Thesis for the Degree of Master of Engineering

Optimization of Cyclodextrin product process by large product of CGTase

by

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CGTase 대량생산을 통한 CD 생산공정의 최적화

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by

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A Dissertation

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CONTENTS

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ABSTRACT

Bacillus subtilis NA1/pKB1 with its strong activity of cyclodextrin glycosyltransferase(CGTase) was used for the production of cyclodextrin (CD). The enzyme was purified by series of chromatographies or starch adsorption method. The purified enzyme exhibited its maximum CGTase activity in the pH range of 6.0 ~7.0 and temperature range of 60C ~ 70C. Immobilization of purified enzyme was carried out using various immobilizing materials such as Amberlite IRA-900 and Diaion PA418. The immobilized enzyme exhibited its maximum CGTase activity in the pH 6.0 and temperature of 60 C. Although the enzyme activity was high with free enzyme, the activity of immobilized enzyme maintained more than a month. Continuous culture was operated in fluidized bed reactor. Free CGTase activity was remained 0.01 unit/ml for 20days. Immobilized CGTase activity was about 3.2 unit/ml for initial3days but that decreased

to by 2.5 unit/ml. After 30days, immobilized CGTase activities decreased rapidly to 0.3 unit/ml. Continuous production of CD in fluidizedbed reactor showed maximalproductivity of immobilized CGTase at a relatively to optimal condition and substrate concentration. Productivity of immobilized CGTase was maintained by about 1.2 g/l more than free CGTase for 25 days. Optimal concentration of substrate was 10%.

Keywords: CGTase, Immobilization, Amberlite IRA-900, Cyclodextrin, *Bacillus sub.*

I. INTRODUCTION

Cyclodextrin glucanotransferase (CGTase) is a unique enzyme capable of converting starch and related substrate into cyclodextrins (CDs) possessing a hydrophilic outside and hydrophobic central cavity. CDs are homogeneous cyclic non-reducing oligosaccharides in which from 6-12 glucose units are joined by means of a -1,4-glucosidic bond. Most of the bacterial CGTases produce mainly α-CD, β-CD and a trace amount of γ-CD consisting of six, seven, or eight glucose units, respectively [11]. Recently, these cyclic CDs have been widely utilized in food, pharmaceutical, chemical, cosmetic and agricultural industries because of their ability to form inclusion complexes with wide variety of chemicals by partially encapsulating them into their cavity, thereby altering the physical and chemical properties of those compounds. The bacterial CGTase is a multifunctional enzyme. Besides cyclization reaction that is the conversion of starch and related α-1,4-glucans into CDs through an intramolecular transglycosylation reaction, the enzyme also catalyzes a decoupling reaction that is opening of the rings of CDs and transfer of the linear maltooligosacchrides formed to acceptors and a disproportion reaction that is transfer of linear maltooligosacchrides to acceptors through intermolecular

transglycosylation reactions. Also, the CGTase possesses a weak-hydrolyzing activity [8].

CDs are relatively stable molecules in comparison to the corresponding linear maltooligosaccharides because of the absence of reducing and non-reducing end glucose. In this work, the CGTase from *Bacillus sp*.NA1/pKB1 was adsorbed on Amberlite IRA-900 for the optimum conditions of CGTase immobilization, such as the pH of the solution and the mixing ratio of the enzyme to the Amberlite resin, were determined. Stability and activity of the immobilized enzyme were also evaluated. The effect of the variation of operating conditions of the fluidizedbed reactor containing immobilized CGTase on the yield of cyclodextrin was investigated along with stability of the immobilized enzyme in a fluidized bed reactor.

This research will not only facilitate in the development of an effective method for the immobilization of CGTase but also improve the process for the optimal product of cyclodextrin and using CGTase through immobilized enzyme system.

Ⅱ. MATERIALS AND METHODS

1. Microorganism and materials

Bacillus sp. NA1/pKB1 was obtained from Dongeui University (Prof. B. K. Kim). Culture components used soluble starch (1%, DF co., USA), polypeptone (0.5%, BD co., USA), yeast extract (0.5%, DF co., USA), dipotassium hydrogenphosphate (0.1%, Katayama co., JAPAN), magnesium sulfate (0.02%, St. Louis, Sigma chem., USA) [1,9].

2. Growth conditions

Cell grown in slant culture was inoculated to petridish and was grown 37 C. After 5 ml rest tube culture and the 50 ml culture used carried out using 5 ml culture as an inoculum. The cultures were grown in 250 ml Erlenmeyer flasks each containing 50 ml of sterile medium. Aliquots were drawn periodically to assess the growth of cell. The cells obtained from the logarithmic phase of the growth were used for the immobilization experiments.

3. Measurements of CGTase activities

The α-CD synthesis activity was examined by measurement of liberated α-CD by the methyl orange method [10]. The reaction mixture (3ml), containing 600 μl of 5% (w/v) soluble starch in 50 mM sodium acetate buffer (pH 5.5) and 105 μl of 1mM methyl orange solution in 50 mM sodium acetate buffer (pH 5.5), 1,795 l of 50mM sodium acetate buffer (pH 5.5) and 500 μl of enzyme solution, was incubated at 40℃ or 60℃ for 5min. The reaction was terminated by adding 150 μl of 6N HCl at 16℃. The optical density at 505nm was measured. One unit of CGTase activity was defined as the amount of enzyme, which released 1μmol of α-CD per min.

4. Isolation of CGTase

In this work, CGTase was purified from cell by gel chromatography (DEAE sephadex G-100, Sigma) and starch adsorption method. Starch adsorption and desorption methods show Fig.1. After removed cell from culture broth, it is treatment ammonium sulfate (30%) for an increase in starch adsorption and purification yield of gel chromatography. [4]

5. Immobilized of CGTase using Amberlite IRA-900 and Diaion PA 418

Amberlite IRA-900 and Diaion PA 418, the strong basic anion exchanger, was washed with 0.5N HCl solution and 0.5N NaOH solution to activatethe resin, and then suspended in 100ml of 10mM Tris-phosphate (pH6.0) for equilibration. The mixing ratio of the CGTase to the resin in the buffer solution varied from 200 to 1,200 units of CGTase per gram of resin.

6. pH and thermal stabilities of immobilized CGTase

The pH stability was investigated by measuring the residual coupling activities of immobilized CGTase and the free CGTase in various buffer solutions at 60℃ for 2h. To measure the thermal stability of an enzyme, the residual activity was measured after a 30 min pre-incubation at temperature ranging from 40℃ to 100℃ [3].

7. Operation of fluidized bed reactor

The fluidized bed reactor was set up with a water-jacketed acryl column (14cm ID, 30cm height), flow-rate controller, and water bath. The temperature was maintained at desired

temperature with thermostat (Fig. 2). The acryl column was loaded with 20g of Amberlite IRA-900 adsorbing 88.5 units of CGTase per gram of resin itself [6].

8. Analytical Methods

α-CD was analyzed by HPLC system (HewlettPackard, Inc., Palo Alto, USA): using Sugar-Pak column (Waters Associate Inc., Milford, USA) with acetonitrile/water (67/35 as mobile phase at the flow rate of), 1.0 ml/min, and RI detector. The protein concentration was analyzed by the Bradford method [2,7].

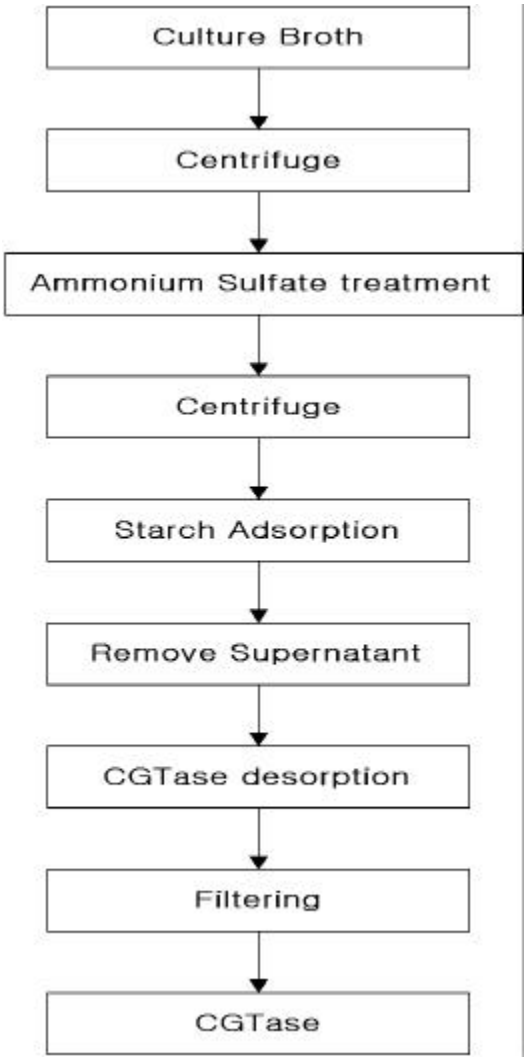

Figure 1. The protocol of starch adsorption and desorption

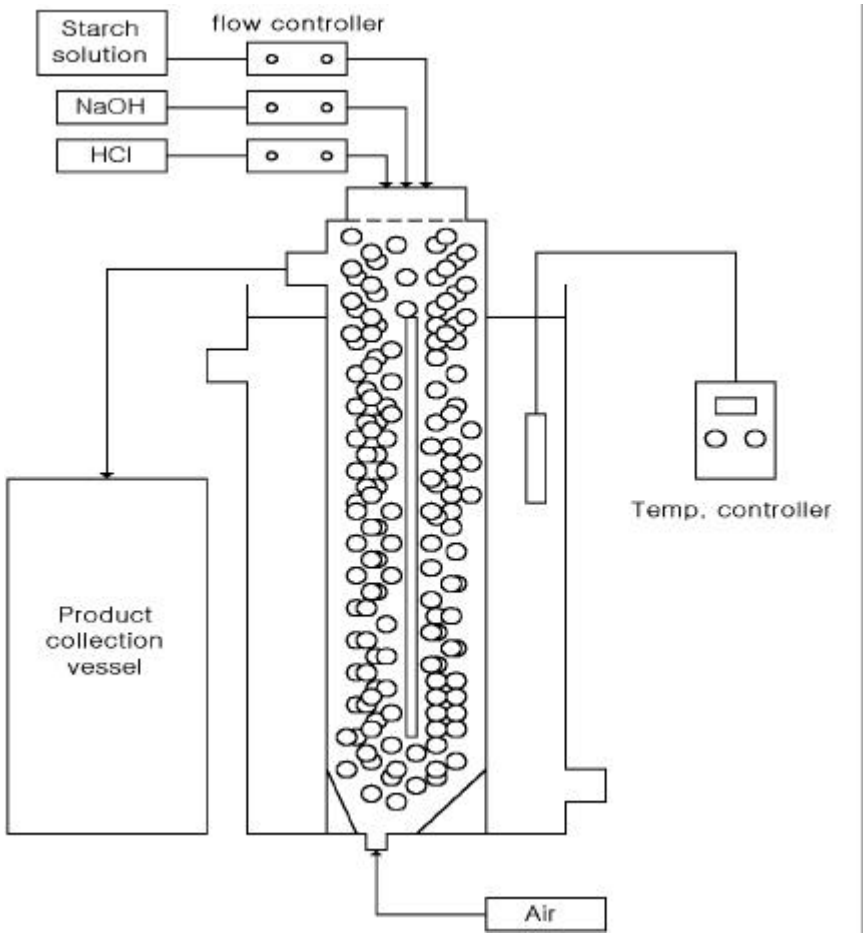

Figure 2. Fluidized bed reactor using immobilized CGTase

Ⅲ. RESULTS AND DISCUSSION

1. Optimal culture conditions for CGTase production

In order to optimize conditions for the activity of CGTase by the *Bacillus subtilis* NA1/pKB1, the activity of CGTase by cell growth the CGTase was produced in late stationary phase as shown in Fig. 3 and the effect of pH on CGTase production was evaluated and the optimum initial pH for the production of CGTase was pH 6.0 as shown in Fig.4. The optimum temperature for the production of CGTase was 60℃ (Fig.5).

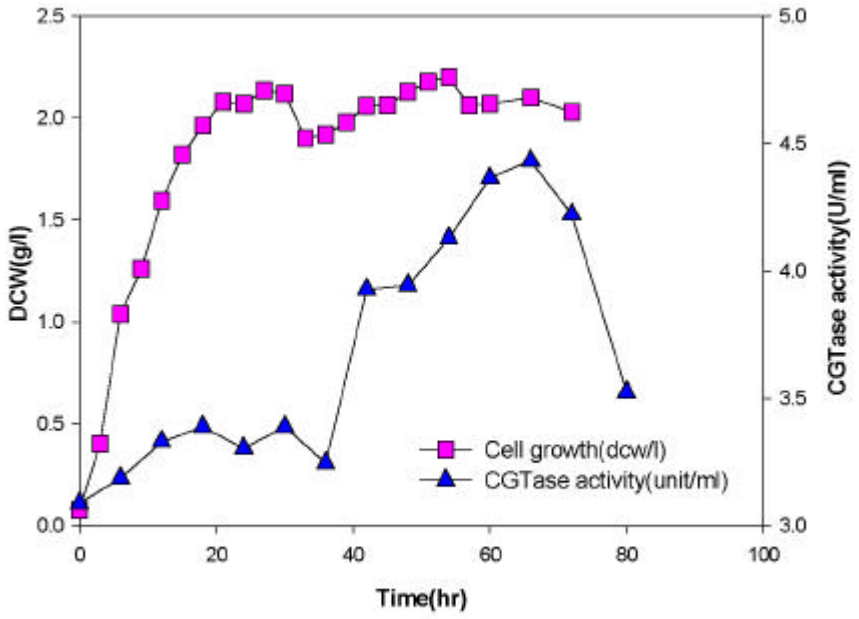

Figure 3. Fluidized bed reactor using immobilized CGTase

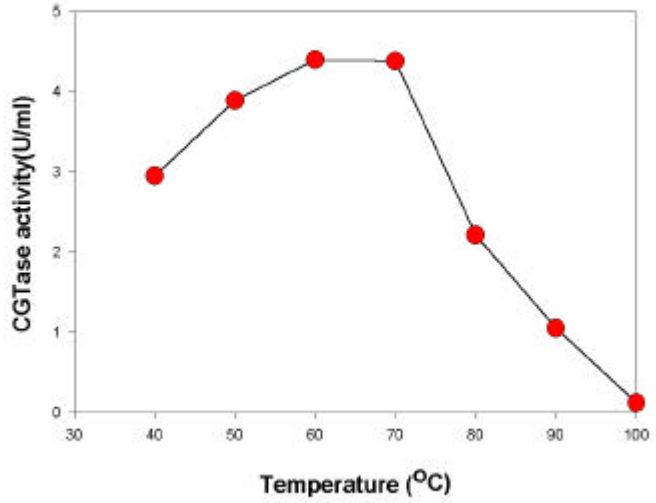

Figure 4. Effect of temperature on CGTase activity

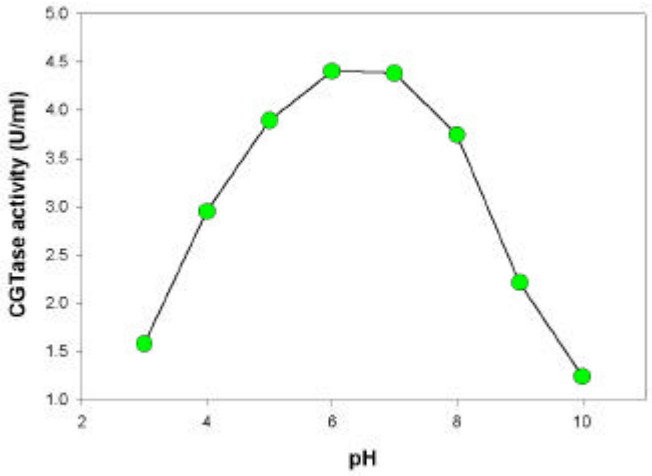

Figure 5. Effect of pH on CGTase activity

2. Purification of CGTase (gel chromatography and starch adsorption method)

The crude CGTase was purified to homogeneity in two methods gel chromatography and starch adsorption method. After CGTase purification using gel chromatography (Table.1) and starch adsorption method (Table. 2), There are not many differences in the CGTase yield between starch adsorption and gel chromatography method. In this study, starch adsorption method was selected because of simplicity comparison to gel chromatography and the similar yield of CGTase activity. The purification protocol resulted in a pure protein giving a single band on a SDS-PAGE gel (Fig.6). Starch adsorption and difference purification methods were shown to same band.

Table 1. CGTase purification using gel chromatography

	Total vol. (ml)	Total activity (U)	Activity (U/ml)	Protein Conc. (mg/ml)	Total Protein (mg)	Specific activity (U/mg)
Culture broth	1500	19,875	13.25	40.59	60,885	0.33
Ammonium sulfate	45	2,319	51.54	0.81	36.45	63.62
Gel chromatography	22.5	1,042	46.33	0.28	6.3	165.40

Table 2. CGTase purification using starch adsorption method

	Total vol. (ml)	Total activity (U)	Activity (U/ml)	Protein conc. (mg/ml)	Total Protein (mg)	Specific activity (U/mg)
Culture broth	1500	19,875	13.25	40.59	60,885	0.33
Ammonium sulfate	45	2,319	51.54	0.81	36.45	63.62
Starch adsorption	45	2,183	48.52	0.75	33.75	64.68
Phosphate buffer	20.4	922	45.21	0.48	9.79	94.17

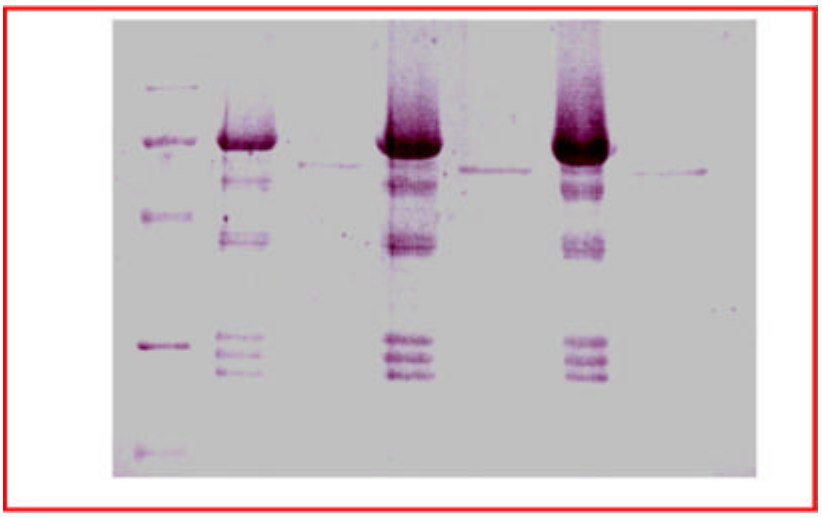

Figure 5. Electrophoretic analysis of the purification CGTase from *B. subtilis* NA1/pKB1.

A; ammonium sulfate treatment, B; Ion exchange chromatography (DEAE sephadex A-25) C; Gel chromatography (DEAE Sephadex G-1000), D; Starch adsorption (1% Starch)

3. Recovery of CGTase using starch adsorption and

desorption method between the number of adsorption and desorption treatment

After the cell removed from culture broth, culture broth was treated with ammonium sulfate 30%(w/v) for the increase of starch adsorption yield [4]. 80% CGTase was adsorbed more than by three times of starch adsorption as shown in Fig. 7. In CGTase desorption, desorption reactions were carried out after three times desorption reactions by the treatment by phosphate buffer in three times (pH 6.0). CGTase recovery yield was over 80% (Fig.8). CGTase was purified simply and short time by the starch adsorption and desorption method comparing to different purification methods such as ion exchange chromatography and gel chromatography.

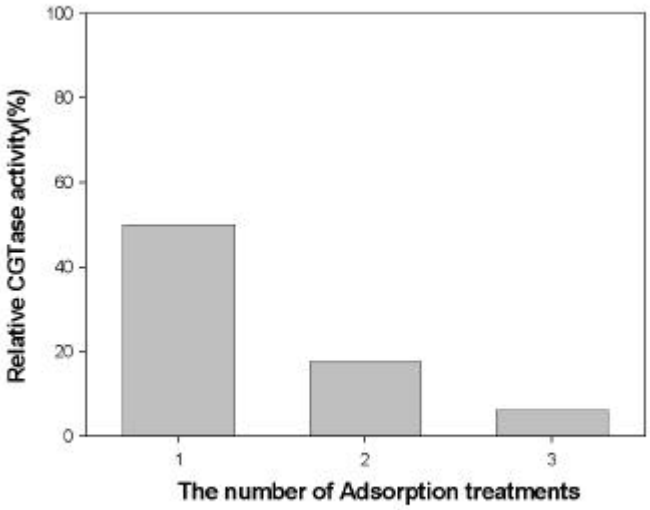

Figure 7. Adsorption rate of CGTase using starch adsorption method

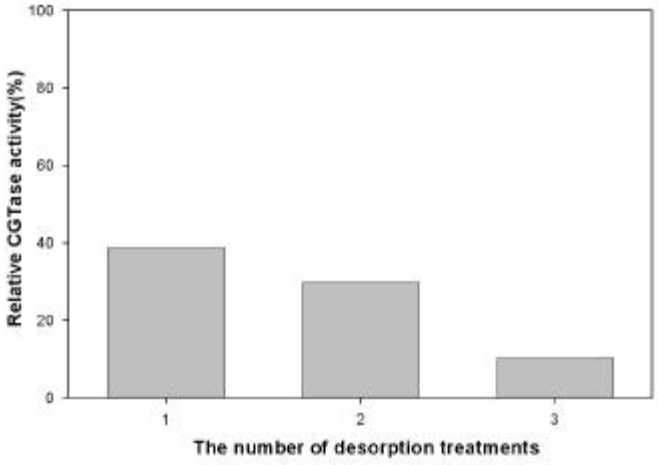

Figure 8. Recovery rate of CGTase using starch desorption method

4. Optimal activity condition for compare immobilized CGTase with free CGTase

In order to optimize conditions for the immobilized CGTase using the two resins (Amberlite IRA-900 and Diaion PA 418), The optimum condition was determined with pH and temperature. The activities and stabilities of the free and immobilized CGTases at different pH ranged from 4.0 to 10.0 were measured and shown in Fig.9. We found that the optimum pH and pH stability did not change significantly even after immobilization from that of the free enzyme. Thermal stabilities of the free and immobilized CGTase was determined in temperatures ranged from 40℃ to 100℃ as shown in Fig.10. It was among the higher activity free CGTase (3.5 unit/ml). It was revaluated activity immobilized CGTases using the two resins (Amberlite IRA-900 and Diaion PA418) respectively, It was high activity about 3.0 unit/ml immobilized CGTase using Amberlite IRA-900 better than Diaion PA418. Immobilized CGTase using anion resin not affect binding site of enzyme and substrate better than different immobilization methods. For that reason, it makes little difference from free CGTase activity. Also, it was known more high-immobilized CGTase activity using Amberlite IRA-900 compare with immobilized CGTase activity using Diaion PA418. Immobilized CGTase using

the two resins (Amberlite IRA-900 and Diaion PA 418) with strong ion charge and free CGTase revaluated activity for about 120 hours on optimal condition (pH6.0, Temp. 60℃) (Fig.11.).

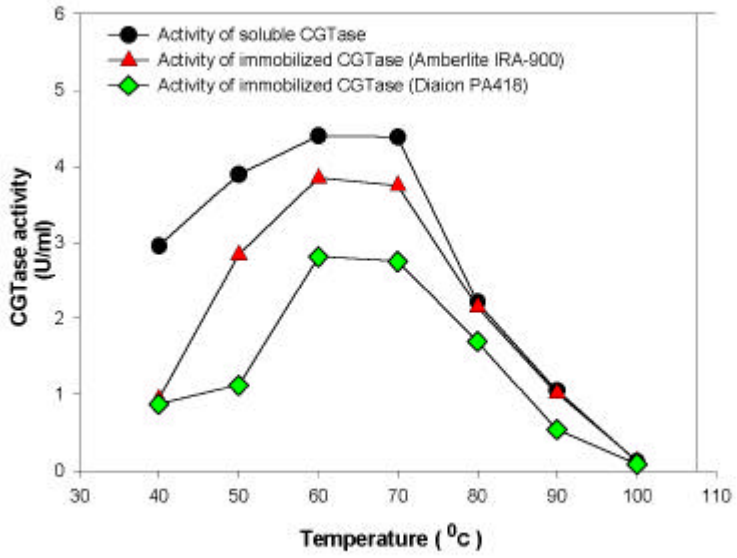

Figure 9. Effect of temperature on immobilized CGTase and soluble CGTase activity

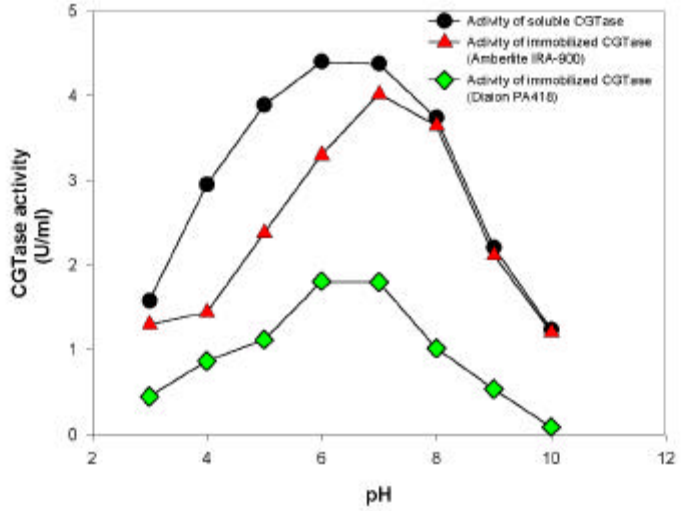

Figure 10. Effect of pH on immobilized CGTase and soluble CGTase activity

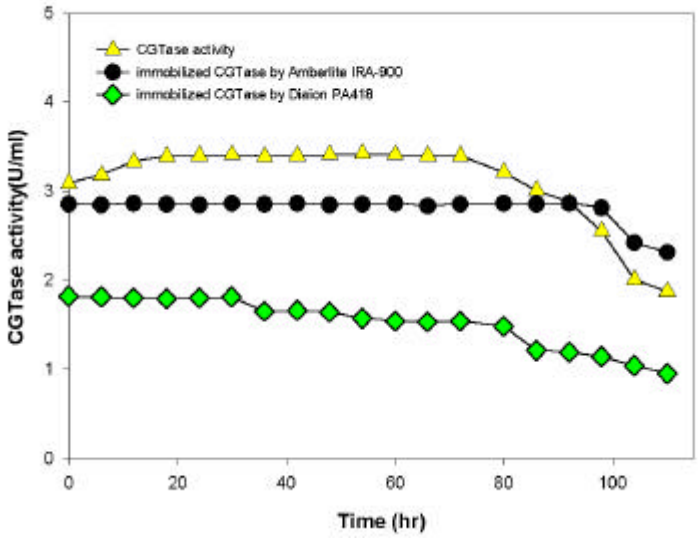

Figure 11. Immobilized CGTase activity using various resins

5. Continuous production of cyclodextrin using immobilized CGTase in fluidized-bed bioreactor

Activity of immobilized CGTase by continuous reaction and productivity of cyclodextrin from immobilized CGTase were studied in fluidized bed bioreactor. The immobilized CGTase was reused at 2days interval in fluidized bed reactors (Fig.12). Immobilized CGTase reused by filter set (Whattman filter #4) after operated on bioreactor. Immobilized CGTase wasalso found a high degree of efficiency in the collection of enzyme aspect. In case of design-immobilized bioreactor, it will design process have economical efficiency as soon as high reuse yield of enzyme including withdraw yield of enzyme.

The continuous operation of a fluidized bed bioreactor for 30 days with immobilized CGTase using Amberlite IRA-900 is shown in Fig.13. Immobilized CGTase and free CGTase wereoperated for about 30 days on same condition (Temperature: 60C. pH: 6.0). Substrate feeding rate was 200ml/day and dilution rate was 200ml/day. In this work, Immobilized CGTase activity was about 0.25 unit/ml for initial 3days, and decreased to the enzyme activity of 0.17 unit/ml and maintained the activity until 30days similar to free enzyme. Free CGTase activity was remained 0.01 unit/ml for 20days. Immobilized CGTase activity was remained longer than

free CGTase activity for 35days. Free CGTase activity was decreased to because wash out effect on bioreactor. But, immobilized CGTase activity was remained on bioreactor because possible reuse of enzyme.

Productivity of immobilized CGTase was maintained by about 1.2 g/l more than free CGTase for 25 days (Fig.14). Main product making certain of the α-CD by HPLC for identify product produced on the bioreactor using immobilized CGTase (Fig. 15). Fig.16 shows the effect of variation of the substrate concentration from 1.0 to 15%(w/v) on the productivity of the fluidized bed reactor containing immobilized CGTase. The cyclodextrin production by immobilized CGTase in the fluidized bed reactor wasalso dependent on the substrate concentration. Optimal concentration of substrate was 10% and productivity was about 1.17 (g/l/day). In case of over about 10% substrate concentration, a substrate inhibition was observed.

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