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# Characterization of α-Cyclodextrin Glucanotransferase from *Bacillus licheniformis*

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## Abstract

**Objectives**: Cyclodextrin glucanotransferase (CGTase) is a multifunctional industrial enzyme, which undergoes cyclization reaction to converts starch into Cyclodextrin (CD). Due to their potential properties, CDs had been discovered to have numerous application in food industries, pharmaceutical, agriculture and also environmental engineering. To improve the production of cyclodextrin (CD) in the future, characterization of α-CGTase on the effect of operating conditions was investigated. **Methods/Statistical Analysis:** The α-CGTase from *Bacillus licheniformis* was characterized by examining their cyclization activity. The enzymatic activity of the enzyme towards the temperature and pH was determined by methyl orange method. **Findings:** The enzyme was estimated to be 70 kDa by the gel electrophoresis. The cyclization activity of α-CGTase was highest at a temperature of 40°C and pH 6.0. The α-CGTase enzyme was able to extend its thermostability up to 60°C with pH stability between pH 6.0 and pH 8.0. **Application/Improvements:** High production of CD is expected to obtain by using the optimal conditions which in turn may be beneficial for the industrial purpose.

**Keywords:** Cyclodextrin Glucanotransferase, *Bacillus licheniformis*, Characterization, Cyclization Activity, Cyclodextrin

## 1. Introduction

Cyclodextrins (CDs) are produce by an amylolytic enzyme known as Cyclodextrin glucanotransferase (EC 2.4.1.19) that undergoes cyclization process[1,2]. This enzyme is generally produced in bacteria and some in archae. There are several types of bacteria that have been recorded in the production of CGTase such as anaerobic thermophilic, aerobic thermophilic bacteria, aerobic mesophilic bacteria, aerobic halophilic and aerobic alkalophilic[3].

The most common CDs produced by CGTase are α-, β- and γ-cyclodextrins. Even the current market price for cyclodextrins are pricey, the market demand for cyclodextrins as encapsulating agents and additives are huge[4]. To date, the cheapest cyclodextrin would cost about US$ 3-4 per kilogram (β-CD) while the most expensive are γ-CD which about US$ 80-100 per kilogram[5.] The α-CD costs about US$ 20-25 per kilogram[5]. The real reason behind the price gap between these three types of cyclodextrin is due to their difficulty in separation and purification. Both α-CD and γ-CD have higher water solubility compare to β-CD[5].

CDs are toroidal shaped molecules that are composed of α-1,4-glycosidic linked glucose as shown in Figure 1[5,6]. CDs are also well-known in drug and pharmaceutical application as useful excipients, due to their properties of hydrophobic inside and hydrophilic outside[7]. With these properties, inclusions complexes can be formed when the interaction of cyclodextrins and hydrophobic molecules occur[5,8]. Therefore, the physical and chemical properties of cyclodextrin will change.

CDs have been majorly applied in pharmaceutical industries, to increase the solubility, stability and bioavailability of drugs[7]. Nevertheless, CDs play a major role in preventing drug-drug interaction, reduce gastrointestinal irritation and also in producing microcrystalline drugs[7]. In food industries, CDs mainly used as dietary fiber and

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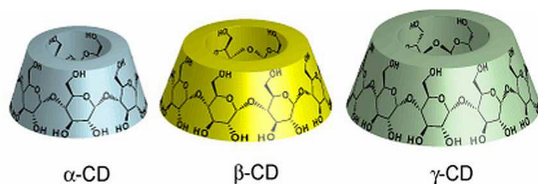


**Figure 1.** The toroidal shape of α-, β- and γ-cyclodextrin respectively[6].

calories substitute agent for weight control[9], reduction of unwanted and odour[9] and as thickening agent[10]. A significant analysis presented by Astray et al.[10] showed that CDs act excellently as a process aid agent especially in removing cholesterol from dairy products. Since CDs are growing popular due to their numerous applications in industries, their production and study have been a great interest to the researcher. However, the type of host producing CGTase and the reaction conditions might affect the production of α-, β- and γ-CD. Moreover, by using a selective strain as a CGTase producer, it would produce a specific CGTase of α-, β- or γ-CD. This would help in the downstream process as much as, reducing the cost of production.

The present study was performed to characterize the CGTase from *Bacillus licheniformis* on the future CD production.

## 2. Materials and Methods

The standard of α-cyclodextrin (98%) was purchased from Sigma (St. Louis, USA). Commercial CGTase (Toruzyme 3.0L) isolated from *Bacillus licheniformis* was purchased from Novozymes A/S (Bagsvaerd, Denmark).

### 2.1 Physical Properties of α-CGTase

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used in this study to estimate the molecular weight of α-CGTase and Page ruler prestained protein ladder (Thermo Scientific) as a standard.

### 2.2 α-CGTase standard Assay

The cyclization activity of α-CGTase was performed by the methyl orange assay according to the previous method with some modifications[8]. Briefly, 0.1 mL of enzyme was added into 0.9 mL of 3% (w/v) soluble starch in 50 mM phosphate buffer (pH 6.0), before incubated at 40 °C for 10 min. To stop the reaction, 1.0 mL of 1.0 M HCl and 0.1 mL of 0.01 M methyl orange in 50 mM phosphate buffer was added. After 20 min incubated at 16 °C, the amount of α-cyclodextrin was spectrophotometrically at 507 nm. The activity of α-CGTase shows the amount of enzyme that able to produce 1 mol of α-cyclodextrin per min[11].

### 2.3 Activity of CGTase at Different Temperature and Thermostability

Different temperatures ranging from 30 to 70 °C were used to determine the effect of temperature on the α-CGTase activity. The thermostability of the enzyme was measured by incubating 0.1 mL of enzyme at different temperatures of 40 to 90 °C in phosphate buffer (0.2 M, pH 6.0). After 30 min, the enzyme was removed and the standard CGTase assay was performed for enzyme relative activity.

### 2.4 Activity of CGTase at Different pH and pH stability

The cyclization activity of α-CGTase at different pH was studied by incubating the enzyme into various buffer including citrate-phosphate (pH 3, 4, 5), sodium phosphate (pH 6, 7, 8) and glycine-NaOH (pH 9 & 10). After 10 min reaction, followed with the standard α-CGTase assay as mentioned above. The 0.1 mL enzyme was incubated in various buffers (pH 3-10) for 30 min, to determine the stability of the enzyme towards different pH values. The remaining enzymatic activity was continued by the standard α-CGTase assay[11].

### 2.5 Relative Activity of α-CGTase

The relative activity of the enzyme was calculated from the following equation:

$$\text{Relative activity } (\%) = \frac{\text{Current activity}}{\text{Highest activity}} \times 100 \quad (1)$$

## 3. Results and Discussion

### 3.1 Physical Properties

The molecular weight of α-CGTase was determined by SDS-PAGE. As shown in Figure 2, it is estimated that the molecular mass of CGTase was 70 kDa. This molecular mass was within the range of the majority molecular weight reported in previous literature. An experimental study of α-CGTase by Li et al.,[8] has demonstrated a

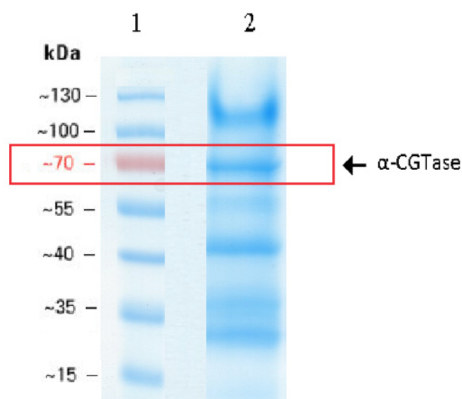


**Figure 2.** SDS-PAGE analysis of the α-CGTase. (1) molecular weight marker (Thermo Scientific); (2) crude enzyme solution.

molecular weight of 72 kDa. Furthermore, a study by Yu et al.,[12] has shown the similar molecular weight of CGTase isolated from *Bacillus licheniformis.* Several studies have also revealed that most isolated CGTase from *Bacillus sp*. has molecular weight range from 33 to 110 kDa[13]. For instance, CGTase from *Bacillus lentus* has molecular weight of 33 kDa[14], *Bacillus firmus* (75kDa)[15] and *Bacillus agaradhaerens* LS-3C (110 kDa)[14].

## 3.2 Effect of Temperature

The influence of reaction temperature on the CGTase enzymatic activity was determined by varying the temperature from 30 to 70 °C at pH 6.0. Figure 3 shows the highest enzyme activity was 96.4 U/ml at 40 °C. When the temperature reached 60 °C, the enzyme activity was rapidly declined to 30.9 U/ml and the enzyme near to completely inactive at 70 °C. A study by Li et al.[8] shows that 45 °C was the optimum temperature of α-CGTase. Most of the reported CGTase origin *Bacillus sp.*, showed a higher activity at temperatures from 45 to 80 °C[11,16].

The thermo stability of the α-CGTase was also examined. The activity of CGTase was retained from 30 to 60 °C, representing that the enzyme exhibit a good thermostability (Fig. 4). Nevertheless, as the temperature reached 70 °C, it began to lose 42% of its total activity. As the temperature reached 80 °C, it was only able to maintain 30% of its activity. Sian et al.[11] recorded that CGTase was stable up to 60°C for 30 min before its lose almost 50% of their total activity at 75°C. Moreover, the CGTase was completely inactive as the temperature reached 90 °C. Being protein in nature, enzymes are generally not the

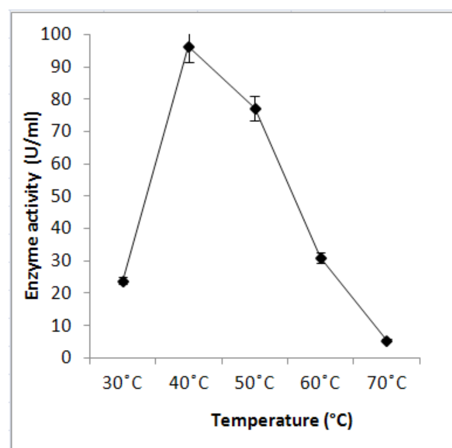


**Figure 3.** The cyclization activity of α-CGtase at various temperature, from 30 – 70 °C.

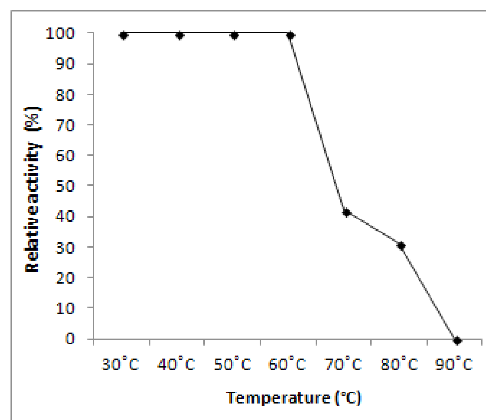


**Figure 4.** The effect of temperature on the stability of CGTase was determined by incubating the enzyme at various temperature for 30 min.

stable compound and have a high tendency to inactivate at a higher temperature[17]. An extreme temperature could alter the size and shape of the active site[18]. Therefore, the substrate molecules can no longer bind and the enzyme is no longer usable.

## 3.3 Effect of pH

The effect of CGTase cyclization activity on different pH was investigated by incubated at various pH values (pH 3 to 10) for 10 min[19]. As shown in Figure 5, the highest α-CGTase activity was observed at pH 6.0. In contrast, the enzyme shows a very low activity when incubating at pH 3.0 which suggested that CGTase from *Bacillus licheniformis* did not favorable to acidic conditions.

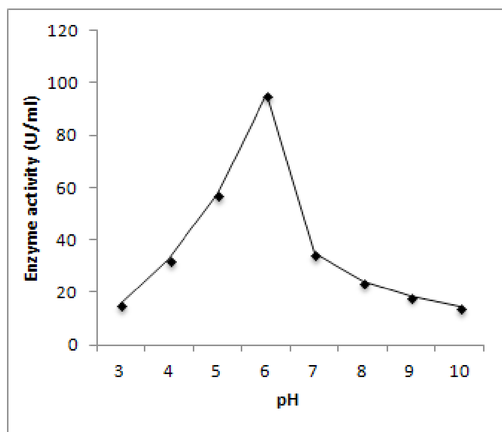


**Figure 5.** Effect of pH on α-CGTase activity. The enzymatic activity was measured under the standard conditions at various pH.

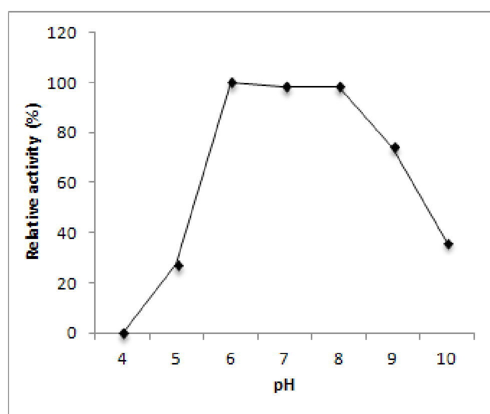


**Figure 6.** The pH stability of α-CGTase was determined by incubating the enzyme at various pH values (pH 4 - 10) for 30 min.

A study by Li et al.[8] recorded pH 5.5 was the optimum pH for α-CGTase *Paenibacillus maceran*[11]. The previous studies have showed that CGTase was known to have higher activity around pH 5.0 to 8.0. For instance, optimum pH for CGTase originated *Bacillus macerans* was pH 5.2[20], *Bacillus stearothermophilus* (pH 6.0)[21] and *Bacillus subtilis* (pH 8.0)[16]. Very little CGTase are reported to exhibit the optimum pH of 4.0 except from Alkaliphilic *Bacillus sp*. ATCC21783[22]. It is suggested that extreme pH values would inhibit the enzyme from carrying out their activity.

The pH stability of α-CGTase was measured by using relative activity equation as shown in Equation 1[11]. From the result below (Fig. 6), the α-CGTase exhibit a wide range of pH stability from pH 6.0 to 8.0, before it began to lose 20% of their relative activity when incubating in pH 9.0. At pH 10.0, the enzyme was only able to retained 40% of their activity.

## 4. Conclusion

The present study was successfully characterized α-cyclodextrin glucanotransferase (α-CGTase) from *Bacillus licheniformis* with highest cyclization activity at different temperature and pH. The enzyme exhibits a wide range of pH stability and good thermostability at high temperature. The enzyme properties recorded was significantly different from other previous studies, due to different host of origin.

## 5. Acknowledgement

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