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Properties of Commercial CGTase in Enzymatic Production of Cyclodextrin from Ungelatinized Sago Starch

Zulfikri[1], Rosli Md Illias[2] , Osman Hassan[1*]

[1]Food Science Department, School of Chemical Sciences and Food Technology
Science and Technology Faculty, Universiti Kebangsaan Malaysia, Bangi 43600 Malaysia

[2]Department of Bioprocess Engineering, Faculty of Chemical and Natural Resource Engineering,
Universiti Teknologi Malaysia, 81310 Skudai, Malaysia

*Corresponding author. E-mail address; *osman@pkrisc.cc.ukm.my*
Telp: +603 891 3805 Fax: +603 8921 5410

Abstract

Cyclodextrin glucanotransferase (CGTase) is the enzyme catalyzing the production of cyclodextrin (CD). This study was conducted using a commercial CGTase enzyme (Toruzyme™) produced from genetically modified strain of *Bacillus* carrying the CGTase gene of *Thermoanaerobbacter*. Although this enzyme catalyses the formation of alpha, beta and gamma cyclodextrin from starch but beta-cyclodextrin is the major product. The result showed that the reaction behavior of the enzyme on ungelatinized sago starch was markedly different when compared with its reaction to gelatinized starch. Ungelatinised sago starch was annealed and reacted at 65°C. The optimum condition for the reaction occurred at pH 9 (0.05M Glycine-NaOH buffer) with concentration enzyme and sago starch was 0.5% (v/v) and 15 % (w/v), respectively. The highest amount of total cyclodextrin (13.17 g/L) was produced when the reaction mixture was agitated at 200 rpm for 4 hours consisting of α-CD: β-CD: γ-CD at ratios of 28: 64: 8. The CGTase lost almost all its dextrinizing activity in the presence $CuSO_4$, $FeSO_4$ and $Co(NO_3)_2$ in substrate. Addition of n-pentane and ethanol to the reaction mixture, shifted the reaction toward an increased of yield of α-cyclodextrin and eventually becoming the main product of the reaction. The *V*max and *K*m value of CGTase Toruzyme™ were 0.09 g β-CD/min and 16.695 %(w/v), respectively.

Abstrak

Cyclodekstrin glucanotranferase (CGTase) merupakan enzim yang mengkatalis produksi cyclodekstrin (CD). Penelitian ini menggunakan satu enzim CGTase komersial (Toruzyme™) yang dihasilkan secara rekayasa genetika menggunakan bakteri *Bacillus* yang telah disisipkan gen GTase dari bakteri termofilik *Thermoanaerobbacter*. Walaupun enzim in dapat menghasilkan alpa, beta, dan gamma siklodekstrin (α-CD, β-CD, γ-CD), namun beta siklodekstrin merupakan produk terbesar. Hasil penelitian memperlihatkan bahwa reaksin enzim CGTase ini dengan pati sagu mentah sangat berbeda jika menggunakan pati sagu tergelatinkan. Temperatur anneling dan temperatur reaksi enzimatik ditetepkan pada 65°C. Kondisi optimum diperoleh pada pH 9 (bufer glisin-NaOH 0.05M), 15% (b/v) pati sagu dan 0.5% konsentrasi enzim. Total siklodekstrin maksimum (13.17 g/L) diperoleh selama 4 jam pada kelajuan agitasi 200 rpm.dengan perbandingan produk adalah 28%: 64%: 8% masing-masing untuk α-CD: β-CD: γ-CD. Adanya $CuSO_4$, $FeSO_4$ and $Co(NO_3)_2$ didalam substrat mampu menghambat aktivitas enzim secara keseluruhan sedangan pemberian n-pentene dan etanol akan menghasilkan α-CD sebagai produk utama. Nilai *V*max dan *K*m CGTase Toruzyme™ adalah 0.09 g β-CD/min and 16.695 %(w/v), secara berurutan

Keywords: Cyclodextrin, Enzymatic, Ungelatinized Sago Starch, Cyclodextrin Glucanotranferase

1. INTRODUCTION

Cyclodextrins (CDs) also known as schardinger dextrin (cycloamylose) is an important polysaccharide due its unique hydrophobic interior cavity and hydrophilic surface. CD is a cyclic and nonreducing oligosaccharide consisting of α-D-glucose joined by α-1,4-glucosidic linkage. According to the number of glucose unit, the three major CD are α-cyclodextrin

(α-CD) with 6 glucose, 7-glucose unit for β-cyclodextrin (β-CD) and γ-cyclodextrin (γ-CD) with 8 glucose units (Szejtli, 1982).

Among of the three types of CDs, β-CD is more suitable for practical use because the inclusion complexes are more easily prepared and separated from reaction mixture without organic solvent because its low solubility in water (Lee, 1991)

CD can encapsulate other hydrophobic organic compound, thus aiding solubility in water. This property allows CD to play useful functions in food, pharmaceutical, cosmetic, and agriculture applications (Szejtli, 1998). Cyclodextrin are produced from starch or other polysaccharide by cyclodextrin glycosyltranferase (EC. 2.4.1.19; CGTase).

Cyclodextrin glucanotransferase can be found in several bacterial species, catalyzes the inter- and intramolecular transglycosylation of α(1,4)-gluean. The reaction will produce cyclodextrins with 6, 7, and 8 glucosyl residues and maltooligosaccharides of various degrees of polymerizations. Besides cyclization (the conversion of starch and related α-1,4-glucans into CDs through an intramolecular transglycosylation reaction), the enzyme also catalyzes coupling reaction (opening of the rings of CDs and transfer of the linear maltooligosaccharides to acceptor) Through intermolecular transglycosylation reactions (Blackwood et al, 2000). CGTase also possesses a weak-hydrolyzing activity (Tonkova, 1998). Major producers of CGTases belonged to *Bacillus* sp. especially aerobic alkalophilic types. Other psychrophilics, mesophilic and thermophilic microorganims that have been reported able to produce CGTase enzymes are *Bacillus stearothermophilus*, *Klebsiella pneumoniae* (Gawande & Patkar, 1999), *Klebsiella oxytoca* 19-1 (Lee, *et al*, 199; Feederle *et al*, 1996), *Brevibacterium* sp. (Mori, 1999) and hyperthermophilic archaea-bacteria (Tachibana, 1999). To date, all known CGTases will produce α-CD: β-CD: γ-CD from starch in different ratio depending on the reaction conditions and the nature of the CGTase.

Production of cyclodextrin from starch usually proceeds through liquefaction and gelatinization of starch at high temperature, followed by reaction with CGTase. Glucose, maltose, maltotriose, and low molecular weight maltodextrin produced from this reaction will accelerate the coupling reaction of CGTase, degradation CD and reduced CD produced (Kim, T.J., 1997)

In this study, we investigated the production of CD, especially β-CD, from ungelatinised sago starch with CGTase.

2. MATERIAL AND METHODS

Material

Sago starch purchased from Nee Seng Ngeng & Sons Sago Industries Sdn. Bhd. The enzyme cyclodextrin glycosyltranferase (CGTase) Toruzyme™ was obtained from Novozyme Sdn. Bhd. This heat stable CGTase from *Thermoanaerobacter* sp. was expressed and produced by a recombinant strain of *Bacillus*. Cyclodextrin standards (α-CD, β-CD and γ-CD) were purchased from Wacker-Chemie Gmbh, Germany. Other chemical according methods were analytical grade.

Activity of cyclodextrin glycosyltransferase

CGTase activity was determined using the method of Kaneko et al. (1987) with slight modifications. This method is based on the reduction of color intensity of phenolphthalein after complexation with β-CD. The reaction mixture containing 1 ml of 0.04 g starch in 0.05 phosphate buffer (pH 6.0) and 0.1 ml enzyme solution suitably diluted with deionized water. The mixture was incubated at 60°C for 30 min. Reaction was stopped by an addition of 3.5 ml of 0.03M NaOH and 0.5 ml of 0.02% (w/v) phenolphatelin in 0.005M Na_2CO_3 solutions. The color intensity was measured at 505 nm. One unit of enzyme activity was defined as the amount of enzyme that forms 1 µmol of β-CD per minute at the optimum condition. Standard curve was plotted with β-CD concentration at less than 1 mg/ml. The activity of cyclodextrin glucanotranferase is 43.46±1.48 U/ml

Cyclodextrin Production

The mixture containing 15% (w/v) sago starch in 0.05M glycine-NaOH buffer pH 9 and 2% (v/v) CGTase was incubated at 65°C with agitation rate of 200 rpm for 5 hours. After 5 hours sample solution was centrifuged at 6000 rpm for 15 minutes, and the supernatant was analyzed for cyclodextrin concentration by HPLC

Analysis Of β-CD

The concentration of β-CD was determined by HPLC (Water Assoc.) with Shodex Asahipak NH2P-50 (4.6mm x 250mm). The eluent was a mixture of 70% acetonitril and 30 % water and flow rate was 1 ml min^{-1} with the column temperature of 30°C. *Eurosep Instrument Evaporative Light Scattering*

Detector, ELSD was used, with nebulizer temperature 50°C and evaporation temperature 60°C.

3. RESULTS AND DISCUSS

Reaction Temperature

Ungelatinized sago starch normally gelatinize at 69°C – 70°C (Fasihuddin et al., 1999). The gelatinization temperature of starch increased with the increase in starch concentration. Gelatinization temperature of starch sago was determined with DSC (differential scanning calorimeter). Table 1 showed the gelatinization temperature at various concentrations of sago starch.

Table 1. The Gelatinize temperature at various concentrations of sago starch

Concentration Sago starch (%)	Start Gelatinize	End Gelatinize
5	69.25°C	77.53°C
10	69.14°C	77.45°C
15	68.63°C	77.90°C

No significant correlation between concentration of sago starch and the gelatinize temperature was detected, but increase of sago starch concentration caused small decrease the gelatinization temperature. From this data, the highest reaction temperature was fixed at 65°C, which was lower than the gelatinization temperature.

The pH and Thermal stability cyclodextrin glucanotrasferase

The thermal stability the CGTase was measured by incubating 0.2 ml CGTase (in 10 ml phosphate buffer, pH 8.5) without starch at a temperature range of 40°C - 100°C for 30 min. The remaining activity of the enzyme was assayed by the standard assay methods. The pH stability of the CGTase was measured by incubating 0.2 ml enzyme with 10 ml of 0.05M phosphate-citrate buffer pH 3, 0.05 M sodium acetate buffer (pH 4-6), 0.05 M potassium phosphate buffer (pH 6-8) and 0.05 M glycine-NaOH buffer (pH 9-11) respectively at 65°C without starch for 30 min. the CGTase standard assay method was used for determine residual activity enzyme.

As seen in Figure 1 the enzyme in phosphate buffer (pH 8.5) was found to be stable up to 80°C. However it began to lose 18% of its activity at 90°C and at 100°C only 25% remained. It was estimated that the CGTase would completely inactive at 110°C.

The effect pH on the stability CGTase is shown in Figure 2. The enzyme did not show any activity and remained inactive when incubated at pH 3, 65°C for 30 min. The enzyme was stable at pH 6-9. However at pH 10 and 11 CGTase loss about 60-70% of its activity.

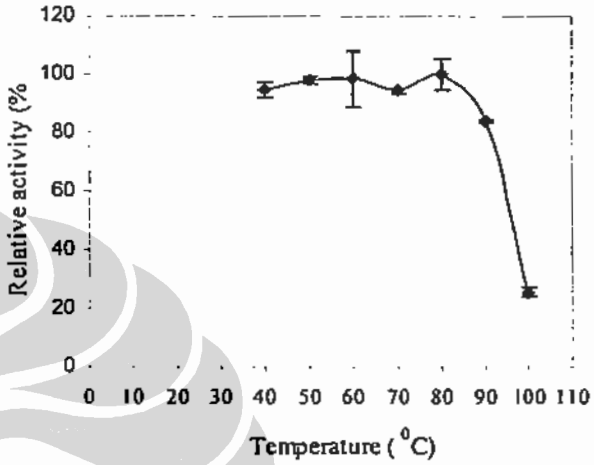

Figure 1. Effect of temperature on stability CGTase

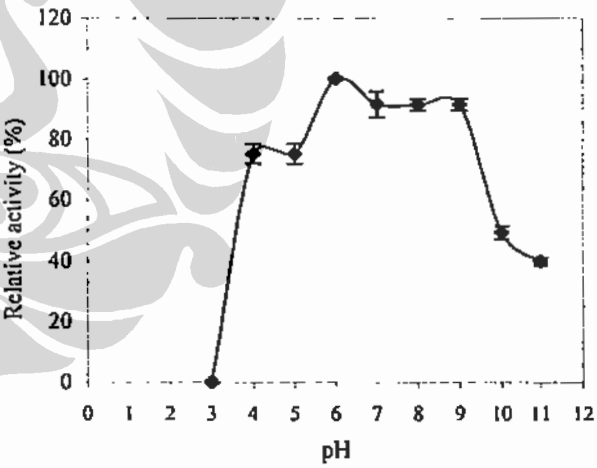

Figure 2. Effect pH on the stability Enzyme.

Effect Temperature on CGTase activity

A sago starch solution (6 %) in phosphate buffer pH 8.5 was incubated at temperature range of 40°C – 90°C for 5 hours. After 5 h the sample was centrifuged at 8000 rpm for 10 min. And the supernatant was analyzed.

At 40°C, the CGTase did not show any CD-producing activity. However, the activity increased as

the temperature was increased, until the maximum was attained at 80°C. At temperature 80°C, 15 g/L β-CD was produced compared to only 4g/L β-CD at 65°C. The higher yield of β-CD at 80°C was probably due to the structural opening of sago starch granule resulting from starch gelatinization. Although that yield was higher, the separation of product was more difficult due to the interference of starch fragments and viscosity of the reaction mixture. Thus, the reaction temperature of 65°C was retained because of the possible advantage associated with ease of separation of the product from ungelatinised starch.

The Effect pH on CGTase activity to β-CD production

The enzyme activity was measured at varying pH values ranging from 4-10 at 65°C with 6%(w/v) sago starch and 2% (v/v) enzyme. 0.05 M sodium acetate buffer for pH 4-6, 0.05 M potassium phosphate for pH 7-8 and 0.05 M Glycine-NaOH buffer for pH 9-10 was used. The pH profile CGTase Toruzyme™ on ungelatinized sago starch show on figure 4.

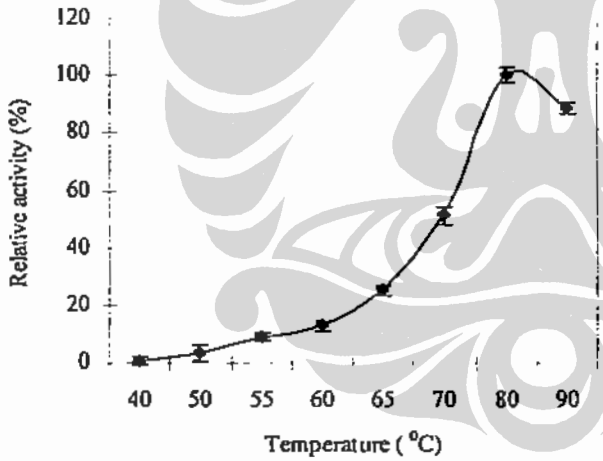

Figure 3. The Effect Temperature on Activity Production β-CD CGTase

The optimum activity was seen at pH 9.0 in glycine-NaOH buffer. Activity of CGTase at pH 4 - 7 were less than 50 % compared to reaction at pH 9. The CGTase Toruzyme™ more suitable in alkaline buffer, pH 8-10. This data contradicted with the claim stated in the technical specifications of product of Toruzyme™, where the pH optimum was claimed to be pH 5.5 (Novo Nordisk, 2004). The difference could be due to the change of substrate (sago starch), reaction condition (ungelatinised, pH 9).

Rita (2003) reported CGTase from *Bacillus agaradhaerens* LC-3C also has pH optimum of 9 in glycine-NaOH buffer. Most other CGTase have pH optimum at acid condition and neutral (Higuti, 2003; Kim, 1995). Enzyme activity at pH 9 was significantly different than the other pH ($p<0.05$). Hence pH 9 in 0.05M glycine-NaOH buffer would be chosen as the optimum pH for the next experiment.

Reaction of Toruzyme™ on gelatinized sago starch occurred optimally at pH 8.6 in phosphate buffer (Suzana et al., 2005).

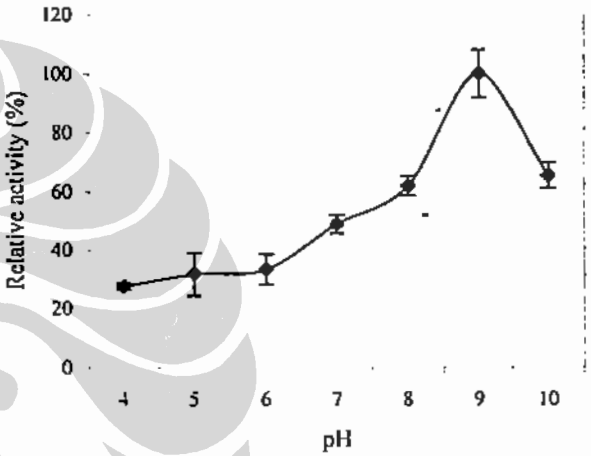

Figure 4. Effect of pH on production activity β-CD CGTase Toruzyme

Effect Concentration of the sago starch on β-CD production

According the above result, the optimum reaction pH was pH 9 in 0.05 Glycine-NaOH buffer. The reaction condition further investigated to include the effect of substrate concentrations (ungelatinised sago starch). In this study, concentration range of sago starch used was 1% - 30% (w/v). The enzyme concentration was 2% (v/v). Figure 5 showed the effect of sago starch concentration on the concentration of β-CD. As the concentration of sago starch exceeded 20%, production of β-CD by CGTase slight increased.

This result was similar with results reported by other investigators, but using different type of starch. Lee (1991) reported that the production yield of cyclodextrin from unliquefied corn-starch of 15 % reached 35 %, while Slomiska (2002) reported concentrations of 5% - 15%.

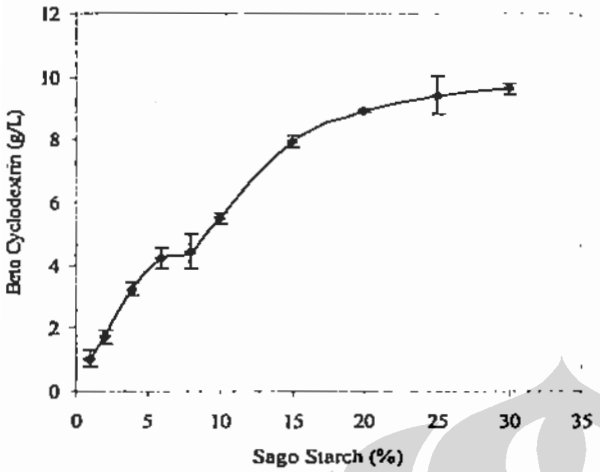

Figure. 5 Optimum concentration Sago starch for β-CD ▲; Concentration β-CD (g/L) condition reaction was pH 9 in 0.05M glycine-NaOH buffer, 2% (v/v); concentration of enzyme and 65°C; temperature

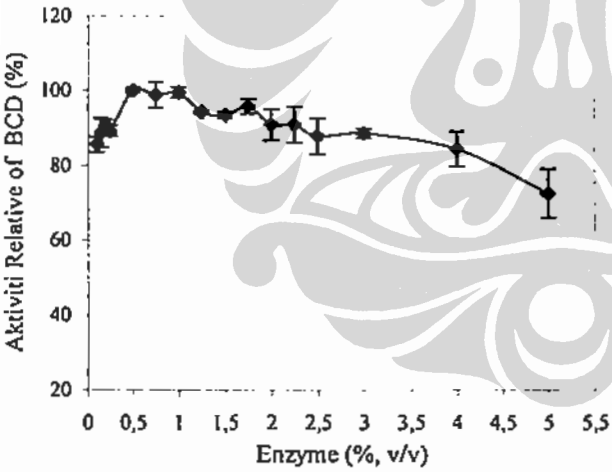

Figure 6. Effect the concentration of CGTase to relative yield β-CD. Condition reaction was pH 9 in 0.05M glycine-NaOH buffer, 10%; sago starch and 65°C; temperature

Effect Concentration of the enzyme on β-CD production

In order to determine of the optimum concentration of enzyme, the effect of the enzyme concentration to the production β-CD was investigated. As seen in the Figure 6, at 0.5% (v/v) enzyme concentration, production of β-CD reached the maximum. The yield was not significantly different with 0.75% but significantly different with the other concentrations. Relative yield of β-CD decreased slightly when the enzyme concentration on exceeded 1.25%.

These studies have proved that this method was highly efficient for the enzyme. Suzana (2005) reported the optimum concentration enzyme for production β-CD through use of debranching enzyme and CGTase from gelatinized sago starch was 2 %. Thus the efficiency obtained using this method was 4 times more efficient.

Effect of the agitation β-CD to production

Agitation was needed in enzymatic reaction system to facilitate the distribution of reactants, media and products. Figure 7 showed the effect of agitation on the relative yield of β-CD from ungelatinised sago starch. The reaction was run at 100 – 250 rpm with range 50 rpm for mixture 10 % sago starch in 0.05 M Glycine-NaOH buffer pH 9, 0.5% (v/v) enzyme and temperature 65°C for 5 hour.

Agitation rate of 200rpm was found to be suitable for the reaction of ungelatinized ago starch with CGTase Toruzyme™. At higher agitation speed the reaction rate was lower. The effect of agitation rate was 200 rpm different significantly ($p<0.05$) with the other rate of agitations in production β-CD.

Cyclodextrin production

According the studies above, the cumulative optimum conditions was found as follows: temperature, 65°C; pH 9 in buffer glycine-NaOH; the substrate concentration was 15% (w/v); the enzyme concentration, 0.5% (v/v) and the agitation rate was 200 rpm. Based on these data, the profile of cyclodextrin production can be studied in detail. Figure 8 showed the cyclodextrin production during 24 hours reaction.

Cyclodextrin production was increased significantly during the first 4-hour; beyond that the production of cyclodextrin decreased slowly until 12-hour. The highest cyclodextrin production was reached at 4-hours reaction with the ratio product was 28: 64: 8 for α-CD: β-CD: γ-CD. β-CD was the major product with more 50% after 12-hour reaction, where production cyclodextrin reached saturation, thus limiting further CGTase reaction. β-CD saturated at concentration about 5 g/L.

CGTase Toruzyme™ was β-CGTase; this fact was shown in our experiments where the major product of reaction was β-CD. During the first 8-hours, more than 50% β-CD was produced. However, beyond this time, the production was reduced (about 49%), however β-CD still was the major product (figure 8). Mahat *et al.* (2004), Ibrahim *et al* (2004), Sian *et al* (2005) and Rahman *et al* (2004) also reported various strains *Bacillus sp* was β-CGTase

Decreased cyclodextrin production after 4-hours, may be caused by the cyclodextrin produced during 4 hours, inhibited the cyclization reaction, and the coupling reaction and the disproportions more actived caused the degraded CD to the linear carbohydrates (Kim et al., 1995).

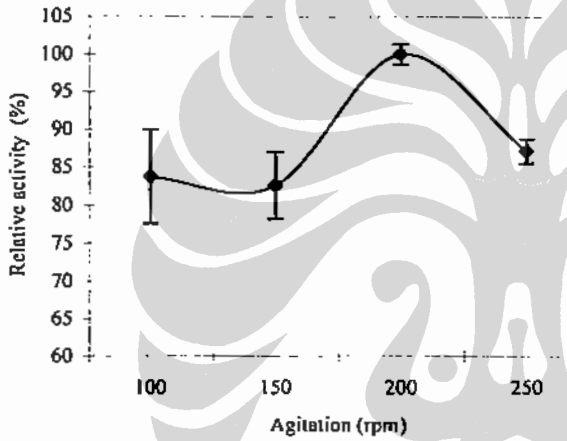

Figure 7. The effect Agitation on the relative activity production β-CD

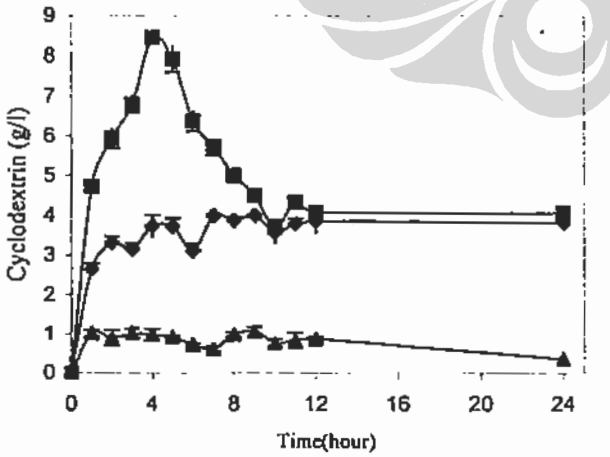

Figure 8. Cyclodextrin production scale 5 liter under condition optimum ▲;γ-CD ■; β-CD ◆;α-CD

The effect of metal ion and reagent on activity production β-CD

The effect of metal ion on the activity enzyme to production β-CD determined by incubating 0.5% (v/v) enzyme in 0.05M glycine-NaOH pH 9 containing different concentration final ion 1mM with 10% (w/v) sago starch at 65^{0}C for 5 hours. Supernatants were analyzed with HPLC. The enzyme activity to β-CD production is summarized in Table 2. The activity of CGTase was strongly inhibited by almost of all the metal and cyclodextrin except $MgCl_2$ and $CoCl_2$ was slightly inhibited. However the $CuSO_4$, $ZnCl_2$, $FeSO_4$, and $Co(NO3)_2$ caused the enzyme was inactivated, may be these metal binding active site enzyme strongly so the conformation of enzyme changed.

Although the presence of Ca^{+2} or Mg^{+2} ions was reported to be able to enhance the stability of enzyme, but most the CGTase no influenced by ion Ca^{++} (Chao, X et al., 2005; Higuti, et al., 2003). In this study, the Ca^{+2} ion also not enhanced the enzyme activity, as shown in Figure 10.

Table 2. Effect of various reagents on CGTase activity

Reagent	Relative Production β-CD (%)
NaCl	59.3 ± 5.82
KCl	51.7 ± 6.08
$MgCl_2$	79.4 ± 4.63
$MnCl_2$	38.5 ± 4.32
$CuSO_4$	1.3 ± 0.91
$ZnCl_2$	6.9 ± 2.64
$FeCl_2$	30.3 ± 4.65
$FeSO_4$	2.5 ± 0.66
$MnSO_4$	46.6 ± 4.11
$FeCl_3$	47.0 ± 3.60
$CoCl_2$	70.8 ± 7.98
$Co(NO_3)_2$	5.5 ± 2.06
ZnSO	27.5 ± 6.30
SDS	24.7 ± 4.63
EDTA	47.9 ± 7.41
α-CD	15.9 ± 6.39
β-CD	42.2 ± 6.03
γ-CD	49.9 ± 2.30

Effect the organic solvent to production of cyclodextrin

Effect of various organic solvents to the production yield of cyclodextrin by CGT toruzyme was studied. Table 3 show the relative production of β-CD and product ratio during 5 hours incubation at

65^{0}C with 15% sago starch in 0.05M glycine-NaOH buffer pH 9.

In the absence of solvent (control), CGTase Toruzyme produces α-CD: β-CD: γ-CD ratios of 34.4:58.8:6.8. When solvent were added, only cyclohexane, 2-propanol, methanol and iso octanol caused decreased production of α-CD, β-CD and γ-CD however the other increased product selectively.

Production of α-CD can be improved by about 8 times than none if the n-pentane was added to the reaction mixture. Similarly, ethanol also improved the production of α-CD by about 306.8 % compared to control, whereas the highest selectivity for β-CD production was obtained with added the isoamyl alcohol. Also with 2-octanol and isobutyl alcohol. The addition of acetonitrile doubled the production of γ-CD compared to control.

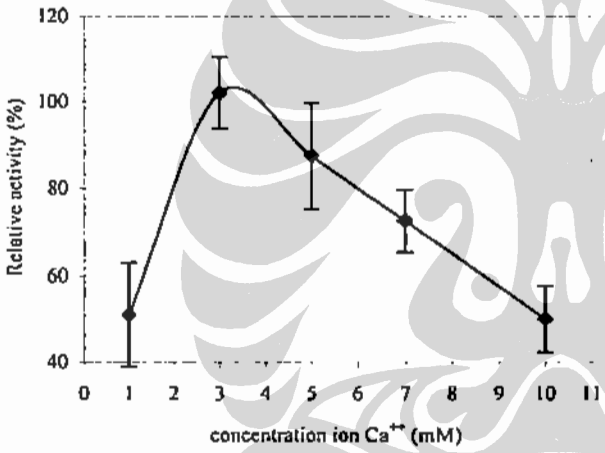

Figure 9. Relative activity enzyme CGTase to production β-CD in presence various ion Ca^{+2}

Kinetic Enzymatic reaction

The *K*m and *V*max value for the CGTase Toruzyme activity on β-cyclodextrin production from ungelatinized sago starch were determined by incubating 0.5% (v/v) CGTase Toruzyme in 0.05M glycine-NaOH buffer at various concentration of sago starch, 1% to 50% (w/v) at 65^{0}C for 4 hours. The values of Km and Vmax determined from Lineweaver-burk plot

Figure 10 show on the cyclodextrin production at various concentration sago starch under optimum conditions at 1% to 50% (w/v). The increase in sago starch was followed by increased in cyclodextrin production. At the concentration of sago starch of less than 20%, concentration of product increased rapidly, but at concentration of more than 20%, the production rate was slower.

From Lineweaver-Burk plot (Figure 11) the *K*m and *V*max values obtained were 16.70 % sago starch (w/v) and 0.09 g β-cyclodextrin/L/min (21.68 g/L for 4 hours), respectively. *K*m values 16.70% mean that 16.70 % sago starch was needed to achieve half of the *V*max, 21.68 g/L. The *K*m Value was specific to each enzyme, depending on the kind enzyme and the substrate, as well as the reaction condition. The same enzyme with different substrate would have the different *K*m, also with condition reaction (Lehninger, 1982).

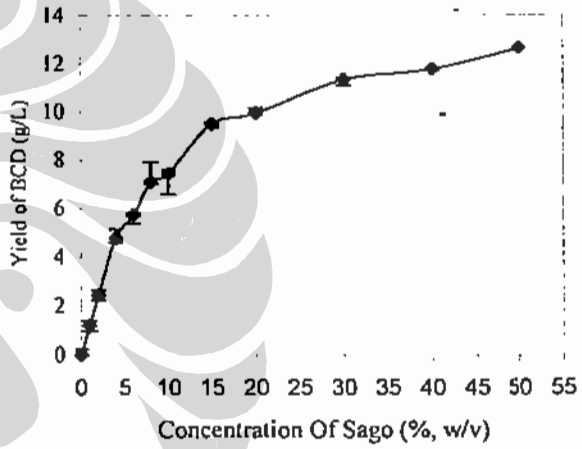

Figure 10. Production β-cyclodextrin at various concentration sago starch under optimum condition to determined Vmax and Km values

DISCUSSION

Cyclization reaction for cyclodextrin production can be achieved using various methods, e.g. with liquefaction (Okada, 1983; horikoshi, 1981), moderate heat-treatment methods (Kim, 1995), solvent methods (Yang, 1990; Blackwood, 2000; Lee, 1991).

In this study, cyclodextrin was produced Enzymaticaly with CGTase and ungelatinized sago starch as substrate. Reaction of CGTase and sago starch at neutral and alkaline pH can be significantly affecting the production of cyclodextrin; while at the acid condition (pH 4-6) the reaction will form low molecule weight molecules saccharide, such as glucose, maltose, and maltotriose (results not shown).

The glucose, maltose, maltotriose can accelerate the coupling and disproportionation reactions of

CGTase, where the cyclic cyclodextrin produced can be degraded to form linear saccharide and caused the decrease the cyclodextrin production.. These conditions have advantages, where residual, undegraded sago starch can be separated from cyclodextrin in reaction mixture by centrifugation.

Table 3. Relative production cyclodextrin and product ratio in presence organic solvents

Solvent [a]	Product concentration (g/L)		
	α-CD	β-CD	γ-CD
Control	3.961± 0.154	6.766 ± 0.402	0.776 ± 0.035
Isobutyl alcohol	8.176 ± 0.398	26.58 ± 6.310	0.923 ± 0.082
Isoamyl alcohol	10.66 ± 0.938	40.86 ±13.261	1.284 ± 0.364
1-oktanol	5.645 ± 0.453	4.912 ± 0.154	0.228 ± 0.217
n-heptane	3.496 ± 0.125	7.124 ± 1.043	0.090± 0.020
Toluene	3.969 ± 0.139	7.716 ± 0.318	0.942 ± 0.231
Cyclohexane	3.365 ± 0.358	6.713 ± 0.360	0.750 ± 0.097
2-propanol	3.196 ± 0.368	6.588± 0.514	0.642 ± 0.061
Methanol	2.187 ± 0.290	2.162 ± 0.707	0.334 ± 0.016
Ethanol	8.707 ± 0.361	1.160 ± 0.380	0.281 ± 0.004
Iso Octanol	0.503 ± 0.13	0.693± 0.319	0.261 ± 0.028
Acetonitril	5.301 ± 0.451	6.202 ± 0.211	1.480 ± 0.154
Ethyl Ether	2.622 ± 0.379	6.709 ± 0.198	0.779 ± 0.290
2-Octanol	1.351 ± 0.142	13.77 ± 1.091	0.278 ± 0.062
n-Pentane	23.48 ± 3.981	4.862 ± 0.373	0.671 ± 0.126
n-Hexane	2.052 ± 0.382	3.572 ± 0.027	0.633 ± 0.110
Acetone	2.383± 0.334	7.479 ± 0.288	0.568 ± 0.00

[a] initial concentration of organic solvent was 10% (v/v).

Enzymaticaly, production process of β-CD using CGTase and the ungelatinized granular sago starch occurred on surface of granular of sago starch and enzymatic reaction will stopped at branch of amylose or amylopectine chain because CGTase cannot hydrolyze the α-1.6 gylcosidic of starch. Hence the activity of cyclodextrin production using this method is lower than methods with gelatinized sago starch

The gelatinization temperature of sago starch is initiated at 69°C and completed at 77°C (Table 1). Cyclodextrin production with gelatinized sago starch gave higher result than ungelatinized starch. 10.65g/L β-CD was produced using gelatinized sago starch while reaction of CGTase on ungelatinized starch produced only 8.451 g/L. However, the recovery of CD was expected to be easier because the unreacted, granular ungelatinized sago starch can be easily separated.

Using the ungelatinized sago starch as substrate for β-CD production exhibit some difference significantly with the soluble starch and the gelatinized sago starch. The first, pH optimum to β-CD production occurred at pH 5.5 in Na-Acetate buffer to soluble starch (Novo Nordisk, 2004) and pH 8.5 in phosphate buffer to the gelatinized sago starch, whereas for ungelatinized sago starch occurred at pH 9 in glycine-NaOH buffer.

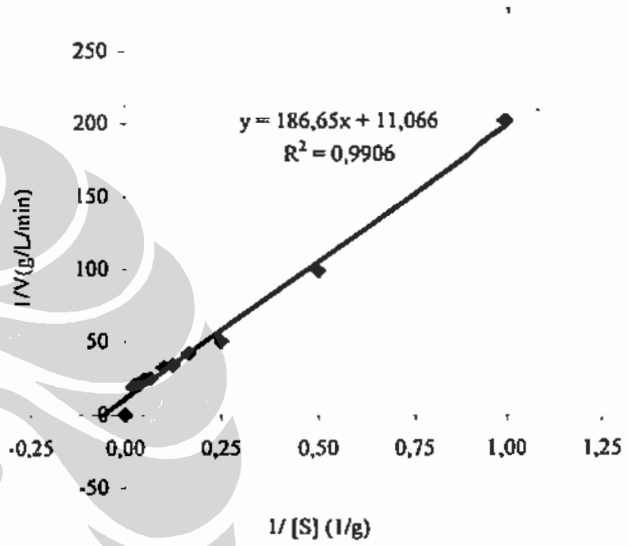

Figure 11. Lineweaver-burk plot; Reciprocal Substrate vs. Reaction rate production β-CD between CGTase Toruzyme and Sago starch ungelatinized

The second difference was enzyme concentration needed to production of β-CD. To achieve maximum productivity for β-CD production, enzymatic reaction between CGTase Toruzyme™ with the ungelatinized starch require 0.65 Unit CGTase /gram starch whereas the gelatinized sago starch require 14.5 Unit/gram starch (Suzana, *et al*, 2005), this mean only 5% than used gelatinized starch. Even though, productivity CGTase Toruzyme™ to production of β-CD with the ungelatinized sago starch only 1/3 than gelatinized sago starch.

At the optimum condition, Ca^{++} and Mg^{++} did not affect cyclodextrin production using the CGTase Toruzyme™ at the low concentration (1 mM). CGTase Toruzyme™ is a heat-stable enzyme and this enzyme does not require the presence of Ca^{++} or Mg^{++} ions to retain the activity catalytic. However, at high concentration of Ca^{++} (3-7mM) activity CGTase increased. Wind, *et al.* (1995) also reported that the

heat-stable CGTase enzyme from *Thermoanaro-bacterium thermosulfurigenes* EM1, in the presence of 10 mM calcium chloride, the enzyme retained 100% activity at 90°C for 2 hours. While the other metal ions tested showed negative influence to CGTase reaction on ungelatinised sago starch. Cyclodextrin productions on scale of 1 liter and 5 liters have different optimum time for cyclodextrin production. Reaction in 1-liter reactor, achieved the highest cyclodextrin production at 5 hours reaction while for 5 liter, it was 4 hours reaction. These results suggested that the reaction time of 4 hours was more efficient than 5 hours for the production of β-CD at bigger capacity.

These methods to cyclodextrin production show the efficient utilization of enzyme CGTase. This study used only 0.5% (v/v) enzyme for 15 % sago starch, while 2% enzyme was needed for the reaction with 6% (w/v) gelatinized starch.

4. SUMMARY

Cyclodextrin glucanotransferase (CGTase) Toruzyme™ is β-CGTase and exhibits good thermostability with high affinity for substrate to production of cyclodextrin with β-CD as the major product. This CGTase show the difference activity if the ungelatinized sago starch used to production of β-CD than the gelatinized sago starch and soluble starch. The optimum condition for the reaction occurred at pH 9 (0.05M Glycine-NaOH buffer) with concentration enzyme and sago starch was 0.5% (v/v) and 15%(w/v), respectively. The highest amount of total cyclodextrin (13.17 g/L) was produced when the reaction mixture was agitated at 200 rpm for 4 hours consisting of α-CD: β-CD: γ-CD at ratios of 28: 64: 8, with ratio mixture CGTase and sago starch 0.65Unit/gram sago starch

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