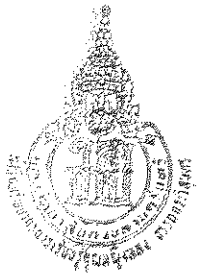

Kinetic Study of β-Cyclodextrin Production from Sago Starch by Cyclodextrin Glycosyltransferase of *Bacilus* sp. C26

Sri Rezeki Muria

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ABSTRACT

In this study, CGTase from *Bacillus* sp. C26 was used to produce β-CD from sago starch as a substrate. The maximum β-CD production was obtained with an enzyme concentration of 10 U/g of sago starch at pH 8.5 and temperature 50°C. The effect of temperature on kinetics of β-CD production by CGTase was investigated by varying temperature in the range of 40-70°C. K_m and V_{max} were calculated from the Lineweaver–Burk plot using different concentrations of substrate at all tested temperatures. It was found that initial rate of β-CD production increased when temperature increased. The increase in initial rate of β-CD production along with increasing temperature at high substrate concentration was higher than that at low substrate concentration. The V_{max} increased from 2.35 to 6.78 g β-CD/L/h when temperature increased from 40 to 65°C while K_m decreased from 39.2 to 9.78 g starch/L when temperature increased from 40 to 60°C. The lower value of K_m indicated that CGTase has higher affinity for the substrate. The catalytic efficiency (V_{max}/K_m) shows the optimum temperature at 65°C. The energy activation (E_a) of CGTase calculated by Arrhenius equation was 8.85 kcal/mol·K. The denaturation constant (K_d) values for CGTase was high at low substrate concentration while the denaturation energy constants (E_d) decreased when substrate concentration increased. It was found that CGTase was more stable in high substrate concentration. The mathematical models for catalytic efficiency and half life show the optimum temperature at 51, 52, 53 and 54 °C for 5, 10, 20, and 30 g starch/L, respectively, at 10 U/g enzyme concentration of CGTase. It was also found that the hydrolysis activity of CGTase was much lower than the activity of β-CD production.

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CHAPTER I

INTRODUCTION

Background

One of the areas of importance in biotechnology and bioengineering is the phenomenon of molecular complexation, which is useful in selecting, separation and solubilization of various bio-molecules. Cyclodextrins (CD) are advantageous molecular complexation agents. It can be synthesized enzymatically by cyclodextrin glycosyltransferase or 1,4-α-D-glucopyranosyl transferase (CGTase). CGTase is the unique enzyme able to convert starch and related sugars into CD via a cyclization reaction. CD are non-reducing cyclic structures consisting of 6, 7 or 8 glucose residues, joined by α-(1,4) linkages, for α-, β- and γ-CD, respectively. These compounds have an exclusive ability to act as molecular containers by entrapping hydrophobic molecules in their internal cavity. This property has been used for stabilization, solubilization and masking odors and tastes of a wide variety of interesting compounds used in food, pharmaceutical, cosmetic, agricultural and chemical industries. Most of the CGTase produce β-CD as the main product (Goh, *et al.*, 2009). The β-CD yield obtained by the action of CGTase is very important for a potential industrial application. And β-CD is found to be widely used in industries (Popova and Pishtiyski, 2001). This is because β-CD is able to form inclusion complexes easily within its low solubility to water. Furthermore, the separation process of β-CD from other reaction mixture is easily done without involving the usage of organic solvent (Lee *et al.*, 1992). CGTase activity is affected by several factors such as temperature, pH, and the others factor. As temperature rise the rate of chemical reactions increases because temperature increases the rate of motion of

molecules. This leads to more interactions between an enzyme and its substrate. However, if the temperature is too high, enzymes can be denatured and they can no longer bind to a substrate and catalyze reactions. Furthermore, the thermal stability of enzymes is a very important parameter in enzyme reactor designs, as it determines the limits for use and reuse of the enzyme, and therefore process costs (Santos *et al.*, 2007). Therefore, finding a working temperature that gives high enzyme activity with good stability represents a compromise between lower process costs and higher productivities.

Sago palms are considered economically acceptable and environmentally friendly, and promote a socially stable agro forestry system. Sago starch is an interesting alternative substrate for bio-production, because sago starch has a low cost of production and high yield compared to other kinds of starch. It is estimated that approximately 60 Mtons of starch are produced from sago palms annually in South East Asia (Charoenlap *et al.*, 2004). In this study, alkalophilic bacteria isolated from farms soil and identified as *Bacillus* sp. C26 which produces high yield of CGTase using sago starch as carbon source was used for CGTase production (Kitcha, 2007). The kinetics of CGTase for cyclodextrin production from sago starch was studied. The model including the effects of temperature and substrate concentration was constructed and the parameters of the model were determined. In addition, the suitable operational temperature was simulated based on the thermal stability and activity of CGTase.

Review of literature

1. Sago starch

Sago palm (*Metroxylon sagu* Rottb.) is a single species of the genus *Metroxylon* belonging to the Palmae family. Sago is the common name of the species but it is also locally called embolong, bagsang, langdang, or lumban in the Visayas, or lumbia in Bagobo. It is estimated that approximately 60 Mtons are produced from sago

palms annually in South East Asia. Sago palms are considered economically acceptable and environmentally friendly, and promote a socially stable agro forestry system (Charoenlap *et al.*, 2004). Sago, reaching a maximum height of 25 m and a diameter of 40 cm, grows in clumps. It has pinnate leaves 6-9 m long and very thick stems similar to those of the buri palm (*Corypha elata* Roxb.). The stem of a full-grown sago is 20 m long. The fruit, in clusters, is scaly, round and golden brown when mature. Sago palm has various uses. The trunk contains plenty of starch deposits with high food value. A healthy mature tree may have a trunk, 6-7 m long. During World War II, sago starch served as a good substitute for rice and corn. At present, it can be a substitute for flour. Its demand in the world market is high. The starch is extracted from the trunk by cutting down the plant. The tree is felled and the interior fibrous part of the trunk is sliced into chips or small pieces (which are eventually crushed or pulverized). These chips are then dried under the sun. The estimated starch yield per tree ranges from 50 to 75 kg (Zuniga, 2000). Sago starch is a well-known and abundant renewable raw material. Starch from the sago palm is a natural raw material alongside that from other important starch-producing plants such as potato, corn, tapioca, rice, and wheat. Besides its use as a foodstuff, sago starch can also be utilized to produce adhesives for paper, textiles, and plywood; as a stabilizer in pharmaceuticals; or converted to other types of food. It is now widely employed together with other starches in the production of noodles, monosodium glutamate, and soft drinks. New uses for sago include in biodegradable plastics, fuel alcohol, and ethanol (Singapore Zoological Gardens Docents). Table 1 summarized the utilization of sago starch and its residues. Various types of starch can be used as substrate for CGTase including corn and potato starch. However, sago starch is an interesting alternative substrate for CD production, because sago starch has a low cost of production and high yield compared to other kinds of starch (Charoenlap *et al.*, 2004). Sago is one of the many rediscovered palm species with nutritive and industrial values.

So far, the research conducted has been on the potentials of sago for food and feed, or for industrial purposes (Aziz, 2002).

Table 1. Utilization of sago starch

Sago palm part	Usage / Utilization
Refined sago starch	An ingredient of noodles, vermicelli (beehoon), Kuah-Tiau, biscuits, and many other foods Used industrially in products such as monosodium glutamate, glucose, caramel (color milk), fructose, syrups, etc.
Sago fiber	Provides bulk for rumen fermentation
Sago pitch	Used as an animal feedstuff and in the livestock Industry
Sago fronds	Used in the pulp and paper industries

Source : Aziz, (2002).

2. Cyclodextrins

Cyclodextrins (CD), as they are known today, were called "cellulosine" when first described by A. Villiers in 1891. Soon after, F. Schardinger identified the three naturally occurring cyclodextrins -α, -β, and -γ. These compounds were therefore referred to as "Schardinger sugars". For 25 years, between 1911 and 1935, Pringsheim in Germany was the leading researcher in this area, demonstrating that cyclodextrins formed stable aqueous complexes with many other chemicals. By the mid 1970's, each of the natural cyclodextrins had been structurally and chemically characterized and many more complexes had been studied. Since the 1970s, extensive work has been conducted by Szejtli and others exploring encapsulation by cyclodextrins and their derivatives for industrial and pharmacologic applications (Wikipedia, 2008a). Figure 1 shows the chemical structure of the three main types of CD, the first is α-CD have six

glucose, β-CD have 7 glucose and the last is γ-CD have 8 glucose. The most stable three dimensional structures of cyclodextrins is a toroid with the larger and smaller openings presenting hydroxyl groups to the external environment and mostly hydrophobic functionality lining the interior of the cavity (Figure 2). It is this unique configuration that gives cyclodextrins their interesting properties and creates the thermodynamic driving force needed to form host-guest complexes with apolar molecules and functional groups. Table 2 summarized the properties of the main cyclodextrins (mass, outer diameter, cavity diameter and solubility).

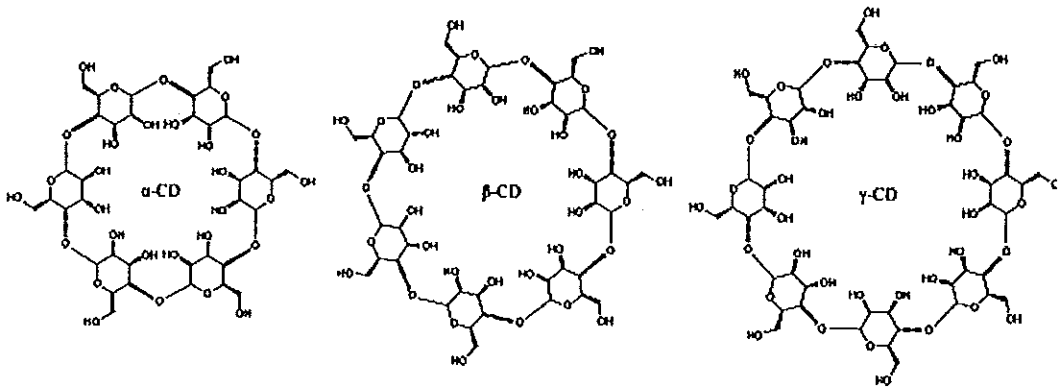

Figure 1. Structure of alpha, beta, and gamma Cyclodextrin

Source : Wikipedia (2008a)

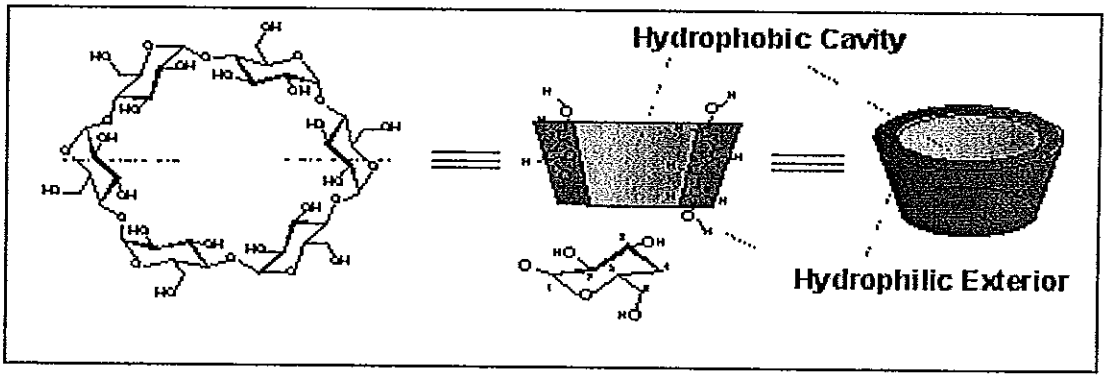

Figure 2. Structure of cyclodextrin

Source : Ispcorp (2006)

Tabel 2. Properties of cyclodextrin

Cylodextrin	Mass	Outer diameter (nm)	Cavity diameter (nm)		Solubility g/kg H2O
			Inner rim	Outer rim	
α-CD	972	1.52	0.45	0.53	129.5
β-CD	1134	1.66	0.60	0.65	18.4
γ-CD	1296	1.77	0.75	0.85	249.2

Source : Chaplin (2008)

CD can be synthesized enzymatically by cyclodextrin glycosyltransferase (CGTase). CD are non reducing cyclic structures consisting of 6, 7 or 8 glucose residues, joined by α-1,4 linkages for α-, β- and γ-CD, respectively (Jemli *et al.*, 2007). The CD molecules have a unique structure with a hydrophobic cavity and a hydrophilic surface. Due to this feature, they can form inclusion complexes with a wide variety of solid, liquid and gaseous compounds (Valle, 2004). In the complexes, a guest molecule is held within the cavity of the CD host molecule. Complex formation is a dimensional, geometrically limited fit, between host cavity and guest molecule (Singh *et al.*, 2002). Generally, hydrophobic molecules or even hydrophilic ones have greater affinity for the CD cavity when they are in a water solution. Moreover, the molecular encapsulation changes the physical and chemical properties of the included molecules. This property has been used for stabilization, volatilization and masking odors and tastes of a wide variety of interesting compounds used in food, pharmaceutical, cosmetic, agricultural and chemical industries (Jemli *et al.*, 2007). Inclusion in cyclodextrins exerts a profound effect on the physicochemical properties of guest molecules as they are temporarily locked or caged within the host cavity giving rise to beneficial modifications of guest molecules, which are not achievable otherwise (Schmid, 1989). These properties are: solubility enhancement of highly insoluble guests, stabilization of labile guests against the degradative effects of

oxidation, visible or UV light and heat, control of volatility and sublimation, physical isolation of incompatible compounds, chromatographic separations, taste modification by masking off flavors, unpleasant odors and controlled release of drugs and flavors. Therefore, cyclodextrins are used in food (Fujishima *et al.*, 2001), pharmaceuticals (Bhardwaj *et al.*, 2000), cosmetics (Holland *et al.*, 1999), environment protection (Lezcano *et al.*, 2002), bioconversion (Dufosse *et al.*, 1999), packing and textile industry (Hedges, 1998).

In addition to the common use of CD, they are used in separation science because they allow discriminating between positional isomers, functional groups, homologues and enantiomers. This property makes them a useful agent for a wide variety of separations (Moriwaki *et al.*, 2007). They have countless applications in the pharmaceutical, food, textile, and cosmetic industries, because they have a nonpolar cavity, which favors the encapsulation of a great variety of organic molecules conferring them improved physicochemical properties, such as greater chemical resistance to environmental factors, higher solubility and reduced volatility (Tardioli, *et al.*, 2006). The natural CD can be used orally, buccally, nasally and in suppositories. γ-CD should not be used parenterally because of its propensity to produce precipitable complexes of cholesterol. The other natural CD does not show this propensity for their complexes to precipitate. The chemically modified CD (methyl-, hydroxypropyl-, sulfobutyl-) are equally as safe for topical use as the natural CD, but are more suitable for parenteral use because of their greatly enhanced water solubility (Cyclodextrin Technologies Development, 2008). In the food industry CD are employed for the preparation of cholesterol free products: the bulky and hydrophobic cholesterol molecule is easily lodged inside CD rings that are then removed, leaving behind a "low fat" food. Other food applications further include the ability to stabilize volatile or unstable compounds and the reduction of unwanted tastes and odor. Reportedly CD was used in alcohol powder, a powder for mixing alcoholic drinks.

The strong ability of complexing fragrances can also be used for another purpose: first dry, solid CD micro particles are exposed to a controlled contact with fumes of active compounds, and then they are added to fabric or paper products. Such devices are capable of releasing fragrances during ironing or when heated by human body. Such a device commonly used is a typical 'dryer sheet'. The heat from clothes dryer releases the fragrance into the clothing. The ability of CD to form complexes with hydrophobic molecules has led to their usage in supramolecular chemistry. In particular they have been used to synthesize certain mechanically-interlocked molecular architectures, such as rotaxanes and catenanes, by reacted the ends of the threaded guest (Answers, 2008). The natural CDs, in particular β-CD, are of limited aqueous solubility meaning that complexes resulting from interaction of lipophiles with these CDs may also be poorly soluble resulting in precipitation of the solid CD complexes from water and other aqueous systems. In fact, the aqueous solubility of the natural CDs is much lower than that of the comparable acyclic dextrins (Brewster, 2007). The interaction of cyclodextrins (CD) with an acid-base indicator gives a stable 1:1 complex, and the equilibrium concentration can be monitored in solution by its absorbance characteristic. The method based on complexation of phenolphatalein is easy to carry out, sensitive and relatively specific to β-CD. The decolorization of the indicator is probably due to the destruction of the planar conjugated structure of the phenolphthalein molecule. The concentration of β-CD was analyzed by the decrease in absorbance at 550 nm due to phenolphtalein-CD complex formation. The colorimetric determination of β-CD was based on molecular complexation of dyes that undergoes spontaneous decolorization (Higuti *et al.*, 2004).

3. Cyclodextrin glycosyltransferase

Cyclodextrin glycosyltransferase (CGTase) is a bacterial enzyme belonging to the same family of the α-amylase specifically known as glycosyl-

hydrolase family 13. This peculiar enzyme is capable of catalyzing more than one reaction with the most important being the synthesis of non-reducing cyclic dextrins known as CD starting from starch, amylose, and other polysaccharides. CGTase is an enzyme common to many bacterial species, in particular of the *Bacillus* genus (e.g. *B. circulans*, *B. macerans* and *B. stearothermophilus*), as well as to some archaea, but it is not known to be present in any other species (Wikipedia, 2008b). CGTase is an enzyme that produces CD from starch via an intramolecular transglycosylation reaction. All known CGTases convert starch into a mixture of α-, β-and γ-CD in different ratios. Depending on the main CD produced, CGTases are classified as α, β or γ-CGTases. Among the three types of CD, β-CD is of high interest due to the size of its non-polar cavity which is suitable to accommodate many molecules such as aromatics and drugs; its low solubility in water which facilitate its separation from the reaction mixture. Furthermore, β-CD inclusion complexes are easily prepared and stable. The reported β-CGTases generate various CD yield and proportion, which depends on the microbial source of the enzyme and the bioconversion conditions. To improve the CD production yield and selectivity, organic complexing agents can be added. However, the application of organic solvents has several disadvantages limiting the use of CD in food and pharmaceutical industries. Hence, it is of interest to set out a CGTase activity producing high concentration of β-CD in the absence of organic solvent. Additionally, CGTases were reported to improve baked product characteristics such as loaf volume and to delay the bread staling during storage. Staling phenomenon, caused by the retrogradation of starch, refers to various undesirable changes that occur in the loaf during storage, thereby lowering the consumer's acceptance (Jemli *et al.*, 2007).

Major producers of CGTase are belongs to *Bacillus* sp. especially aerobic alkalophilic types. Other psychrophilics, mesophilic and thermophilic microorganisms that have been reported able to produce CGTase enzymes is *Bacillus*

stearothermophilus (Mahat *et al.*, 2004). The roles of CGTase production in various microorganisms (*Thermoanaerobacterium*, some species of *Bacillus*, such as *Bacillus megaterium*, *Bacillus macerans*, *Bacillus stearothermophilus*, *Bacillus Klebsiella*, *Bacillus firmus* and *Bacillus lentus*) are still unclear, however some researchers believe that starch is converted by CGTase into CD which can not be used by other competing organisms. In this way, the CD produced can be used as substrate by CGTase producer. The CGTase from most microorganisms are extracellular enzymes (Uitdehaag *et al.*, 2002). All of the CGTases can catalyze up to four reactions: cyclization, coupling, disproportionation and hydrolysis. All these activities share the same catalytic mechanism which is common to all glycosyl-hydrolases. A fascinating feature of cyclodextrin glycosyltransferase is its capability to produce cyclodextrins from linear starch oligosaccharide chains. Cyclization activity is a special form of acceptor specificity, in which the non-reducing end of a donor chain is used as acceptor, leading to cyclic products (see Figure 3) (Uitdehaag *et al.*, 2002). The coupling reaction can be easily described as the reverse process of cyclization: the enzyme cleaves a cyclodextrin to produce a linear dextrin which is subsequently joined to a linear oligosaccharide. Disproportion is very similar to coupling, but the cleaved dextrin is not a cyclodextrin, but a linear oligosaccharide that is then joined to a second oligosaccharide. CGTase also has a weak hydrolyzing activity which consists in cleaving the longer polysaccharidic chains into shorter fragments (Wikipedia, 2008c). The molecular weight of the CGTase enzyme was determined by SDS-PAGE and found to be 77.6 kDa. This value is within the usual range of molecular weight obtained for CGTases from different microorganisms (66–80 kDa) (Matioli *et al.*, 2001).

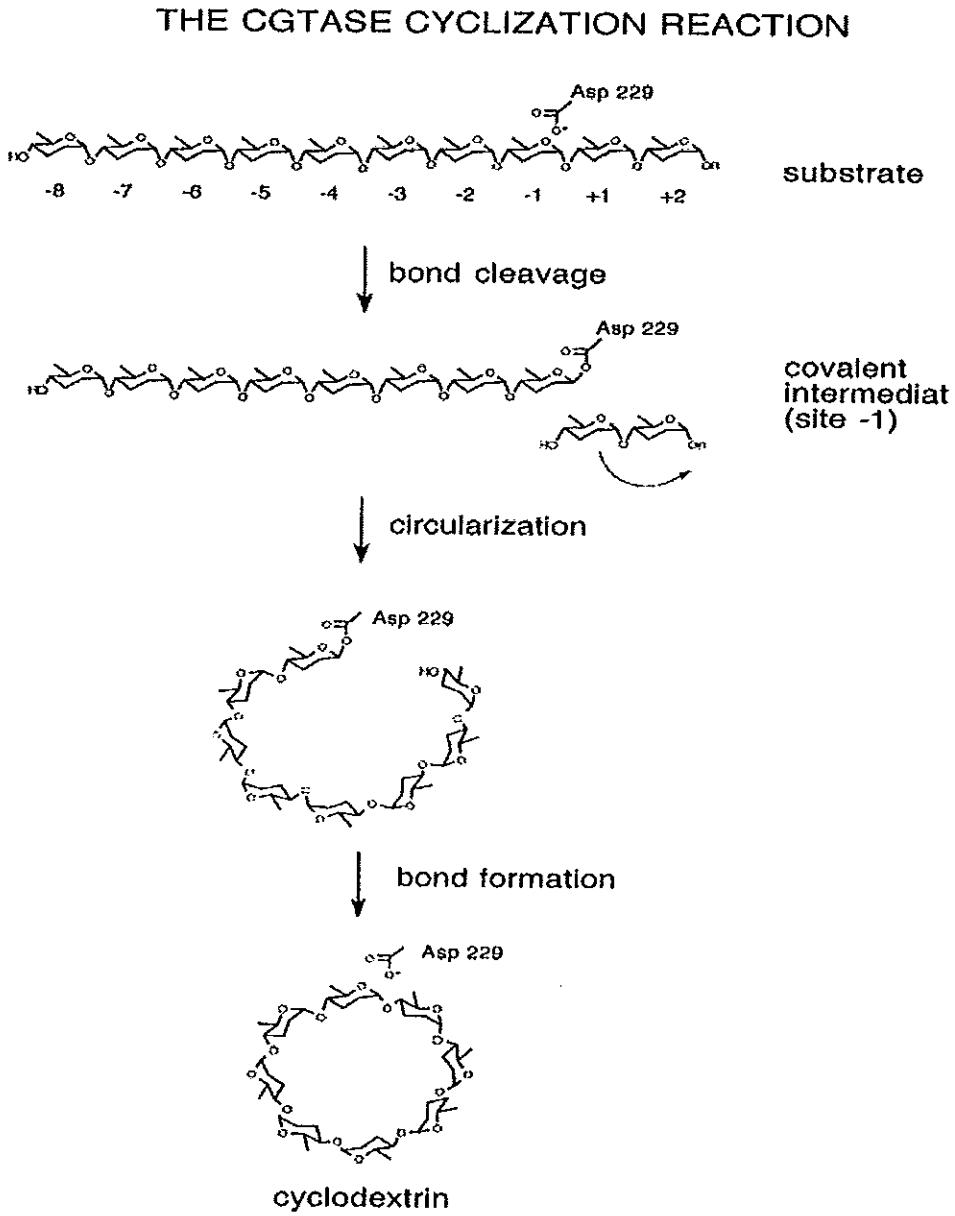

Figure 3. The CGTase Cyclization Reaction

Source : Uitdehaag *et al.* (2002)

4. Cyclodextrin production

The production yield and ratio of the different CD formed by CGTases is dependent not only on the microbial source producing the enzyme but also on the

nature of the substrate and the bioconversion conditions (such as enzyme concentration, substrate concentrations, temperature, pH and time) (Cao *et al.*, 2005).

4.1. Effect of enzyme concentration

The CD yield obtained by the action of CGTase is very important for a potential industrial application. CD yield would determine the suitable enzyme concentration. Kim *et al.* (1997) determined the optimum concentration of enzyme for CD production from 10% corn starch at pH 6.0 and 65°C. They found that the optimal enzyme concentration was 22 U/g starch because the produced CD was nearly the same as when the enzyme concentration was above 22 U/g starch. Gawande and Patkar (2001) studied about the effect of enzyme concentration on CD production. They found that the optimal enzyme concentration was 10 U/g starch and addition of more enzyme did not show any improvement in CD production yield. Martins and Hatti-Kaul (2002) studied the CD production by varying the amount of enzyme per gram of the substrate using 5% (w/v) maltodextrin incubated at 50°C and pH 8.0. With increasing the enzyme concentration the production of β-CD decreased drastically and became undetectable at enzyme concentration of 30 U/g (Table 3). On the other hand, α-CD production increased to over 2 mg/ml at the enzyme concentration of 5-10 U/g, before falling again at 30 U/g. The total amount of CD produced was significantly lowered at 10 U/g and above, which was accompanied by formation of low levels of γ-CD (0.5% conversion). Jemli *et al.* (2007) determined the suitable enzyme concentration for a maximal productivity, by varying the amount of enzyme per gram of the substrate (50-300 U/g). The conversion rate of starch into CD was about 34, 42, 30 and 28% for 50, 100, 200 and 300 U/g, respectively. This result indicated that the enhancement of CD production was not closely correlated with the increase in the enzyme concentration; there by the best concentration to be used is 100 U/g of substrate.

Tabel 3. Effect of enzyme concentration on the production of CDs by *B. agaradhaerens* LS-3C CGTase.

U/g[a]	mg/ml			Conversion into CD (%)	Reducing sugar content (mg glucose/ml)
	α-CD	β-CD	γ-CD		
2	0.79	4.37	-	10.3	13.8
5	2.16	3.06	-	10.4	16.6
10	2.13	0.47	0.27	5.7	16.0
30	0.28	-	0.26	1.1	19.2

[a] Units of CGTase per gram of maltodextrin (the concentration of maltodextrin used was 5% (w/v)).

Source : Martins and Hatti-Kaul (2002)

4.2. Effect of substrate concentration

According to Kim *et al.* (1995), the concentration of gelatinized starch used as substrate cannot be increased above 5% (w/v) because the solution becomes very viscous, which hinder the procedures and decrease the yield of CD. Kim *et al.* (1997) studied the effect of starch concentration using the concentration of enzyme at 22 U/g starch, pH 6.0 and 65°C. According to an increase in the concentration of substrate, the relative content of cyclodextrin after production and centrifugation was decreased and the concentration of cyclodextin was increased. The relative content of cyclodextrin from soluble starch after centrifugation was from 29-38%. Because soluble starch does not have particle structure, the residual substrate could not be separated by centrifugation. In order to get 50% CD, 7.5% substrate was chosen. The maximum concentration of substrate would be chosen for CD production within the allowable relative content of CD. Gawande and Patkar (2001) investigated the substrate concentration which would give the maximum concentration of α-CD. The optimal substrate concentration of gelatinized soluble starch and raw wheat starch were found to be 100 g/L and 125 g/L, respectively. Starch solutions, at concentration

higher than 150 g/L become highly viscous and are difficult to handle. Some industrial processes use up to 300 g/L starch concentration, but in these processes the starch is liquefied with either CGTase before CD production. Matioli *et al.* (2002) measured the influence of substrate concentration in relation to the production of β- or γ-CD, and determined substrate inhibition. The substrate solution for the β-CD and γ-CD assays were prepared in the ranges of (0.05% - 30% (w/v)) and (0.1% - 20% (w/v)), respectively. In both cases, substrate inhibition is clearly seen, because activity initially increases with substrate concentration, but it reaches a maximum and then decreases with a further increase in substrate concentration. The point at which the maximum occurs is different for each CD, and substrate concentrations at these points are about 0.02 and 0.03 M for β- and γ-CD, respectively. Substrate inhibition reduces the initial rate of CD production by about 10% at the substrate concentrations of 0.0075 and 0.023 M for the production of β- and γ-CD, respectively. These results show that CGTase from *B. firmus* is inhibited by substrate to a greater degree for β-CD production.

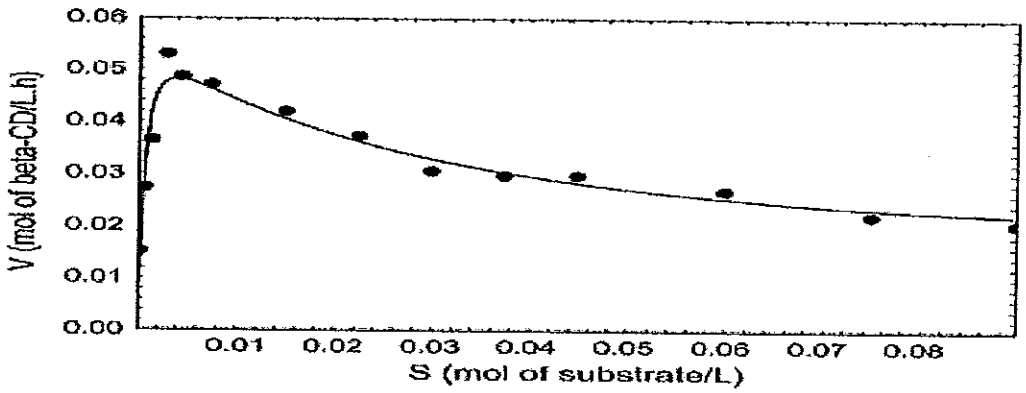

Figure 4. Initial velocity of β-CD production (V) by CGTase of *Bacillus firmus* strain 7B as a function of substrate concentration (S), and model comparison. (Conditions were: pH 8.0, 50°C, 50 mM Tris–HCl buffer and the substrate was maltodextrin (DE 10).)

Source : Moriwaki *et al.* (2002)

Figure 4 shows data at the initial rate of reaction (V) as a function of the substrate concentration. The substrate inhibition is clearly seen because the initial rate of CD production at the reaction beginning increased until a maltodextrin concentration of 0.0029 mol/L was reached, and then, decreased with further increase in substrate concentration (Moriwaki *et al.*, 2002). The evaluation of different pretreatments of starch that could allow the use of higher concentrations of starch was a convenient step to improve the concentration of CD obtained in the reaction. Szerman *et al.* (2007) found that the concentration of β-CD increased with the increase of pretreatment temperature from 55°C to 70°C with up to 20 min of treatment. They also studied the effects of the concentration of substrate on the starch bioconversion to CD. They calculated the percentage of bioconversion from the concentration of β-CD obtained in each assay and the initial concentration of cassava starch. Although the highest concentration of β-CD was achieved with 15% starch, the maximum bioconversion results were obtained using 5% starch and 15 CGTase U/g starch. Prado *et al.* (2007) used CGTase produced by *Bacillus clausii* strain E16 to produce CD from maltodextrin, soluble starch and different starches (corn, cassava, sweet potato, and waxy corn starches) at 2.5% of substrates concentration under agitation of 100 rpm at 55°C for 24 h. Soluble starch was a better substrate than maltodextrin for CD production because the better conversions were observed on 1.0% soluble starch, which showed a major conversion 80% or in 2.5% maltodextrin 41%. Effects of starches: cassava starch, sweet potato starch, corn starch and waxy corn starch from different botanical sources on CD production by CGTase from *B. clausii* strain E16, homogenized by heating in a boiling water bath have conversion 22%, 21%, 7.3% and 1.5%, respectively.

4.3 Effect of temperature

Matioli *et al.* (2001) studied the activity of CGTase strain no. 37 as a function of temperature for β-CD and γ-CD production. As the temperature increased the ratio of γ-CD/β-CD produced increased (i.e., an increase in the temperature favors the production of γ-CD in relation to β-CD). At 70°C the ratio of γ-CD/β-CD production (0.15) was nearly double that observed at 50°C (0.077). In the determination of the β-CD produced by the CGTase of strain no. 37, as a function of temperature, the maximal specific activity was found at 65°C (71.5 mmol of β-CD/[min · mg of protein]). For the γ-CD, the maximal specific activity was observed at 70°C (9.1 mmol of γ-CD/[min · mg of protein]). It was found that both the optimal pH and optimal temperature values, of maximal specific activity of the enzyme, were different for the products β- and γ-CD. The values of optimal temperature for CGTase strain no.37 are in agreement with those found in the literature, and the optimal temperature for known *Bacilli* is found in the range of 45–70°C. Illias *et al.* (2002) studied the effect of different temperatures on the activity of CGTase by incubating the reaction mixture at temperatures ranging from 20-80°C. Optimum temperature for maximum CGTase activity was found to be 70°C. At temperature higher than 70°C, activity of CGTase declines sharply may be because the CGTase could be denatured above 70°C. Relative activity also decreased by about 60% at 80°C. However the crude CGTase from *Bacillus* sp. G1 was found to be quite stable at temperature ranging between 20°C to 60°C. Qi *et al.* (2003) compared the effect of different reaction temperatures on the cyclization and coupling reactions of the CGTase from *B. macerans* influencing the yield of largering CD synthesis products. The maximum yield of large-ring CD after 2 h of incubation at 60°C reached about 50% of the total glucan and remained almost constant during longer incubation time. The results show an optimum of the cyclization reaction at 60°C, while the optimum for the coupling activity with γ-cyclodextrin as substrate was found around 45°C. A comparison of the

kinetic parameters of the cyclization reaction of the CGTase showed a higher rate of β-cyclodextrin formation at 60°C compared to 40°C. Sian *et al.* (2005) found that the optimum temperature for CGTase from *Bacillus* sp. G1 was 60°C with soluble starch as substrate. Studies done by other researchers on CGTase from *B. autolyticus* 11149, *B. stearothermophilus* and *B. circulans* E 192 also discovered 60°C as the optimum temperature, which is in agreement with CGTase from *Bacillus* sp. G1. It is interesting to note that this enzyme exhibited a minor peak at 80°C (28% relative activity), which was never reported before. The enzyme was stable up to 60°C at pH 6.0 for 30 min incubation. However, it began to lose 50% of its total activity at around 76°C and was only able to retain 14% of its remaining activity at 80°C. The CGTase was completely inactive at 90°C. The activity of the purified CGTase was measured at different temperatures at pH 6.0 by the standard assay method. The optimum temperature for CGTase from *Bacillus* sp. G1 was 60°C with soluble starch as substrate (Figure 5).

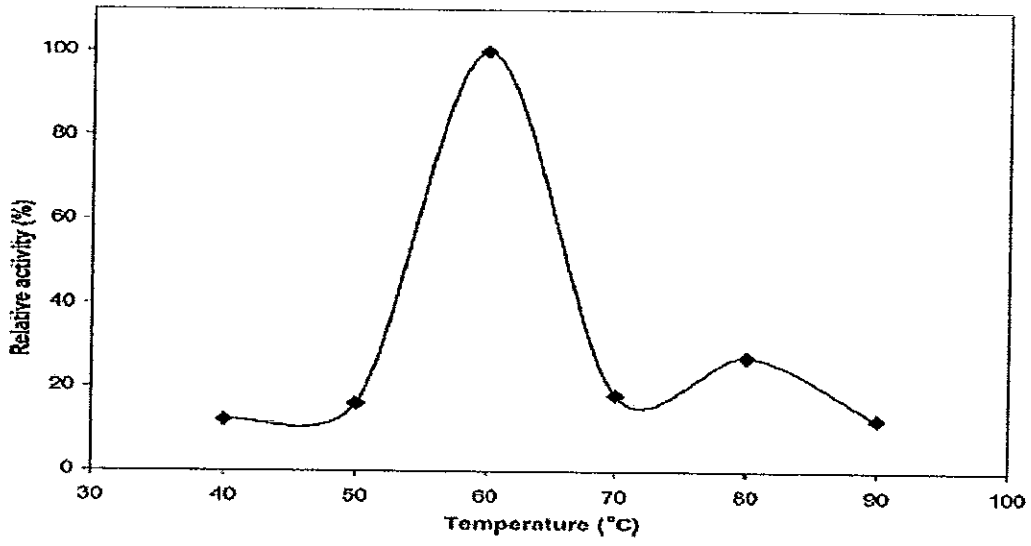

Figure 5. Optimum temperature of the CGTase from *Bacillus* sp.G1 with soluble starch as substrate at pH 6.0.

Source : Sian *et al.* (2004)

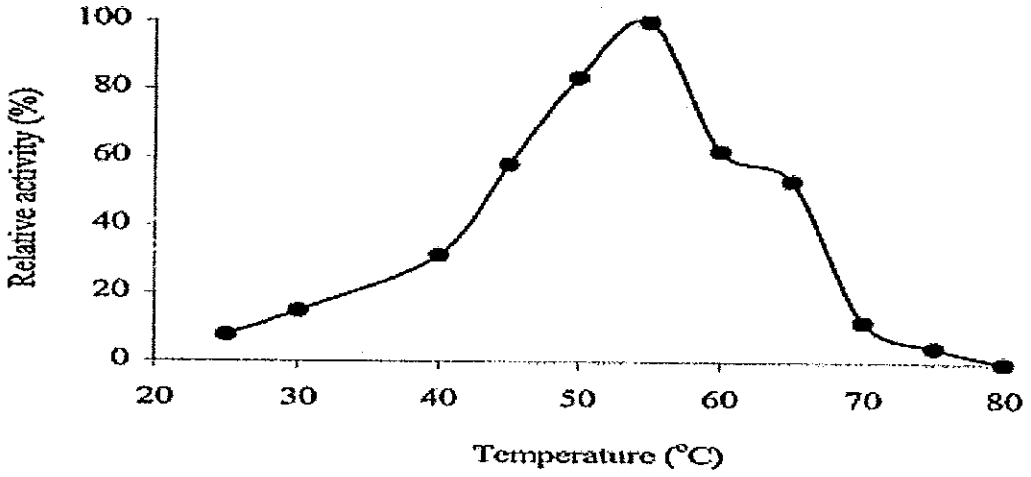

Figure 6. Effect of temperature on the activity of CGTase purified from *B. agaradhaerens* LS-3C.

Source : Martins and Hatti-Kaul (2002)

The activity of the purified CGTase was measured at different temperatures at pH 8.0. Figure 6 shows the enzyme to be optimally active at 55°C and completely inactive at 80°C. It is evident that improvement in activity of the *B. agaradhaerens* LS-3C CGTase with respect to temperature would be desirable for conversion of starch to CD (Martins and Hatti-Kaul, 2002). Szerman *et al.* (2007) reported considering that the gelatinization temperature of cassava starch ranged from 52°C to 65°C and 5% (w/v) suspension in 25 mM phosphate buffer (pH 6.4) was incubated for 60 min in a range of temperatures from 55°C to 75°C. Then, lyophilized enzyme (10 U/g starch) was added and incubated at 56°C for 60 min. The β-CD concentration was measured by the phenolphthalein method, the concentration of β-CD increases with the increase of pretreatment temperature from 55°C to 70°C. They concluded that 70°C is the minimum temperature required to obtain a reactive structure of starch granules. The data presented in Figure 7 show that the enzyme from *Bacillus firmus*, strain 7B, retained almost 100% of its initial activity in a wide range of

temperatures, between 35 and 60°C for a reaction period of 4 h. Comparison with other alkalophylic bacilli CGTases revealed that this range is practically the same. At 65°C and above, the enzyme presented increasingly higher thermal deactivation, particularly for 75°C, where it did not show any residual activity after 240 min. While enzyme CGTase from *Bacillus* sp. C26 kept in buffer at pH 8.5 showed a wide thermal stability from 30 – 50°C, though the enzyme rapidly lost its activity above 55°C (Kitcha, 2007).

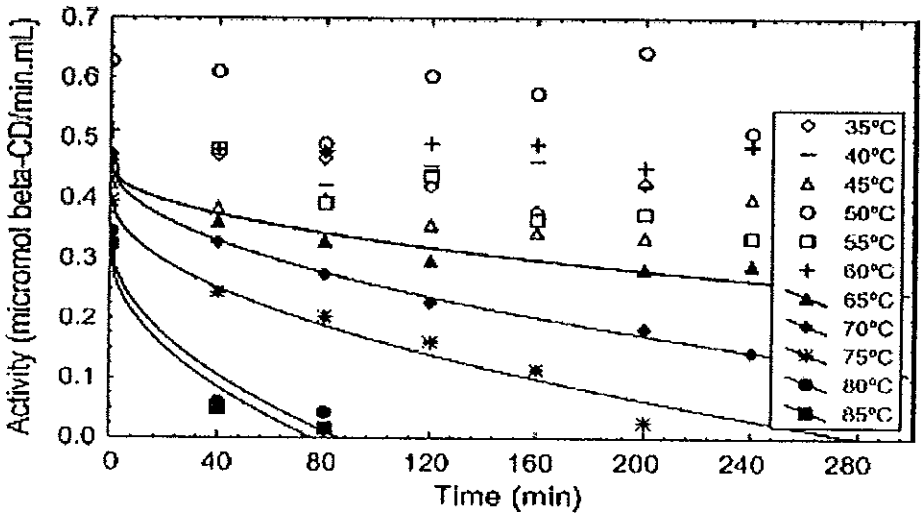

Figure 7. *Bacillus firmus*, strain 7B, CGTase activity for the production of β- CD as a function of time. (Conditions: substrate is 10% (w/v) maltodextrin, in 50 mM Tris–HCl buffer and 5 mM $CaCl_2$, pH 8.0.)

Source : Moriwaki *et al.* (2007)

5. Kinetic study

5.1 The effect of substrate concentration

Matioli *et al.* (2002) presented CGTase activity as a function of substrate concentration, the kinetic parameters K_m, V_{max}, and K_s were obtained. When substrate concentration is low enough to neglect substrate inhibition, Michaelis-Menten kinetics are adequate to fit the data and K_m and V_{max} are obtained.

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$$V = V_{max}/(1 + K_m/S) \quad (1)$$

The cyclization reaction, Eq. 1, implies an increase in the number of moles. Consequently, according to thermodynamics, the conversion of substrate to CDs should decrease with higher substrate concentration. This was experimentally observed by Horikoshi *et al.*(1982) refered by Matioli *et al.* (2002). They used CGTase from *B.* alkalophilic no. 38-2 and 1–30% potato starch solutions hydrolyzed by CGTase to DE2. They observed that β-CD yield increased from 10 to 50% when the initial starch concentration decreased from 25 to 2% (Matioli *et al.*, 2002). Figure 8 illustrates the initial rate of reaction (*V*) as a function of the substrate concentration for β-CD production (Figure 1A) and for γ-CD production (Figure 1B). In both cases, substrate inhibition is clearly seen, because activity initially increases with substrate concentration, but it reaches a maximum and then decreases with a further increase in substrate concentration. The point at which the maximum occurs is different for each CD, and substrate concentrations at these points are about 0.02 and 0.03 *M* for β- and γ-CD, respectively. Substrate inhibition reduces the initial rate of CD production by about 10% at the substrate concentrations of 0.0075 and 0.023 *M* for the production of β- and γ-CD, respectively. These results show that CGTase from *B. firmus* is inhibited by substrate to a greater degree for β-CD production. The observed reduction in the initial rate of CD production is accentuated at higher substrate concentrations. This type of enzymatic kinetic behavior for different substrate concentrations is characteristic of reactions that show inhibition at high substrate concentration.

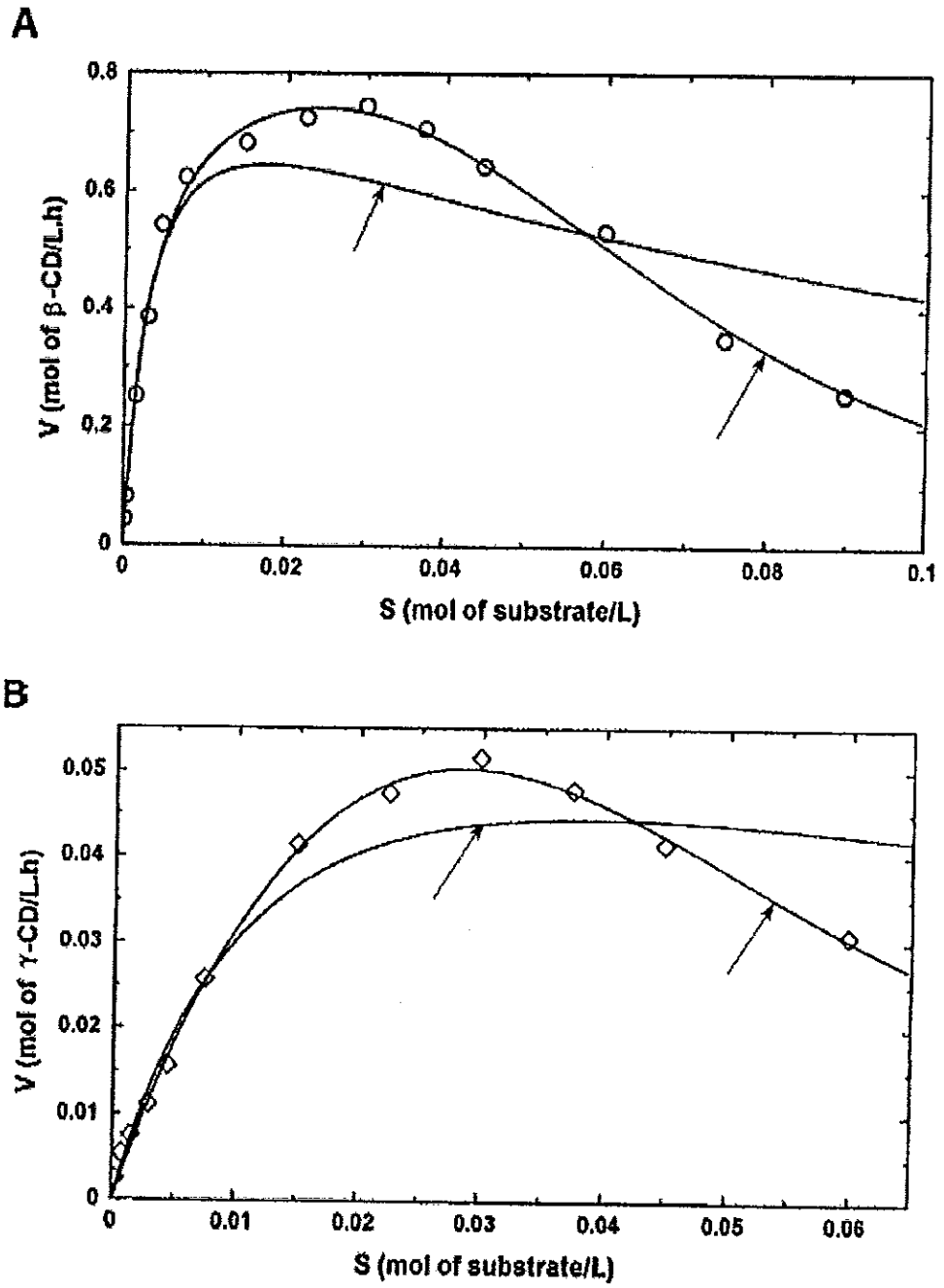


Figure 8. Initial velocity of CD production (V) by the CGTase of *B. firmus* as a function of substrate concentration (*S*), and model comparison. (Conditions were pH 8.0, 50°C, 0.05 *M* Tris-HCl buffer, 5 m*M* CaCl2, and the substrate was maltodextrin (DE 10). (A) β-CD production ; (B) γ-CD production.)

Source : Modified from Matioli *et al.* (2002)

Matioli *et al.* (2002) presented for higher substrate concentrations, the data were used to initially fit to the standard model of inhibition by substrate:

$$V = V_{max}/(1 + K_m/S + S/K_s) \quad (2)$$

in which V is the initial velocity for CD production, V_{max} is the maximal reaction velocity, K_m is the Michaelis-Menten parameter, *S* is the substrate concentration, and K_s is the substrate inhibition constant. The values for K_m and V_{max} with the lower substrate concentration data were obtained by Lineweaver-Burk method as :

$$1/V = K_m/V_{max} \cdot 1/S + 1/V_{max} \quad (3)$$

At the higher concentration data, consequently, K_s was the single adjusting parameter it was determined by the Hanes method as :

$$(S/V) = (K_m/V_{max}) + (1/V_{max})\, S + [1/(K_s V_{max})]\, S^2 \quad (4)$$

In this experiment only using Lineweaver-Burk method to measure V_{max} and K_m because did not find substrate inhibition from the result. Figure 9 show the Hanes plots used with the low substrate concentration points to determine V_{max} and Km. The adjusted straight lines gave the following values for V_{max} and K_m:

For β-CD:

V_{max} = 0.8878 mol of β-CD/(L·h); K_m = 0.0033 mol/L; r^2 = 0.9607

For γ-CD:

V_{max} = 0.0791 mol of γ-CD/(L·h); K_m = 0.0151 mol/L; r^2 = 0.9785

In Figure 8, the initial velocity data for CD production is compared with the standard substrate inhibition model, Eq. 3, adjusted to the data as indicated in Materials and Methods. As Figure 8 shows, this model does not follow the data trend very well at high substrate concentration and the fit is not entirely satisfactory. The adjusted equations and K_s values are as follows:

For β-CD: $(S/V) = 0.0037 + 1.126\, S + 12.14\, S^2$ and K_s = 0.0928 mol/L; r^2 = 0.8210

For γ-CD: $(S/V) = 0.1912 + 12.64\, S + 128.35\, S^2$ and K_s = 0.0985 mol/L; r^2 = 0.9415

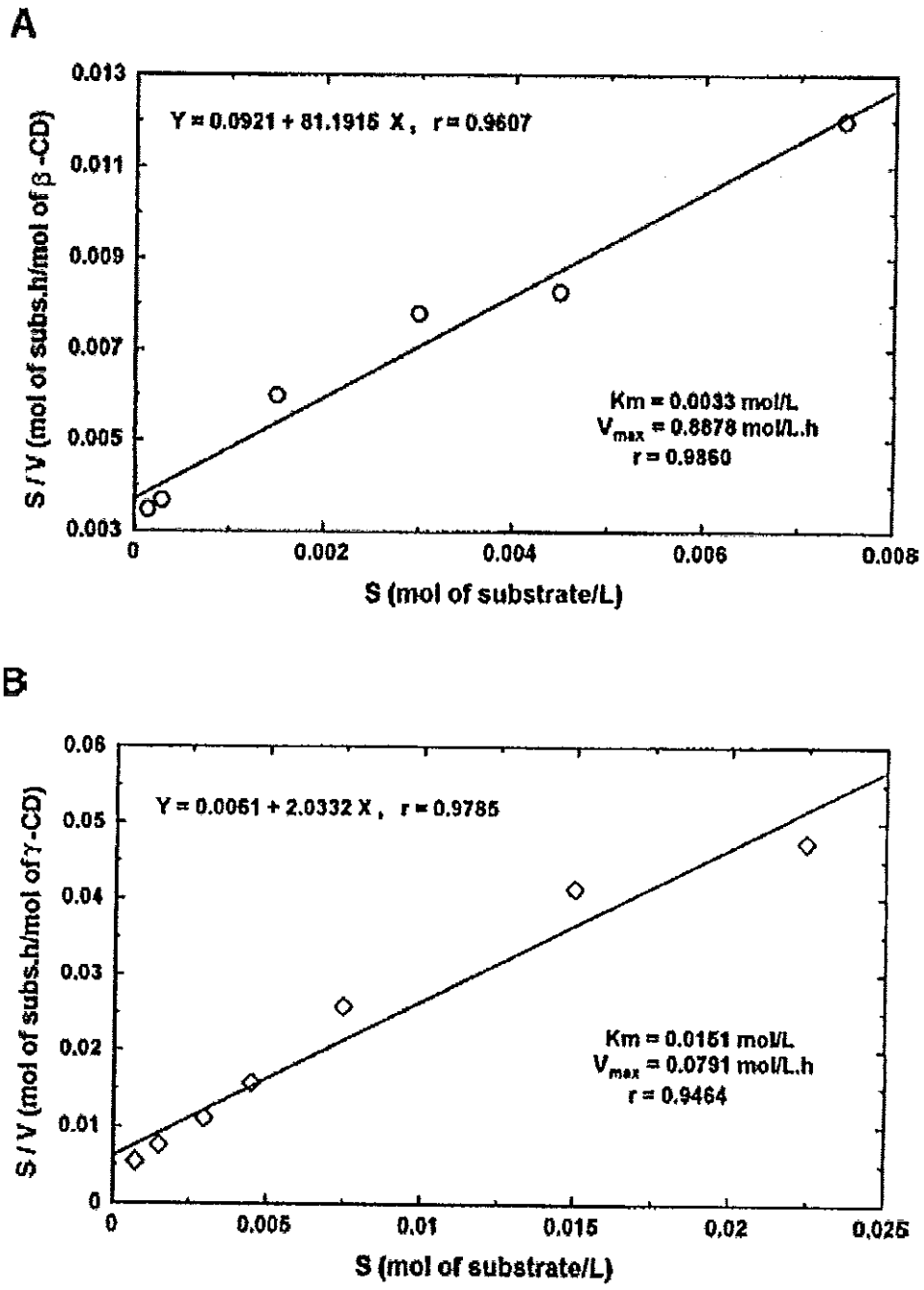


Figure 9. Determination of K_m and V_{max} by the Hanes method for the CGTase of *B. firmus*. (Only the points shown in Figure 8 with low substrate concentration were used. (A) β-CD production; (B) γ-CD production. Experimental conditions were as in Figure 8.)

Source : Matioli *et al.* (2002)

Figure 10 shows the Lineweaver-Burk plots for free and immobilized enzymes. The K_m of the enzyme increased from 2.5 to 4.5μg starch/ml upon immobilization, which is true for most of the immobilization systems. The slight increase in V_{max} from 475 μg β-CD/(ml min) for free enzyme to 515 μg β-CD/(ml min) for the immobilized enzyme is some what unusual (Arya and Srivastava, 2006).

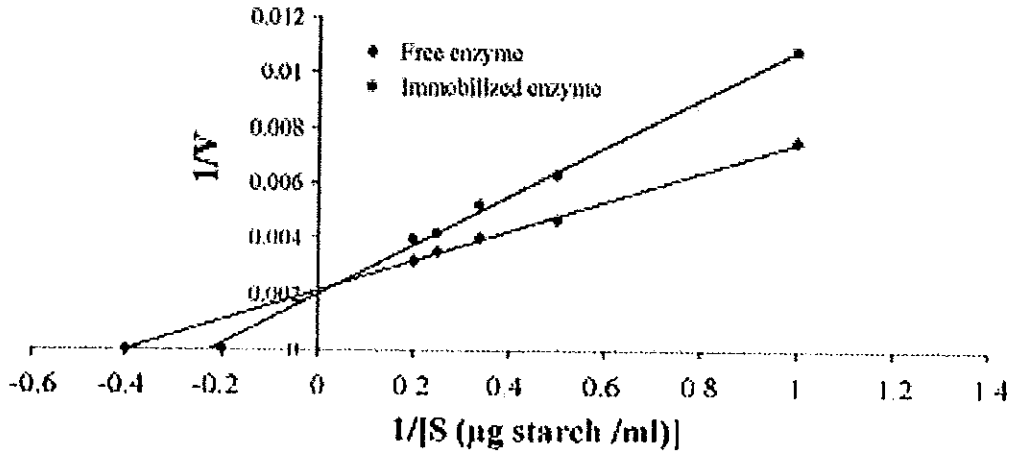


Figure 10. Lineweaver-Burk plot for estimation of K_m and V_{max} enzyme CGTase from *Bacillus macerans* ATCC 8244 at temperature 60°C and pH 7.5.

Source : Arya and Srivastava (2006)

The V_{max} and K_m values obtained by maltodextrin as substrate were 0.0706 mol of (-CD/(L h) and 0.0011 mol/L, respectively. Figure 11 shows the Hanes plots used with points of low substrate concentration to determine V_{max} and K_m. The K_m value (0.0011 mol/L) of this work is smaller than the K_m from *Bacillus firmus* strain 37 CGTase (0.0033 mol/L). Considering that the K_m parameter is correlated to the affinity of the enzyme for its substrate, the low value observed in this work indicates that the enzyme has comparatively higher affinity for the substrate. V_{max} value for this study is 0.0706 mol/L.h (Moriwaki *et al.*, 2007).

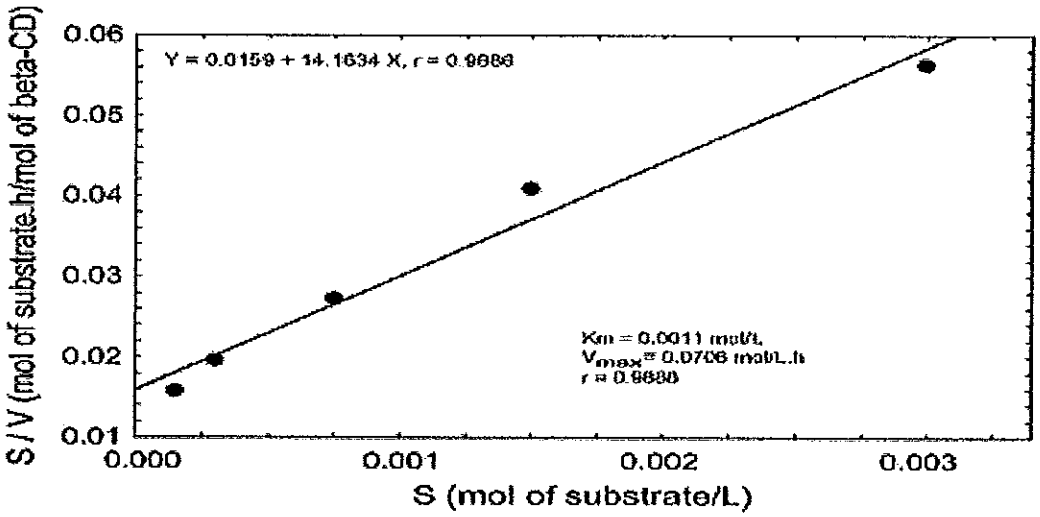


Figure 11. Determination of K_m and V_{max} by the Hanes method for the CGTase from *Bacillus firmus*, strain 7B.

Source : Moriwaki *et al.* (2007)

5.2 Effect of temperature

Every enzyme has a temperature range of optimum activity. Outside that temperature range the enzyme is rendered inactive and is said to be totally inhibited. This occurs because as the temperature changes this supplies enough energy to break some of the intramolecular attractions between polar groups (hydrogen bonding, dipole-dipole attractions) as well as the hydrophobic forces between non-polar groups within the protein structure. When these forces are disturbed and changed, this causes a change in the secondary and tertiary levels of protein structure, and the active site is altered in its conformation beyond its ability to accommodate the substrate molecules. Matioli *et al.* (2001) studied the effect of temperature on an CGTase reaction through the Arrhenius equation :

$$k = k_o \, . \exp (-E_a/RT) \qquad (5)$$

where k is the rate constant (or the specific reaction rate), T is the absolute temperature, R is the ideal gas constant, k_o is the frequency (or pre-exponential) factor and E_a is the activation energy. Both k_o and E_a are the parameters of the Arrhenius

equation, usually estimated from experimental data by plotting the Ln of the experimental initial rate constant at different temperatures, against 1/T as linear equation:

$$\ln k = \ln k_o - E_a/R \cdot 1/T \quad (6)$$

This equation was adjusted to the experimental points and it allowed the determination of the activation energy for the reaction of β- and γ-CD production, giving 7.5 and 9.9 kcal/mol, respectively. The larger activation energy found for the reaction of γ-CD production, together with the results, which show that larger temperatures favor the production of γ-CD, confirms the following general rule: "larger temperatures favor the reactions of larger activation energy". Within the range of validity, the enzymatic activity for the production of β- and γ-CD and their ratio can be calculated with the adjusted Arrhenius equation (Matioli *et al.*, 2001). The effect of the temperature on enzymatic activity was further analyzed, using the Arrhenius equation by plotting activity (Ae expressed in mmol of β-CD/(min mL)) against the inverse of temperature (1/T expressed in K^{-1}), which were further adjusted to the equation $Ae = Q \exp(-E_a/RT)$ ($r^2 = 0.998$ for $T \leq 50$ °C). The activation energy (E_a) was determined as 9.4 kcal/mol (Figure 12) (Moriwaki *et al.*, 2007). The activation energy (E_a) were 9.4 kcal/mol for *Bacillus firmus* and the reported E_a of CGTase from *Bacillus sp.*, *B. agaradhaerens* and *B. macerans* are 8.1 kcal/mol, 17.0 kcal/mol and 27.4 kcal/mol, respectively (Moriwaki *et al.*, 2001). Therefore, the E_a of the CGTase depends on their source. The high energy of activation value shows that high temperatures favour the formation of β-CD by CGTase (Martins and Hatti-Kaul, 2002).

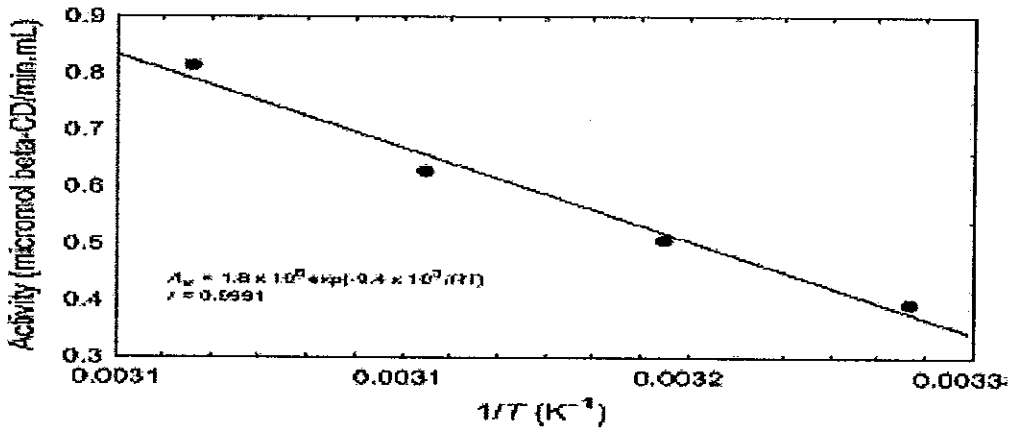


Figure 12. Arrhenius plot of the activity for the production of β-CD as a function of the inverse of the absolute temperature for the CGTase from *Bacillus firmus*, strain 7B. (Conditions: substrate is 1% (w/v) maltodextrin, in 50 mM Tris–HCl buffer, pH 8.0.)

Source : Moriwaki *et al.* (2007)

5.3 Inactivation enzyme

Most of the current mechanisms of enzyme action in chemical kinetics assume that the enzyme is stable and maintains its activity during the time course of the reaction. However, it is not uncommon for an enzyme to rapidly lose its activity in vitro conditions. Under temperature changes, diluted conditions, or changes in the reaction medium (pH or buffer), enzymes can undergo progressive loss of activity. Because of this, the current mechanisms are often not adequate for predicting chemical kinetics in industrial or in vitro conditions. Neglecting enzyme inactivation can result in errors in both estimating the kinetics parameters and reporting the mechanisms of enzyme action. For example, the observed low enzyme activity due to enzyme inactivation can be incorrectly attributed to cooperativity, substrate or product inhibition, presence of a competitive inhibitor, or two enzymes acting on the same or different substrates. Attempting to describe a system using any of the above

mechanisms when the mechanism involved is actually enzyme inactivation can result in grievous errors in describing the behavior of the system, such as incorrect estimation of the kinetics parameters. Therefore, it is very important to be able to know when enzyme inactivation is affecting a reaction (Schnell *et al.*, 2006).

The model for enzymatic denaturation is assumed as a first order process, enzyme decay can be expressed by the following equations:

$$\frac{dE}{dt} = -K_d . E \tag{7}$$

or

$$E = E_o . \exp(-K_d.t) \tag{8}$$

Where E_o is the active enzyme concentration at the starting point, usually zero time.

Since the denaturation constant, K_d, is a kinetic parameter and temperature dependent, it may also be represented by an Arrhenius-type equation.

$$K_d = K_{do} . \exp(-E_d/RT) \tag{9}$$

The values for K_d at different temperatures are obtained by linearisation of Eq. (7) whereas the values for E_d and K_{do} are estimated by plotting the Ln of K_d at different temperatures against 1/T.

Figure 13 shows the slope of the adjusted straight line that correlates the natural logarithm of K_d with the inverse of the absolute temperature (T) and the resulting deactivation energy is 37.8 kcal/mol (Moriwaki *et al.*, 2007). Another important parameter in enzyme reactor design is the thermal enzyme stability, which can be expressed by its half-life (τ) as shown in Eq. (10), which is obtained from Eq. (8) by substituting E by $E_o/2$ and K_d by its respective expression from Eq. (9).

$$\ln\left(\frac{E_0/2}{E_0}\right) = -K_d\tau$$

or

$$\tau = \frac{0.69}{K_{d0} \cdot e - \frac{E_d}{RT}} \tag{10}$$

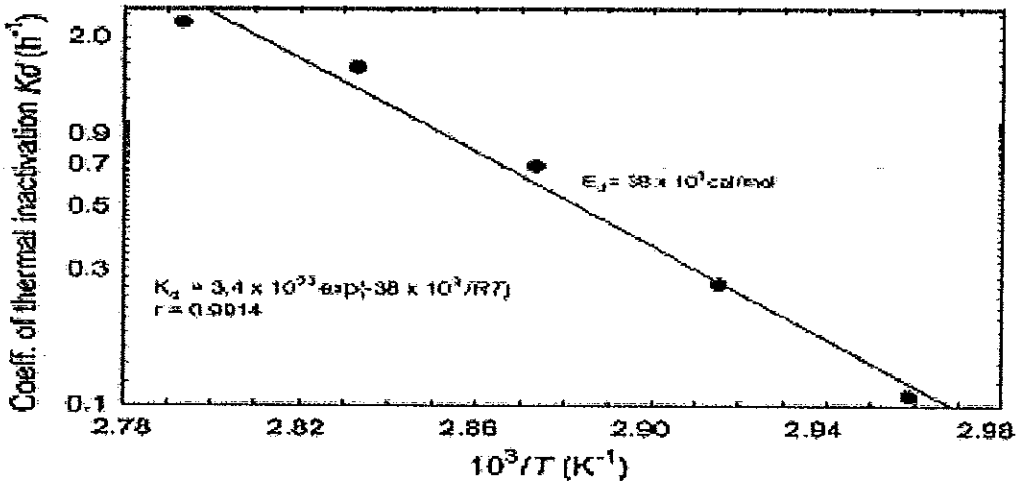


Figure 13. Arrhenius plot of the residual activity for the production of β-CD as a function of the inverse of the absolute temperature for the CGTase from *Bacillus firmus*, strain 7B. (Conditions: substrate is 1% (w/v) maltodextrin, in 50 mM Tris–HCl buffer, pH 8.0.)

Source : Moriwaki *et al.* (2007)

Objectives

1. To study kinetic reaction of CGTase.
2. To obtain the model of CGTase reaction.
3. To simulate the suitable operational condition for cyclodextrin production using the model.

Scope of research

1. The effects of CGTase amount, temperature and sago starch concentration on cyclodextrin production were studied.
2. The model including the effects of temperature and substrate concentration was constructed and the parameters in the model were determined.
3. The suitable operational temperature for cyclodextrin production by CGTase was simulated.

CHAPTER 2

RESEARCH METHODOLOGY

Material and Methods

1. Material

Sago starch was used for enzyme CGTase and cyclodextrin production. Alkalophilic bacteria isolated from farms soil and identified as *Bacillus* sp. C26 which produces high yield of CGTase using sago starch as carbon source was used for CGTase production. The maximum activity of CGTase produced by this strain was obtained at 37°C and 200 rpm for 48 h. The ratio of each CD production from this CGTase was 0.35:0.65:0 for α-CD, β-CD and γ-CD, respectively. The optimum pH for this CGTase was 8.5 and had stability over 80% at temperature 50°C (Kitcha, 2007).

2. Methods

2.1 Production of enzyme CGTase from *Bacillus* sp.C26

2.1.1 Preparation of bacterium inoculum and enzyme production

Bacteria *Bacillus* sp.C26 used for enzyme CGTase production was kept as a stock culture in glycerol 50% (0.5 ml of bacteria and 0.5 ml of glycerol) at -20°C and 0.5 ml of bacterial culture was inoculated into 5 ml Horikoshi II broth containing (w/v) 1.0% sago starch, 0.5% yeast extract, 0.5% peptone, 0.1% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$ and 1.0% Na_2CO_3 (Illias *et al.*, 2002) and incubated at 37°C, 200 rpm for 24 h. Five percent of the cells with optical density of about 1.0 (660 nm) was inoculated into a 250 ml conical flask containing 50 ml medium and incubated at 37°C, 200 rpm for 48 h. Experimental studies were carried out and after cultivation cells

were removed by centrifugation at 8,000 rpm 4°C for 15 minutes. The supernatant was used as crude enzyme solution for assaying enzyme activity.

2.1.2 Partial purification of enzyme CGTase

The broth was harvested after 48 h fermentation. After removing the bacterial cells by centrifugation at 8,000 rpm 4°C for 15 minutes, solid ammonium sulfate was added to the supernatant at 4°C, and the precipitate obtained on 80% saturation was collected by centrifugation. The precipitated was dissolved in 20 ml phosphate buffer pH 7.0 at 4°C and the solution was dialyzed by using a membrane with an 8,000 Da molecular cut-off at 4°C in the same buffer. The partial purified enzyme was used in the next study.

2.1.3 Determination of activity of enzyme CGTase

CGTase activity in the supernatant was measured using 4% (w/v) soluble starch in phosphate buffer pH 8.0 and incubated at 70°C for 1 hour for gelatinization process. 0.5 ml of gelatinized soluble starch was incubated at 60°C for 10 min and 0.5 ml enzyme was added and mixed then incubated at 60°C for 10 min the reaction was stopped by boiling at 100°C for 5 min. The production of β-CD was determined using phenolphthalien method of Keneko *et al.* (1987) referred by Sian *et al.* (2005). One unit of CGTase is defined as the amount of enzyme that formed 1 μmol of β-CD min^{-1} under standard assayed condition.

2.1.4 Determination of β-cyclodextrin concentration

The sample was taken and the reaction was stopped by boiling at 100°C for 5 min. β-CD was determined using phenolphthalien method assayed by the method of Keneko *et al.* (1987) referred by Sian *et al.* (2005) based on the reduction in the color intensity of phenolphthalien after complexation with β-CD. 0.2 ml of the sample was added into 0.7 ml of 30 mM NaOH and 0.1 ml of 0.02% (w/v) phenolpthalien in 5 mM Na_2CO_3 solution. The color intensity was measured at 550 nm. A standard curve was plotted with various β-CD concentration.

2.1.5 Determination of reducing sugar

Determination of reducing sugar was carried out according to the DNS method (Miller, 1959) 200 µl of sample was mixed with 600 µl of DNS solution (dinitrosalicylic acid reagent solution, dinitrosalicylic acid 10 g, phenol 2 g (optional), sodium sulfite 0.5 g, sodium hydroxide 10 g and add water to 1 liter) and boiled at 100 °C for 15 minutes after that cooling on ice immediately and then continue diluted with 1.2 ml of distilled water. The colour intensity was measured at 550 nm against blank of distilled water treated in the same manner.

2.2 Production of cyclodextrin from sago starch using CGTase

Sago starch was gelatinized in 50 ml glycine-NaOH buffer pH 8.5 at 70°C. Sago starch was reacted with partial purified enzyme CGTase obtained from 2.1.2 for 24 h at 100 rpm. The sample was taken at 0, 30 min, 1, 2, 4, 6, 12, 18 and 24 h. The sample was centrifuged at 8,000 rpm for 10 min. The supernatant was used to measure the remained enzyme activity, amount β-CD, conversion of β-CD and reducing sugar.

The factors affecting cyclodextrin production was investigated as follows:

2.2.1 The effect of enzyme concentration

The effect of enzyme concentration was examined in the range of 5, 10, 20, 30, and 40 U/g sago starch in triplicates. The 10 g/L of sago starch in 50 ml glycine-NaOH buffer pH 8.5 was used in this step. The optimum enzyme concentration with the highest yield of cyclodextrin was chosen.

2.2.2 The effect of sago starch concentration

The effect of sago starch concentration was examined at 5, 10, 20 and 30 g/L sago starch in glycine-NaOH buffer pH 8.5 in triplicates. The optimum enzyme concentration from step 2.2.1 was used in this step. The optimum sago starch concentration with the highest yield of cyclodextrin was chosen.

2.2.3 The effect of temperature

The effect of temperature on CD production at various sago starch concentrations was examined at 40, 45, 50, 55, 60, 65 and 70°C. The optimum enzyme and sago starch concentration from step 2.2.1 and 2.2.2 was used in this step.

2.2.4 Determination of kinetic parameters

The kinetic parameters for CGTase catalyzed reaction was determined using initial production rates of CD (V_o) at 5 – 30 g/L sago starch concentrations. The initial velocity at each substrate concentration was calculated by slope of β-CD production versus time. Kinetic parameters in Michaelis-Menten model, K_m and V_{max} were determined by Lineweaver-Burk method using Eq. (3).

The effect of temperature on the kinetic parameters was analyzed using the Arrhenius equation and non-linear regression method.

a. Energy activation (E_a)

The activation energy (E_a) in the temperature range between 40 - 65°C was determined by Eq. (5).

$$V_{max} = V_o \exp(-E_a/RT)$$

b. Michaelis-Menten constant

The effect of temperatures on K_m was determined by non-linear regression method. $K_m = \exp(A)\exp(BT)$, where A and B are constants.

c. Energy deactivation (E_d)

The deactivation energy of the enzyme, in the temperature range between 45 - 70°C, was determined with the values of thermal inactivation coefficient (K_d) obtained by the method described Eq. (8) and (9).

$$E = E_o \exp(-K_d \cdot t)$$

$$K_d = K_{do} \exp(-E_d/RT)$$

d. Half-life

The thermal stability of the CGTase enzyme was expressed by its half-life (τ). The half-life was calculated by Eq. (10).

Half-life (τ) = ln $[(E_o/2)/E_o] = -K_d\tau$

$$\tau = \frac{0.69}{K_{d0} \cdot e - \frac{E_d}{RT}}$$

2.2.5 Simulation of optimum condition

The suitable temperature that gives high CGTase activity with good stability represents the highest cyclodextrin production will be determined. This temperature can be estimated from a double plot of CGTase half-life and the activity against temperature.

CHAPTER 3

RESULTS AND DISCUSSION

To obtain CGTase from *Bacillus* sp. C26 the cultured broth of *Bacillus* sp. C26 was centrifuged at 8,000 rpm, 4°C for 15 min. The supernatant was collected as a crude enzyme. The CGTase was then partially purified by ammonium sulfate precipitation at 80% saturation. The effects of enzyme amount, substrate concentration, temperature and kinetic parameters on cyclodextrin production were studied.

1. Effect of enzyme amount on cyclodextrin production

The effect of enzyme amount on yield and initial rate of β-CD production was examined to select the most suitable enzyme amount for β-CD production. The reaction was carried out in 10 g/L sago starch in glycine-NaOH buffer pH 8.5. The enzyme amounts were 5, 10, 20, 30 and 40 U/g sago starch. The reaction mixture was incubated at 50°C. The results are shown in Figure14. It was found that 10 U/g sago starch gave higher β-CD production than 5, 20, 30 and 40 U/g sago starch. This result was consistent with the result from other research using CGTase from *Klebsiella pneumoniae* AS-22 (Gawande and Patkar, 2001). They found that the optimal enzyme amount was 10 U/g starch and addition of more enzyme did not show any improvement in CD production yield. When increasing the enzyme amount from 5 – 10 U/g the β-CD production also increased. This is indicated that the enzyme amount below than 10 U/g still not yet suitable with substrate concentration used. For a fixed amount of enzyme molecules, the rate of reaction is directly proportional to substrate concentration. If substrate concentration is high then the active sites of the enzyme are used up already and still have many substrate not yet used by enzyme. Therefore, to

produce CD from sago starch the minimum required enzyme amount was 10 U/g. However, Kitcha (2007) found that the optimum enzyme concentration for β-CD production from tapioca starch by CGTase of *Bacillus* sp. C26 was 48 U/g starch.

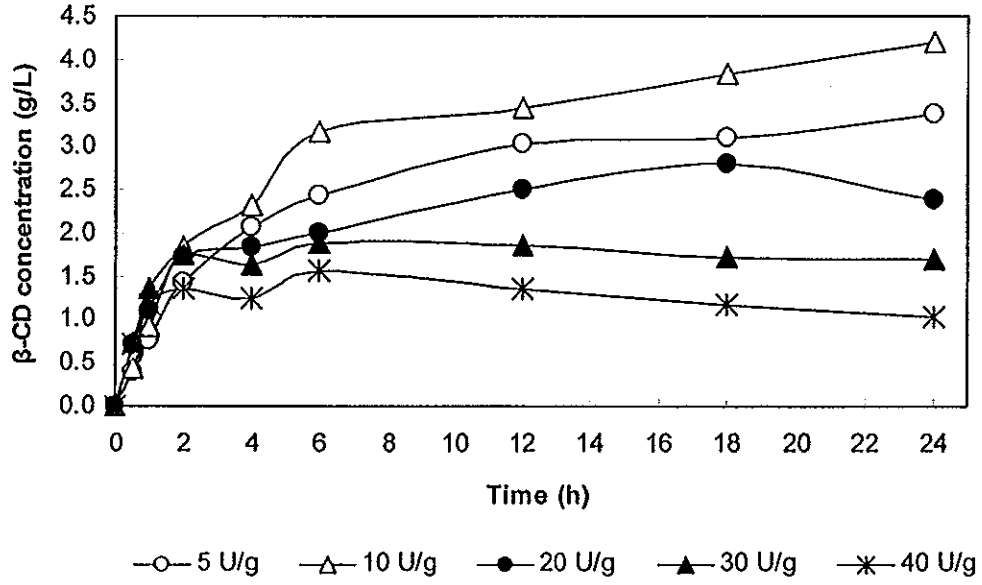


Figure 14. Effect of CGTase amount on β-CD production using sago starch as substrate. The reaction mixture contained 10 g/L sago starch in 50 ml glycine-NaOH buffer pH 8.5 was incubated at 50°C.

Figure 15 shows the effect of CGTase amount on yield and the initial rate of β-CD production. The increasing in enzyme amount from 5 U/g to 10 U/g increased yield of β-CD. However, when the enzyme amount increased more than 10 U/g, the yield of β-CD decreased. The results indicate that reactions other than the cyclization are dominating when the enzyme concentration is increased like hydrolysis process proofed by increasing reducing sugar concentration. Besides cyclization, CGTase is able to catalyse coupling and disproportionation reactions, and also CD ring opening and starch hydrolysis (Van der Veen *et al.*, 2000). An increasing in the reducing sugar content with increasing enzyme concentration was attributed to the hydrolysis of CD to reducing sugar products (Martins and Hatti-Kaul, 2002). On the

other hand, the initial rate increased with increasing enzyme amount. This happened only on the initial process but for continued process the velocity decreased significantly with increasing enzyme amount as shown in Figure 14. Martins and Hatti-Kaul (2002) also found that increasing the enzyme amount the production of β-CD decreased drastically and became undetectable at enzyme amount of 30 U/g. The β-CD concentration was increased monotonically in the region of low enzyme concentration but at a sufficiently high enzyme concentration, it was decreased rapidly after being increased until about 3 h (Shiruishi *et al.*, 1989).

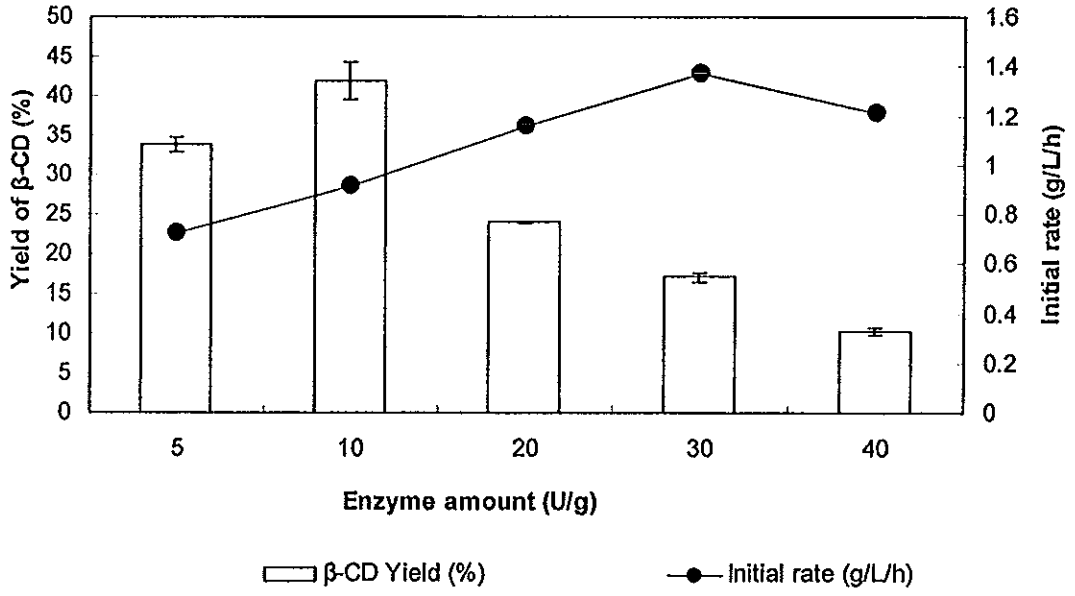


Figure 15. Effect of CGTase amount on yield and initial rate of β-CD production using sago starch as substrate. The reaction mixture contained 10 g/L sago starch in 50 ml glycine-NaOH buffer pH 8.5 was incubated at 50°C.

2. Effect of substrate concentration and temperature

One of the objectives of this research project was to use sago starch as a substrate in the production of β-CD. CD is mainly produced from starch or its derivatives via enzyme reaction. The yield and selectivity of CD produced highly

depend on the type of CGTase and substrate. Amylose, amylopectin, maltose, maltohexose, maltotriose, maltoheptose and starches have been reported as substrate for CD production. Above all, starches are reported to be the best substrate for CD productions including corn, tapioca, rice, wheat, potato and sago (Zain, 2005). Recently, only potato starch has been industrially applied in CD production (Biwer *et al.*, 2002). Amylopectin is reported to be the best substrate for cyclodextrins production compared to amylose. Better yield of CD is obtained using pure amylopectin (Zain, 2005). However, pure amylopectin is very expensive, therefore starches containing high percentage of amylopectin are preferable for CD production (Biwer *et al.*, 2002). Since sago starch contains 73% amylopectin and 27% amylose (Singhal *et al.*, 2008), it was thought to be the suitable substrate for CD production. Beside enzyme amount, temperature and substrate concentration were the important thing in enzyme catalyze process. The temperature gave affects nearly all chemical reaction rates. Rising temperature increases enzyme reactions rates until, at high temperatures, enzymes fail to work. Moreover, the amount of substrate can control an enzyme catalytic reaction. Therefore, both temperature and substrate concentration are important factors to determine the optimal condition for CD production. In this study, the effect of substrate concentration using amount of enzyme at 10 U/g sago starch and 5-30 g/L sago starch in the mixture at various temperatures 40-70°C was investigated. The results are shown in Figure 16-19. With increasing the substrate concentration the β-CD production was also increased *at all* temperature. Kim *et al.* (1997) also found that according to an increase in the concentration of substrate, the concentration of β-CD was increased. It is ideal that the maximum concentration of substrate would be chosen for CD production within the allowable relative content of cyclodextrin.

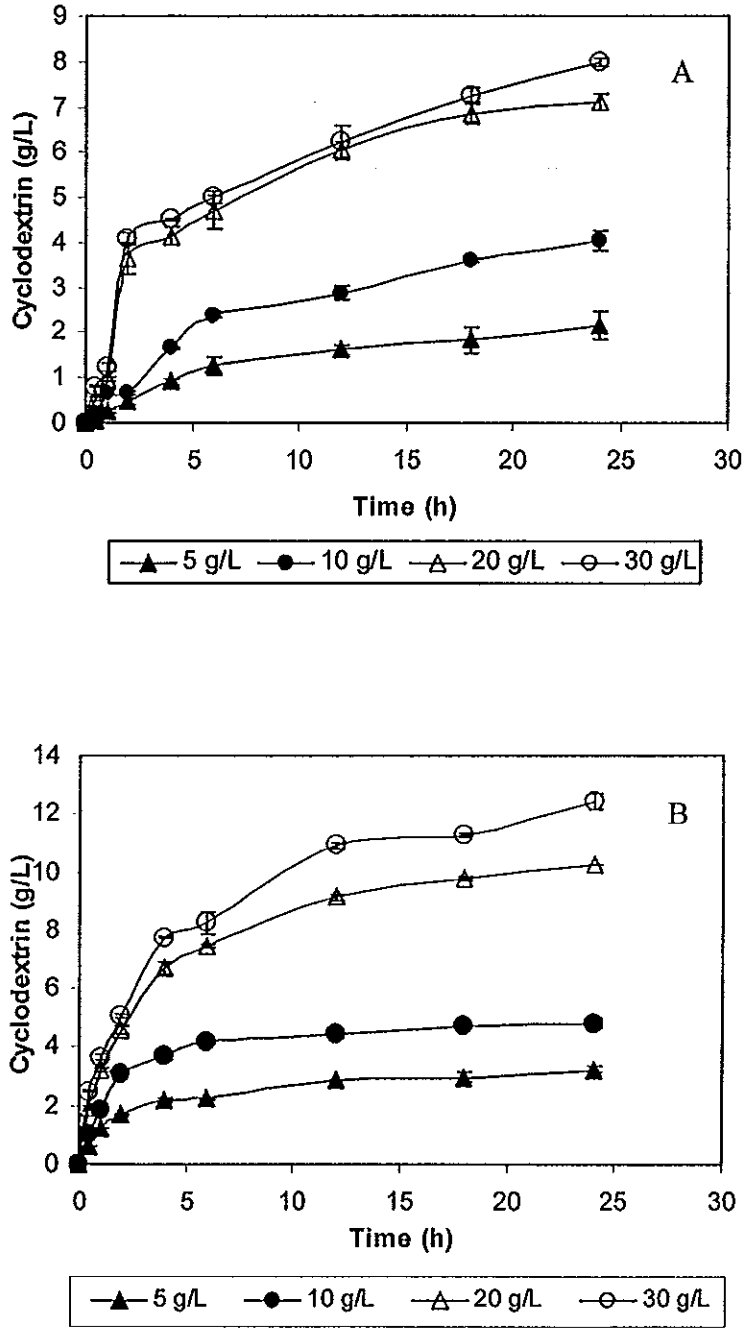


Figure 16. Effect of sago starch concentration on cyclodextrin production using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. The reaction mixture was incubated at 40°C (A) and 45°C (B), 100 rpm for 24 h.

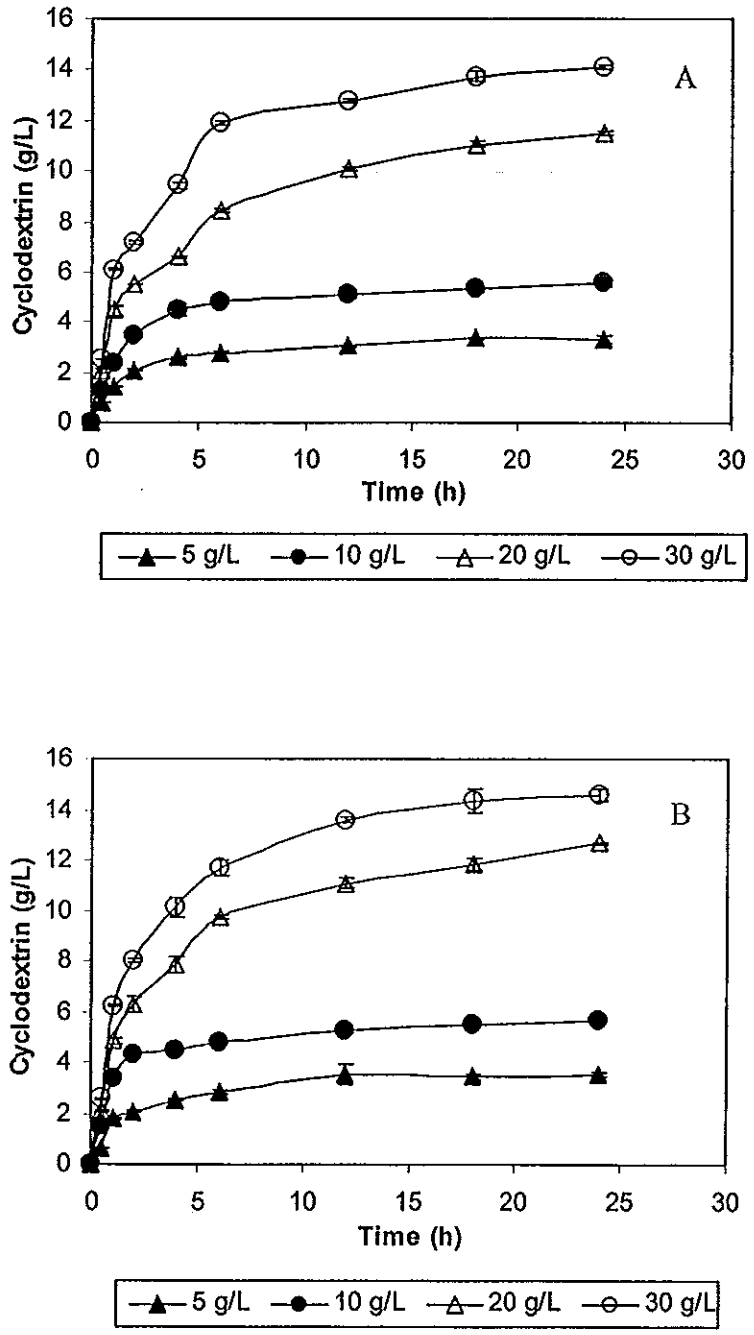


Figure 17. Effect of sago starch concentration on cyclodextrin production using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. The reaction mixture was incubated at 50°C (A) and 55°C (B), 100 rpm for 24 h.

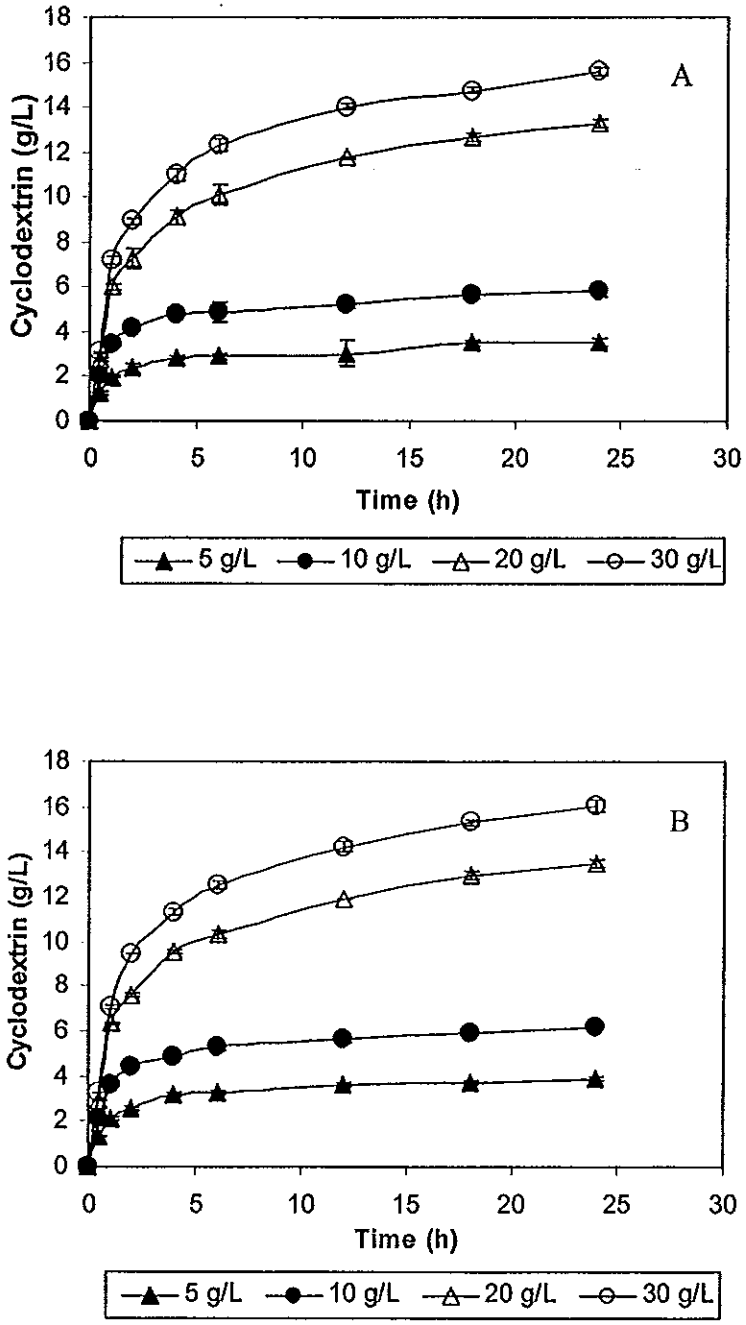


Figure 18. Effect of sago starch concentration on cyclodextrin production using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. The reaction mixture was incubated at 60°C (A) and 65°C (B), 100 rpm for 24 h.

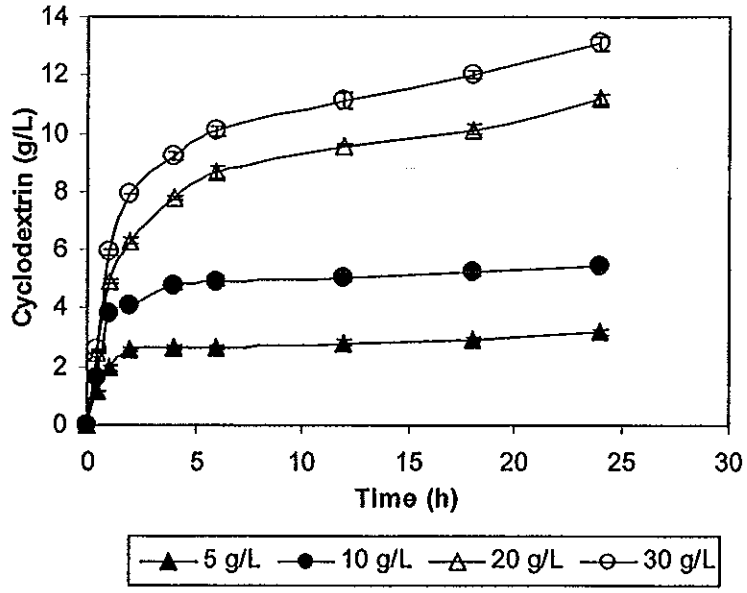


Figure 19. Effect of sago starch concentration on cyclodextrin production using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. The reaction mixture was incubated at 70°C, 100 rpm for 24 h.

In this study, we found that the optimum temperature for β-CD production was 65°C because when the temperature increased higher than 65°C the β-CD production decreased almost 20% at 70°C (Figure 20). This might be because the enzyme already denatured at high temperature proofed by decreased the yield of β-CD production. Matioli *et al.* (2001) also reported that the greatest stability of CGTase enzyme from *B. firmus* strain 37 occurred at 60 °C and the highest specific activity was obtained at 65 °C. The activity and the stability in high temperatures indicate industrial potential for this CGTase. For the industrial conversion of starch to CDs, it would be desirable an enzyme active at higher temperatures, because this would reduce the risk of microbial contamination. In the literature, few enzymes are reported with optimum activity at temperatures above 60°C, as the CGTases from *Bacillus amyloquefaciens* (Yu *et al.*, 1988), *Bacillus stearothermophilus* (Chung *et al.*, 1998) and *Thermoanaerobacter* sp. (Tardioli *et al.*, 2006), with maximal activity at 70, 80 and 85

°C, respectively. Kim *et al.* (1997) found that the variation in CD content was due to the increased CGTase activity by increased temperature. Moreover, the amount of maltodextrin released from starch by hydrolysis reaction was also increased by an increase in reaction temperature. The optimum temperature for maximum β-CD production from raw corn starch was found to be 65°C at temperatures ranging from 55-75°C. Illias *et al.* (2002) reported that a temperature higher than 70°C, activity of CGTase from *Bacillus* sp. G1 declined sharply this may be because the CGTase could be denatured above 70°C. Relative activity also decreased by about 60% at 80°C. Therefore, the crude CGTase from *Bacillus* sp. G1 was found to be quite stable at temperature ranging between 20°C to 60°C (Illias *et al.*, 2002).

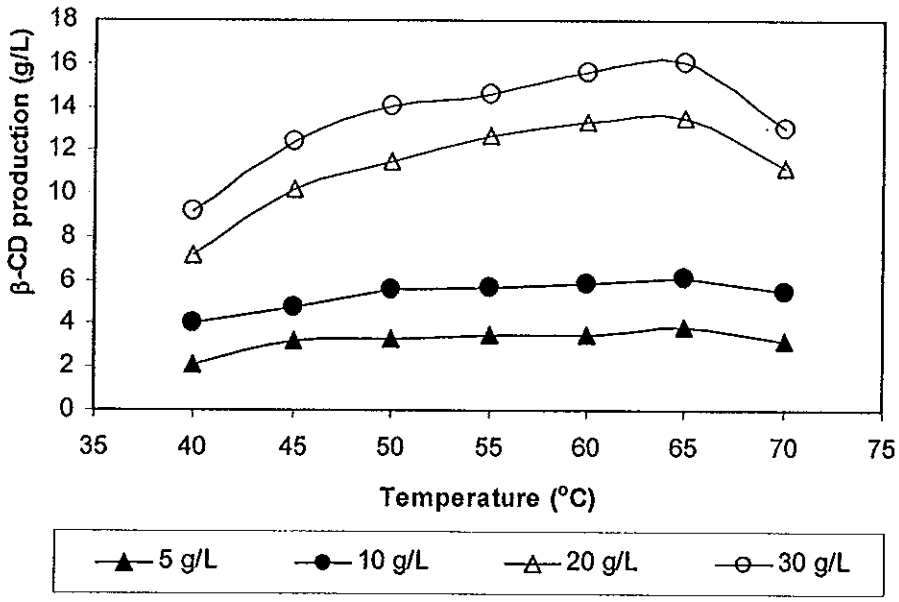


Figure 20. β-CD production in 50 ml glycine-NaOH buffer pH 8.5 at 24 h incubation time using 5-30 g/L sago starch as a substrate and temperature 40-70°C.

The β-CD concentration was dependent on starch concentration. It increased when substrate concentration increased from 5 to 30 g/L at temperature 40-65°C. However, when temperature increased until 70°C the β-CD concentration

decreased. In terms of β-CD productivity, optimal sago starch concentration will be more than 30 g/L because 30 g/L sago starch has still not yet shown substrate inhibition. With increasing starch concentration, however, stirring of the suspension was more difficult and energy-consuming in a larger scale. Therefore, optimal starch concentration should be determined by CD productivity and working efficiency (Yamamoto *et al.*, 2000).

3. Effect of temperature on kinetic parameters

All chemical reactions respond to temperature. It is the same reason as temperature affecting enzyme reaction. Enzyme are not active on the temperature which below 0°C. Mean while, at the higher temperature, the velocity of the reaction increases because more energy are supplied to break the intramolecular attraction of the protein structure. However, the enzyme characterized as protein, when temperature increases the protein begin to denature and loose its function making it inactive to operate properly. The optimal temperature of enzyme is different for every enzymes and the enzyme can be destroyed as the temperature more than optimum condition (Oppapers.com, 2006). Temperature can affect a lot of different factors hence its effect on enzyme activity is very complex. It affects the speeds of molecules, the activation energy of the catalytic reaction and the thermal stability of the enzyme and substrate. At low temperatures (say at around 0°C) the rate of enzyme reaction is very slow. The molecules have low kinetic energy and collisions between them are less frequent and even if they do collide the molecules do not posses the minimum activation energy required for the reaction to occur. It can be said that the enzymes are inactivated at low temperatures. An increase in temperature increases the enzyme activity since the molecules now possess greater kinetic energy (Blurtit, 2007).

The effect of temperature on kinetic of β-CD production was determined. The initial rate of β-CD production was calculated using β-CD production during 0 to 6 h. The results are shown in Figure 21. It was found that the initial velocity (V_0) of β-CD production increased when temperature increased. The increase in initial velocity on β-CD production along with increasing temperature at high substrate concentration was higher than that at low substrate concentration. As temperature rise the rate of chemical reactions increases because temperature increases the rate of motion of molecules. Increasing the substrate concentration also increases the collision frequency of the enzyme and substrate. This leads to more interactions between an enzyme and its substrate. Matioli *et al.* (2001) studied the activity of CGTase from *B. firmus* strain no. 37 as a function of temperature for β-CD production. They also found that as the temperature increased the β-CD production also increased.

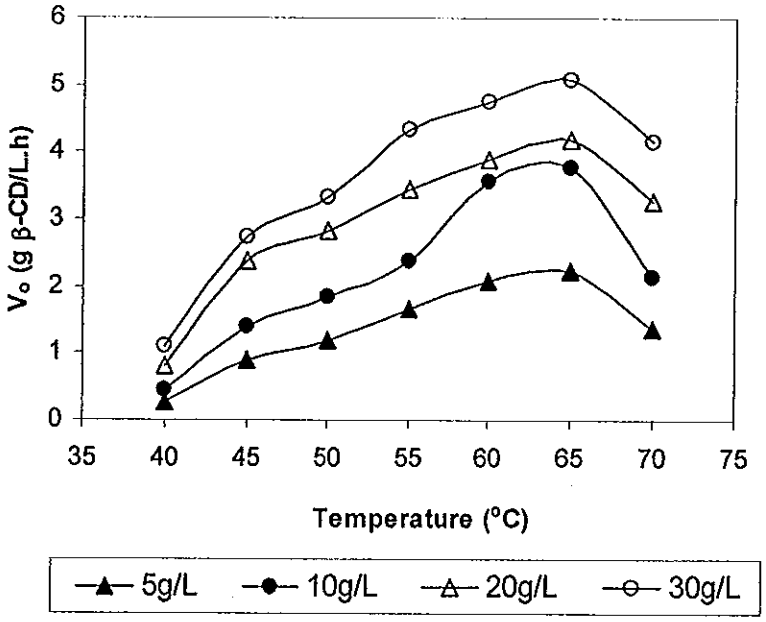


Figure 21. Effect of temperature on initial rate of β-CD production using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 40-70°C.)

The kinetic parameters were determined using the Michealis-Menten equation. The initial production rate of CD, V_o (g/L.h) for various initial substrate concentration, S (g/L), was fitted to the Michealis-Menten equation. Where V_{max} is the maximum reaction rate (g/L.h) and K_m is the Michaelis-Menten constant (g/L). In Michaelis-Menten kinetics, V_{max} value corresponds to the condition where every enzyme molecule present is saturated with substrate. K_m value represents the affinity between substrate and enzyme. The Michaelis-Menten equation was further transformed into Eq. (3) for Lineweaver–Burk plot according to which a linear plot between 1/V and 1/*S* was attempted. The intercept on the Y-axis is equal to $1/V_{max}$ and the slope K_m/V_{max}. Both intercept and slope were determined by linear regression.

The Lineweaver–Burk plots for temperature 40-70°C are shown in Figures 22-28. The kinetic parameters obtained from Figures 22-28 are summarized in Table 4. It is quite obvious from Michealis-Menten equation that higher V_{max} and lower K_m are associated with the greater β-CD production as shown in Figure 29. Moreover, the lower K_m value of gelatinized sago starch at high temperature indicates that CGTase has higher affinity. In other words, at higher temperature the gelatinized sago starch exhibited strong substrate binding with CGTase. The greater β-CD production from gelatinized sago starch was associated with its larger V_{max} and smaller K_m values. A possible explanation would be that the greater expansion of the sago starch granule might be induced. Therefore, at high temperature the gelatinized sago starch has a larger surface area for CGTase to react with, which further leads to higher β-CD production. In other words, at high temperature during, the structures of sago starch granules were opened up and became more susceptible to CGTase action.

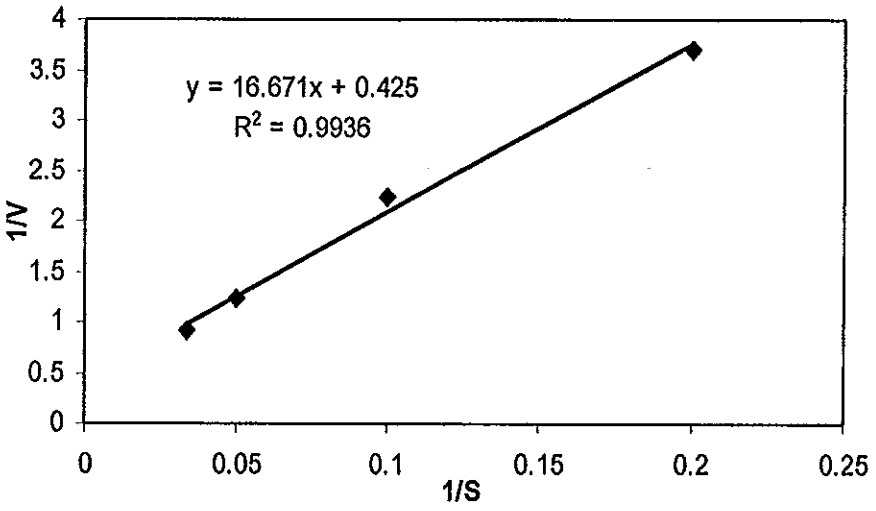


Figure 22. Lineweaver-Burk plot for estimation of K_m and V_{max} at temperature 40°C.

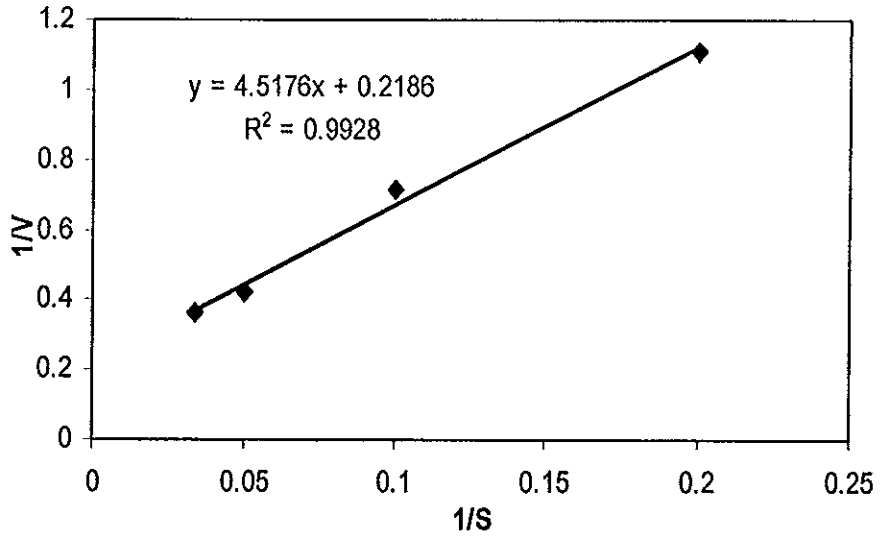


Figure 23. Lineweaver-Burk plot for estimation of K_m and V_{max} at temperature 45°C.

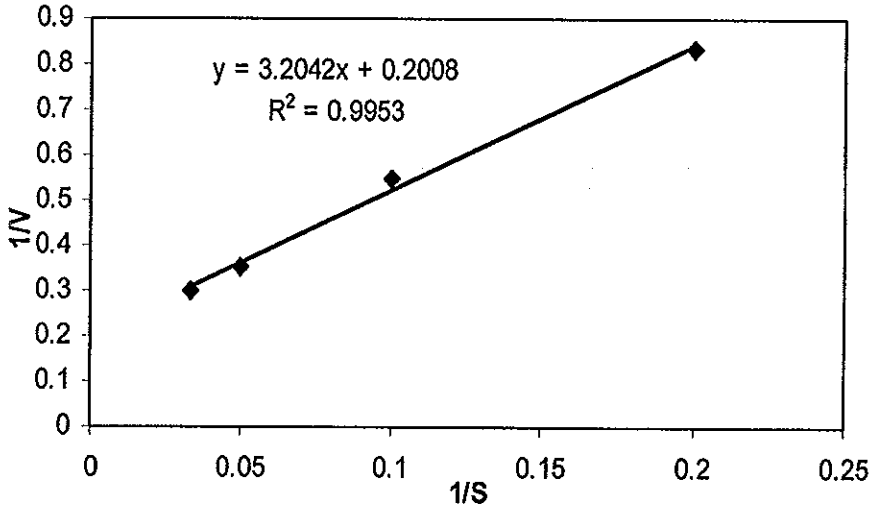


Figure 24. Lineweaver-Burk plot for estimation of K_m and V_{max} at temperature 50°C.

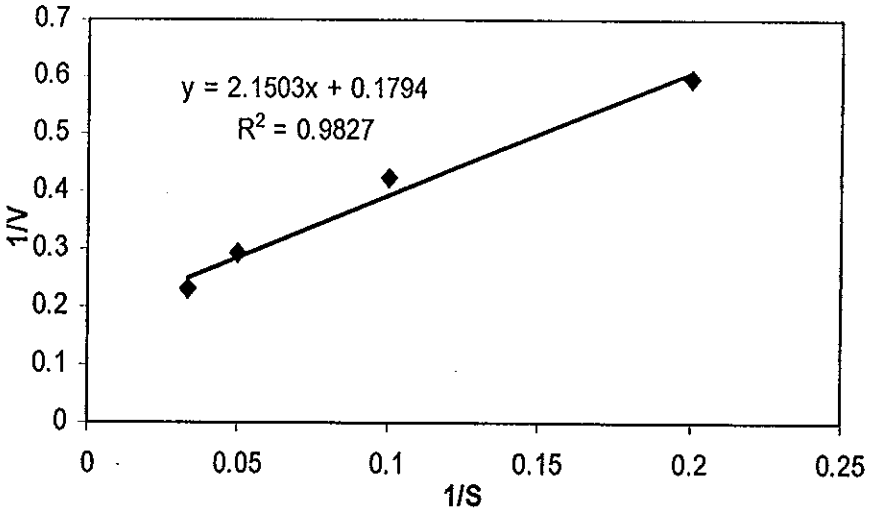


Figure 25. Lineweaver-Burk plot for estimation of K_m and V_{max} at temperature 55°C.

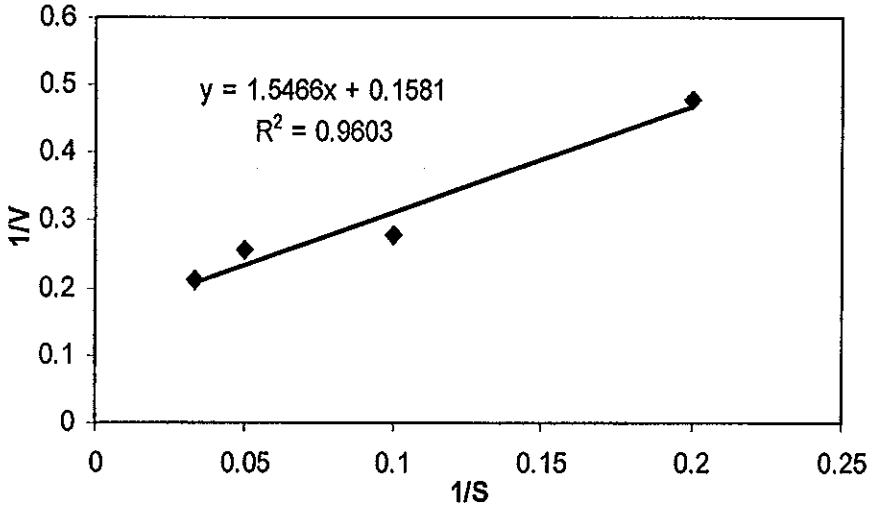


Figure 26. Lineweaver-Burk plot for estimation of K_m and V_{max} at temperature 60°C.

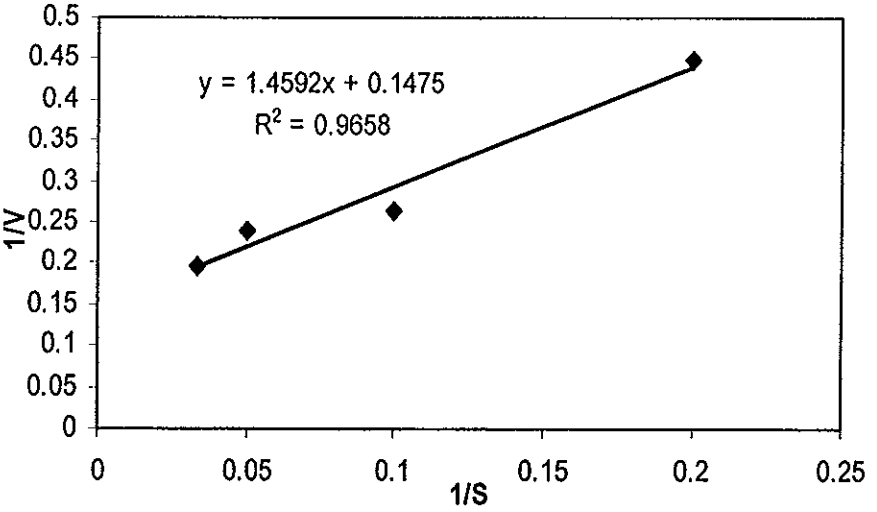


Figure 27. Lineweaver-Burk plot for estimation of K_m and V_{max} at temperature 65°C.

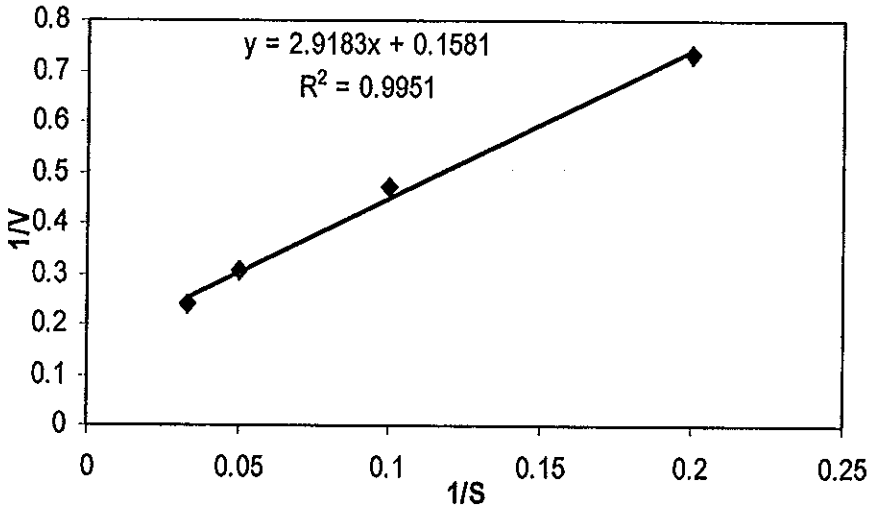


Figure 28. Lineweaver-Burk plot for estimation of K_m and V_{max} at temperature 70°C.

Table 4 shows the effect of temperature on V_{max} and K_m values. When temperature increased V_{max} also increased while K_m was decreased. The V_{max} increased from 2.35 to 6.78 g β-CD/L/h when temperature increased from 40 to 65°C, while K_m decreased from 39.2 to 9.78 g starch/L when temperature increased from 40 to 60°C. At temperature 70°C the opposite result happened where the V_{max} value decreased and K_m value increased because CGTase already denatured at this temperature and the yield for β-CD production decreased from 16.055 g/L at 65°C became 13.085 g/L at 70°C. The lower value of K_m indicates that CGTase has higher affinity for the substrate at higher temperature. It could be concluded that increasing in temperature not only increased reaction rate but also enhanced the ability of enzyme bound to the substrate.

Table 4. Effect of temperature on V_{max}, K_m, V_{max}/K_m and β-CD concentration at various temperatures

Temperature	V_{max}	K_m	V_{max}/K_m	β-CD concentration (g/L)			
(°C)	(g/L h)	(g/L)		5 g/L	10 g/L	20 g/L	30 g/L
40	2.35	39.2	0.06	2.152	4.031	7.126	9.142
45	4.57	20.7	0.22	3.242	4.802	10.225	12.416
50	4.98	15.9	0.31	3.305	5.616	11.513	14.087
55	5.57	11.9	0.47	3.495	5.656	12.668	14.607
60	6.33	9.78	0.65	3.529	5.838	13.296	15.603
65	6.78	9.89	0.69	3.861	6.174	13.496	16.055
70	6.33	18.47	0.34	3.207	5.490	11.168	13.085

The rate of reaction when the enzyme is saturated with substrate is the maximum rate of reaction, V_{max}. The relationship between rate of reaction and concentration of substrate depends on the affinity of the enzyme for its substrate. This is usually expressed as the K_m (Michaelis constant) of the enzyme, an inverse measure of affinity. Relationship between V_{max} and K_m is shown in Figure 29 when V_{max} values increased the K_m values decreased but this condition happened until reached the temperature at 60°C on □-CD production. Increasing temperature more than 60°C until 70°C shown the K_m values increased because of effect high temperature made enzyme denatured and decreased their are affinity to the substrate. Figure 29 also indicates that at 70°C the V_{max} value decreased, while K_m value increased, its mean the CGTase already denatured in this temperature.

The catalytic factor, as a means to quantitate the catalytic power (rate enhancement or catalytic efficiency) of an enzyme, is simple to define as the ratio of the catalyzed rate to the uncatalyzed rate but can be difficult to estimate accurately in many instances (Neet, 1998). The value of V_{max}/K_m in Figure 30 shows the optimum temperature at 65°C, which gives a practical idea of the catalytic efficiency, i.e., how often a molecule of bound substrate reacts to give a product. Since catalytic efficiency (V_{max}/K_m) combines information about reaction products generated per unit of time, as well as how well the substrate is bound to the enzyme, it is more useful than K_m for comparing enzymes (Salami *et al.*, 2008). According to Table 4, the efficiency catalytic (V_{max}/K_m) of CGTase increased about 10 fold when temperature increased 20°C from 40 to 60°C.

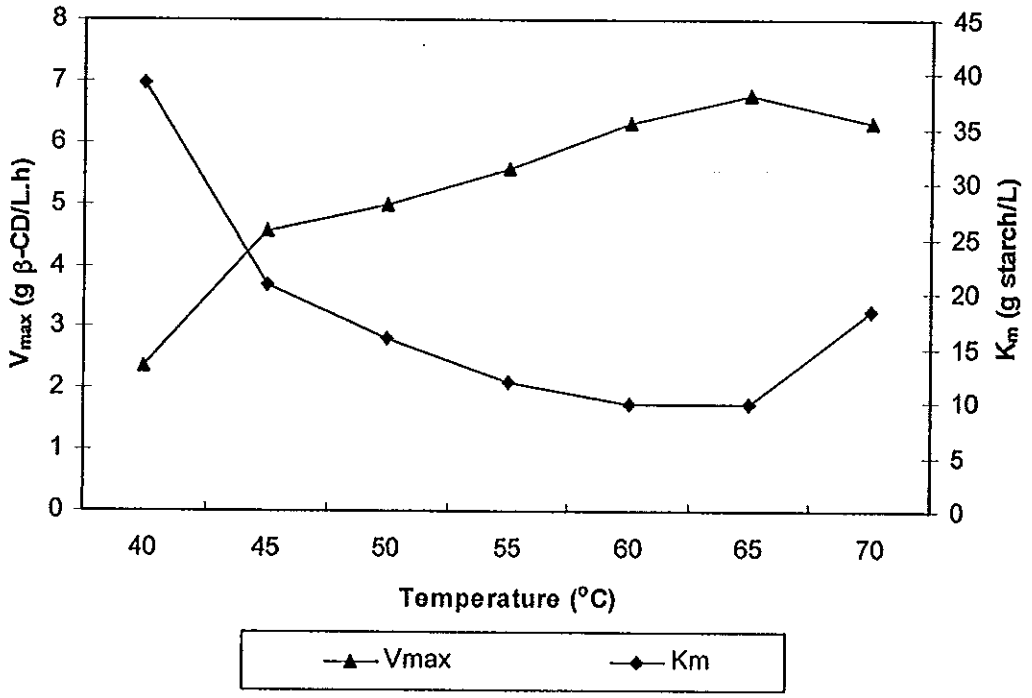


Figure 29. V_{max} and K_m values for β-CD production using sago starch as a substrate at temperature 40-70°C.

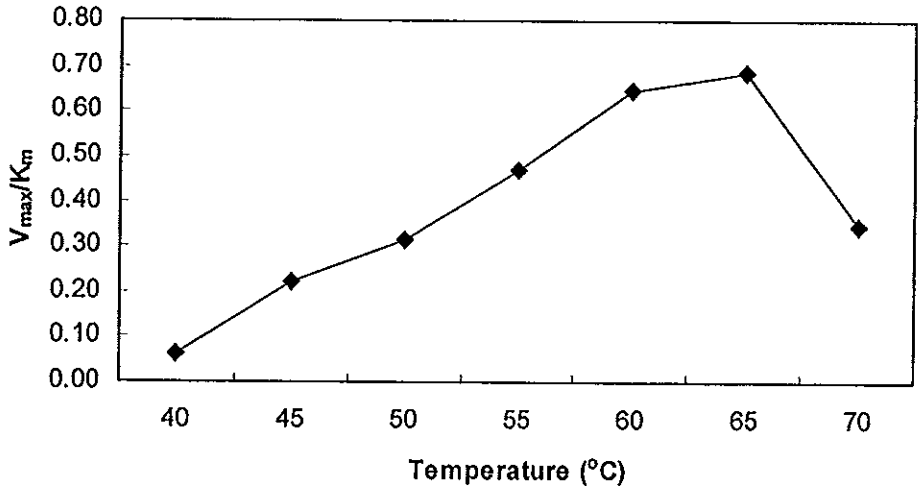


Figure 30. Catalytic efficiency (V_{max}/K_m) of β-CD production at temperature 40-70°C.

4. Energy of activation of CGTase from *Bacillus* sp. C26

The rate of a chemical reaction is affected by several factors, including temperature. As temperature increases, reactions occur faster for two reasons. Increasing temperature increases the kinetic energy of the particles. In addition, because particles are moving faster, there is an increase in the frequency of collisions. Collisions of molecules with higher energy result in an increase in reaction rate (r). Molecules must collide with a certain minimum amount of energy necessary to break bonds and force the molecule through unstable intermediate states to go to product. This minimum amount of energy that must be overcome is called the activation energy (E_a). Arrhenius was seeking to quantify this relationship between temperature and reaction rates. Plotting rate versus temperature in Kelvin (K) results in a nonlinear, exponential curve. Arrhenius found this relationship to be true for most reactions. In considering a variety of reaction data, Arrhenius found that reaction rate data followed a mathematical relationship which is now known as the Arrhenius equation: $k = Ae^{-Ea/RT}$ where k refers to the rate constant for the reaction, A is a proportionality constant that varies depending on the reaction and is related to the frequency of collisions and probability that the collisions have the proper orientation to favor reaction, e is the base of natural logarithms, E_a is the activation energy for the reaction, R is the ideal gas constant = 1.987 cal/mol.K, and T is temperature in Kelvin. In the case of the data presented, the rate of the reaction (r) is given and will be used instead of a rate constant (k).

$$\text{rate} = A \exp(-E_a/RT)$$

Taking the natural log of both sides of the above equation will give:

$$\ln \text{rate} = -E_a/RT + \ln A$$

The equation is now in the form of a straight line, y = mx + b. Plotting the data for V_{max} value from β-CD production using CGTase in the form of ln V_{max} versus 1/T indeed results in a straight line (Figure31) as the following equation:

$$\ln V_{max} = (-E_a/R)(1/T) + \ln A.$$

If ln V_{max} is Y, and 1/T is X, then the slope of the line would be E_a/R. Using the slope shown on the graph (Figure31) and substituting 1.987 cal/mol.K for R, the activation energy (E_a) of the reaction can be determined.

$$\text{Slope} = -4453.7 = -E_a/R$$

$$E_a = 4453.7 \times R$$

$$E_a = 4453.7 \times 1.987 \text{ cal/mol.K}$$

$$E_a = 8849.52 \text{ cal/mol.K or } 8.85 \text{ kcal/mol.K}$$

This value is almost similar to E_a of Higuti *et al.* (2001) who reported the thermodynamic parameter for activation energy, as calculated from Arrhenius equation, was 8.27 kcal/mol.K. This high value reflected a high temperature sensitivity of the CGTase. The E_a of the CGTase also depends on their source. The high energy of activation value (E_a) shows that high temperatures increase the formation of β-CD by CGTase (Martins and Hatti-Kaul, 2002). The rate of an enzymatic reaction is strictly dependent on the activation energy of the catalyzed reaction.

Using Figures 31 and 32, the equations for V_{max} and K_m as fuction of temperature were obtained as follows:

$$V_{max} = \exp(15.21) \times \exp(-4453.7/T) \quad (11)$$

$$K_m = \exp(24.368) \times \exp(-0.0666T) \quad (12)$$

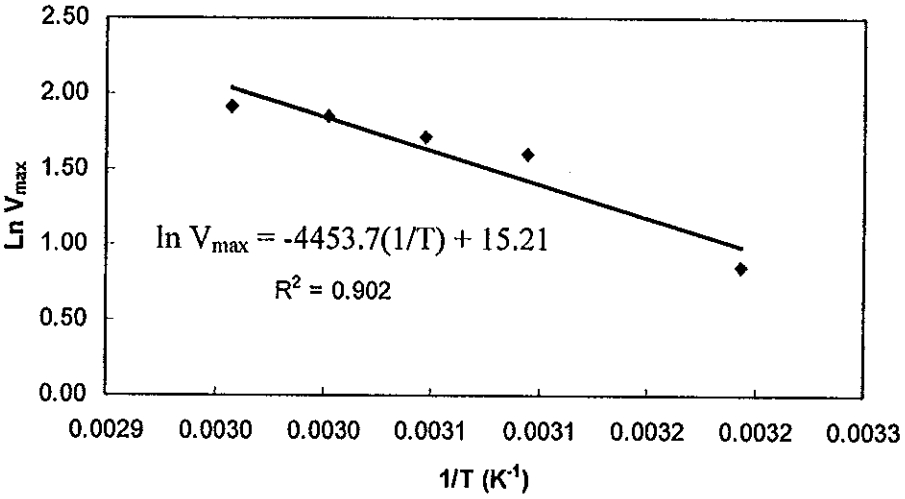


Figure 31. Ln V_{max} - 1/T plot for cyclodextrin production using 5-30 g/L sago starch as substrate in 50 ml glyscine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 40-65°C.)

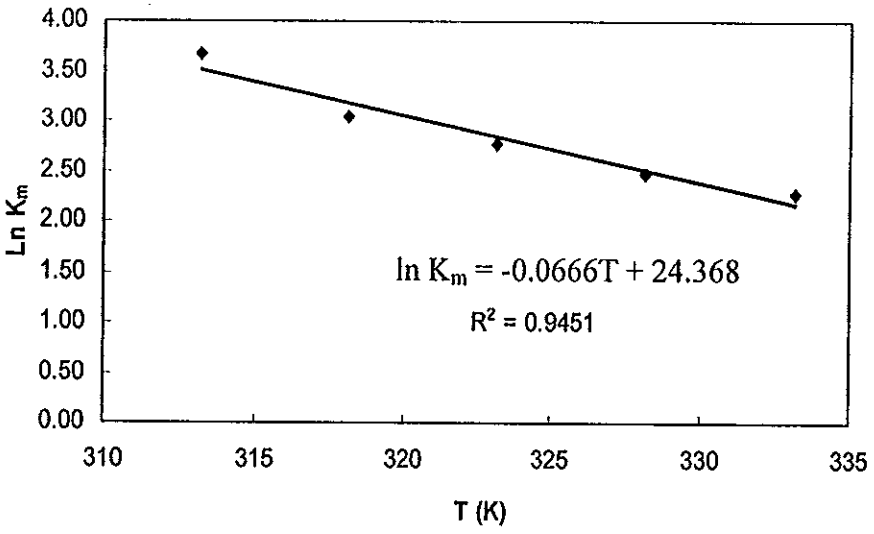


Figure 32. Ln K_m – T (K) plot for cyclodextrin production using 5-30 g/L sago starch as substrate in 50 ml glyscine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 40-60°C.)

5. Thermal stability of CGTase from *Bacillus* sp. C26

Most enzymes from mesophilic microorganisms are generally thermolabile and enzyme loses its activity by heat treatment (Yamamoto *et al.*, 1999). The CGTase activity from *Bacillus* sp. C26 as a function of time from 0 to 24 h at temperature in the range of 45-70°C was determined (Figures 33-35). The activity of CGTase decreased with increasing temperature. Incubation times also have effect on the enzyme CGTase activity and stability as shown in Figures 33-35 where E and E_o are the activity of enzyme during reaction and at initial time, respectively. The enzyme activity becomes decreased during incubation time from 0 to 24 h. At 65°C as the optimum temperature for CGTase, the residual activities at 24 h were 29%, 36%, 49% and 56% when using sago starch concentration at 5 g/L, 10 g/L, 20 g/L and 30 g/L, respectively. The longer time for an enzyme was incubated with its substrate, the greater amount of product will be formed. However, all proteins suffer denaturation, and hence loss of catalytic activity with time (Rha *et al.*, 2005). Possibly, CGTase deterioration was a significant decrease in enzymatic activity with prolonged incubation time. Some enzymes, especially in partially purified preparations, may be noticeably unstable, losing a significant amount of activity over the period of incubation. If the activity of the enzyme is such that much of the substrate is used up during the incubation, then, even if the concentration of substrate added was great enough to ensure saturation of the enzyme at the beginning of the experiment, it will become inadequate as the incubation proceeds, and the formation of product will decrease. Enzyme catalyzed reactions are reversible. Initially, there is little or no product present, and therefore the reaction proceeds only in the forward direction. However, as the reaction continues, so there is a significant accumulation of product, and there is a significant rate of back reaction. As a result, the rate of formation of product slows down as the incubation proceeds, and if the incubation time is too long,

then the measured activity of the enzyme is falsely low.Figures 33-35 also showed the effect of substrate concentration on enzyme stability. A higher substrate concentration gave higher enzyme stability compared to that at low substrate concentration. Earlier reports have showed that the CGTase was more resistant to thermal denaturation in the presence of its substrate (Martins and Hatti-Kaul, 2001). Gawande and Patkar (2001) also reported that the temperature stability of the enzyme was improved in the presence of soluble starch at 40°C.

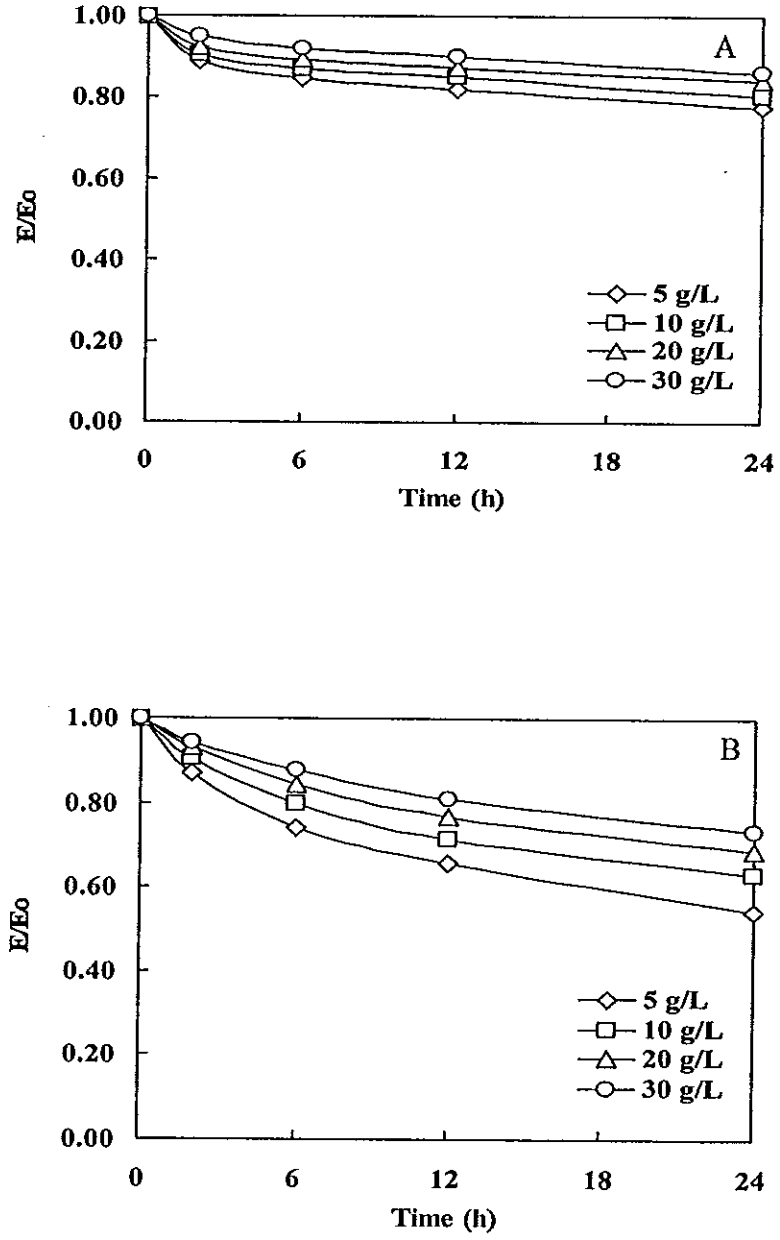


Figure 33. Residual activity (E/E_o) of CGTase in β-CD production from sago starch at 45°C (A) and 50°C (B).

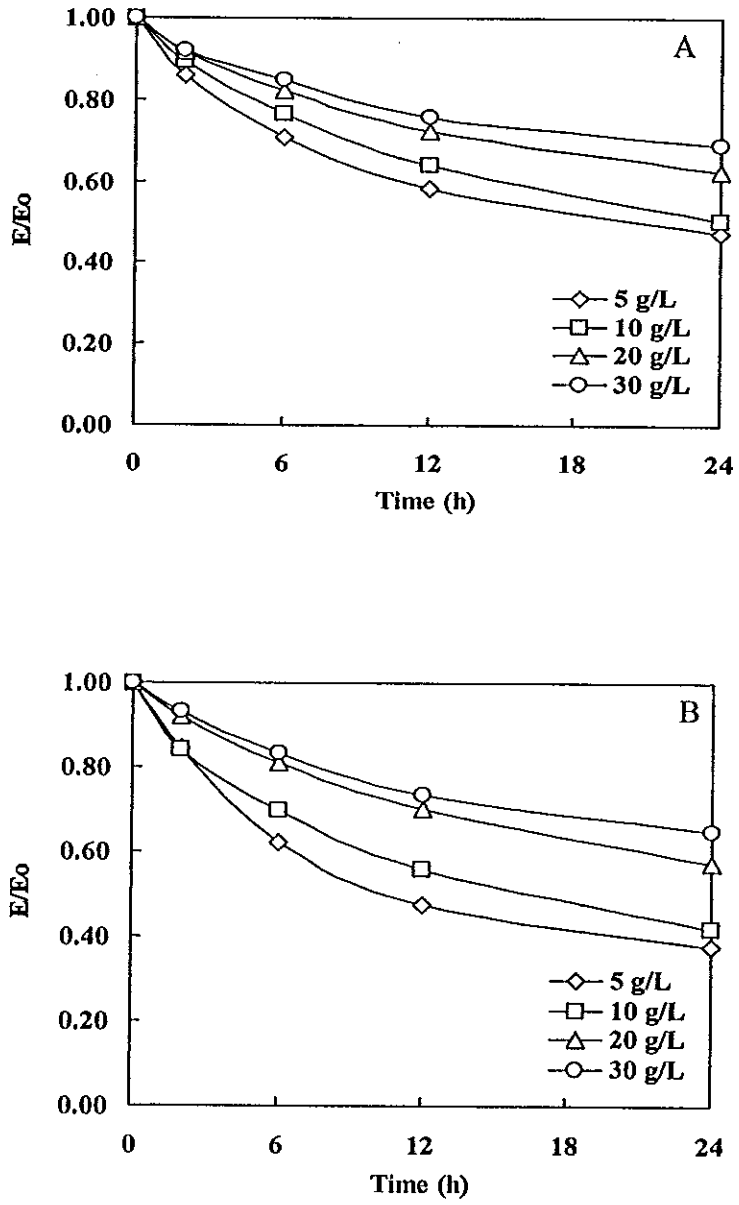


Figure 34. Residual activity (E/E_o) of CGTase in β-CD production from sago starch at 55°C (A) and 60°C (B).

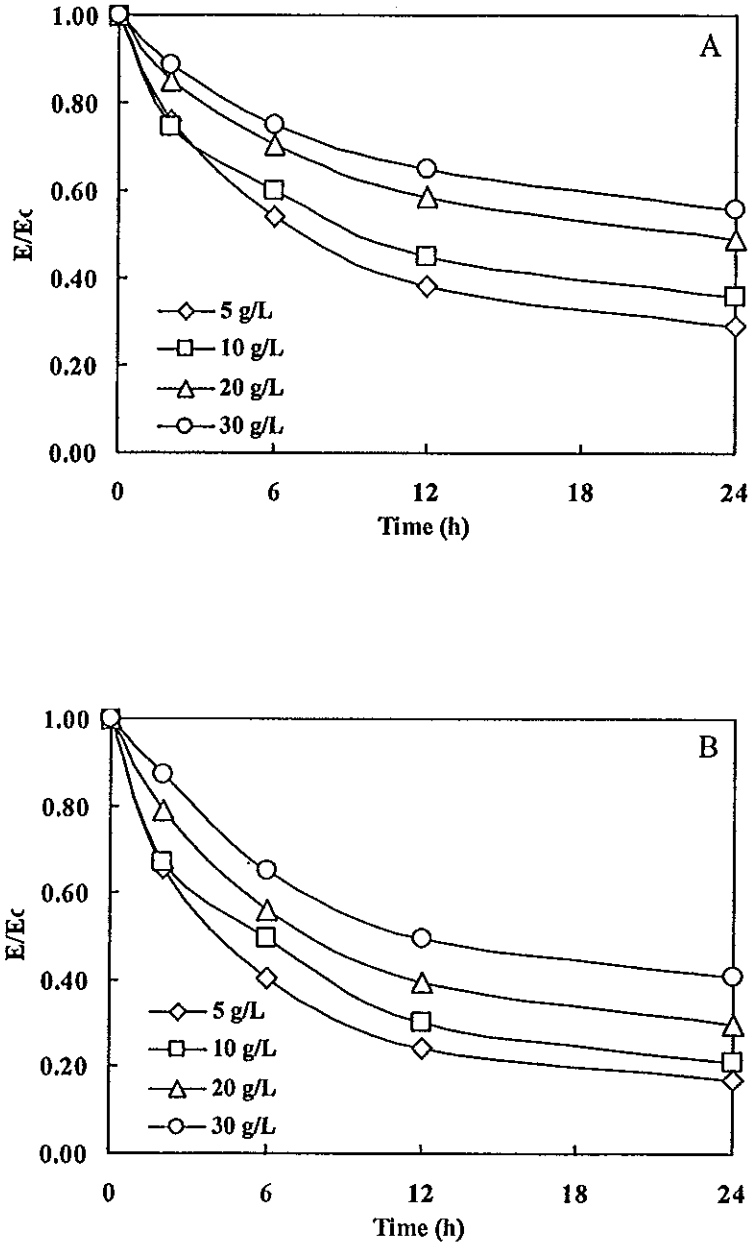


Figure 35. Residual activity (E/E_o) of CGTase in β-CD production from sago starch at 65°C (A) and 70°C (B).

6. The denaturation energy constant

The rate of enzyme activity is highest between 0 until optimum condition and this increase is almost linear. After that the rate of reaction starts to decrease (Figure 21). This is because the increase in temperature after the optimum condition does not increase the kinetic energy of the enzyme but instead disrupts the forces maintaining the shape of the molecule. The enzyme molecules are gradually denatured causing the shape of the active site to change. There are some enzymes known as 'extremophiles' found in thermophillic organisms. They retain activity at 80°C (Blurtit, 2007). The rate of reaction will increase with increasing temperature until the optimum temperature for the reaction is reached. Below the optimum temperature the reaction time will get increasingly slower for each lower temperature. Enzymes are large protein molecules held together by hydrogen bonds of various strengths, able to keep their tertiary structure together to varying degrees, depending upon their surroundings. If the protein's shape changes enough to alter its active site, it will stop working. Denaturation, the general opening up of the molecule, can happen when hydrogen bonds are disrupted in many places at once. This can occur when the temperature gets high enough to vibrate the bonds apart, or when charged particles, such as those associated with acids or bases, interfere with bond attractions. With both of these factors, there are points at which a particular enzyme will be at peak activity - this is called the optimum (McDarby, 2008). Thermal denaturation of enzymes occurs in two steps as shown below:

$$N \leftrightarrow U \longrightarrow I$$

Where N is the native enzyme, U is the unfolded inactive enzyme which could be reversibly refolded upon cooling and I was the inactivated enzyme formed after prolong exposure to heat and therefore cannot be recovered upon cooling. The thermal denaturation of enzymes is accompanied by the disruption of non-covalent linkages, including hydrophobic interactions, with concomitant increase in the enthalpy of

activation. The opening up of enzyme structure is accompanied by an increase in disorder or entropy of activation (Bhatti *et al.*, 2005). In order to determine the thermodynamic parameters for irreversible thermal stability, the energy of activation for thermal denaturation was determined by applying the Arrhenius plots which indicated that high energy is required for thermal denaturation. The denaturation constants, K_d, were determined at different temperatures by plotting ln (E/E_o) against time where E and E_o are the activity of enzyme during reaction and at initial time. The effect of temperature and substrate concentration on K_d was showed in (Figure 36). The denaturation energy constants, E_d, were determined using the same method as for E_a, by first plotting ln (K_d) against temperatures Figures 37-40 and then using linear regression to obtain the values of E_d and K_{do}.

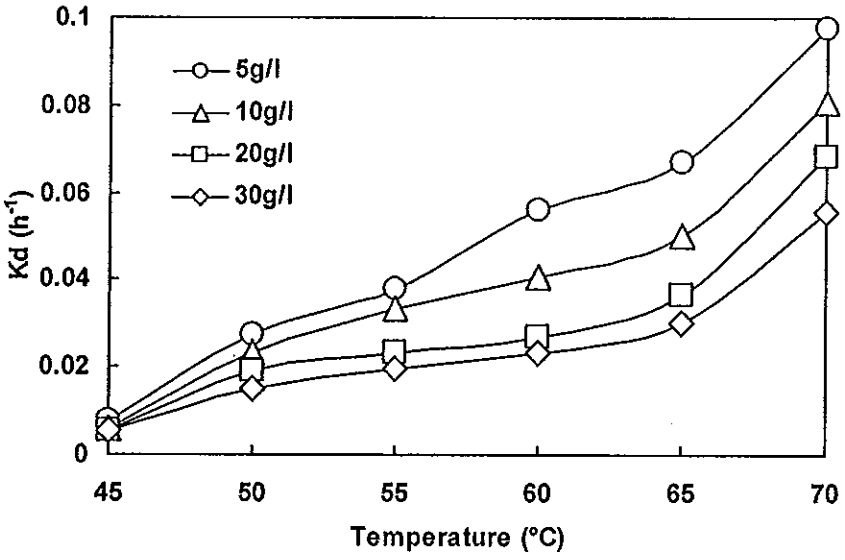


Figure 36. K_d values of CGTase using 5-30 g/L sago starch as substrate in 50 ml glyscine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 40-70°C.)

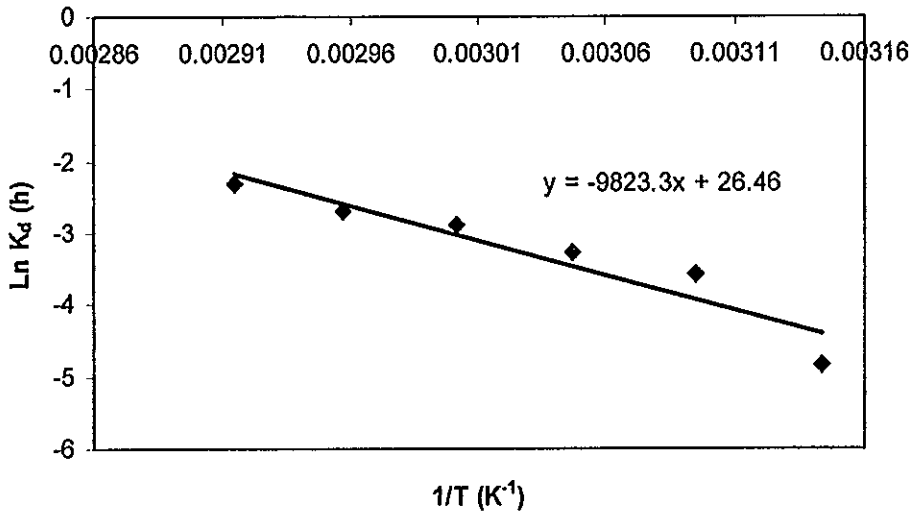


Figure 37. Estimation of the denaturation energy constants (E_d) and initial denaturation constants (K_{do}) for 5 g/L of sago starch as a substrate at temperatures 45-70°C.

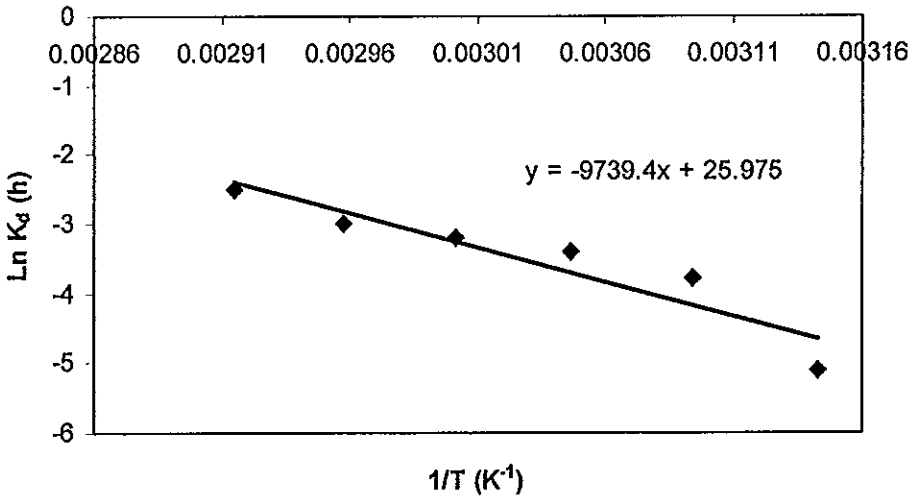


Figure 38. Estimation of the denaturation energy constants (E_d) and initial denaturation constants (K_{do}) for 10 g/L of sago starch as a substrate at temperatures 45-70°C.

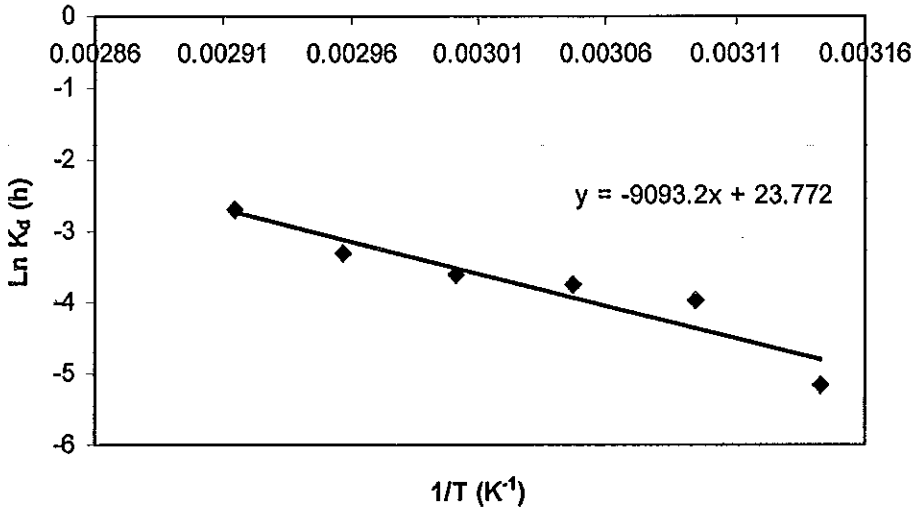


Figure 39. Estimation of the denaturation energy constants (E_d) and initial denaturation constants (K_{do}) for 20 g/L of sago starch as a substrate at temperatures 45-70°C.

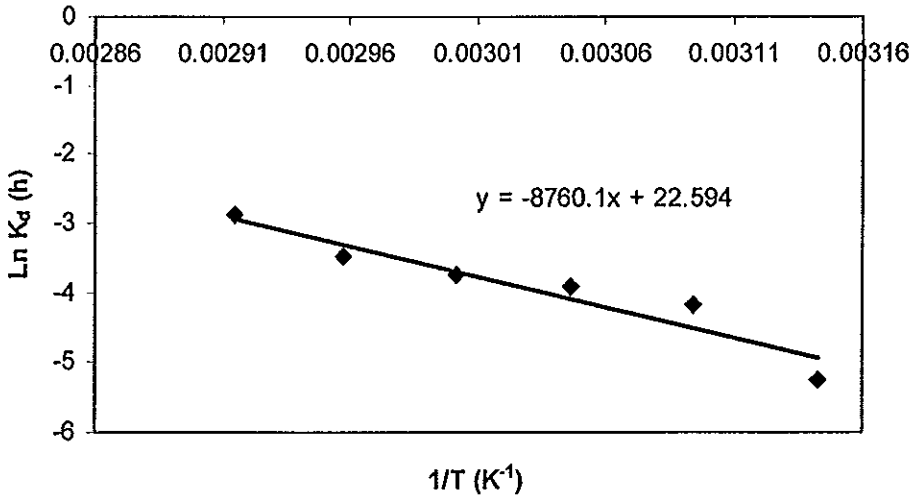


Figure 40. Estimation of the denaturation energy constants (E_d) and initial denaturation constants (K_{do}) for 30 g/L of sago starch as a substrate at temperatures 45-70°C.

Yamamoto *et al.* (2000) found that CD were produced even after 24 h, indicating that CGTase was active at least for 24 h at 50 °C, the enzyme did not completely inactivate even at pH 10.0 and 10.5 and at 65 and 70 °C. These indicate that CGTase might be stabilized in their study by its adsorption to raw starch granules and to heating starch, respectively. The starch concentration was prepared by heating starch suspension in boiling water for 10 min while the suspension was manually shaken. This procedure might be insufficient for the complete gelatinization and retain some crystalline part. Heat-moisture-treated starch adsorbed CGTase (Kobayashi *et al.*, 1978 in Yamamoto *et al.*, 2000) and heat moisture- treated starch showed higher crystallinity than raw starch (Osman, 1967 in Yamamoto *et al.*, 2000).

Table 5 shows the values of denaturation energy constants (E_d) and denaturation constants (K_{do}) of β-CD production at different substrate concentrations. K_{do} decreased when substrate concentration increased, indicated that enzyme was more stable at higher substrate concentration while E_d increased when substrate concentration decreased. High levels of substrates tend to stabilize enzymes, even under non-ideal conditions (Enzymes for education, 2008). Kim *et al.* (1993) also reported that the stability of CGTase increased with the presence of starch. The CGTase stability was enhanced in the presence of starch (Szerman *et al.*, 2007). This phenomenon is due to the adsorption of CGTase on starch surface, which protect the enzyme against denaturation. Enzyme denaturation can be defined as the loss of enough structure to render the enzyme inactive. Changes in the rate of the reaction, the affinity for substrate (K_m), pH optimum, temperature optimum, specificity of reaction, etc., may be affected by denaturation of enzyme molecules (Ohio, 2009). The K_d values of CGTase in β-CD production are shown in Figure 36. It was found that the denaturation constant (K_d) of CGTase in β-CD production increased with increasing temperature. The increase in K_d values along with increasing temperature at high substrate concentration was lower than that at low substrate concentration. This

indicated that CGTase was more stable at high substrate concentration. It is possible that CGTase could maintain their active structure at high temperature by binding with substrate. Pishtiyski *et al.* (2008) reported that the inactivation of CGTase at high temperatures should not be considered a disadvantage for CD production, as an increase in enzyme stability is expected in the presence of a substrate.

Table 5. K_{do} and E_d of CGTase in β-CD production from sago starch using different substrate concentrations

Substrate g/L	$K_{do}(h^{-1})$	E_d(kcal/mol.K)
5	3.10×10^{11}	19.519
10	1.91×10^{11}	19.352
20	2.11×10^{10}	18.068
30	6.49×10^{9}	17.406

As temperature rise the rate of chemical reactions increases because temperature increases the rate of motion of molecules beside that with increasing temperature could inactive the enzyme because enzyme will be denatured at high temperature. Mare`chal *et al.* (1996) also reported that the CGTase stability was enhanced in the presence of starch, since the enzyme in the buffer solution is inactivated under the conditions described. Moreover, Szerman *et al.* (2007) reported that the enzyme adsorbed on the surface of starch would be protected from heat-denaturation. When the temperature increased from 65°C to 70°C, the sharp increase of the K_d was observed at all substrate concentrations. This could explain the lower β-CD production at 70°C (Figure 20).

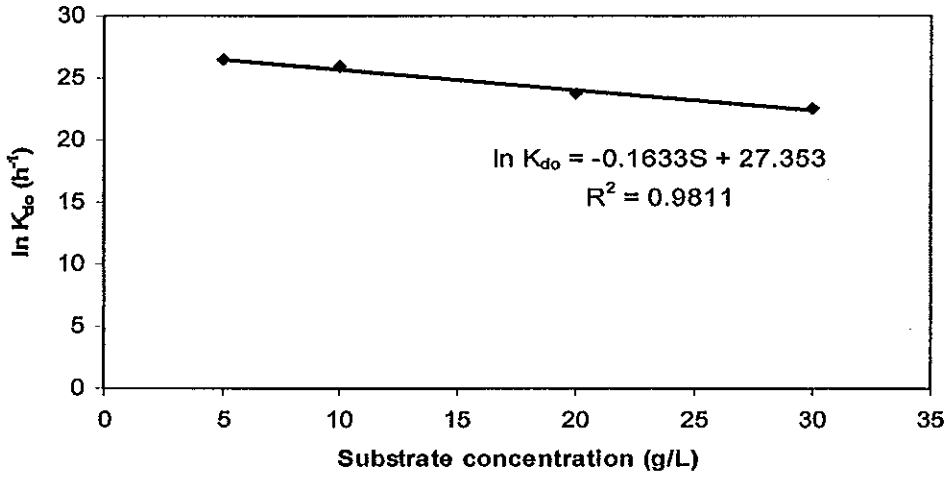


Figure 41. The plot of K_{do} and substrate concentration for CGTase activity using 5-30 g/L sago starch as substrate in 50 ml glyscine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 40-70°C.)

Using Figure 41 and 42, the equations for K_{do} and E_d were obtained as follows:

$$K_{do} = \exp(-0.1633\ S + 27.353) \quad (13)$$

$$E_d = -0.0907\ S + 20.06 \quad (14)$$

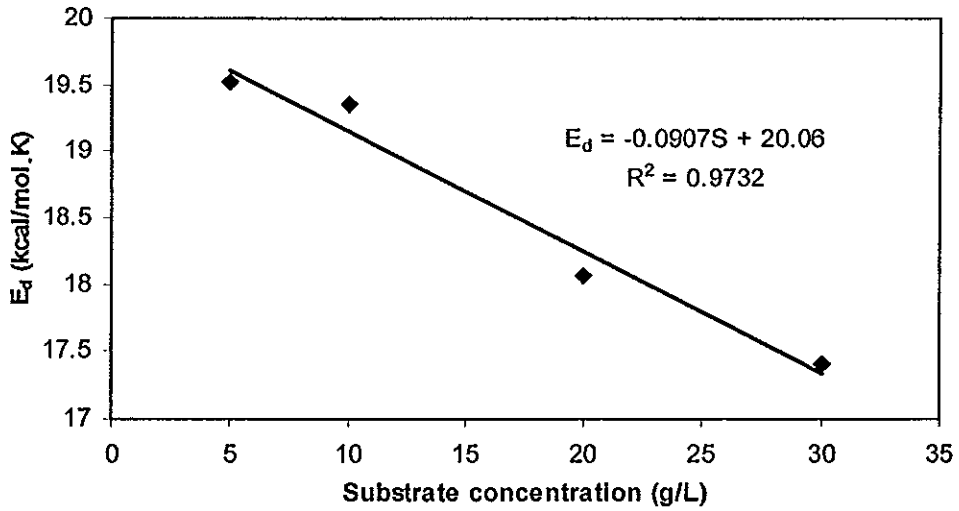


Figure 42. E_d versus substrate concentration of CD production using 5-30 g/L sago starch as substrate in 50 ml glyscine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 40-70°C.)

7. Half-life

A common parameter used in the characterization of enzyme stability is the half-life (τ). The half-life is another useful measure of the rate of a reaction. The half-life has units of time and corresponds to the time required for the loss of half of the original enzyme concentration, or activity. To calculate half-life (τ) E_d and K_{do} values from Table 5 with R = 1.987 cal/mol/K and temperature (K) were used in the following equation:

$$\tau = \frac{0.69}{K_{d0} \cdot e - \frac{E_d}{RT}}$$

The half-life of CGTase in the function of substrate concentration and temperature are showed in Figure 43. An increase in temperature half-life of CGTase from *Bacillus* sp. C26 decreased. And at higher substrate concentration gave higher value for half-life. It could be said that high substrate concentration could stabilize the activity of CGTase and moderate the effect of temperature on the acivity of the enzyme. The substrate protection effect on an enzyme in a reversible reaction was studied by Lin *et al.* (1986) using immobilized glucose isomerase. They found that, first the protection by the reactant was equal to the protection by the product, and secondly, the half-life of the enzyme increased slightly at high sugar concentration. Thus, the experimental method described here appears to be useful for the determination of substrate protection of enzyme deactivation in reversible reactions.

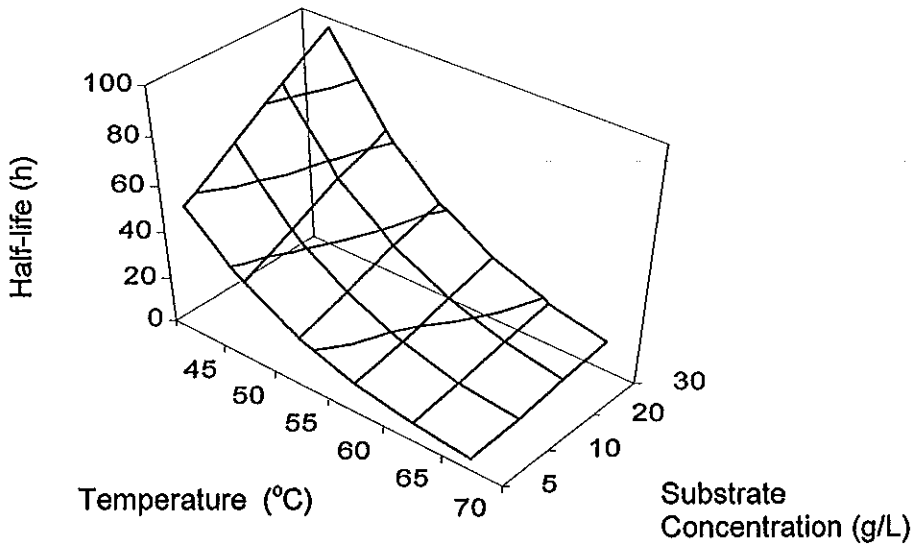


Figure 43. Correlation between half-life (h), temperature (45-70°C) and substrate concentration (5-30g/L).

8. Simulation of optimum condition

In industrial processes, finding a working temperature that gives high enzyme activity with good stability represents a compromise between lower process costs and higher productivities. This temperature can first be estimated from a double normalized plot of the enzyme half-life (τ) and the enzymatic catalytic activity (V_{max}/K_m) against temperature. The mathematical model of both activity and half-life was shown to be a very useful procedure in order to obtain the optimum conditions to operate the enzyme reactor, mainly when an objective function was to be determined (Santos *et al.*, 2007). Santos *et al.* (2007) also concluded that according to the method using a double normalized plot, using either the experimental results or model predictions for the activities and half-life, it is possible to determine the most appropriate operational temperature for an enzyme reactor. The half-lives of CGTase from *Bacillus* sp. C26 were estimated by Eq. (10) and presented together with V_{max}/K_m against temperatures in a double normalized plot of enzyme CGTase from *Bacillus* sp.

C26. Figure 44 showed the optimum condition at different concentrations of substrate in the range of 5-30 g/L. From Eq. (11) and (12) the catalytic activity (V_{max}/K_m) is the function of temperature. And from Eq. (13) and (14) the half-life is the function of both temperature and substrate concentration. The optimal temperature for catalytic activity was found to be 65°C. However, the half-life of CGTase was very short in the range of 6 to 96 h. At higher substrate concentration the half-life of CGTase was higher compared to that at low substrate concentration. As can be seen in this plot, the process temperatures are not necessarily those corresponding to maximum enzyme activity, since at this point the denaturation rate of the enzyme is very high. Therefore, the process temperature must be some what lower a that giving maximum activity in order to increase the enzyme lifetime, but to an extent that will not affect process productivity as a whole. The values predicted by the model and the experimental data were plotted in Figure 44. In this figure, the ordinates were normalized, represented by the relative catalytic activity $(V_{max}/K_m)/(V_{max}/K_m)_{max}$ and relative half-life (τ/τ_{max}) where $(V_{max}/K_m)_{max}$ is the maximum catalytic activity at temperature 65°C and τ_{max} is the maximum half-life at the temperature 45 °C and substrate concentration of 30 g starch/L. The optimum temperature was determined from the interception of the lines below the optimum point in Figure 44. From Figure 44 the optimum temperature for CGTase activity would be 51, 52, 53 and 54 °C at 5, 10, 20, and 30 g starch/L, respectively. At this point, enzyme stability is significantly higher, which means that the loss of enzyme is greatly diminished. On the other hand, the reaction rate is lowered, which can be circumvented by a proportional increase in the enzyme concentration in the reaction medium, so that productivity will be less affected. However, since half-lives decrease very rapidly with temperature increases, the selection of an even lower temperature may improve enzyme stability without any considerable loss of activity. This optimum point could be used for continuous process design. It is possible that the double intercept plot methodology described above may

need a complementary assay, such as, for example, a target function involving the costs of the enzyme and the production, subsequently using standard optimization calculations to find the optimum point.

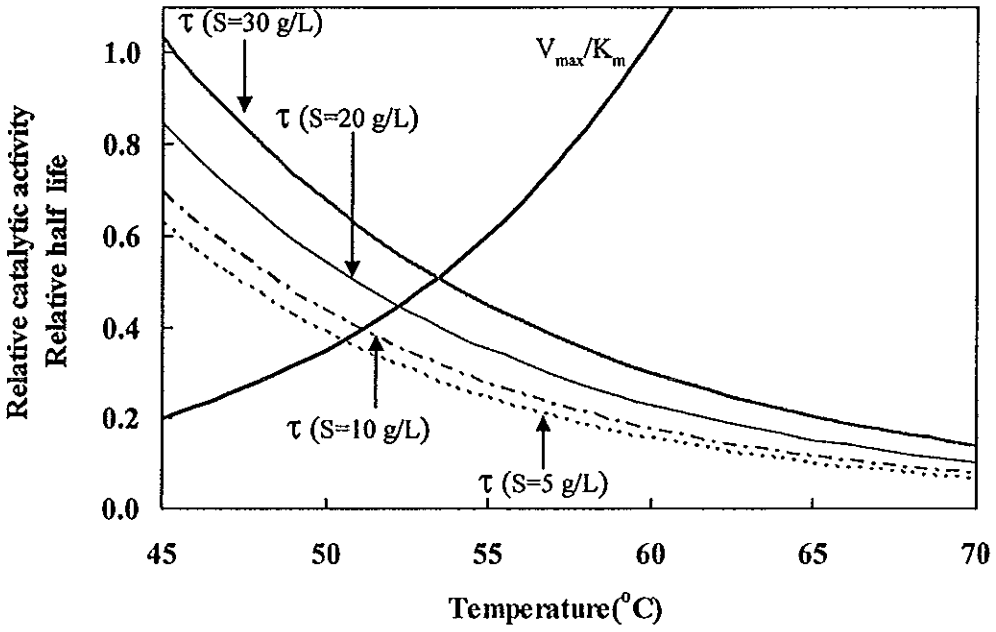


Figure 44. Simulation of optimum condition from relative half-life (τ) and relative V_{max}/K_m of CGTase in β-CD production using 5-30 g/L sago starch as substrate (S) in 50 ml glyscine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 40-70°C.)

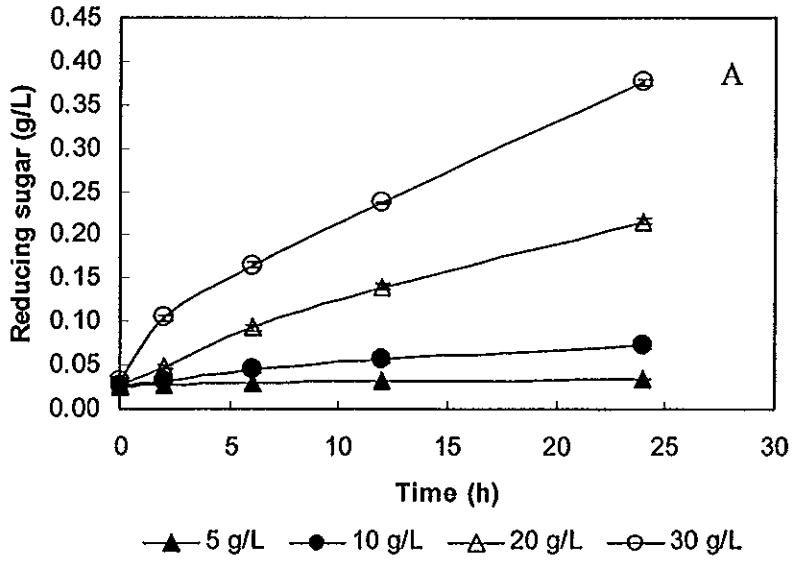
9. Effect of temperature on hydrolytic activity of CGTase

CGTases are the exo-acting enzymes. Exo-acting enzymes cleaved at α-(1,4) bonds at the non-reducing end in starch molecule and produce only low molecular weight products mainly glucose and maltose. Hydrolysis involves the evacuation of newly made reducing end to water molecule resulting in hydrolysis of oligosaccharides. This reaction increased the number of the reducing end of

oligosaccharides (Zain, 2005). The hydrolysis activity of CGTases is much lower than the disproportionation and cyclization activities. Only CGTases from *Thermoanaerobacter* and *Thermoanaerobacterium thermosulfurigenes* strain EM1 (Tabium) have relatively high hydrolysis activities, although still very low compared to α-amylases (Wind *et al.*, 1998). The hydrolysis activity of CGTase from *Bacillus* sp. C26 was determined by the formation of reducing sugar. Figure 45-48 shows the reducing sugar formation in reaction mixture at temperature range 40°C-70°C using substrate concentration of 5 – 30 g/L. The reducing sugar formation increased with increasing temperature and substrate concentration. Martins and hatti-Kaul, (2003) reported that enzyme CGTase was seen to have a significant starch hydrolysis activity, resulting in an unusually high ratio (0.52) of hydrolysis to cyclization specific activities. Raising the assay pH and/or temperature could, however, reduce this ratio. For example, ratio of 0.17 was obtained at 55 °C when the cyclization activity was promoted, while hydrolysis activity was reduced to 3.3 U/mg from 7.8 U/mg at 50 °C. The product profile obtained upon degradation of soluble starch (1% w/v) by the CGTase revealed G6 to be the predominant product at about four fold higher concentrations than β-CD. Shorter polysaccharides were also produced although at a significant lower extent. Minor CD ring-opening activity was detected for the enzyme only at high concentrations of the substrates. Figure 49 shown the effect of substrate concentration and temperature on initial velocity of reducing sugar production and it was found that initial velocity (V_H) for reducing sugar production increased when the substrate concentration and temperature increased. Figure 50 shows the relationships between initial velocities of β-CD production (V_{CD}) and reducing sugar production (V_H) by CGTase and it can be derived as the following equation:

$$V_H = 0.0085\ V_{CD} - 0.0118 \qquad (15)$$

From the equation the ratio of hydrolysis activity to β-CD production activity of CGTase was 0.0085. This low ratio means that the linear polysaccharides were produced at low extent.



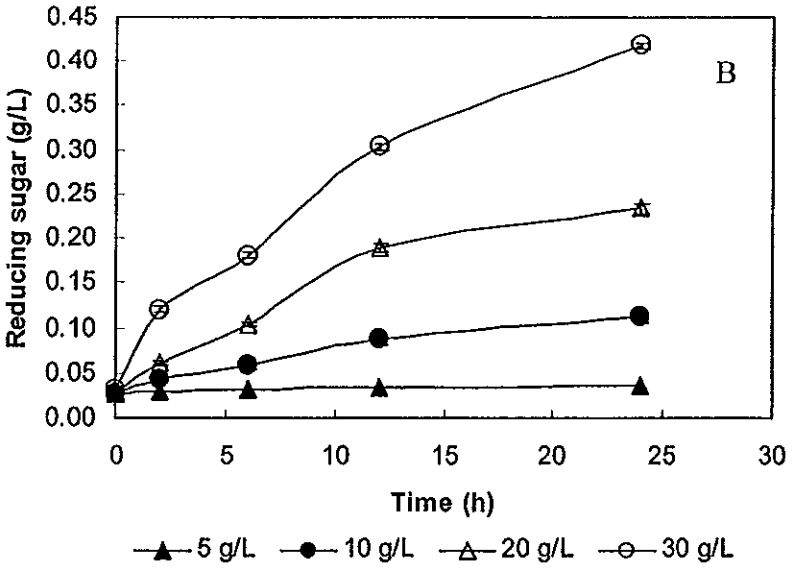


Figure 45. Effect of sago starch concentration on reducing sugar concentration using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 40°C (A) and 45°C (B).)

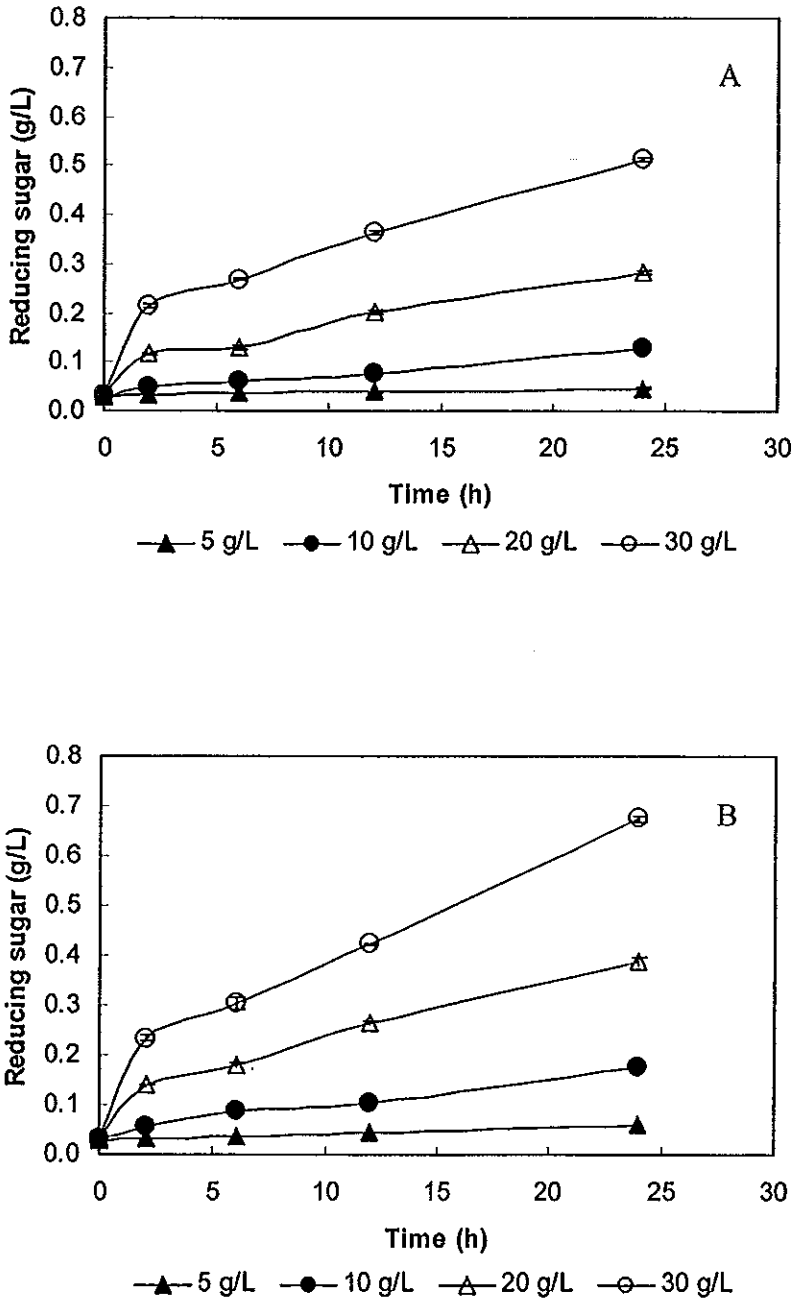


Figure 46. Effect of sago starch concentration on reducing sugar concentration using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 50°C (A) and 55°C (B).)

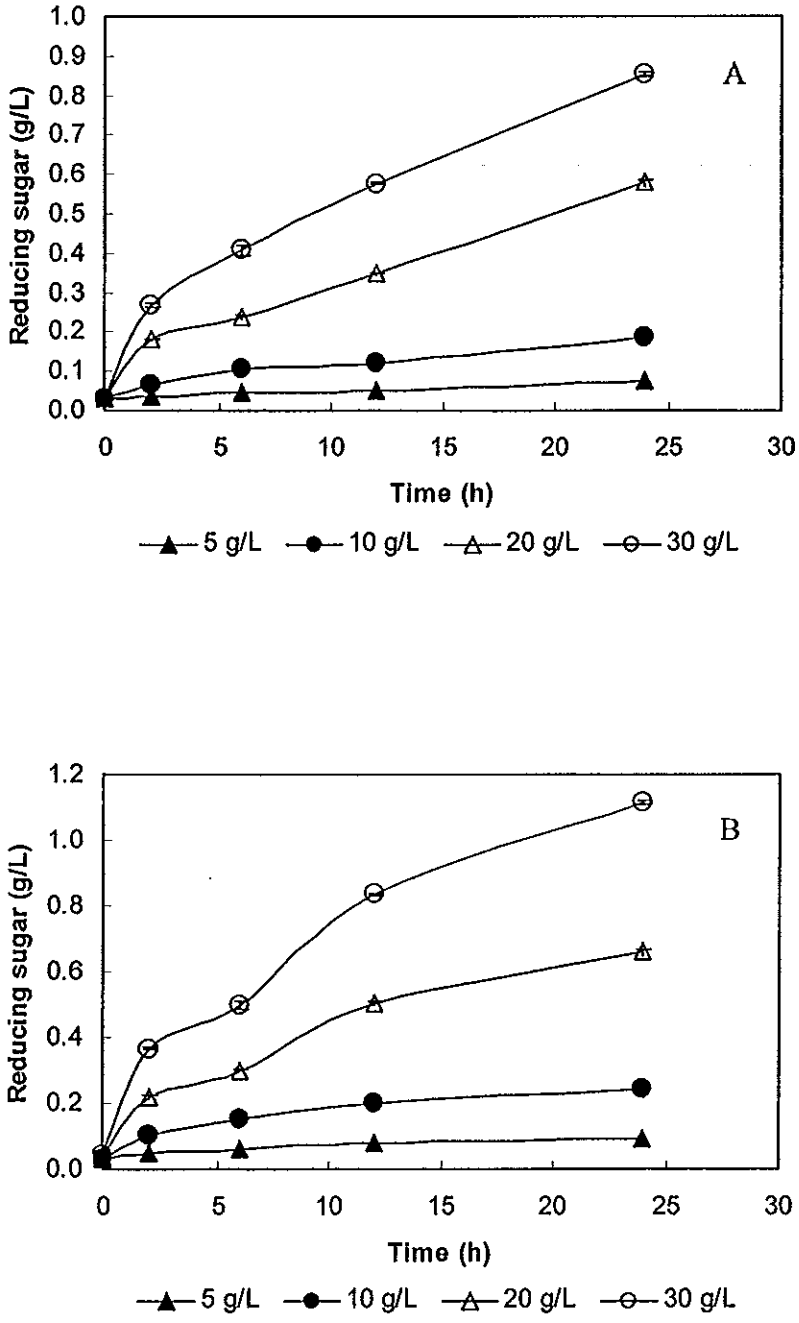


Figure 47. Effect of sago starch concentration on reducing sugar concentration using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 60°C (A) and 65°C (B).)

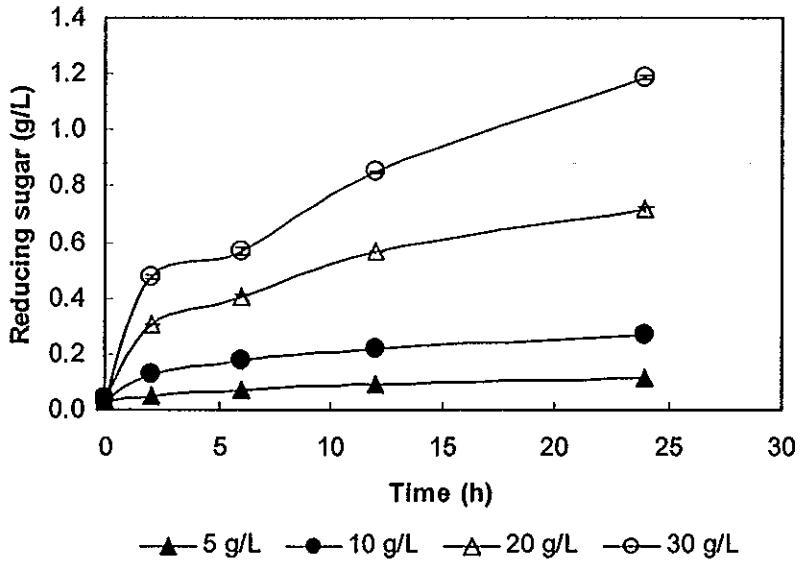


Figure 48. Effect of sago starch concentration on reducing sugar concentration using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 70°C.)

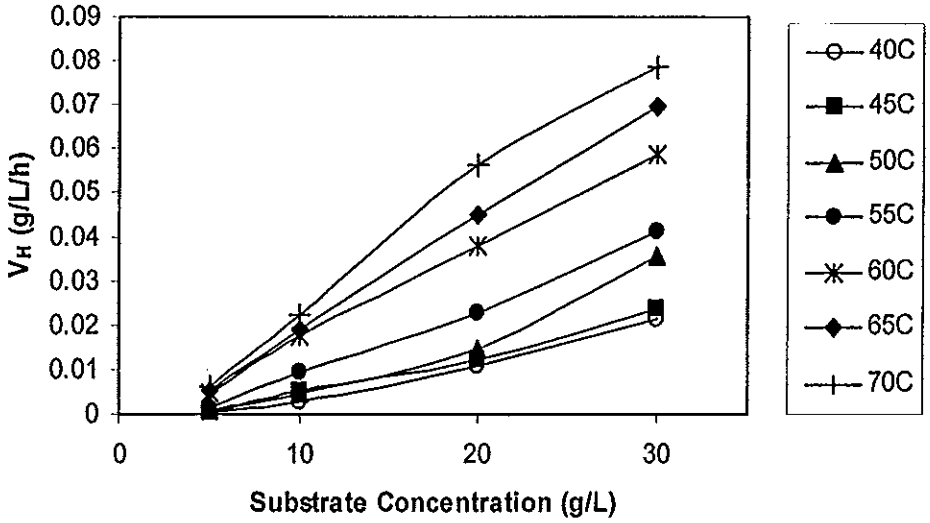


Figure 49. Initial velocity of reducing sugar production on cyclodextrin production using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 40-70°C.)

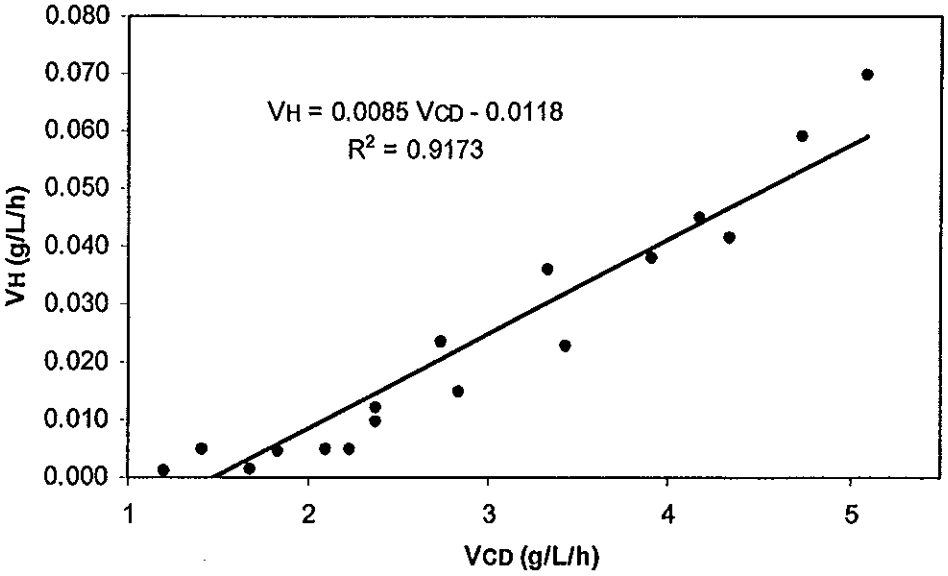


Figure 50. Relationships between initial velocity of cyclodextrin production and initial velocity of reducing sugar production from sago starch using enzyme CGTase at 40-65°C.

CHAPTER IV

CONCLUSIONS

The kinetics of β-CD production by CGTase from *Bacillus* sp. C26 was studied. It was found that with increasing CGTase amount the reaction rate was increased but the concentration of β-CD was decreased. The optimum CGTase amount for high production of β-CD was 10 U/g starch. The effects of substrate and temperature on the production of β-CD and the activity of CGTase were investigated. With increasing the substrate concentration the β-CD production was also increased *at al*l examined temperatures. The kinetic parameters were determined using the Michaelis–Menten equation, where the V_{max} increased from 2.35 to 6.78 g β-CD/L/h when temperature increased from 40-65°C, while K_m decreased from 39.2 to 9.78 g starch/L when temperature increased from 40 to 60°C. It is quite obvious from Michealis-Menten equation that higher V_{max} and lower K_m are associated with the greater β-CD production. The catalytic efficiency (V_{max}/K_m) shows the optimum temperature at 65°C. The energy activation (E_a) calculated using Arrhenius equation was 8.85 kcal/mol.K, where the high value of E_a reflected a high temperature sensitivity of the CGTase. The activity of CGTase decreased with increasing temperature and prolonging incubation time. A higher substrate concentration gave higher enzyme stability compared to that at low substrate concentration because CGTase was more resistant to thermal denaturation in the presence of its substrate. The denaturation constant (K_d) of CGTase in β-CD production increased with increasing temperature while at high substrate concentration, it was lower than that at low substrate concentration. This also indicated that CGTase was more stable at high substrate concentration. An increase in temperature half-life of CGTase from *Bacillus* sp. C26 decreased because of denaturation effect at high temperature. However, higher

substrate concentration gave longer half-life. Although the optimal temperature for catalytic activity was found to be 65°C, the half-life of CGTase was very short. To solve this problem, the mathematical models for catalytic efficiency and half-life was constructed and it shows the optimum temperature at 51, 52, 53 and 54 °C for 5, 10, 20, and 30 g starch/L, respectively using 10 U/g of CGTase concentration. It was also found that the hydrolysis activity of CGTase from *Bacillus* sp. C26 was much lower than the activity of β-CD production.

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APPENDICES

APPENDIX 1

Medium

1. Inoculum in this research using Horikoshi II broth, composition of medium (w/v) (Illias *et al.*, 2002) :

1. 1.0% sago starch
2. 0.5% yeast extract
3. 0.5% peptone
4. 0.1% KH_2PO_4
5. 0.02% $MgSO_4 \cdot 7H_2O$
6. 1.0% Na_2CO_3

2. DNS solution to measure of reducing sugar concentration.

DNS solution dinitrosalicylic acid reagent solution:

1. Dinitrosalicylic acid 10 g
2. Phenol 2 g (optional)
3. Sodium sulfite 0.5 g
4. Sodium hydroxide 10 g
5. Add water to 1 liter

APPENDIX 2

Standard Curve

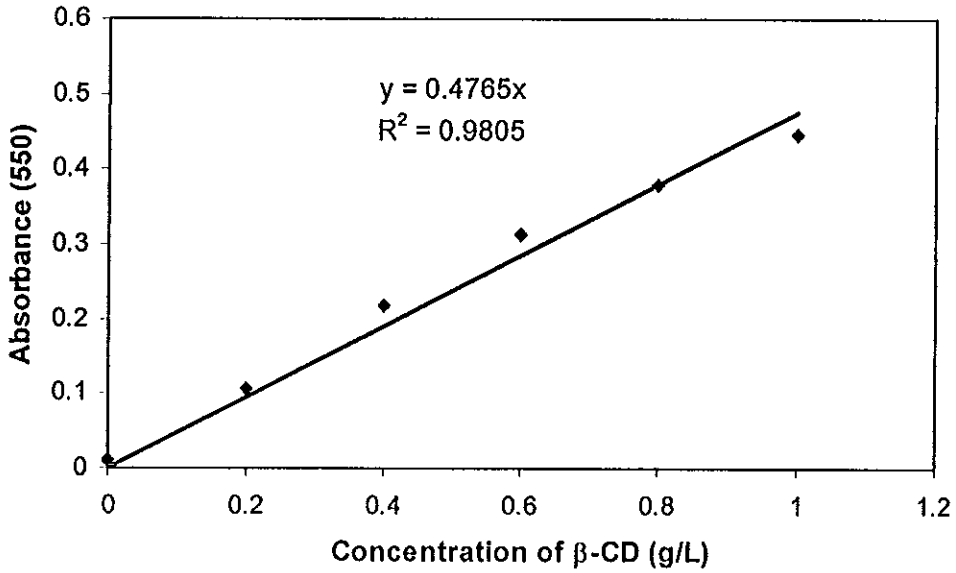

Figure 51. Standard curve for β-cyclodextrin concentration.

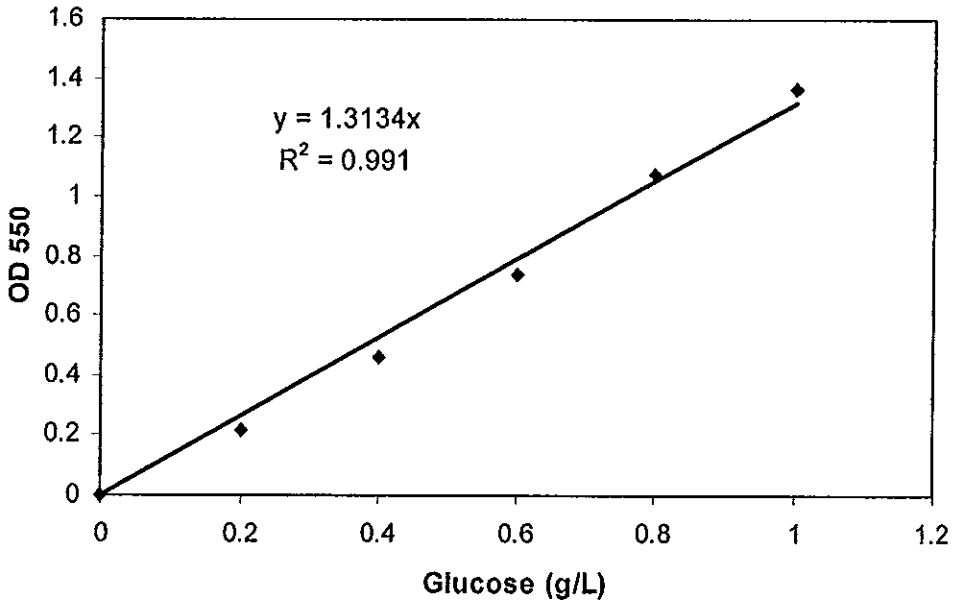

Figure 52. Standard curve for reducing sugar concentration.

APPENDIX 3

Effect of enzyme CGTase concentration

Table. 6 Effect of CGTase amount on β-CD production using sago starch as substrate. (The reaction mixture contained 10 g/L sago starch in 50 ml glycine-NaOH buffer pH 8.5 was incubated at 50°C.)

Time (h)	5 U/g	10 U/g	20 U/g	30 U/g	40 U/g
0	0	0	0	0	0
0.5	0.383	0.431	0.708	0.732	0.703
1	0.768	0.919	1.096	1.351	1.162
2	1.429	1.830	1.717	1.749	1.361
4	2.057	2.314	1.836	1.637	1.247
6	2.439	3.163	1.988	1.881	1.564
12	3.034	3.436	2.501	1.865	1.354
18	3.100	3.826	2.798	1.725	1.174
24	3.384	4.192	2.395	1.706	1.028

Table. 7 Effect of CGTase amount on yield and initial rate of β-CD production using sago starch as substrate. (The reaction mixture contained 10 g/L sago starch in 50 ml glycine-NaOH buffer pH 8.5 was incubated at 50°C.)

CGTase (U/g)	β-CD Yield (%)	Initial rate (g/L/h)
5	33.84	0.7273
10	41.920	0.9134
20	23.950	1.1602
30	17.060	1.3736
40	10.280	1.2108

APPENDIX 4

Effect of substrate concentration and temperature

Table. 8 Effect of sago starch concentration on cyclodextrin production using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 40°C.)

Time (h)	5 g/L	10 g/L	20 g/L	30 g/L
0	0	0	0	0
0.5	0.056	0.205	0.535	0.806
1	0.256	0.664	0.945	1.227
2	0.493	0.651	3.637	4.065
4	0.905	1.660	4.136	4.508
6	1.254	2.390	4.680	4.999
12	1.609	2.870	6.042	6.238
18	1.834	3.601	6.860	7.252
24	2.152	4.031	7.126	7.994

Table. 9 Effect of sago starch concentration on cyclodextrin production using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 45°C.)

Time (h)	5 g/L	10 g/L	20 g/L	30 g/L
0	0	0	0	0
0.5	0.620	1.045	1.886	2.484
1	1.240	1.824	3.207	3.631
2	1.698	3.098	4.554	5.026
4	2.175	3.678	6.684	7.749
6	2.241	4.168	7.442	8.235
12	2.849	4.451	9.130	10.896
18	2.954	4.723	9.784	11.270
24	3.242	4.802	10.225	12.416

Table. 10 Effect of sago starch concentration on cyclodextrin production using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 50°C.)

Time (h)	5 g/L	10 g/L	20 g/L	30 g/L
0	0	0	0	0
0.5	0.790	1.231	2.027	2.512
1	1.425	2.355	4.458	6.101
2	2.059	3.463	5.507	7.177
4	2.569	4.496	6.592	9.425
6	2.791	4.835	8.447	11.899
12	3.059	5.131	10.088	12.782
18	3.358	5.339	11.068	13.750
24	3.305	5.616	11.513	14.087

Table. 11 Effect of sago starch concentration on cyclodextrin production using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 55°C.)

Time (h)	5 g/L	10 g/L	20 g/L	30 g/L
0	0	0	0	0
0.5	0.652	1.459	2.104	2.556
1	1.765	3.389	4.835	6.233
2	2.073	4.327	6.302	7.964
4	2.533	4.446	7.864	10.115
6	2.812	4.795	9.745	11.685
12	3.506	5.250	11.073	13.596
18	3.414	5.526	11.845	14.338
24	3.495	5.656	12.668	14.607

Table. 12 Effect of sago starch concentration on cyclodextrin production using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 60°C.)

Time (h)	5 g/L	10 g/L	20 g/L	30 g/L
0	0	0	0	0
0.5	1.244	2.047	2.744	3.061
1	1.990	3.450	5.990	7.190
2	2.350	4.208	7.264	8.921
4	2.844	4.772	9.104	11.004
6	2.910	4.877	10.087	12.323
12	3.057	5.242	11.750	14.050
18	3.522	5.708	12.659	14.739
24	3.529	5.838	13.296	15.603

Table. 13 Effect of sago starch concentration on cyclodextrin production using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 65°C.)

Time (h)	5 g/L	10 g/L	20 g/L	30 g/L
0	0	0	0	0
0.5	1.304	2.152	2.913	3.249
1	2.132	3.646	6.325	7.039
2	2.572	4.421	7.589	9.402
4	3.151	4.839	9.542	11.304
6	3.277	5.263	10.342	12.525
12	3.596	5.633	11.929	14.250
18	3.739	5.901	12.950	15.347
24	3.861	6.174	13.496	16.055

Table. 14 Effect of sago starch concentration on cyclodextrin production using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 70°C.)

Time (h)	5 g/L	10 g/L	20 g/L	30 g/L
0	0	0	0	0
0.5	1.167	1.671	2.492	2.586
1	1.966	3.856	4.890	5.915
2	2.597	4.099	6.307	7.903
4	2.641	4.768	7.775	9.220
6	2.686	4.944	8.688	10.073
12	2.809	5.075	9.580	11.131
18	2.935	5.230	10.132	12.036
24	3.207	5.490	11.168	13.085

Table. 15 Yield of β-CD production in 50 ml glycine-NaOH buffer pH 8.5 at 24 hours incubation time using 5-30 g/L sago starch as a substrate and temperature 40-70°C.

	Yield of β-CD production (g/L)			
Temperature (°C)	5 g/L	10 g/L	20 g/L	30 g/L
40	2.152	4.031	7.126	9.142
45	3.242	4.802	10.225	12.416
50	3.305	5.616	11.513	14.087
55	3.495	5.656	12.668	14.607
60	3.529	5.838	13.296	15.603
65	3.861	6.174	13.496	16.055
70	3.207	5.49	11.168	13.085

Table. 16 Effect of temperature on initial rate of β-CD production using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 40-70°C.)

	Initial velocity (V_o) of cyclodextrin production			
Temperature (°C)	5g/L	10g/L	20g/L	30g/L
40	0.2701	0.4463	0.8101	1.0948
45	0.9026	1.4047	2.3747	2.7366
50	1.2012	1.8314	2.8299	3.3373
55	1.6728	2.3677	3.4312	4.3445
60	2.0891	3.5789	3.905	4.7385
65	2.2274	3.7775	4.178	5.0914
70	1.3637	2.1256	3.2612	4.1544

Table. 17 Data to plot Lineweaver-Burk plot for estimation of K_m and V_{max} at temperature 40°C.

substrate (g/L)	velocity g β-CD/L/h	1/S	1/V
5	0.2701	0.2	3.7023
10	0.4463	0.1	2.2406
20	0.8101	0.05	1.2344
30	1.0948	0.0333	0.9134

Table. 18 Data to plot Lineweaver-Burk plot for estimation of K_m and V_{max} at temperature 45°C.

Substrate (g/L)	Velocity (g β-CD/L/h)	1/S	1/V
5	0.9026	0.2	1.1079
10	1.4047	0.1	0.7119
20	2.3747	0.05	0.4211
30	2.7366	0.0333	0.3654

Table. 19 Data to plot Lineweaver-Burk plot for estimation of K_m and V_{max} at temperature 50°C.

Substrate (g/L)	Velocity (g β-CD/L/h)	1/S	1/V
5	1.2012	0.2	0.8325
10	1.8314	0.1	0.5460
20	2.8299	0.05	0.3534
30	3.3373	0.0333	0.2996

Table. 20 Data to plot Lineweaver-Burk plot for estimation of K_m and V_{max} at temperature 55°C.

Substrate (g/L)	Velocity (g β-CD/L/h)	1/S	1/V
5	1.6728	0.2	0.5978
10	2.3677	0.1	0.4224
20	3.4312	0.05	0.2914
30	4.3445	0.0333	0.2302

Table. 21 Data to plot Lineweaver-Burk plot for estimation of K_m and V_{max} at temperature 60°C.

Substrate (g/L)	Velocity (g β-CD/L/h)	1/S	1/V
5	2.0891	0.2	0.4787
10	3.5789	0.1	0.2794
20	3.905	0.05	0.2561
30	4.7385	0.0333	0.2110

Table. 22 Data to plot Lineweaver-Burk plot for estimation of K_m and V_{max} at temperature 65°C.

Substrate (g/L)	Velocity (g β-CD/L/h)	1/S	1/V
5	2.2274	0.2	0.4490
10	3.7775	0.1	0.2647
20	4.178	0.05	0.2393
30	5.0914	0.0333	0.1964

Table. 23 Data to plot Lineweaver-Burk plot for estimation of K_m and V_{max} at temperature 70°C.

Substrate (g/L)	Velocity (g β-CD/L/h)	1/S	1/V
5	1.3637	0.2	0.7333
10	2.1256	0.1	0.4705
20	3.2612	0.05	0.3066
30	4.1544	0.0333	0.2407

APPENDIX 5

Thermal stability of CGTase from *Bacillus* sp. C26

Table. 24 Residual activity (E/E_o) of CGTase in β-CD production from sago starch at 45°C

Time (h)	E/E_o			
	5 g/L	10 g/L	20 g/L	30 g/L
0	1.000	1.000	1.000	1.000
2	0.889	0.903	0.925	0.950
6	0.847	0.871	0.895	0.918
12	0.819	0.849	0.872	0.899
24	0.778	0.806	0.842	0.862

Table. 25 Residual activity (E/E_o) of CGTase in β-CD production from sago starch at 50°C

Time (h)	E/E_o			
	5 g/L	10 g/L	20 g/L	30 g/L
0	1.000	1.000	1.000	1.000
2	0.871	0.905	0.933	0.943
6	0.743	0.800	0.843	0.879
12	0.657	0.716	0.769	0.809
24	0.543	0.632	0.687	0.732

Table. 26 Residual activity (E/E_o) of CGTase in β-CD production from sago starch at 55°C

Time (h)	E/E_o			
	5 g/L	10 g/L	20 g/L	30 g/L
0	1	1	1	1
2	0.858	0.894	0.916	0.920
6	0.708	0.766	0.822	0.849
12	0.585	0.638	0.723	0.756
24	0.472	0.504	0.624	0.689

Table. 27 Residual activity (E/E_o) of CGTase in β-CD production from sago starch at 60°C

Time (h)	E/E_o			
	5 g/L	10 g/L	20 g/L	30 g/L
0	1	1	1	1
2	0.844	0.841	0.920	0.932
6	0.624	0.697	0.811	0.835
12	0.477	0.559	0.701	0.737
24	0.376	0.421	0.572	0.648

Table. 28 Residual activity (E/E_o) of CGTase in β-CD production from sago starch at 65°C

Time (h)	E/E_o			
	5 g/L	10 g/L	20 g/L	30 g/L
0	1	1	1	1
2	0.757	0.746	0.851	0.887
6	0.542	0.599	0.706	0.752
12	0.383	0.451	0.587	0.651
24	0.290	0.359	0.493	0.559

Table. 29 Residual activity (E/E_o) of CGTase in β-CD production from sago starch at 70°C.

Time (h)	E/E_o			
	5 g/L	10 g/L	20 g/L	30 g/L
0	1	1	1	1
2	0.654	0.671	0.790	0.873
6	0.402	0.497	0.560	0.653
12	0.243	0.301	0.395	0.496
24	0.168	0.210	0.300	0.407

APPENDIX 6

The denaturation energy constant of CGTase from *Bacillus* sp. C26

Table. 30 K_d values of cyclodextrin production using 5-30 g/L sago starch as substrate in 50 ml glyscine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 40-70°C.)

Temperature (°C)	K_d(h)			
	5 g/L	10 g/L	20 g/L	30 g/L
45	0.0079	0.006	0.0057	0.0053
50	0.0276	0.0231	0.0190	0.0152
55	0.0378	0.0334	0.0235	0.0196
60	0.0561	0.0406	0.0269	0.0232
65	0.0673	0.0502	0.0366	0.0303
70	0.0978	0.0806	0.0684	0.0557

APPENDIX 7

Half-life of CGTase from *Bacillus* sp. C26

Table. 31 Data of half-life (h) at temperature 45-70°C using sago starch as substrate with concentration 5-30 g/L.

Tempearure (K)	τ 5g	τ 10g	τ 20g	τ 30g
318.15	57	71	85	96
323.15	35	44	54	63
328.15	22	28	35	42
333.15	14	18	23	28
338.15	9	12	16	19
343.15	6	8	11	13

APPENDIX 8

Effect of temperature on hydrolytic activity of CGTase from *Bacillus* sp. C26

Table. 32 Effect of sago starch concentration on reducing sugar concentration using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 40°C.)

Time (h)	5 g/L	10 g/L	20 g/L	30 g/L
0	0.026	0.027	0.028	0.032
2	0.028	0.033	0.047	0.104
6	0.029	0.045	0.093	0.165
12	0.031	0.057	0.140	0.237
24	0.034	0.073	0.215	0.376

Table. 33 Effect of sago starch concentration on reducing sugar concentration using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 45°C.)

Time (h)	5 g/L	10 g/L	20 g/L	3 g/L
0	0.026	0.027	0.029	0.032
2	0.028	0.042	0.061	0.120
6	0.030	0.058	0.103	0.181
12	0.034	0.089	0.189	0.303
24	0.036	0.113	0.233	0.418

Table. 34 Effect of sago starch concentration on reducing sugar concentration using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 50°C.)

Time (h)	5 g/L	10 g/L	20 g/L	30 g/L
0	0.028	0.029	0.030	0.032
2	0.032	0.047	0.115	0.215
6	0.035	0.059	0.130	0.268
12	0.039	0.078	0.201	0.365
24	0.046	0.128	0.282	0.514

Table. 35 Effect of sago starch concentration on reducing sugar concentration using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 55°C.)

Time (h)	5 g/L	10 g/L	20 g/L	30 g/L
0	0.028	0.029	0.031	0.032
2	0.032	0.056	0.139	0.233
6	0.038	0.088	0.181	0.303
12	0.043	0.105	0.264	0.422
24	0.060	0.174	0.388	0.675

Table. 36 Effect of sago starch concentration on reducing sugar concentration using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 60°C.)

Time (h)	5 g/L	10 g/L	20 g/L	30 g/L
0	0.029	0.030	0.031	0.032
2	0.035	0.065	0.181	0.266
6	0.045	0.106	0.236	0.408
12	0.050	0.119	0.346	0.577
24	0.077	0.186	0.579	0.853

Table. 37 Effect of sago starch concentration on reducing sugar concentration using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 65°C.)

Time (h)	5 g/L	10 g/L	20 g/L	30 g/L
0	0.031	0.032	0.036	0.040
2	0.049	0.104	0.218	0.366
6	0.063	0.152	0.300	0.495
12	0.076	0.203	0.502	0.834
24	0.090	0.243	0.660	1.116

Table. 38 Effect of sago starch concentration on reducing sugar concentration using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 70°C.)

Time (h)	5 g/L	10 g/L	20 g/L	30 g/L
0	0.031	0.032	0.039	0.041
2	0.053	0.131	0.303	0.475
6	0.070	0.178	0.409	0.567
12	0.090	0.218	0.565	0.851
24	0.114	0.271	0.719	1.189

Table. 39 Initial velocity of reducing sugar on cyclodextrin production using 5-30 g/L sago starch as substrate in 50 ml glycine-NaOH buffer pH 8.5. (The reaction mixture was incubated at 40-70°C.)

gr/L	40°C	45°C	50°C	55°C	60°C	65°C	70°C
5	0.000	0.000	0.001	0.001	0.003	0.002	0.003
10	0.002	0.004	0.004	0.006	0.007	0.009	0.006
20	0.008	0.008	0.010	0.013	0.023	0.028	0.019
30	0.013	0.014	0.015	0.020	0.031	0.035	0.026

VITAE

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List of Publication and Proceedings

Proceedings

Muria, S. R., Cheirsilp, B. 2008. Kinetic Study of β-Cyclodextrin Production from Sago Starch by Cyclodextrin Glycosyltransferase of *Bacilus* sp. C26. The 20th Annual Meeting of the Thai Society for Biotechnology "Biotechnology for Global Care". October 14-17, 2008. Mahasarakham, Thailand.