3^-	40	138	3,450	46.0
	50	140	3,500	46.7
	60	153	3,825	51.0
	70	155	3,875	51.7
SO_4^{--}	40	191	4,775	63.7
	50	175	4,375	58.3
	60	180	4,500	60.0
	70	207	5,175	69.0
OH^-	50	203	5,075	67.7
	55	181	4,525	60.3
	60	182	4,550	60.7
BO_4^{--}	50	221	5,525	73.7
	55	225	5,625	75.0
	60	188	4,700	62.7

Total raw enzyme activity: 7,500 XIU.

Immobilization yield

As Table 4 indicates, Amberlite IRA 93 and Diaion HP 20 showed the highest immobilization yield, to the native raw enzyme activity, 80.6% and 79.3%, respectively.

Table 6. Effect of regeneration type on immobilization of xylose isomerase into Diaion HP 20.

Resin Type (HP 20)	Column (IXIU/ ml)	Activity (IXIU/ Total)	Immobilization Yield (%)
HCO_3^-	248	6,200	62.0
SO_4^{--}	225	5,625	45.0
Cl^-	227	5,675	43.0
OH^-	215	5,374	43.0
BO_4^{--}	210	5,249	42.0

1. Total enzyme activity: 10,000 XIU
2. Immobilization was conducted at 55°C.

Effect of regeneration type of ion exchange resin

Amberlite IRA 93 in BO_4^{--} form recored maximum yield of 75%(Taqle 5) whereas Diaion HP 20 showed best in HCO_3^- form(Table 6).

immobilization conditions

55°C and pH 8.0 were effective on immobilization of the enzyme into Amberlite IRA 93. No significant differences were noticed between 50~60°C and in pH range of 6.0~8.0(Table 7).

Activity decay test(Fig. 4) **and reuse of carrier**

Table 7. Effect of temperature and pH on immobilization of xylose isomerase into Amberlite IRA 9.3.

Experimental Temp(°C)	pH	Conditions Resin Type	Column IXIU/ml	Activity IXIU/Total	Immobilization Yield (%)
25	6.0	HCO_3^-	115	2,875	41.1
55	6.2	SO_4^-	167	4,175	59.6
55	8.0	SO_4^-	188	4,700	67.1
60	8.0	SO_4^-	128	3,200	45.7
50	6.0	HCO_3^-	184	4,600	65.7
50	7.0	HCO_3^-	186	4,650	66.4
50	8.0	HCO_3^-	169	4,225	60.4
60	6.0	HCO_3^-	183	4,575	65.4
60	7.0	HCO_3^-	180	4,500	64.3
60	8.0	HCO_3^-	183	4,574	64.4

Total raw enzyme activity: 7,000 XIU

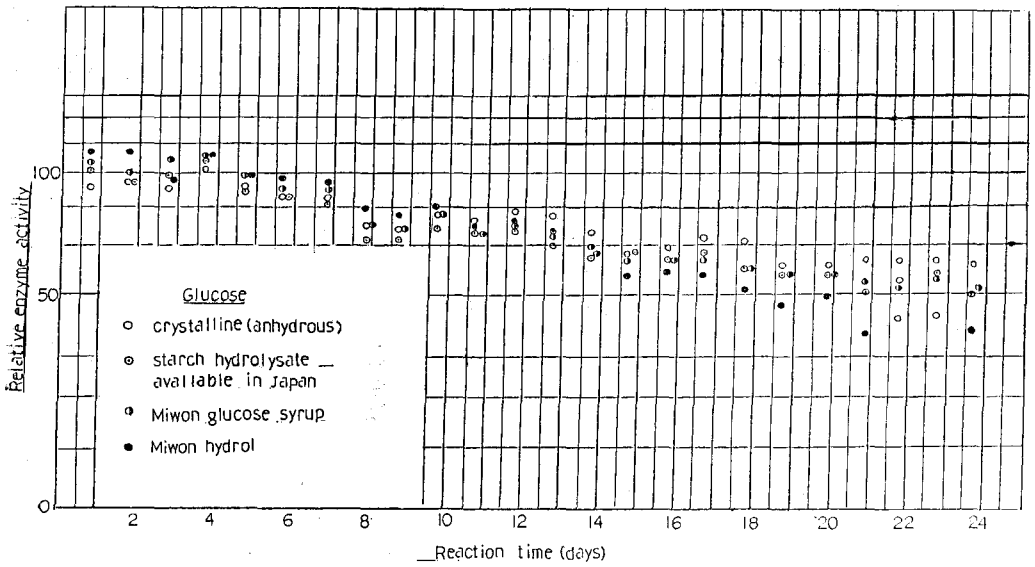

Fig. 4. Activity decay profile of immobilized enzyme

Three kinds of substrate showed very stable decreasing curves over 24 days while hydrol substrate was proved to adversely influence the half life of the immobilized enzyme. Crystalline glucose showed the best stablity, therefore it is recommendable to use purified substrate material for sustaining the long half life of immobilized enzyme.

Resue of the carrier

Table 8 shows both IRA 93 and HP 20 can be resuable more than five times.

Table 8. Relative activity of xylose isomerase immobilized on reused carrier

(Unit: %)

Resin \ Cycle	1	2	3	4	5
Amerlity IRA 93	100	98.2	93.3	90.7	87.5
Diaion HP 20	100	96.4	9139	87.4	82.7

Characteristics for immobilized enzyme

Immobilized xylose isomerase showed slightly different characteristics from those of native enzyme. Optimum temperature ranged widely between 60°C to 75°C and the activity decreased rapidly at higher temperatures than 80°C(Fig. 5). Fig. 6 showed that the optimal pH range was narrowed and shifted to the alkaline side,

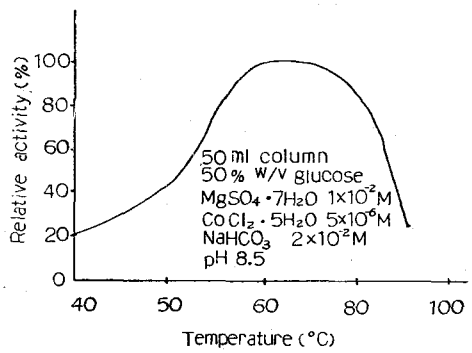

Fig. 5. Enzyme relative activity % versus temperature plot

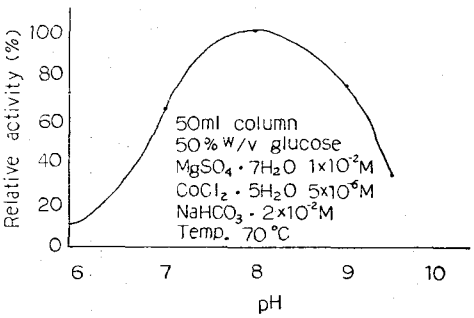

Fig. 6. Enzyme relative activity % versus pH plot

which is simillar to Smiley, et al[14] who reported that when the enzyme is immobilized into an anion exchange resin its optimum pH moves in the alkaline direction.

Purification degree of substrate is also an important factor to the half life of immobilized enzyme as shown in Fig. 6. This result was also reported by Zittan, et al[1].

Production of high fructose corn syrup in pilot scale

13.65*l* of 50% product solution(fructose equivalent 40%) was prepared at a first trial production of high fructose corn syrup through the immobilized xylose isomerase. This quantity is equivalent to 9.5k*l* of 75% high fructose syrup prepared over a 24 day period. This means one liter of immobilized enzyme can produce about 304*l* of 75% fructose syrup over a 24 day period.

Judging from the activity decady curve(Fig. 4) a 30 day half life of immobilized enzyme can be assumed to produce approximately 293*l* of 75% high fructose syrup; this volume is much lower than the theoretical 382*l* estimated based on 350 IXIU per milliliter of the insouble enzyme in 30 days.

To achieve this theoretical production volume, more effective immobilizing conditions should be developed.

Abstract

This study was designed to develop a process for the immobilization of xylose isomerase(D-xylose ketol isomerase, EC 5.3.1.5) from *Streptomyces griseolus* previously isolated by the authors and its application on a pilot plant scale for the production of high fructose corn syrup.

The biomass which has endo-excreted xylose isomerase was homogenized under a pressure of 500kg/cm^2 ,and 90.8% of the enzyme recovery of the native activity was obtained as compared to 54.7% recovery by the lysozyme treatment.

Ionic bonding method was adopted for the enzyme immobilization due to its many reported merits. It was found that the porous resins such as Diaion HP 20, Duolite A-7, Amberlite IRA 93 and 94 were effective in immobilizing the enzyme. In addition, it was disclosed that the regeneration form of BO_4^{--} is effective for Amberlite IRA 93 and HCO_3^- for Diaion HP 20.

Optimal immobilization condition for Amberlitc IRA 93 was pH 8.0 and 55°C yielding 80.6% of immobilization.

Activity decay test showed half life of the immobilized enzyme with Amberlite IRA 93 was more than 24 days at 65°C. The carrier was evaluated to be resuable and its result showed the relative immobilization yields were 98.2, 93.3, 90.7 and 87.5%, respectively at second, third, forth and fifth rebinding test of the enzyme on Amberlite IRA 93.

Optimal temperature of the immobilized enzyme was slightly lowered and the range widened to 60~70°C, while optimal pH moved toward 8.0~8.3 in its isomerization reaction.

The trial production result of high fructose corn syrup in pilot scale immobilization showed that one liter of immobilized xylose isomerase (350 IXIU/ml-R) is capable producing about 293*l* high fructose corn syrup(75% dry substance) in 30 days.

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