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Optimization of Cyclodextrin Glucanotransferase Immobilization on Amberlite IRA-900

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Cyclodextrin glucanotransferase (CGTase) produced by *Bacillus subtilis* NA1/pKB1 was used for the production of cyclodextrin (CD). The enzyme was purified by ion exchange and gel filtration chromatography. The purified enzyme exhibited its maximum activity in the pH range of 6.0 to 7.0 and temperature range of 60 to 70℃. Immobilization of purified CGTase was carried out with various immobilization matrices. Amberlite IRA-900, a strong basic anion exchange resin, showed the highest immobilization ability (38 units per gram resin). Optimal pH and temperature for enzymatic reaction of the immobilized CGTase were pH 6.0 and 60℃. The activity of immobilized CGTase maintained more than a month and could be reused for a month in a continuous enzyme reactor for the production of CD.

Key words – CGTase, Immobilization, Amberlite IRA-900, Cyclodextrin, *Bacillus subtilis*

Cyclodextrin glucanotransferase (CGTase) is an unique enzyme capable of converting starch and related substrates into cyclodextrins (CDs) [1,2,6,9,13,17]. CDs are homogeneous cyclic non-reducing oligosaccharides in which 6 to 12 glucose units are joined by means of α-1,4-glycosidic linkage. 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 [8,17]. Recently, these cyclic CDs have been widely used in food, pharmaceutical, chemical, cosmetic and agricultural industries due to their ability to form inclusion complexes with wide variety of chemicals into cavity of CDs, thereby altering the physical and chemical properties of those compounds. CDs are relatively stable molecules in comparison to the corresponding linear maltooligosaccharides due to the lack of reducing and non-reducing ends of glucose [2,3,17]. The bacterial CGTase is a multifunctional enzyme [17]. Besides cyclization reaction by an intramolecular transglycosylation reaction, the enzyme also catalyzes a decoupling reaction which opens the rings of CDs and the disproportion reaction of the linear maltooligosacchrides by intermolecular transglycosylation reactions [11,12,16,17].

Immobilized enzyme system have been applied for various biochemical processes [3,7,9,15]. The application of this system offers many advantages such as the ability to separate the enzyme from reaction solution for possible reuse facilitating continuous operation over a prolonged period and enhanced reactor productivity. The production of cyclodextrin has been mainly performed with batch-type operation using soluble CGTase. However, a continuous production of cyclodextrin using immobilized CGTase would have potential advantages of permitting reuse of expensive CGTase for an extended period of time, simplification of product purification process and scale-up availability [3,7,10].

In this work, the CGTase from *Bacillus subtilis* NA1/pKB1 was immobilized on various macroreticular ionic binding matrices. The various operating conditions were evaluated for the optimal production of cyclodextrin using immobilized CGTase.

Materials and Methods

Bacterial strain and growth conditions

Bacillus subtilis NA1/pKB1 was constructed at Dong-Eui University (Prof. Byung- Woo Kim) [1,4]. The medium was composed of 1% soluble starch (Difco Co., USA), 0.5% polypeptone (Difco Co., USA), 0.5% yeast extract (Difco Co., USA), 0.1% dipotassium hydrogenphosphate (Katayama Co., Japan) and 0.02% magnesium sulfate (Sigma Co., USA) [2,16]. Initial pH of medium was adjusted at 7.2. A colony of *Bacillus subtilis* NA1/pKB1 was inoculated into a 5 ml liquid medium and then incubated for 12 hr at 37℃ and 200 rpm. It was used as an inoculum (5%) for 200 ml

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culture in 1 L Erlenmeyer flasks with the same condition. Aliquots were drawn periodically and the cell growth was detected by optical density at 660 nm using spectrophotometer (UV 1201, Shimazdu. Co., Japan).

Measurements of CGTase activities

The activity of CGTase was examined by the measurement of liberated α-CD with methyl orange method[4]. The reaction mixture (3.0 ml), containing 600 μl of 5% (w/v) soluble starch in 50 mM phosphate buffer (pH 6.0), 105 μl of 1 mM methyl orange stock solution in 50 mM phosphate buffer (pH 6.0), 2,245 μl of 50 mM phosphate buffer (pH 6.0) and 50 μl of enzyme solution was incubated at 50℃ for 10 min. The reaction was stopped by the addition of 150 μl of 6 N HCl and stabilized at 16℃ for 30 min. Then, the optical density of the reaction mixture was measured spectrophotometrically at 505 nm. One unit of CGTase activity was defined as the amount of enzyme, which producing 1 μmole of α-cyclodextrin per minute.

Purification of CGTase with ion exchange and gel chromatography

After the removal of cells from culture broth by the centrifugation at 15,000×g for 15 min, the precipitate containing cell debris was separated by 30% ammonium sulfate treatment, and then the cell debris were removed by centrifugation at 15,000×g for 20 min. The supernatant were treated again with 80% ammonium sulfate precipitation at 4℃. The precipitate was obtained by centrifugation at 15,000×g for 20 min and dialyzed for 24 hr with changing buffer (pH 6.0, 50 mM phosphate buffer) every 2 hr and freeze-dried. Then, CGTase was purified by ion exchange chromatography (DEAE sephadex A-25, Sigma Co., USA) and gel filtration chromatography (DEAE sephadex G-100, Sigma Co., USA). Ion exchange chromatography was carried out using DEAE sephadex A-25 column equilibrated with 50 mM phosphate buffer (pH 6.0) and CGTase was eluted with 0.5 M NaCl solution. Elute was dialyzed with 50 mM phosphate buffer (pH 6.0) and then used for the immobilization of CGTase.

Determination of optimal immobilization condition for CGTase

For the immobilization of purified CGTase, various anionic and cationic exchange resins such as Amberlite IRA-900, Diaion PA418, Amberlite IRA-96, Amberlite 200 and Amberlite IRC-50 were tested. Classification and type of these matrices were shown in Table 1. Pretreatment and activation of the resins were carried out with 0.5 N HCl/ 0.5 N NaOH or 0.2 N HCl/ 0.2 N NaOH and then equilibrated in 50 mM phosphate (pH 6.0) at 4℃ for 12 hr[15].

The immobilization ability of these resins was evaluated with 5 ml (40 units) of purified CGTase solution and one gram of each resin. Immobilization of CGTase was performed under the condition of 37℃ for 1 hr. The activity of the immobilized CGTase was measured indirectly by the detection of decreased total activity in the reaction mixture. And then, the mixing ratio of CGTase to Amberlite IRA-900 was tested for the determination of optimal immobilization ability of the resin. One gram of resin was mixed with 20 to 100 units of CGTase in 50 mM phosphate buffer (pH 6.0) at 4℃ for 1 hr. To measure the thermal stability of the free and immobilized CGTase, the residual activity was measured after 30 min incubation at temperatures ranged from 40 to 100℃ [5]. The pH stability was determined by measuring the residual activities of free and immobilized CGTase after 2 hr incubation in various pHs ranged from 3 to 10 at 50℃.

The effect of the substrate concentration to the cyclodextrin production was evaluated. Soluble starch with concentrations of 1 to 15%(w/v) in 50 mM phosphate buffer (pH 6.0) was used as a substrate for immobilized CGTase and the productivity of α-cyclodextrin was determined.

Operation of continuous reactor with immobilized CGTase

Continuous reaction of immobilized CGTase was performed with a 2.5 L Bioflow III bioreactor (New Brunswick Scientific Instrument Co., USA) and the reaction volume was 2 L. The operating condition of immobilized enzyme reactor was adjusted to a pH of 6.0, a temperature of 60℃ and 10% substrate concentration. Ten grams of Amberlite

Table 1. Classification and type of various immobilization matrices used in this study

Classification	Type	Commercial name
strongly anionic	macroreticular	Amberlite IRA-900
	macroreticular	Diaion PA418
weakly anionic	macroreticular	Amberlite IRA-96
strongly cationic	macroreticular	Amberlite 200
weakly cationic	macroreticular	Amberlite IRC-50

IRA-900 resin with 400 units of CGTase were used. The continuous reactor was operated for 30 days and the flow rate was adjusted at 200 ml/day. The activity changes of the immobilized CGTase were compared to free CGTase at the same operating condition, and the cyclodextrin productivity with immobilized CGTase was evaluated.

Results and Discussion

Optimal culture conditions for CGTase production

In order to optimize the conditions to produce CGTase by *Bacillus subtilis* NA1/pKB1, the activity of CGTase was determined along with the cell growth. CGTase activity was maximal in a late stationary phase of the cell growth as shown in Fig. 1. From this result, the optimal incubation time for the production of CGTase was determined as 60 hr of cultivation and the maximum CGTase activity of culture broth was 0.43 unit/ml.

Purification of CGTase

For the purification of CGTase in the culture broth, ammonium sulfate precipitation was carried out. The precipitates of 30 to 80% fraction were recovered, followed by dialysis and freeze-drying. Ion exchange and gel filtration chromatographies were performed for further purification. The purified CGTase was analyzed by 10% SDS-PAGE and single band of purified CGTase was shown in Fig. 2. As shown in Fig. 2, molecular weight of the CGTase used in this study had about 65 kDa and most of the previously purified CGTases from various *Bacillus sp.* had a molecular weight range of 60-80 kDa [14,16]. Finally, the purified CGTase had activity of 8.2 units/ml, and it

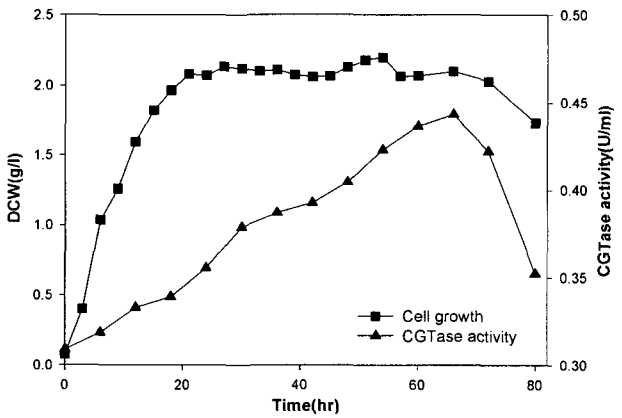

Fig. 1. Growth of *Bacillus subtilis* NA1/pKB1 and the CGTase activity.

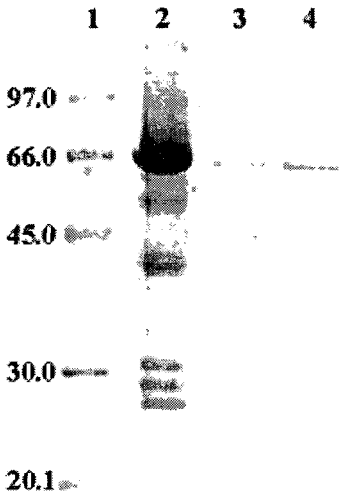

Fig. 2. SDS-PAGE analysis of purified CGTase from *B. subtilis* NA1/pKB1.
Lane 1, molecular weight marker; lane 2, partially purified culture broth with ammonium sulfate precipitation; lane 3, purified CGTase with ion exchange chromatography (DEAE sephadex A-25); and lane 4, purified CGTase using Gel chromatography (DEAE Sephadex G-100).

was used for experiments of CGTase immobilization.

Immobilizations of CGTase on various matrices

Immobilizations of CGTase using various anionic and cationic exchange resins were carried out with 40 units of CGTase and 1 gram resin. As a result, Amberlite IRA-900, one of the strong anionic exchange resin showed the maximum immobilization ability with 38 units of CGTase on 1 gram resin (Fig. 3). Another strong anionic exchange

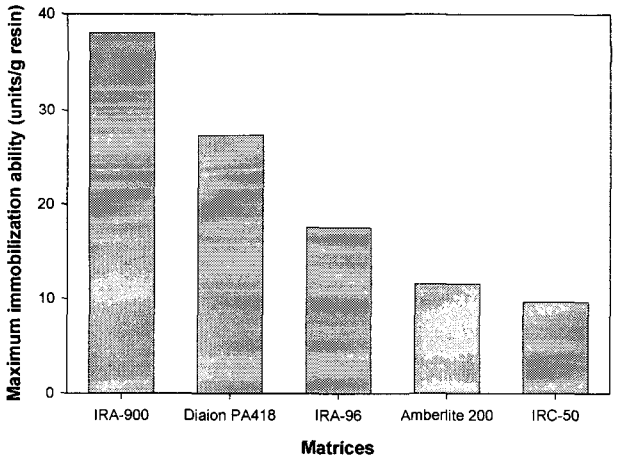

Fig. 3. Comparison of maximal immobilization abilities using various immobilization matrices with 40 units of CGTase.

resin, Diaion PA418 had high immobilization ability with 27 units of CGTase per gram resin. Therefore, Amberlite IRA-900 was selected for CGTase immobilization matrix. According to several reports, good yield of enzyme activity and stability were obtained with CGTase immobilized on Amberlite IRA-900 [7,9]. Since the CGTase has net negative charges at pH 6.0, CGTase showed low adsorption to the cationic exchange resins. The immobilization yield of CGTase on Amberlite IRA-900 was determined as shown in Fig. 4. The ability of the CGTase immobilization increased with amounts of the loaded CGTase up to 40 units per gram of resin, but further increase of loaded CGTase did not increase the immobilization ability significantly. The immobilization yield about 95% was obtained with the mixing ratio of 40 units of CGTase and 1 gram of resin mixed condition for Amberlite IRA-900. And the resin had 38 units of immobilized CGTase activity (Fig. 4). Further

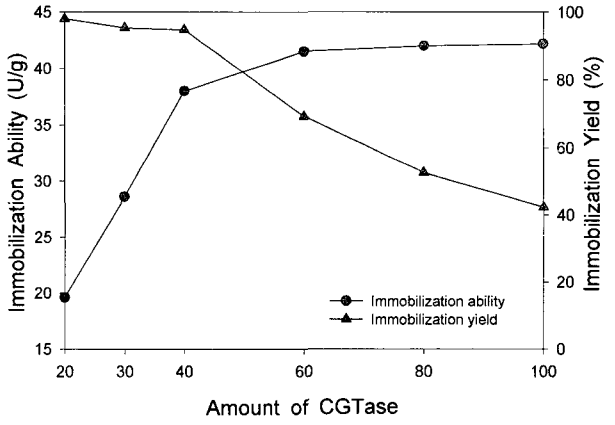

Fig. 4. Effect of mixing ratio between CGTase and Amberlite IRA-900 on immobilization ability and yield.

increase of loaded CGTase decreased the immobilization yield. It could be assumed that adsorption of CGTase on the resin was saturated at a mixing ratio of 40 units per one gram of resin. Therefore, the optimal mixing ratio of CGTase to Amberlite IRA-900 for the maximum activity of immobilized CGTase was determined as 40 units of CGTase per gram of resin with the corresponding adsorption yield of 95%. The rest of the experiments were executed under these conditions.

Optimal conditions for the immobilized CGTase activity

In order to obtain optimal enzyme reaction conditions for the immobilized CGTase in various ranges of pH, temperature and substrate concentration were tested. Thermal stabilities of the free and immobilized CGTases were determined in the temperature range from 40 to 100 ℃ as shown in Fig. 5 (a). The maximal activity was obtained at 60℃ for both free and immobilized CGTases. Most of the operation temperatures of CGTase were about 50℃ in the reports described by Lee *et al* [3,9,17]. However, the operation temperature of CGTase in this study was 60℃. The thermal stability of the CGTase used in this study was confirmed by the thermophilic origin of CGTase gene from *Bacillus stearothermophilus* NO2 [1,4]. High operation temperature is essential for commercial application due to low risk of contamination. The effect of pH on CGTase activity was evaluated with various pHs ranged from 3.0 to 10.0 as shown in Fig. 5 (b). The activities of free and immobilized CGTase show similar result and the maximal activity was obtained from pH 6.0 to 7.0. Although the maximal activity of the CGTase immobilized on Amberlite IRA-900 was lower than that of free CGTase,

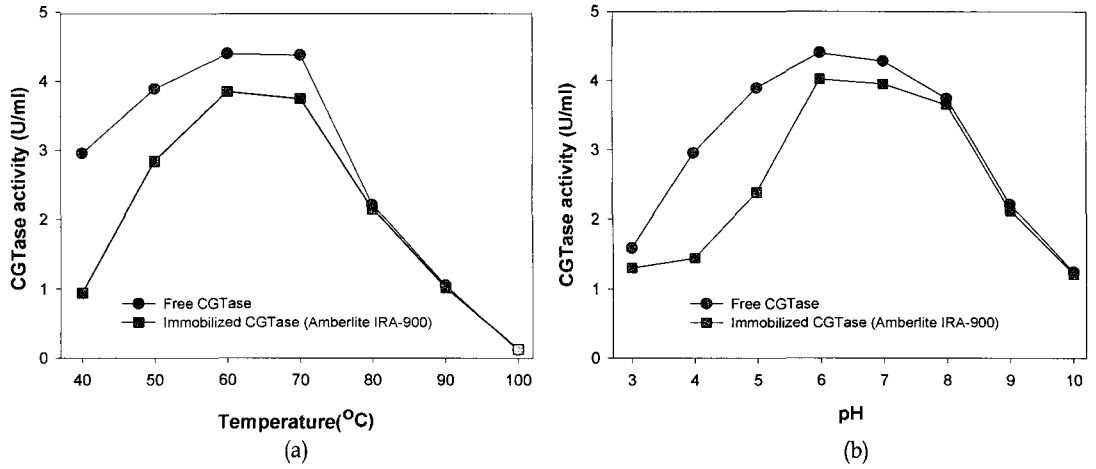

Fig. 5. Effect of temperature (a) and pH (b) on the activities of free and immobilized CGTase activity.

immobilized CGTase showed almost same optimum pH and 80% of activities compared to that of free CGTase. Therefore, it is obvious that the immobilization on Amberlite IRA-900 did not affect the functional groups of CGTase. Determination of optimal substrate concentration for immobilized CGTase was carried out. As shown in Fig. 6, the maximal productivity of α-cyclodextrin was obtained from 10% soluble starch. A substrate inhibition was observed with further increase in the substrate concentration.

Continuous reaction of immobilized CGTase for cyclodextrin production

Activity at continuous reactor and productivity of cyclodextrin by soluble and immobilized CGTase were studied with a 2.5 L bioreactor. The results from the continuous operation of enzyme reactor for 30 days with soluble and immobilized CGTase on Amberlite IRA-900 was shown in Fig. 7. The activities of the soluble CGTase were decreased dramatically after beginning the operation. On the other hand, continuous reactor operation with immobilized CGTase on Amberlite IRA-900, the activities could be maintained until 30 days. Lee *et al.* were reported that Amberlite IRA-900 seems to be more proper carrier than other materials for the industrial production of CD by immobilized CGTase bioreactor[9]. Immobilized CGTase activity was remained in bioreactor and the productivity of immobilized CGTase was maintained at 1.2 g/L/day for 25 days as shown in Fig. 7. In cases of previous works, immobilized CGTases from *B. macerans* were mainly used for continuous operation [3,9]. But in our experiment, CGTase originated from *B. stearothermophilus* was produced

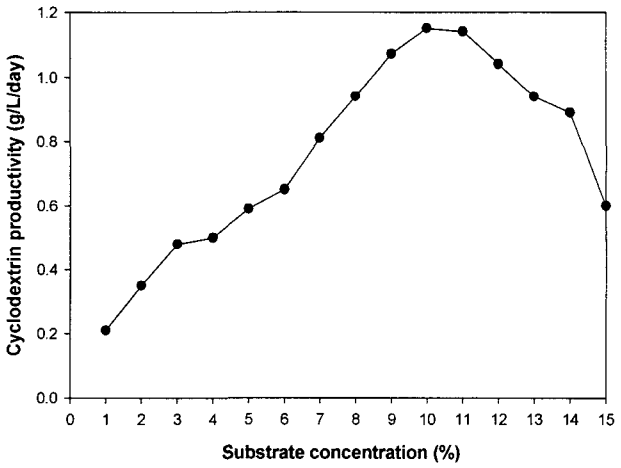

Fig. 6. Effect of substrate concentration on the productivity with immobilized CGTase.

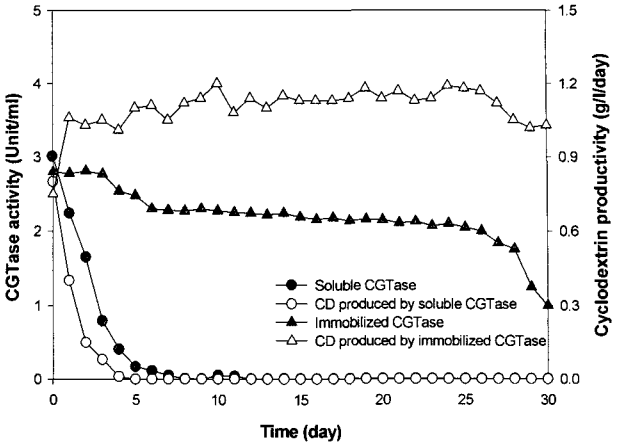

Fig. 7. Free and immobilized CGTase activity changes and the cyclodextrin concentration produced from continuous reactor.

by recombinant *B. subtilis* and then immobilized on Amberlite IRA-900. Continuous operation with this immobilized enzyme maintained its stabilities and activities until 30 days.

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초록 : Amberlite IRA-900을 이용한 cyclodextrin glucotransferase의 최적 고정화

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Bacillus subtilis NA1/pKB1으로부터 생산된 cyclodextrin glucanotransferase (CGTase)는 cyclodextrin (CD)의 생산에 이용되었으며, 이에 사용된 CGTase는 ion-exchange chromatography와 gel filtration chromatography에 의해 정제되었다. 정제된 CGTase는 pH 6.0-7.0 범위, 60-70℃에서 최대 활성을 나타내었으며, 다양한 이온결합성 고정화 담체를 이용하여 정제 효소의 고정화를 실시한 결과, 강염기성 음이온교환수지인 Amberlite IRA-900이 가장 우수한 고정화 효율을 나타내었다. 고정화된 효소는 pH 6.0, 60℃에서 최대 활성을 나타내었고, 그 활성이 약 1개월간 유지되어 cyclodextrin을 생산하기 위한 연속반응기내에서 장기간 사용이 가능함을 알 수 있었다.