Comparative Studies on the Synthesis of Cyclodextrin from Two Bacterial CGTases in the Presence of Organic Solvents

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Abstract

The synthesis of cyclodextrins (CDs) by cyclodextrin glycosyltransferase (CGTase) from *Paenibacillus* sp. A11 (A11) and *Bacillus macerans* (BM) in the presence of polar organic solvents was investigated. The results showed that the addition of 20% (v/v) polar organic solvents had effects on the yield and the product selectivity of CD production. With BM CGTase, when compared with the control, all solvents increased CD production, the highest yield of 66% being obtained with the addition of ethanol. Moreover, the product selectivity of BM CGTase depended on the type of organic solvent used; the synthesis of CD_7 was promoted in the presence of n-propanol and N, N-dimethyformamide while the synthesis of CD_6 was promoted in the presence of methanol. In contrast, A11 CGTase produced relatively smaller increases in overall CD yield (41-48 %), but generated the highest product selectivity for CD_7 up to 82%. In the coupling activity assay, addition of 20% (v/v) ethanol reduced the degradation of CD_7 by BM and A11 CGTases even in the presence of maltose as an acceptor, resulting in the increased percentage of remaining CD_7. The during CD production, as incubation progressed, both A11 and BM CGTases produced higher amounts of small-ring CDs (CD_{6-8}) and large-ring CDs (CD_{9-24}) (LR-CDs) in the presence of 20% (v/v) ethanol. Interestingly, BM CGTase significantly produced LR-CDs with a higher yield than A11 CGTase. These LR-CDs are very important in the pharmaceutical industries and are currently not available commercially.

Keywords: *Bacillus macerans*, Cyclodextrin (CD), Cyclodextrin glycosyltransferase (CGTase), Large-ring Cyclodextrin (LR-CD), Organic solvents, *Paenibacillus* sp. A11

1. Introduction

Cyclodextrin glycosyltransferase (CGTase; EC 2.4.1.19) plays a role in the starch utilization of some bacteria via cyclodextrin (CD) metabolic pathway. The action of CGTase consists of four reactions: cyclization, coupling, disproportionation, and hydrolysis reactions [1]. Beginning from the cleavage of one α-1,4-linkage within the glucan molecule, the newly produced reducing end is then transferred either to its own non-reducing end (cyclization reaction; Fig. 1A) or to the non-reducing end of another carbohydrate molecule (disproportionation reaction; Fig. 1C). CGTase also catalyzes the reverse of the cyclization reaction in which CD is opened and a linearized fragment is transferred to an acceptor (coupling reaction; Fig. 1B). In addition, the newly produced reducing end is transferred not only to a carbohydrate acceptor but also to a water molecule, which results in either the hydrolysis of starch or the linearization of large-ring CD_S ($LR\text{-}CD_S$) (hydrolysis reaction; Fig. 1D).

CGTase is produced by various microorganisms, for example *Klebsiella pneumoniae* M5al [2], *Micrococcus* sp. [3] and mainly *Bacillus* sp. [4]. All CGTases produced are mainly CDs with the degrees of polymerization (DP) of 6, 7, and 8 (CD_6, CD_7, and CD_8), which are generally called α-, β-, and γ-CD, respectively. The molecular structures of CD_S resemble a hollow, truncated cone with a hydrophobic cavity [5,6]. Due to this structure, CD_S can form inclusion complexes with suitable guest molecules. Various properties of the guest molecules, such as their water solubility, stability, and bioavailability can be efficiently manipulated. Therefore, CDs are widely used in the food, cosmetics, and pharmaceutical industries. In addition to producing CD_6, CD_7,

and CD_8, CGTases also produce LR-CD with DP from 9 to more than 60, each of which has a specific property for different guest molecules [7]. Importantly, the type of CD obtained in synthesis reactions depends on the type of CGTase, the reaction conditions, and the production processes.

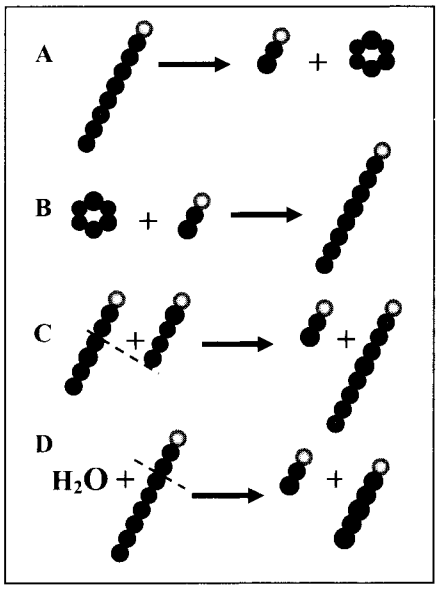

Fig. 1 Schematic representation of the CGTase-catalyzed reaction [1]

The solid circles (•) represent glucose residues; the open circles (○) indicate the reducing end sugars. (A) Cyclization, (B) coupling, (C) disproportionation, and (D) hydrolysis.

In this study, high-performance anion-exchange chromatography (HPAEC) was used to quantify CD synthesis from two different CGTases: *Paenibacillus* sp. A11 and *Bacillus macerans* CGTases, in the presence of several polar organic solvents. To obtain the highest yield of CDs, the best organic solvent was further investigated, including its optimum concentration and incubation period. These studies were performed in order to understand the effects of polar organic solvents on CD production for industrial applications.

2. Materials and methods

2.1 Chemicals and materials

CD_6, CD_7, CD_8, soluble potato starch, maltose and phenolphthalein were purchased from Sigma (St. Louis, USA). *Rhizopus* sp. glucoamylase was obtained from Toyobo Co., Ltd. (Osaka, Japan). Samples of CD_9 to CD_{24} were kindly provided by T. Endo and H. Ueda, Hoshi University, Tokyo, Japan. All anhydrous organic solvents of analytical grade were purchased from Aldrich Chemical Co. (Gillingham, U.K.), Merck (Darmstadt, Germany) and Bayer (Leverkusen, Germany).

2.2 CGTase preparation

Paenibacillus sp. A11 was isolated from South-East Asian soil as described in previous work [4]. *Paenibacillus* sp. A11 was tested for CGTase activity by growing on a screening medium that contained 1% (w/v) soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4$ and 0.75% Na_2CO_3 with starting pH 10.0 at 37 °C for 72 h. A positive colony which showed a surrounding clear zone with added iodine solution, was inoculated into Horikoshi medium in a 500 ml flask and cultivated at 37 °C with 250 rpm rotary shaking for 72 h [4]. Cells were separated by centrifugation at 3,000 rpm for 30 min at 4 °C. Culture broth with crude CGTase enzyme was collected and partially purified by using starch adsorption [4]. The enzyme solution was then concentrated by Vivaflow 50 and applied into the prepared DEAE-Toyopearl 650M column at 4 °C. The column was washed with 15 mM Tris-HCl buffer, pH 8.5 until A_{280} was negligible and subjected to 250 ml of 0-0.3 M NaCl linear gradient elution at 30 ml/h flow rate. Fractions of 3 ml were collected. The active CGTase fractions were pooled, concentrated, and dialyzed against 50 mM acetate buffer pH 6.0 containing 10 mM $CaCl_2$ at 4°C. The specific activity of purified A11 CGTase was 5,000 U/mg protein by dextrinizing activity [8]. Commercial CGTase from *Bacillus macerans* was the product of Amano Enzyme, Inc. (Nagoya, Japan) and had a specific activity of 1,003 U/mg protein by dextrinizing activity.

2.3 CGTase assay and protein determination

Cyclization activity of *Paenibacillus* sp. A11 and *Bacillus macerans* CGTase was determined as CD_7 forming activity by the phenolphthalein method [9]. One unit of enzyme was defined as the amount of enzyme able to produce 1 μmole of CD_7 per min. The same unit of both enzymes (2 U/ml by cyclization activity) was used to study their biochemical properties.

Protein concentration was determined by the Coomassie blue method [10].

2.4 Effects of polar organic solvents on the yield and product selectivity of CGTase

One half milliliter (2 U/ml by cyclization activity) of each CGTase was incubated with 2.5 ml of soluble potato starch at 2% (w/v) in 20% (v/v) of the tested polar organic solvent in 0.2 M potassium phosphate buffer, pH 6.0 at 40 °C for 24 h. The reaction was stopped by boiling for 10 min. Glucoamylase (10 µl, 38.5 U/ml) was added to convert the linear oligosaccharide to glucose (3 h). Subsequently, the glucoamylase activity was deactivated by boiling for 10 min. The reaction mixture was then analyzed for CD_S by HPAEC as described below. For the control, the CGTases were incubated with soluble potato starch in 0.2 M potassium phosphate buffer without organic solvent. The percentage of converted starch to CD was calculated according to Malai [11]:

$$\%\text{conversion to } CD_S = \frac{\text{CD detection (g/l) x 100}}{\text{Starch (g/l)}}$$

To study the time course of CD synthesis, CGTase was incubated with soluble potato starch for various reaction times under the same conditions described above.

2.5 Coupling activity

Coupling reactions were determined using 2% (w/v) CD_7 in the presence of 2.5 g/l maltose as acceptor with or without 20% (v/v) organic solvent in 0.2 M potassium phosphate buffer, pH 6.0. The coupling activity was tested by adding CGTase (0.5 ml, 2 U/ml) to each reaction and then was stopped by boiling for 10 min. Glucoamylase (10 µl, 38.5 U/ml) was then added at 40 °C for 3 h to convert the linearized oligosaccharides to glucose. The percentage of remaining CD_7 after 30 min incubation at 40 °C was determined by HPAEC analysis as described below.

2.6 Analysis of cyclodextrins

High performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was carried out using a DX-600 system (Dionex Crop., Sunnydale, USA) with a Carbopac PA-100 analytical column (4x250 mm, Dionex Crop., Sunnydale, USA) to analyze and quantify CD products. A sample (25 µl), containing maltose (G2) as an internal standard, was injected and eluted with a linear gradient of sodium nitrate (0-10 min, increasing from 0% to 4%; 10-12 min, 4%; 12-32 min, increasing from 4% to 8%; 32-48 min, increasing from 8% to 9%; 48-59 min, increasing from 9% to 18%; 59-79 min, increasing from 18% to 28%) in 150 mM NaOH with a flow rate of 1 ml/min. The amounts of CD_6 to CD_{24} were identified and quantified by comparison with standard curves of authentic CD_6 to CD_{24}.

2.7 Statistical analysis

Results were shown as mean±SD. Statistical significance was determined by one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test for effect of ethanol on the intermolecular transglycosylation reaction. Differences were considered to be statistically significant at $p<0.5$. Student's t test was used to construct 95% confidence intervals for the differences in the presence and absence of organic solvents.

3. Results and discussion

3.1 Effects of polar organic solvents on the yield and product selectivity of CGTase from *Paenibacillus* sp. A11 and *Bacillus macerans*

The effects of 20% (v/v) concentration of various polar organic solvents on the yield and product selectivity of CD products by A11 and BM CGTases were studied (Table 1). For this study, 1 U of each CGTase was incubated with 2% (w/v) soluble potato starch in 20% (v/v) polar organic solvents at 40 °C for 24 h. The amounts of CD_S produced were quantified by HPAEC and the percentage conversion of soluble potato starch to CD_S was calculated by the equation described in method 2.4. In the absence of any polar organic solvent, A11 and BM CGTases synthesized CD_6: CD_7: CD_8 at the ratios of 10:65:25 and 45:35:20, respectively. In addition, A11 CGTase produced the highest proportion of CD_7 while BM CGTase produced the highest proportion of CD_6. In the presence of all polar organic solvents, the overall yield of CD production was increased with both CGTases. BM CGTase produced the highest conversion yield (66%) in the presence of ethanol, which was two times higher than the yield obtained in the absence of ethanol.

Moreover, BM CGTase with addition of DMF and n-propanol as co-organic solvents resulted in an increase of CD_7 formation with decreases in the respective ratios of both CD_6 and CD_8 products. Therefore, DMF and n-propanol could be the best organic solvents used to improve the selectivity for CD_7 synthesis by the BM CGTase. Additionally, the BM CGTase with addition of methanol increased CD_6 yield from 45 to 58% which was the highest CD_6 production when compared with addition of other organic solvents (Table 1).

Table 1 Effects of organic solvents on yield and product selectivity of CDs by *Paenibacillus* sp. A11 and *B. macerans* CGTases

Solvents	*Paenibacillus* sp. A11 CGTase				*B. macerans* CGTase			
	Yield	Product ratio (%)			Yield	Product ratio (%)		
	(%)	CD_6	CD_7	CD_8	(%)	CD_6	CD_7	CD_8
None	41±0.57	10	65	25	36±2.08*	45	35	20
Methanol	45±2.00	15	66	19	60±2.00*	58	27	15
Ethanol	48±2.64*	10	82	8	66±3.00*	47	38	15
t-Butanol	47±4.35	15	75	10	57±2.00*	43	42	15
n-Propanol	44±2.64	15	70	15	55±3.00*	34	48	18
DMF[a]	42±0.57	11	67	22	51±2.00*	32	57	11
Acetonitrile	45±2.00*	8	75	17	58±1.15*	48	42	10
THF[b]	43±3.00	10	76	14	58±2.00*	48	43	9

[a] N,N-Dimethylformamide
[b] Tetrahydrofuran
Statistical values are shown as mean ± SD (n=3)
*significantly differ from control $p<0.05$.

With A11 CGTase, although the addition of co-organic solvents produced an overall increase in product formation in each case, it was not significant when compared with that of the BM CGTase. Interestingly, all cases of the A11 CGTase mainly generated CD_7 with maximum conversion achieved, 82%, with addition of ethanol. The product ratio of this highest conversion is 10:82:8, which has nearly pure CD_7, thereby helping reduce the process of CD purification. This highest product selectivity of A11 CGTase for CD_7 was similar to that of *B. circulans* 251 CGTase with t-butanol added [12]. Although the overall yield of A11 is relatively smaller than that of BM CGTase, it produced a marked increase in CD_7 selectivity. Among the three kinds of CDs, CD_7 is highly desirable for practical applications because its inclusion complexes are stable and the size of its non-polar cavity is optimum for many molecules such as drugs and preservatives [13, 14]. Additionally, CD_7 can be easily prepared and separated from reaction mixtures due to its low solubility in water. As a result, the A11 CGTase can be used to improve CD_7 formation along with the addition of ethanol.

3.2 Optimum concentration of ethanol on CD production

Since addition of ethanol at 20% (v/v) concentration showed significant increase in the overall yield of both BM and A11 CGTases and in CD_7 selectivity of A11 CGTase (Table 1), the effects of ethanol at various concentrations on CD production by these two enzymes were further investigated (Fig. 2). For this study, 1 U of each CGTase was incubated with 2% (w/v) soluble potato starch in ethanol concentrations ranging from 0 to 50% (v/v) at 40 °C for 24 h. The amounts of CD_S produced were quantified by HPAEC as described in method 2.4. The result showed that the highest percentage of starch converted to total CDs by both CGTases was at 20% (v/v) ethanol. Moreover, ethanol concentrations at lower or higher than 20% (v/v) caused a decrease in CD production by both enzymes, especially BM CGTase. Consistent with this data, Doukyu and colleagues [15] reported that 50% (v/v) ethanol could decrease the percentage of starch conversion to CDs because the higher ethanol concentrations had negative effects on the *Paenibacillus illinoisensis* ST-12K CGTase activity and

stability. For BM and A11 CGTases, it appears that addition of 20% (v/v) ethanol as co-solvent produced the highest CD formation.

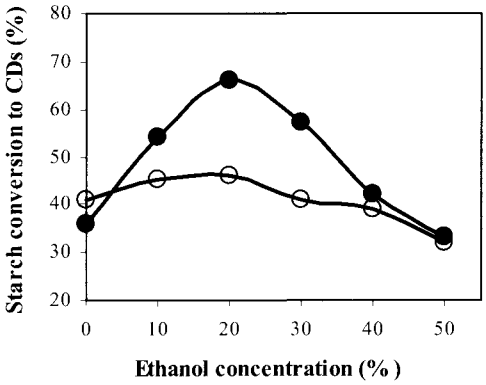

Fig. 2 The percentage of soluble potato starch converted to total CDs by *Paenibacillus* sp. A11 (○) and *B. macerans* CGTases (●) in the presence of ethanol at different concentrations

3.3 Time course of CD production

To investigate the effect of 20% (v/v) ethanol on CD production at various time points, timed trials, time of A11 and BM CGTases were analyzed. For this, each CGTase was incubated with soluble potato starch in the presence or absence of ethanol for various time points (30 min, 1 h, 3 h, 6 h, 24 h) and the amounts of CDs produced were quantified by HPAEC (methods 2.4, 2.6). The results shown in Fig. 3 clearly revealed that 20% (v/v) ethanol affected an increase in the amounts of both small-ring CDs (CD_{6-8}) and large-ring CDs (CD_{9-24}) by both A11 and BM CGTases, and the amounts of these CD products by the two enzymes increased with longer incubation times (Fig. 3B and D). Obviously, BM CGTase produced LR-CDs with a higher yield than A11 CGTase. It is possible that the addition of ethanol may slow down rates of coupling and hydrolysis reactions of LR-CDs, resulting in an increase of cyclization reaction of these CDs.

Without ethanol, most of the CD produced by A11 and BM CGTases was synthesized at the early stage of the reaction (30 min-3 h) (Fig. 3A and C). With prolonged incubation times (6 h-24 h), the amount of LR-CD gradually decreased because of their conversion into CD_6, CD_7 and CD_8. Additionally, it is important to note that the type of enzymes appears to be a major factor involved in the production of CDs. As seen in Fig. 3C and D, BM CGTase with or without ethanol preferentially produced all forms of CDs with a higher yield than A11 CGTase with or without ethanol (Fig. 3A and B).

3.4 Effects of ethanol on intermolecular transglycosylation reaction

To demonstrate effects of ethanol on CD remaining, the hydrolysis rate of CD_7 was performed using coupling activity assay. For this, 2% (w/v) CD_7 was incubated with CGTase in the presence of maltose as an acceptor with or without 20% (v/v) ethanol. This is explained in more detail in method 2.5. The percentage of CD_7 that remains at the end of 30 min incubation was monitored by HPAEC. As shown in Table 2, both A11 and BM CGTases could not hydrolyze CD_7 in the absence of maltose, whereas these two enzymes increased CD_7 hydrolysis with maltose addition. However, addition of 20% (v/v) ethanol affected the extent of hydrolysis by both CGTases even in the presence of maltose in that it reduced the degradation of CD_7, thereby increasing the percentage of remaining CD_7. Although addition of ethanol as a co-solvent decreased CD_7 hydrolysis, these results cannot clearly explain the real mechanism of ethanol effects at the molecular level such as increased yield and improved product selectivity. These effects may be related to blockage of the intermolecular transglycosylation reaction which causes degradation of CD products. Interestingly, the coupling activity of BM CGTase was lower than that of A11 CGTase regardless of ethanol addition, resulting in the higher percentage of CD_7 remaining. These results are consistent with those in Fig. 3C and D where several peaks of high weight CDs can be noticed with BM CGTase. However, most peaks of all CDs produced by A11 CGTase (Fig. 3A and B) are relatively smaller due to its higher coupling activity.

4. Conclusion

This study demonstrated that several polar organic solvents had an important role in increasing the CD syntheses in parallel with an improvement of product selectivity of the CGTases. Our work might be useful for technological applications in terms of using 2%

(w/v) soluble starch was incubated with (A) *Paenibacillus* sp. A11 CGTase alone, (B) *Paenibacillus* sp. A11 CGTase and ethanol, (C) *B. macerans* CGTase alone, or (D) *B. macerans* CGTase and ethanol. The number listed on each peak indicates the DP of CDs. G2 indicates maltose (0.1 mg/ml) added as an internal standard.

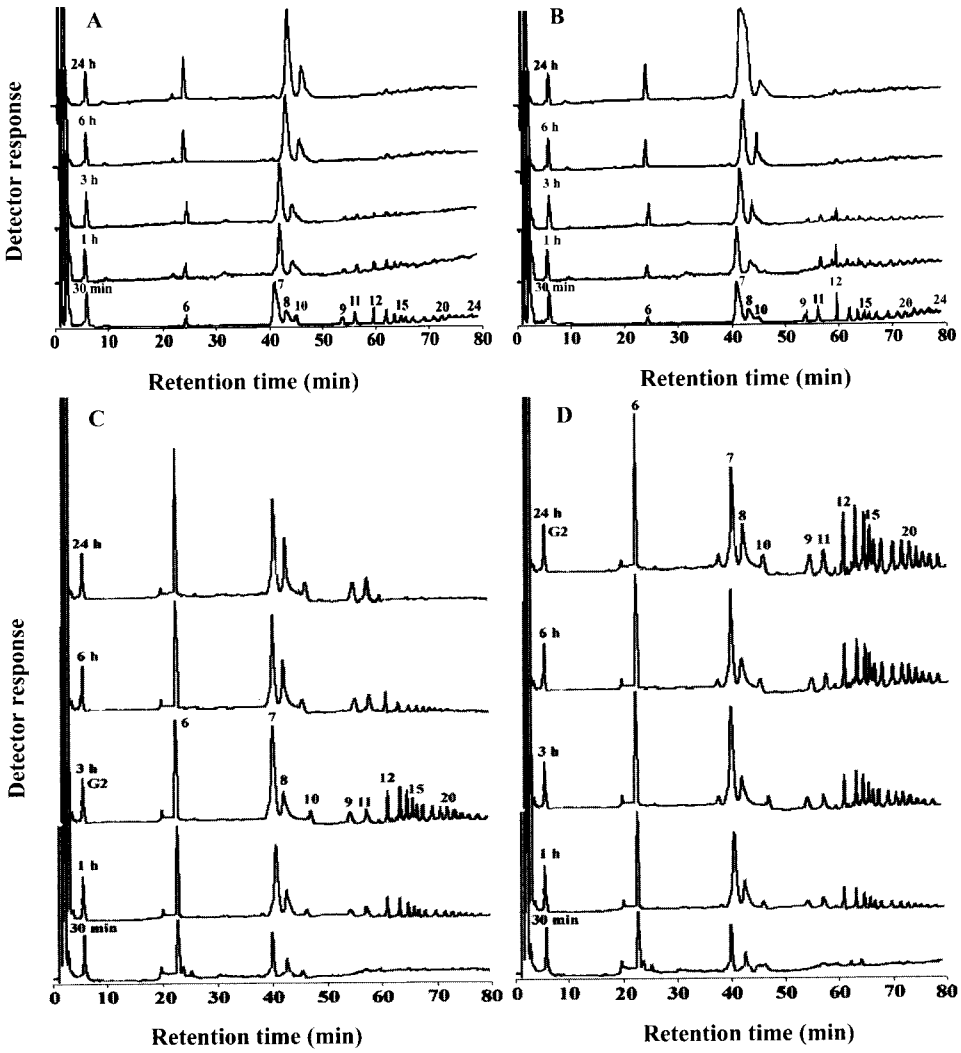

Fig. 3 HPAEC analysis of CDs synthesized by the action of *Paenibacillus* sp. A11 and *B. macerans* CGTases with or without 20% (v/v) ethanol.

Table 2 Percentage CD_7 remaining after 30 min incubation by the *Paenibacillus* sp. A11 and *Bacillus macerans* CGTases under the following conditions

Reaction mixtures	CD_7 remaining (%)
A11 CGTase	100±0.57*
A11 CGTase+maltose	39±3.60*
A11 CGTase+maltose+ethanol	65±1.00*
BM CGTase	100±0.58*
BM CGTase+maltose	55±2.64*
BM CGTase+maltose+ethanol	82±3.60*

Statistical values are shown as mean ± SD (n=3) and *significantly differ from control, $p<0.05$.

organic solvents to obtain increased amounts of CDs. Moreover, addition of polar organic solvents in processes that produce CD_S by some organic solvent-stable bacteria, might reduce contamination from other organic solvent-nonstable strains. This advantage of polar organic solvents was previously reported in CD_S producing from *Paenibacillus illinoisensis* [15].

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6. References

[1] Van der Veen, B.A., Uitdehaag, J.C.M., Dijkstra, B.W. and Dijkhuizen, L., Engineering of CGTase Reaction and Product Specificity, Biochim. et Biophys. Acta, Vol. 1543, pp. 336-360, 2000.

[2] Bender, H., CGTase from *Klebsiella pneumoniae* M5a1; Formation, Purification and Properties of the Enzyme from *Klebsiella pneumoniae*, Arch Microbiol., Vol. 111, No. 3, pp. 271-82, 1977.

[3] Yagi, Y., Sato, M. and Ishikura, T., Comparative Studies of CGTase from *Bacillus* sp. and *Micrococcus* sp. and Production of Cyclodextrins Using those CGTase, J. Jpn. Soc. Starch Sci., Vol. 2, pp. 144-151, 1986.

[4] Rojtinnakorn, J., Kim, P., Laloknam, S., Tongsima, A., Kamolsiripichaiporn, S., Limpaseni, T. and Pongsawasdi, P., Immunoaffinity Purification and Characterization of CGTase from *Bacillus circulans* A11, Science Asia., Vol. 27, pp. 105-112, 2001.

[5] Lindner, K. and Saenger, W. Crystal Structure of the Gamma-cyclodextrin n-Propanol Inclusion Complex; Correlation of Alpha-, Beta-, Gamma-cyclodextrin Geometries. Biochem Biophys Res Commun., Vol. 92, No. 3, pp. 933-938, 1980.

[6] Sabadini, E., Cosgrove, T. and Egídio, F.C., Solubility of Cyclodextrins in H_2O and D_2O: a Comparative Study, Carbohydr. Res., Vol. 341, No.2, pp. 270-274, 2006.

[7] Terada, Y., Yanase, M., Takata, H., Takaha, T. and Okada S., Cyclodextrins are not the Major Cyclic Alpha-1,4-Glucans Produced by the Initial Action of Cyclodextrin Glucanotransferase on Amylase, J. Biol Chem., Vol. 272, No. 25, pp. 15729-15733, 1997.

[8] Fuwa, H., A New Method for Microdetermination of Amylase Activity by the use of Amylose as the Substrate, J. Biochem., Vol. 41, pp. 583-603, 1954.

[9] Goel, A. and Nene, N.S., Modifications in the Phenolphthalein Method for Spectrophotometric Estimation of Beta Cyclodextrin, Starch/starke., Vol. 47, No. 10, pp. 399-400, 1995.

[10] Bradford, M.M., A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-dye Binding, Anal. Biochem. Vol. 72, pp. 248-254, 1976.

[11] Malai, T., Cyclodextrin Production from Rice starch, M.Sc. Thesis, Chulalongkorn University, 1995.

[12] Blackwood, A.D. and Bucke, C., Addition of Polar Organic Solvents can Improve the Product Selectivity of Cyclodextrin Glycosyltransferase: Solvent Effects on

Cgtase, Enzyme. Microb. Technol., Vol. 27, No. 9, pp. 704-708, 2000.

[13] Reddy, M.N., Rehana, T., Ramakrishna, S. and Chowdary, K.P.R., β-Cyclodextrin Complexs of Celecoxib: Molecular-Modeling, Characterization, and Dissolution Studies, AAPS PharmSci., Vol. 6, No. 1, pp. 1-9, 2004.

[14] Garbow, J.R., Likos, J.J. and Schroeder, S.A., Structure, Dynamics, and Stability of Beta-cyclodextrin Inclusion Complex of Aspartame and Neotame, J. Agric. Food Chem., Vol. 49, No. 4, pp. 2053-2060, 2001.

[15] Doukyu, N., Kuwahara, H. and Aono, R., Isolation of *Paenibacillus illinoisensis* that Produces Cyclodextrin Glucanotransferase Resistant to Organic Solvents, Biosci. Biotechnol. Biochem, Vol. 67, No. 2, pp. 334-40, 2003.