EFFECT OF OPERATING CONDITIONS ON CHARACTERIZATION AND STABILIZATION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE (CGTASE)

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Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
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Dedicated to whom that believed in me for the knowledge and encouragement.

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ABSTRACT

Cyclodextrin glycosyltransferase (CGTase) is an enzyme that produces cyclodextrin (CD) from starch under catalytic reaction. It is an important industrial enzyme which mostly used in food and pharmaceutical industry. The purpose of this study is to determine the effect of operating conditions on characterization and stabilization of CGTase to improve the production of cyclodextrin (CD) in the future. Therefore, the effect of starch concentration and pH on the characterization of CGTase were studied. The effect of pH and temperature on stabilization of CGTase were investigated. The best starch concentration and pH on characterization of CGTase were 3% w.v and pH 6 respectively. The most stable conditions of CGTase were detected at pH 6-8 and temperature of 40-60˚C. By using these optimum conditions, the CD production may improve in the future which in turn may be beneficial to industrial uses.

ABSTRAK

CGTase adalah enzim yang menghasilkan cyclodextrin (CD) daripada kanji melalui reaksi pemangkin. CGTase enzim merupakan enzim industri yang penting dan banyak digunakan dalam industri makanan dan farmaseutikal. Kajian penyelidikan ini bertujuan untuk menentukan kesan keadaan operasi pada pencirian dan penstabilan cyclodextrin glycosyltransferase (CGTase) untuk meningkatkan lagi pengeluaran cyclodextrin (CD) pada masa hadapan. Oleh itu, kesan kepekatan kanji dan pH pada pencirian CGTase telah disiasat. Selain itu, kesan pH dan suhu pada penstabilan juga disiasat. Kepekatan kanji dan pH terbaik pada pencirian CGTase adalah 3% w/v dan pH 6. Selain itu, pH 6-8 dan suhu 40-60 ˚C adalah keadaan yang paling stabil untuk CGTase. Dengan menggunakan keadaan optimum ini, pengeluaran CD boleh bertambah baik pada masa hadapan dimana ia boleh memberi manfaat untuk kegunaan industri.

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LIST OF SYMBOLS

g	gram
L	liter
ml	mililiter
w/v	weight solute per volume solution
M	mol

LIST OF ABBREVIATIONS

CGTase	cyclodextrin glycosyltransferase
CD	cyclodextrins
OD	optical density
MW	molecular weight

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Enzyme is a biological catalyst that is used to speed up the biochemical reactions. Nowadays, the uses of enzyme in research and industry applications are increasing due to its ability to increase the rate of reaction. Mostly enzyme is specific and unique according to their function. Thus, each enzyme has its own operating conditions to carry out the biochemical reaction. The operating conditions such as temperature, pH and concentration of substrate have significant effects on enzyme activity. These operating conditions are important to maintain the uses of enzyme as well as to maximize the production of desired product. The range of temperature and pH are different depends on the sources of enzyme. There is enzyme that able to function at high temperature of ˚C but mostly the enzyme is only function under a moderate temperature of 37˚C to 47˚C. Moreover, some of enzyme work at acidic and alkaline pHs while others work at neutral condition. The operating conditions can be determine through the characterization and stabilization of enzyme. The identification of optimum conditions of the enzyme helps to increase the efficiency of the enzyme and to obtain the high production of desired product.

Cyclodextrin glycosyltransferase (CGTase) is an enzyme in α-amylase family that produces cyclodextrin (CD) through the degradation of starch (Hirano et al., 2006). CGTase has the ability to catalyze cyclization, disproportionation, hydrolysis and coupling reactions (Veen et al., 2000). However, CGTase is mainly employed in the industrial production of CD through the cyclization reaction (Brena et al., 2013). There are 3 major types of CD that are commonly known which are α-, β-, and γ-CD. The types of CD is depends on the assembly of glucose units in the CD molecules. (Fretas et al., 2012; Frey et al., 2003). These CDs have the ability to protect the fragile substances from

oxygen and UV, to delay the evolution of volatiles and to aid the ancillary processing of dangerous or volatiles substances in powdered form.

CD also generally employed as a complexing agent due to its chemical and physical properties (Brena et al., 2013). Therefore, the uses of CD in the industries is growing in interest as CD can be used in the different applications such as in pharmaceutical, food and cosmetic. The industrial interest is getting wider by years that lead to the high demand in the production of CD. However, nowadays, the production of CD is still in a small scale due to its low yield and time consuming. There are many studies were conducted on characterization and stabilization of CGTase in order to increase the production of CD. In this study, the commercialized CGTase from *Bacillus* sp. is used and the operating conditions of the enzyme have been identified. The operating conditions such as temperature, pH and concentration of substrate have significant effects on enzyme activity. The characterization and stabilization of the enzymes are important to determine the ideal conditions in order to obtain the high production of desired product. Thus, in order to maximize the production of CD in the future, the characterization and stabilization of CGTase used to produce the CD were carried out.

1.2 Motivation

CGTase is an industrially important group of starch-converting enzyme that synthesises CD (Hirano et al., 2006). CGTase is mainly employed in the industrial production of CD through degradation of starch by cyclization reaction (Brena et al., 2013). The uses of CD in the industrial applications are gaining attentions especially in food, cosmetic and pharmaceutical industry. However, despite of the higher demand, the production of CD nowadays is still in small scale due to the low yield of CD. Thus, in this study, a commercialized CGTase from *Bacillus* sp. is used to produce CD. The best operating conditions of the CGTase was identified in order to maximize the production of CD in the future.

1.3 Problem Statement

CGTase is an enzyme that is used to produce CD from degradation of starch by cyclization reaction. In recent years, the demand for the production of CD is increasing due to the applications in industrial. However, the production of CD is low due to the unstable enzyme at certain condition. Thus, the characterization and stabilization of enzyme must be carried out in order to maximize the production of CD in the future.

1.4 Objectives

The objectives of this research are:

1) To determine the best operating conditions on characterization of CGTase for the future CD production.
2) To investigate the best operating conditions on stabilization of CGTase for the future CD production.

1.5 Scopes of Study

The scopes of this research are:

1) Study the effect of starch concentration (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0% (w/v)) on characterization of CGTase.
2) Study the effect of pH (5, 6, 7, 8, 9 and 10) on characterization of CGTase.
3) Study the effect of pH (5, 6, 7, 8, 9 and 10) on stabilization of CGTase.
4) Study the effect of temperature (20, 30, 40, 50, 60 and 70˚C) on stabilization of CGTase.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Cyclodextrin glycosyltranferase (CGTase) is an enzyme that produce cyclodextrin (CD) from starch via cyclization, coupling, disproportional or hydrolysis reaction. Nowadays, CGTase are widely applied in the industrial production of CD as it given the ability to form inclusion complexes with a great variety of organic molecules (Zanin et al., 2013).

2.2 Cyclodextrin Glycosyltransferase (CGTase)

CGTase is an enzyme which categorize in the α-amylase family known as glycosyl hydrolyse family 13 (Park et al., 2000). The molecular weight of CGTase is in range 60-110 kDa and it commonly found in the bacteria species especially *Bacillus*. CGTase is capable of catalyzing more than one reaction that consists of cyclization, coupling, disproportional and hydrolysis reaction (Alkan et al., 2004) as illustrated in the Figure 2.1. In the industrial production of CD, cyclization reaction is the most often reaction of CGTase that is used to convert starch into CD (Jemli et al., 2007).

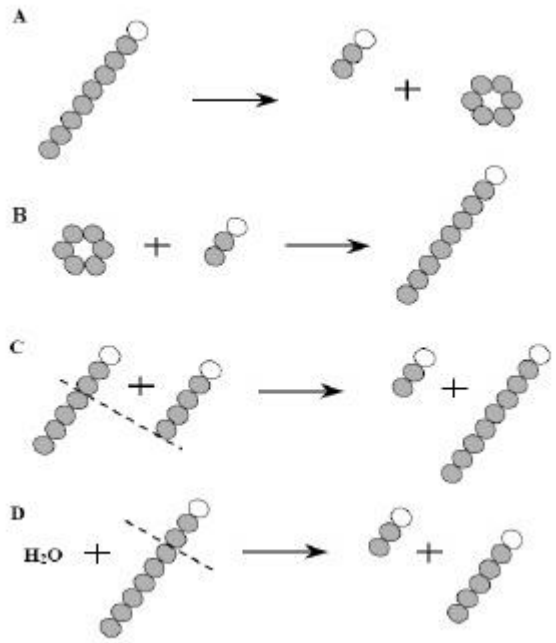

Figure 1.1: Reaction catalyzed by CGTase; (A) cyclization, (B) coupling, (C) disproportional and (D) hydrolysis (Veen et al., 2000)

2.3 Sources of Cyclodextrin Glycosyltransferase (CGTase)

CGTase is generally produced by different species of bacteria that mainly found in *Bacillus* species (Reyad et al., 2000; Bovetto et al., 1992; Martins et al., 2002). There are varieties of *Bacillus* species used in the research such as *Bacillus megaterium* (Nallusamy et al., 2013), *Bacillus macerans* (Steighardt and Klein, 1993), *Bacillus stearothermophilus* (Ahn et al., 1990), *Bacillus klebsiella* (Gawande and Patkar, 1999), *Bacillus firmus* (Goel and Nene, 1995) and *Bacillus lentus* (Sabioni and Park, 1992). Each of the species has different range of operating conditions. Table 2.1 shows the producers of CGTase.

Table 2.1: Producers and operating conditions of CGTase

Origin	Operating condition of CGTase	References
Paenibacillus macerans	Characterization: • Temperature = 45˚C • pH = pH 5.5 Stabilization: • Temperature = 40-50˚C • pH = pH 6–9.5	Li et al. (2010)
Bacillus halodurans	Characterization: • Temperature = 60˚C • pH = pH 7 and 9 Stabilization: • pH = pH 5-11	Sunil et al. (2012)
Bacillus sp. G1	Characterization: • Temperature = 60˚C • pH = pH 6	Ong et al. (2008)
Bacillus agaradhaerens	Characterization: • Temperature = 55˚C • pH = pH 9	Ibrahim et al. (2011)
Bacillus sp. TS1-1	Characterization: • Starch concentration = 3.30% (w/v)	Zain et al. (2007)
Bacillus macerans	Characterization: • pH = pH 5 and 9 • Starch concentration = 5.0% (w/v)	Zhekova et al. (2011)
Bacillus megaterium	Characterization: • Temperature = 27˚C • pH = pH 9	Nallusamy et al. (2013)
Alkaliphilic *Amphibacillus*	Stabilization: • Temperature = 45-70˚C • pH = pH 5-11	Ibrahim et al. (2012)

Bacillus oshimensis	Characterization: • Starch concentration = 1.0% (w/v)	Kamble et al. (2014)
Bacillus cereus	Characterization: • Temperature = 40˚C • pH = pH 6 • Starch concentration = 2.0% (w/v) Stabilization: • Temperature = 30-45˚C • pH = pH 6.5-8	Ismail et al. (2010)
Alkalophilic *Bacillus firmus*	Characterization: • Temperature = 60˚C • pH = pH 7-10.5	Ramli et al. (2010)

2.4 Cyclodextrin (CD)

CD or known as cycloamylose is a cyclic oligosaccharides that composed of six to eight D-glucose units linked by α-(1,4) linkages (Figure 2.2). CD is a family of compounds made up from sugar molecules bound together in a ring creating a cone shape. The molecules of CD have a unique structure of inner hydrophobic cavity and hydrophilic surface due to their peculiar arrangement (Gawade and Patkar, 2001). The number of glucose unit represent in a closed ring structure has it own name where 6 glucose units called α-CD, 7 glucose units called β-CD and 8 glucose units called γ-CD. In a typical production of CD, the reaction of CGTase on starch naturally create a mixture of α, β, and γ-CDs in different ratios depending on the nature of CGTase, the reactions and time conditions (Nallusamy et al., 2011; Ong et al., 2008). The classification of α, β, and γ-CDs are based on the major CD produced (Rahman et al., 2006).

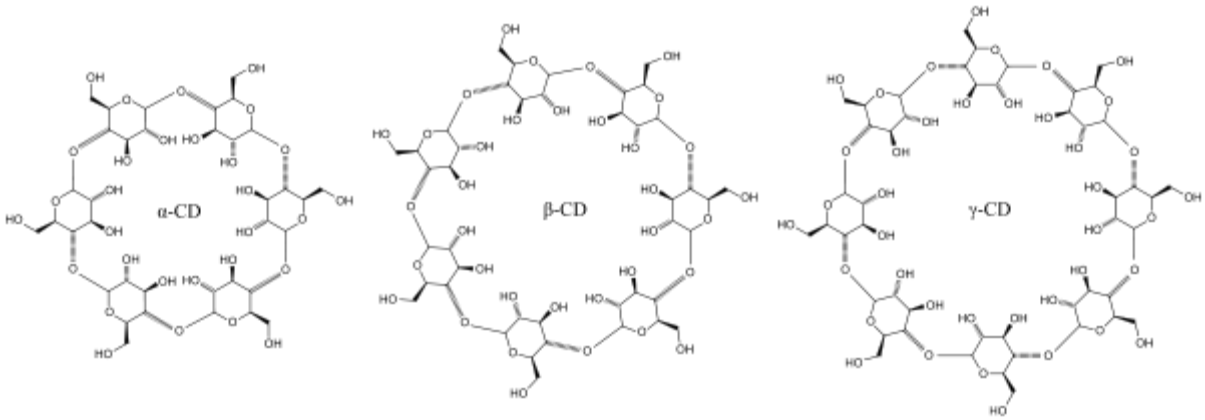

Figure 2.2: Chemical structure of three main types of CD (Veen et al., 2000)

2.5 Application of Cyclodextrin (CD) in Industry

CD and its derivatives are chemically and physically stable, thus they have a wide variety of applications. CD is commonly used in pharmaceutical, food and cosmetic industry (Astray et al. 2009). In food industry, CD is used as food additives, flavors stabilization, eliminator of undesired tastes or other undesired compounds such as cholesterol and avoid microbiological contaminations and browning reactions (Astray et al., 2009).

For pharmaceutical industry, CD is used as complexing agents to improve the solubility, stability, safety and bioavailability of drugs molecules (Loftsson et al., 2006). In addition, CD can be used to reduce or prevent gastrointestinal and ocular irritation, reduce or eliminate unpleasant smells or tastes, prevent drug-drug or drug-additive interactions, or to convert oils and liquid drugs into microcrystalline or amorphous powders (Rasheed et al., 2008).

In cosmetic industry, CD is used as protector and stabilizer of fragrance materials. CD helps to provide controlled release and protect the volatile components from fast evaporation. Thus, the volatile components such as perfumes can be a long lasting product. CD also has the ability to convert fragrance substances from liquid to powder form by preparing the inclusion complexes (Kartal et al., 2007). Moreover, CD is also widely use in toothpaste, skin creams, liquid and solid fabric softeners (Koch, 1982).

2.6 Characterization of CGTase

The process conditions play a critical role in industrial as they influence the yield of the product and process economic (Thombre, 2012). In recent years, there are many studies conducted by the researchers on the process optimization of various parameters for CGTase in order to obtain the high production of CD (Li et al., 2010; Sivakumar et al., 2011). These studies mentioned that the production of CD in the future can be improved by manipulating the operating conditions of the enzyme such as temperature, pH and concentration of starch.

2.6.1 Effect of Starch concentration

The effect of starch concentration on the production of CD is important as the starch is used as substrate and being converted into CD. There are various sources of starch such as corn, soluble, potato, tapioca and rice (Ibrahim et al., 2011). In this study, the tapioca starch is used as substrate, thus the operating conditions of the starch is need to be determined. According to Ibrahim et al. (2011), the highest conversion of starch is into CD was at concentration of 1.5% (w/v). The result was similar to the study reported by Goh et al. (2007) and Zhekova et al. (2008), where the best concentration of starch were 1.5% (w/v) and 2% (w/v) respectively. Ibrahim et al. (2011) also mentioned that the amount of CD produced will increase as the concentration of starch increase.

However, the study carried out by Kamble et al. (2014) and Zain et al. (2007) showed that high concentration of starch gives the highest CGTase activity with 3.30% (w/v) and 3% (w/v) respectively. This finding also similar with Jemli et al. (2007) and Menocci and Goulart (2008), where the optimum concentration of starch was determined at 4% (w/v).

The conversion rate of CD will decrease if the concentration of starch is too high. This statement is supported by Yamamoto et al. (2000) and Sian et al. (2005), where when the starch concentration is at the highest concentration, the yield of CD will be low. This phenomenon occurred due to the inhibition action of CD in the reaction (Ibrahim et al.,

2011). Moreover, when the high concentration of starch is present, the viscosity of starch slurry will become higher. Therefore, the starch need to be liquefied using the high temperature in order which prolong the time taken for the reaction process.

2.6.2 Effect of pH

The pH medium play a vital role in the production of CD as it influences the enzyme condition. According to Prakasm et al. (2004) and Ibrahim et al. (2011), the maximum enzyme activity was obtained at pH 7. It also showed that the conversion and the yield of CD is the highest when pH 7 was applied. The result was quite similar to the study reported by Illias et al. (2003), An-Noi et al. (2008) and Goh et al. (2007), where the optimum pH value for the CGTase was at pH 6 with the highest enzyme activity.

Meanwhile, An-Noi et al. (2008) mentioned that at acidic and alkaline pH, the enzyme activity was low. This is proven by Prakasm et al. (2004), where the pH below pH 4 and above pH 9, the enzyme have lost their activity. This occurrence was probably due to unsuitable condition for CGTase to be active. Thus, it can be concluded that CGTase required neutral pH to convert starch into CD.

2.7 Stabilization of CGTase

The stability of enzyme is an important economic factor. The stability of enzyme can be affected by variety of factors such as temperature and pH. There are many studies were carried out to investigate the effect of temperature and pH on the stability of enzyme.

2.7.1 Effect of pH

pH is one of the parameters that can affected the stability of enzyme. According to Cao et al. (2005), CGTase has a wide range of pH from 6 to 10. The result was supported by Ong et al. (2011) where the CGTase showed a constant enzyme activity at

pH 6-10. Moreover, study conducted by Goh et al. (2007) and Ibrahim et al. (2012) showed a wider range of pH stability within pH 5-11.

In the study reported by Cao et al. (2005) and Ong et al. (2011), the stability of CGTase decreased significantly at pH below 5 and above 10. Meanwhile the study conducted by Goh et al. (2007) and Ibrahim et al. (2012), the CGTase was less stable at pH below 4 and above pH 12. This indicates that the enzyme unable to maintain stable and active at strong acidic and alkaline pH. This probably due to the unsuitable conditions for the enzyme to be function as the enzyme normally requires neutral pH for the reaction process. Table 2.2 shows the effect of pH on stabilization of enzyme based on the origin.

Table 2.2: Effect of pH on the stabilization of CGTase

Origin	pH stbility of CGTase	Reference
Bacillus sp G1	7-9	Sian et al. (2005)
Paenibacillus macerans	6-9.5	Li et al. (2010)
Bacillus megaterium	6-10.5	Zhekova et al. (2008)
Bacillus alcalophilus	4-8.5	Sunil et al. (2010)
Bacillus agaradhaerens	5-11	Martins et al. (2002)
Bacillus firmus	7-11	Gawande et al. (1999)

2.7.2 Effect of Temperature

Temperature is one of the parameters that can affected the stability of enzyme. According to Goh et al. (2007), CGTase was stable at temperature of 40˚C to 70˚C. The result was similar to the study reported by Ibrahim et al. (2012), where the enzyme activity was constant at temperature of 45˚C to 70˚C. However, Jemli et al. (2007) and Kitcha et al. (2008) stated that the CGTase only stable at temperature of 30-55˚C. The result was also supported by Gawande et al. (2001), where the CGTase was able to stable at temperature of 35-50˚C.

The study conducted by Jemli et al. (2012) showed that the enzyme was inactive when the temperature exceed 60˚C. Moreover, in the study reported by Illias et al. (2005) also showed a similar pattern where the enzyme activity was decreased rapidly when the

temperature exceed 70˚C. Meanwhile, the enzyme was started to be inactive when the temperature exceeded 80˚C (Goh et al., 2007; Ibrahim et al., 2012). This probably because of the enzyme started to denature at the high temperature. Table 2.3 shows the effect of temperature on stabilization of enzyme based on the origin.

Table 2.3: Effect of temperature on stabilization of CGTase

Origin	Thermal stability of CGTase (˚C)	Reference
Paenibacillus macerans	40-60	Li et al. (2010)
Bacillus firmus	30-85	Higuti et al. (2003)
Bacillus sp	30-80	An-Noi et al. (2008)
Bacillus sp G1	30-60	Sian et al. (2005)

CHAPTER 3

METHODOLOGY

3.1 Introduction

In this chapter, the effect of operating conditions on characterization and stabilization of CGTase were investigated. Figure 3.1 shows the flowchart for the characterization and stabilization of CGTase.

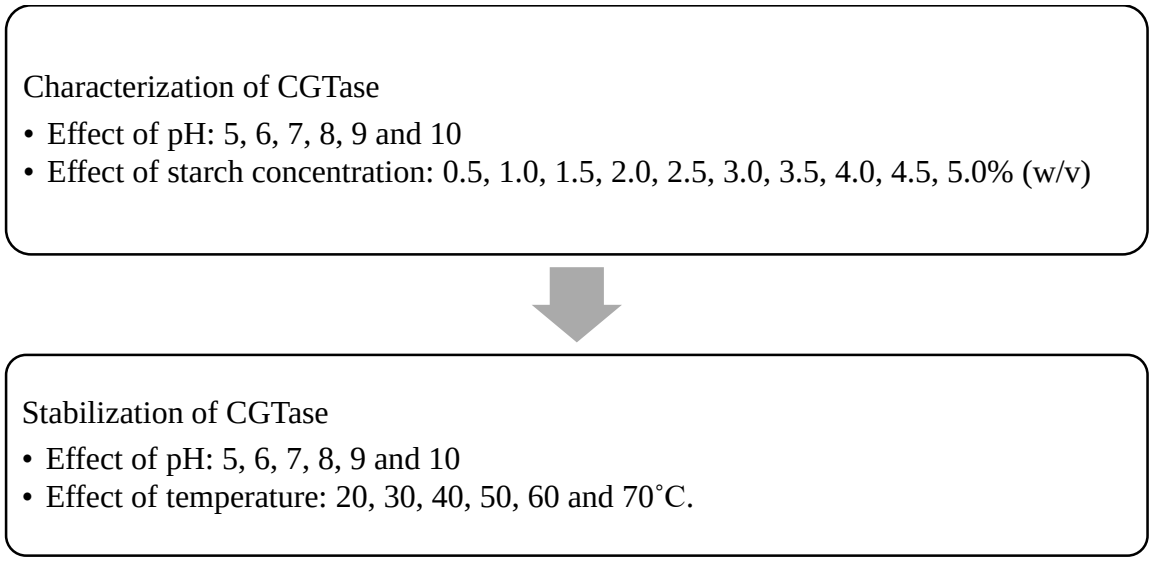

Figure 3.1: Flow chart for characterization and stabilization of CGTase

3.2 Materials

3.2.1 Chemicals and reagents

All the chemicals and reagents were purchased from various companies. The CGTase enzyme from Bacillus licheniformis was purchased from Next Gene Sdn Bhd. The tapioca starch was purchased from Chemolab Supplies. For methyl orange, HCl and pH buffers (Phosphate-citrate buffer (pH 5 and pH 6), Sorensen's phosphate (pH 7 and pH 8) and Glycine-NaOH buffer (pH 9 and 10) were puchased from Sigma-Aldrich.

3.3 CGTase Activity Assay

The enzyme activity was measured by using the methyl orange method by Lejeune et al. (1989) with some modifications. The CGTase solution (0.1 mL, appropriately diluted in 50 mM phosphate buffer) was incubated with 0.9 mL of 3% (w/v) soluble starch in 50 mM phosphate buffer (pH 6) at 40˚C for 10 min. The reaction was terminated by the addition of 1 M HCl (1.0 mL), and then 1 mL of 0.1 mM methyl orange in 50 mM phosphate buffer (pH 6) was added. After the reaction, mixture was incubated at 16 ˚C for 20 min. The amount of α-cyclodextrin in the mixture was spectrophotometrically determined by measuring the absorbance at 505 nm. One unit of α-cyclodextrin-forming activity was defined as the amount of enzyme that was able to produce 1 mol of α-cyclodextrin per min.

3.4 Characterization of CGTase

Screening of the operating conditions on characterization of CGTase was conducted by using one factor at one time method (OFAT).

3.4.1 Effect of Starch concentration

The effect of starch concentration on characterization of CGTase was studied by using concentration of 0.2, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 % (w/v). The pH, temperature and time of CGTase assay were fixed as mentioned in Section 3.3.

3.4.2 Effect of pH

The effect of pH on characterization of CGTase was studied by using various pH values of 5, 6, 7, 8, 9, and 10. The buffer solutions for the different pH were phosphate-citrate buffer (pH 5 and pH 6), Sorensen's phosphate buffer (pH 7 and pH 8) and glycine-NaOH (pH 9 and pH 10). The optimum pH of CGTase was determined by replacing 50 mM phosphate buffer (pH 6.0) in the CGTase assay (Section 2.2) with the different pH values of buffer solutions.

3.5 Stabilization of CGTase

Screening of the operating conditions on stabilization of CGTase was carried out by using one factor at one time method (OFAT) on stabilization of CGTase.

3.5.1 Effect of pH

The effect of pH on stabilization of CGTase was studied by using various pH values of 5, 6, 7, 8, 9, and 10. The stability of pH for the enzyme was measured by incubating 0.1 ml enzymes with 0.2 ml of phosphate-citrate buffer (pH 5 and pH 6), Sorensen's phosphate buffer (pH 7 and pH 8) and glycine-NaOH (pH 9 and pH 10), respectively at 60°C, without substrate for 30 min. The remaining activity of the enzyme was assayed by using the standard CGTase assay and the optimum operating conditions obtained from section 3.4.

3.5.2 Effect of Temperature

The effect of temperature on stabilization of CGTase was studied by using different temperature of 20, 30, 40, 50, 60, 70˚C. The experiment was conducted by incubating 0.1 ml enzyme with 0.2 ml of 50 mM phosphate buffer, pH 6 (optimum Section 4.5.1) without substrate at different temperatures (20–70 ˚C) for 30 min. The remaining activity of the enzyme was assayed by using the standard CGTase assay and the optimum operating conditions obtained from section 3.4.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

The objective of this study is to determine the best operating conditions on characterization and stabilization of CGTase for the future CD production. The effect of operating conditions such as starch concentration and pH for characterization on CGTase were studied. Meanwhile, the effect of pH and temperature for stabilization of CGTase were investigated.

4.2 Characterization of CGTase

4.2.1 Effect of Starch concentration

The effect of starch concentration was studied by varying the concentration from 0.2 to 5.0 % (w/v). In this study, the optimum starch concentration was 3% (w/v) with 140 U/ml of CGTase activity as shown in Figure 4.1. The result was similar to the study conducted by Zain et al. (2014) and Ravinder et al. (2007), whereas 3.30% and 3.0% were the best optimum concentration of starch on the characterization of CGTase respectively.

However, the findings by Cao et al. (2007) and Ismail et al. (2010) were contradicted with the present study whereby, the optimum starch concentration were 1.5% and 2.0% respectively. Moreover, the result obtained by Kamble et al. (2014) also showed that 1.0% was the best optimum concentration of starch. When the concentration of starch increased, the enzyme activity also increased. This phenomenon was due to the rate of reaction increased (Ivanova 2010). When the rate of reaction increased, the products will be formed also increased.

At concentration of 3.5% to 5.0% (w/v), the enzyme activity decreased gradually. This occurrence was due to the high viscosity of starch slurry at the high concentration of starch. Furthermore, the conversion of CD is low when the starch concentration is too high. This statement was supported by Yamamoto et al. (2000) and Sian et al. (2010), whereas the CD yield decreased when the concentration of starch was high. Thus, to maximize the production of CD in the future, the amount of starch concentration must be at the moderate level.

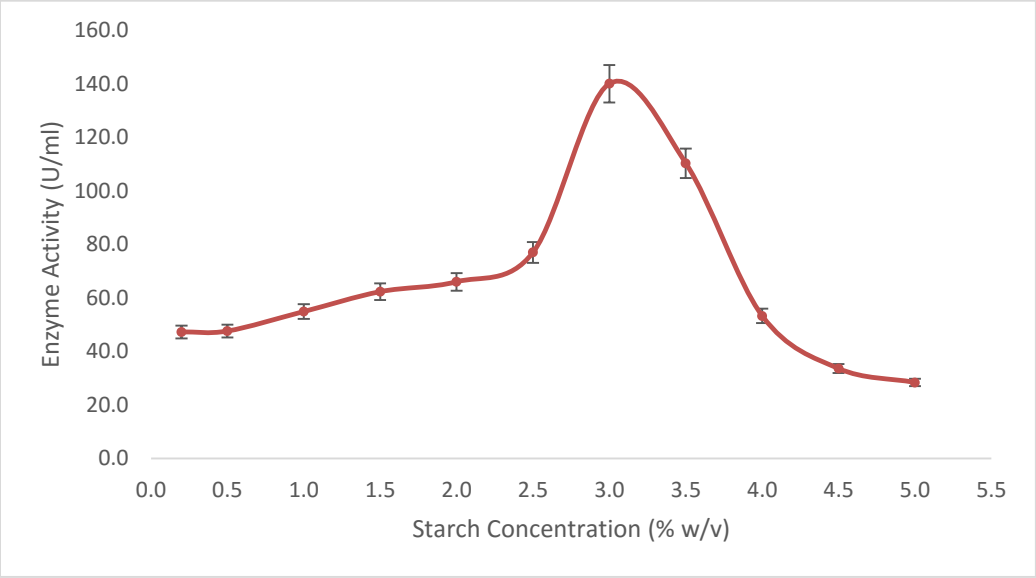

Figure 4.1: Optimum starch concentration of CGTase

4.2.2 Effect of pH

The effect of pH on characterization of CGTase was determined by varying the pH values from pH 5 to 10. The optimum pH of CGTase was determined at pH 6 with the highest enzyme activity of 126 U/ml as shown in Figure 4.2. The result was supported by An-Noi et al. (2008) and Illias et al. (2003), where the maximum enzyme activity was obtained at pH 6. These studies also showed that the conversion and the yield of CD were highest at pH 6. This is because the enzyme typically requires a neutral pH condition to be functioning and to undergo the reaction.

In contrast, Martins et al. (2001) and Ibrahim et al. (2011) stated that the optimum pH value for CGTase was at pH 9. Study conducted by Cao et al. (2005) also has a similar result, whereas the optimum pH value was obtained at pH 8.5.

The enzyme activity keeps decreasing at pH 7 to 10, due to unsuitable condition for enzyme to be active that leads to depletion of the rate of reaction. An-Noi et al. (2008) also mentioned that it was unsuitable to enzyme to carry out the reaction at acidic or alkaline pH because of unstable enzyme. This occurrence was due to the denaturation of CGTase. At acidic or alkaline conditions, the H+ and OH- ions will disrupt the hydrogen and ionic bonds that hold together the enzyme. This interferences will leads to the changed in shape of the enzyme. Therefore, the enzyme cannot bind with the substrate thus its lead to decreases in the enzyme activity. Thus, to avoid the enzyme from denature and lost the function, the neutral pH is required for maintaining the enzyme activity.

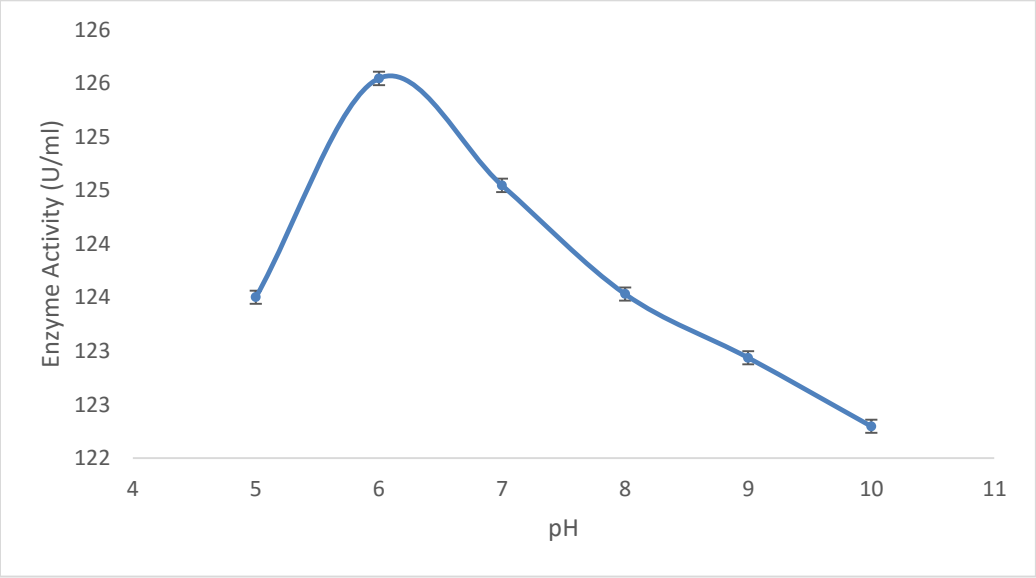

Figure 4.2: Optimum pH of CGTase

4.3 Stabilization of CGTase

4.3.1 Effect of pH

The effect of pH on stabilization of CGTase was determined by varying the pH values from pH 5 to 10. Figure 4.3 shows that the CGTase was stable at pH 6 to 8 at 60˚C after 30 min incubation. The result was similar to the study conducted by Gawande et al. (2012) and Jemli et al. (2007), where the CGTase was stable in range of pH 6 to 9. Li et al. (2010) also stated that the CGTase has a wide pH range with the value of pH 6 to 9.5.

However, the result from the present study was different with the study conducted by Ibrahim et al. (2012) and Goh et al. (2007). These studies mentioned that the CGTase was stable at pH 5 to 11. It could be concluded that the enzyme was able to maintain the activity at wide pH range.

The stability of CGTase decreased significantly at pH 5 (133 U/ml) and above pH 9 (132 U/ml). The inactive enzyme was due to the unsuitable conditions for enzyme to be active. According to Ismail et al. (2010), the enzyme requires neutral pH to perform the reaction. Thus, the strong acidic or alkaline conditions was not suitable for enzyme. Moreover, at acidic and alkaline conditions, the enzyme tends to gain and lose hydrogen ions which will disrupt the hydrogen bonds that holds the enzyme together. The shape of the enzyme becomes distorted when this interferences occurred. Therefore, the enzyme cannot bind with the substrate thus it leads to decreases in the enzyme activity. Thus, to avoid the enzyme from denature and lost the function, the neutral pH is required for maintaining the enzyme activity.

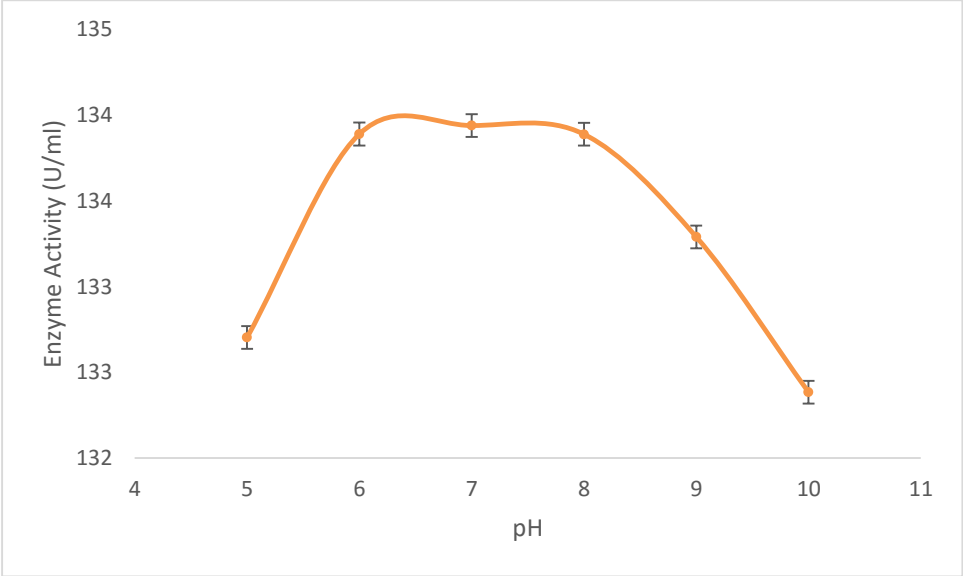

Figure 4.3: pH stability of CGTase

4.3.2 Effect of temperature

The effect of temperature on stabilization of CGTase was determined by varying the temperature from 20˚C to 70˚C. As shown in the Figure 4.4, the CGTase was stable and active in a wide range of temperature starting from 30˚C to 60˚C with the maximum activity of 119 U/ml. The result was in agreement with the study conducted by Ibrahim et al. (2012) and Jemli et al. (2007), where the CGTase was stable at temperature of 30˚C to 55˚C.

However, the study reported by Cao et al. (2005) and An-Noi et al. (2008) discovered that the CGTase exhibited good thermal stability ranging from 30˚C to 80˚C. Otherwise, Ong et al. (2012) stated that the stability of CGTase was in the range of 60-90˚C.

The CGTase started to decrease the activity when the temperature exceeded 60˚C. This is probably because of the enzyme was denatured at the high temperature. According to study conducted by Kitcha et al. (2008) and Cheirsilp et al. (2010), the CGTase unable to be active at high temperature because the disruption of hydrogen bonds. Therefore, the enzyme starts to denature and loose the function. Moreover, at high temperature, the molecules of CGTase have higher chance of hitting each other as they are moving faster

due to the increment of kinetic energy. This movement eventually changed the structure of enzyme thus leads to the denaturation of enzyme.

Meanwhile, at temperature of 20˚C, the enzyme activity is low (118 U/ml) and not stable as the typical temperature for enzyme to be fully function is at 37˚C. Thus, at low temperature, the enzyme was inactive to convert the substrate into the product.

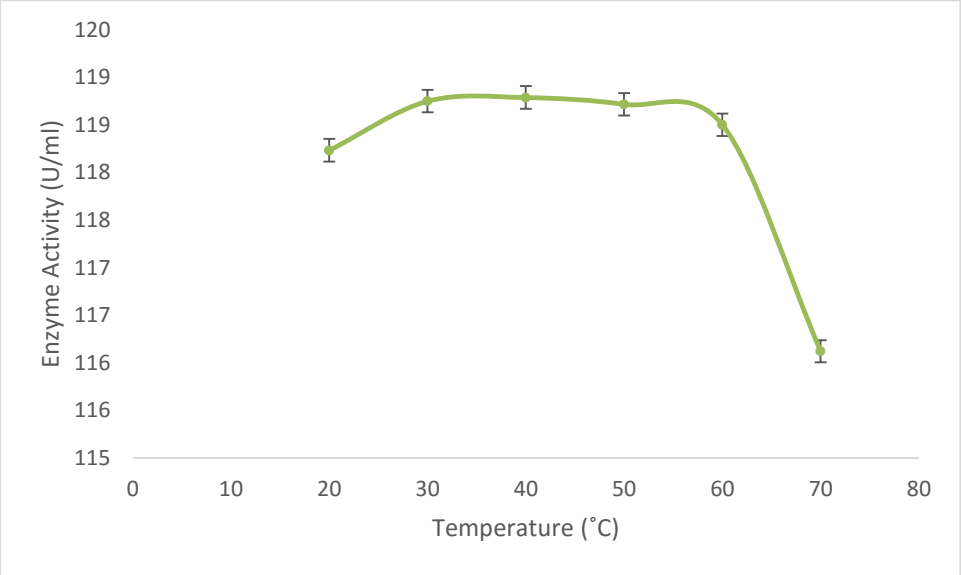

Figure 4.4: Thermal Stability of CGTase

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The objective of this study is to determine the best operating conditions on characterization and stabilization of CGTase for the future CD production. It can be concluded that the best starch concentration and pH on characterization of CGTase were 3% w/v and pH 6 respectively. Meanwhile, pH 6-8 and temperature of 40-60˚C were the most stable conditions for CGTase. Moreover, CGTase showed a constant enzyme activity at the moderate temperature (40-60˚C). However, the enzyme started to denature and lost its function when the temperature was exceeded 60˚C due to the CGTase unable to be active at high temperature.

5.2 Recommendation

There are some recommendations that should be considered in the future in order to obtain the high production of CD. The effect of other sources of starch such as corn, rice, soluble and potato should be implemented in this study. This is because the different sources of starch could affected the reaction process of CGTase. Moreover, the effect of other operating parameters such as time and temperature on characterization and stabilization of CGTase should be taken into consideration in order to produce high production of CD.

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APPENDICES

APPENDIX A.1: Buffers preparation

1. Sodium Phosphate Buffer, 0.1 M

Solution A: 0.2 M sodium phosphate monobasic

Solution B: 0.2 M sodium phosphate monobasic

Mix solution A and solution B in the proportions indicated and adjusted the final volume to 100 ml with distilled water.

Solution A	43.4	19.5	2.7
Solution B	6.6	30.5	47.3
pH	6.0	7.0	8.0

2. Phosphate-citrate Buffer, pH 2.2 – 8.0, pK_A= 7.20/6.40

Add the following to create 100 ml of phosphate-citrate buffer solution. Stock solution are 0.2 M dibasic sodium phosphate; 0.1 M citric acid (Pearse, 1980).

0.2 M Na_2HPO_4	0.1 M citrate (ml)	pH
5.4	44.6	2.6
10.2	39.8	3.0
16.1	33.9	3.6
19.3	30.7	4.0
23.3	26.7	4.6
25.7	24.3	5.0
29.0	21.0	5.6
32.1	17.9	6.0
36.4	13.6	6.6
43.6	6.5	7.0

3. Sorensen's phosphate buffer, pH 5.8 – 8.0, $pK_A = 7.20$

Mix appropriate volume of stocks and add an equal volume of distilled water to make a final 0.1 M Sorensen's phosphate buffer solution (Sorensen, 1909; Gomori, 1955).

Stocks solutions: A = 0.2 M NaH_2PO_4, B = 0.2 M Na_2HPO_4

A (ml)	B (ml)	pH
92.0	8.0	5.8
87.7	12.3	6.0
51.0	49.0	6.8
39.0	61.0	7.0
8.5	91.5	7.8
5.3	94.7	8.0

4. Glysine –NaOH buffer, pH 8.6 – 10.6, $pK_A = 9.78$

Combine 25 ml glysine stock solution with *x* ml 0.2 M NaOH and dilute with DI to make a 100 ml solution (Pearse, 1980).

Stock solutions: A = 0.2 M Glysine , B 0.2 M NaOH

0.2 M NaOH	pH
2.0	8.6
4.4	9.0
11.2	9.6
19.3	10.4
22.75	10.6

APPENDIX A.2: Standard Curve for α-Cyclodextrin

α-CD	0.0	0.2	0.4	0.6	0.8	1.0
OD 1	2.097	1.231	0.958	0.746	0.398	0.363
OD 2	2.585	0.754	0.926	0.852	0.658	0.397
OD 3	0.277	1.173	1.118	0.993	0.620	0.548
Average OD	1.653	1.053	1.001	0.864	0.559	0.436
%OD decrease	0	36.29764	39.44344	47.7314	66.1827	73.62371

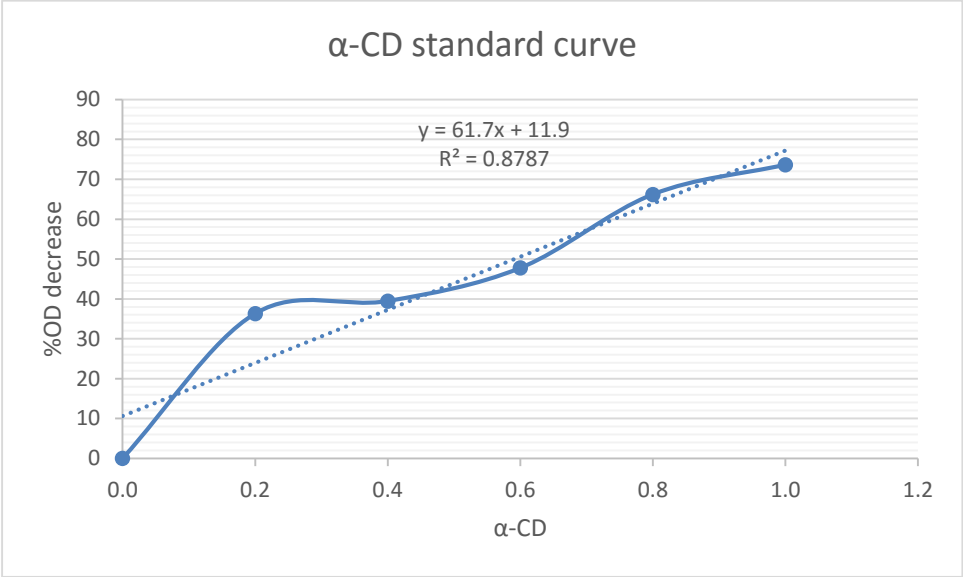

Figure A-2: α-CD standard curve for the calculation of CGTase activity

APPENDIX A.3: Calculation for Enzyme Activity of CGTase

Enzyme activity is calculated as equation below,

$$Enzyme\ activity\ (U/ml) = \frac{(\%OD_{decrease} \times Y_p \times D_f \times 10^3)}{(MW_{\alpha-cyclodextrin} \times t_i)}$$

Where,

%OD decrease = $(\frac{OD\ control - OD\ sample}{OD\ control}) \times 100\%$

Y_p = mg of α-CD equivalent to 100% OD decrease of the standard curve (Figure A-2)

D_f = Dilution factor

MW α-cyclodextrin = Molecular weight of α-cyclodextrin

t_i = Time of incubation

By taking value at 3% (w/v) concentration of starch in the Characterization of CGTase:

a) %OD decrease

OD sample = (0.0398 + 0.0397 + 0.0399)/3

OD control = 0.0401

%OD decrease = $(\frac{OD\ control - OD\ sample}{OD\ control}) \times 100\%$

= $(\frac{0.0773 - 0.0372}{0.0773}) \times 100\%$

= 51.8758

b) Yp

From standard curve of α-Cyclodextrin concentration with OD of 505 nm,

y = 61.7 x + 11.9

When y = 100,

x = 1.43

Therefore, Yp = 1.43

c) Df

Sample taken for assay = 100µl

Therefore, D_f = 10

d) MW α-cyclodextrin

Molecular weight of α-cyclodextrin = 972.84 g/mol

e) t_i

time for assay = 10 minutes

Therefore, t_i = 10

f) Enzyme activity

Enzyme activity (A) = $\frac{(\%OD_{decrease} \times Y_p \times D_f \times 10^3)}{(MW_{\alpha-cyclodextrin} \times t_i)}$

$= \frac{(19.332 \times 1.43 \times 10 \times 10^3)}{(972.84 \times 10)}$

$= 28.4162$

Therefore, the enzyme activity at starch concentration of 5% (w/v) is 28.4162 U/ml.