

Effect of Reaction Parameters on the Production of Cyclodextrin using Cyclodextrin Glucanotransferase from *Bacillus licheniformis*

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ABSTRACT – Cyclodextrin glucanotransferase (CGTase) is an industrial enzyme that converts starch into cyclodextrin (CD). CD has many potential applications in wide range of industries including food, cosmetic, pharmaceutical and plastic industries. Production of CD from CGTase is based on a few process parameters such as concentration of substrate, types of substrate, reaction time, pH and temperature. In this present study, the effect of types of substrate (corn, potato, wheat, rice and tapioca starch), concentration of substrate (1, 2, 3, 4, 5, 6, 7, 8 and 9%) and reaction time (1, 2, 3, 4 and 5 hr) on the CD production using commercial CGTase from *Bacillus licheniformis* were studied. Among the five types of substrate tested, the potato starch was the most suitable substrate for the production of CD with 10.24 mg/ml of CD. In addition, by using 6% (w/v) of potato starch, the production of CD was 15.16 mg/ml. The optimal reaction time was found to be 4 hr with 12.41 mg/ml of CD. Therefore, the best reaction parameters proved to be valuable for the highest CD production. Hence, CGTase from *Bacillus licheniformis* was demonstrated to produce high CD at optimum reaction conditions and it has potential to be used in large scale production for the industrial purposes.

KEYWORDS

Bacillus licheniformis,
cyclodextrin
glucanotransferase,
cyclodextrin,
types of substrate,
concentration of substrate,
reaction time.

INTRODUCTION

CD is produced by the enzymatic reaction between cyclodextrin glucanotransferase (CGTase) and starch (Hao et al., 2017; Fenelon et al., 2018). The other types of substrate used in the CD production are dextrin, amylose, glycogen and amylopectin (Valle, 2004). CD can be divided into three different types which are α -CD, β -CD and γ -CD (Jansook et al., 2018; Fernández et al., 2019). CD consist of hydrophilic surface outside and hydrophobic surface inside which enabling it to form inclusion complexes with organic and inorganic molecules. This allow CD to reduce side effects of drug formulations and to eliminate any unwanted flavours and aroma as well as to keep the guest molecules from degradation under the action of light and heat (Chen et al., 2019). CD has been discovered to have numerous application in the food, cosmetic, pharmaceutical and plastic industries.

CGTase is a unique enzyme produced by the microorganisms, usually *Bacillus* sp. (Rather et al., 2015). It is also called as cyclomalto-dextrin glucanotransferase, cyclomalto-dextrin glycosyltransferase or cyclodextrin glycosyltransferase (Qi and Zimmermann, 2005). CGTase is owned by the transferase enzymes group (EC 2.4.1.19) and classified as hexosyltransferase. It hydrolyze the starch and also catalyzes the reactions of transglycosylation. CGTase is mostly used in food and pharmaceutical industries (Jamil et al., 2018; Suhaimi et al., 2018). Generally, CGTase was produced from various sources such as from *Bacillus obhensis*, *Klebsiella pneumonia*, *Bacillus agaradhaerens* and *Klebsiella pneumonia*. In this present study, commercial CGTase was produced by *Bacillus licheniformis*.

The reaction parameters of CGTase such as types of substrate, concentration of substrate, reaction time, pH and temperature will affect the production of CD. The effect of temperature (70 and 90°C) on the production of CD using CGTase from *Thermoanaerobacter* sp. was studied by Schöffner et al. (2013). The study found that the highest production of CD was obtained at 90°C with 4.93 mg/ml of CD. Meanwhile, the lowest CD production (0.72 mg/ml) was detected at 70 °C of temperature. The temperature has important effect on CD production by enhancing the interaction between enzyme and substrate molecules.

The reaction time also has a major influence on the production process. Qiu et al. (2018) discussed the influence of reaction time on the CD production using CGTase from recombinant *E. coli*. The longer the reaction time, the higher is

the yield of CD. The CD yield increased slowly with the passing of time and the highest yield of about 10% was obtained after 21 hr.

The effect of substrate on the production of CD has shown to be extremely important. The effect of substrate on CD production using CGTase from *Bacillus firmus* strain 37 was carried out by Fenelon et al. (2015). The maltodextrin substrate with a 10 % (w/v) of concentration produced 11.9 mmol/l of CD. Meanwhile, 5 % (w/v) of corn starch substrate gave a higher CD production with 13.5 mmol/l of CD. The different composition of amylose and amylopectin in different types of substrate affected the production of CD.

Most studies discussed the effect of reaction parameters on the CD production using CGTase from various sources such as *Thermococcus* sp., *Thermoanaerobacter* sp., *Bacillus* sp. and recombinant *E. coli* (Goh et al., 2007; Moriwaki et al., 2014). To the best of our knowledge, there have been no studies on the effect of reaction parameters on CD production using the commercial CGTase from *Bacillus licheniformis*. Moreover, the effect of reaction parameters such as substrate types, concentration of substrate and reaction time on the CD production by using commercial CGTase from *Bacillus licheniformis* has not been investigated. Therefore, the effect of types of substrate, concentration of substrate and reaction time on the CD production by using commercial CGTase from *Bacillus licheniformis* was investigated in this present study.

MATERIALS AND METHODS

Materials

Commercial CGTase from *Bacillus licheniformis* (Toruzyme 3.0) was purchased from Novozymes A/S (Bagsvaerd, Denmark). Acetonitrile, methanol, sodium hydroxide, sodium dihydrogen phosphate, glycine, and soluble potato starch were purchased from Merck Sdn Bhd (Darmstadt, Germany). Soluble potato, corn, tapioca, wheat and rice starch were purchased from Chemolab Supplies Sdn Bhd (Selangor, Malaysia).

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Types of substrate

The substrates used in this study were corn, potato, rice, wheat and tapioca starch. About 20 ml of soluble starch solution with concentration of 3% (w/v) (Suhaimi, 2019) was prepared in 50 mM sodium phosphate buffer (pH 6.0) by gelatinize in a steam water bath for 10 min and the solution was allowed to cool at room temperature. After that, 0.1 µg/ml of CGTase was added to the sample and incubated with agitation rate of 100 rpm and 40°C of temperature for 4 hr of reaction time. The experiments were conducted in three replicates. The sample was analyzed using High Performance Liquid Chromatography (HPLC).

Substrate concentration

Effect of substrate concentration on the synthesis of CD was investigated using different concentration of 1, 2, 3, 4, 5, 6, 7, 8 and 9 % (w/v) (Ibrahim et al., 2011). About 20 ml of soluble potato starch solution was prepared in 50 mM sodium phosphate buffer (pH 6.0) by gelatinize in a steam water bath for 10 min and the solution was allowed to cool at room temperature. Then, 0.1 µg/ml of CGTase was added to the sample and incubated in the incubator shaker with agitation rate of 100 rpm at 40°C of temperature for 4 hr of reaction time. All experiments were conducted in three replicates. The sample was analyzed using High Performance Liquid Chromatography (HPLC).

Reaction time

Effect of reaction time on the production of CD using CGTase from *Bacillus licheniformis* was conducted at various reaction times of 1, 2, 3, 4 and 5 hr (Rahim et al. 2015). About 20 ml of soluble potato starch solution with concentration of 6% (w/v) was prepared in 50 mM sodium phosphate buffer (pH 6.0) by gelatinize in a steam water bath for 10 min and the solution was allowed to cool at room temperature. Then, 0.1 µg/ml of CGTase was added to the sample and incubated in the incubator shaker with agitation rate of 100 rpm and 40°C of temperature. The experiments were conducted in three replicates. The sample was analyzed using High Performance Liquid Chromatography (HPLC).

Analytical Analysis

High performance liquid chromatography (HPLC)

The concentration of CD was determined by using high performance liquid chromatography (HPLC) with quaternary pump (Agilent 1260 Infinity Binary LC, California, USA). The column used was Zorbax Eclipse Plus C18, 150 mm x 4.6 mm (Agilent Technologies, California, USA). The mobile phase is a mixture of acetonitrile: water (60:40) at 1 ml/min. The reflective index detector was used to detect the CD. The temperature of column was set at 30°C. All the samples

were filtered with a nylon membrane filter (0.2 μm pore size) with diameter of 13 mm before injection (Sakinah et al., 2014).

EXPERIMENTAL RESULTS

Effect of Types of Substrate on the Synthesis of CD

The effect of starch types on the synthesis of CD was investigated as shown in Figure 1. The highest CD production up to 10.24 mg/ml was detected when the potato starch was used as a substrate. This is due to the highest content of amylopectin in the potato starch compared to other starches (Zhao et al., 2018). The production of CD is depends on the content of amylopectin or amylose of the substrate. Different substrate contains different composition of amylopectin or amylose. The amylopectin contains high branched structure. Meanwhile, the amylose consists of long linear chains structure. The high content of amylopectin is preferred because of more content of α -1, 4-glycosidic bonds (Jeong et al., 2019) that assisted in the production of CD. The α -1,4-glycosidic bonds were cleaved by CGTase between subsite +1 and -1 of the substrate and produced glycosyl-intermediate as shown in Figure 2. Then, the glycosyl-intermediate was formed the hydroxyl group and new α -1, 4-glycosidic bond was produced to form cyclic of CD. This proved that high amount of amylopectin formed high content of α -1,4-glycosidic bonds that contributed in the production of CD. Moreover, the amylopectin with high branched structure allowed the reaction of CGTase occurred at numerous points of substrate, thus greatly enhanced the CD (Leemhuis et al., 2010). In the present study, the potato starch contained the highest amount of amylopectin (about 78%) compared to other starches (Chen et al., 2004). Hence, this proved that the high content of amylopectin contributed in the high production of CD.

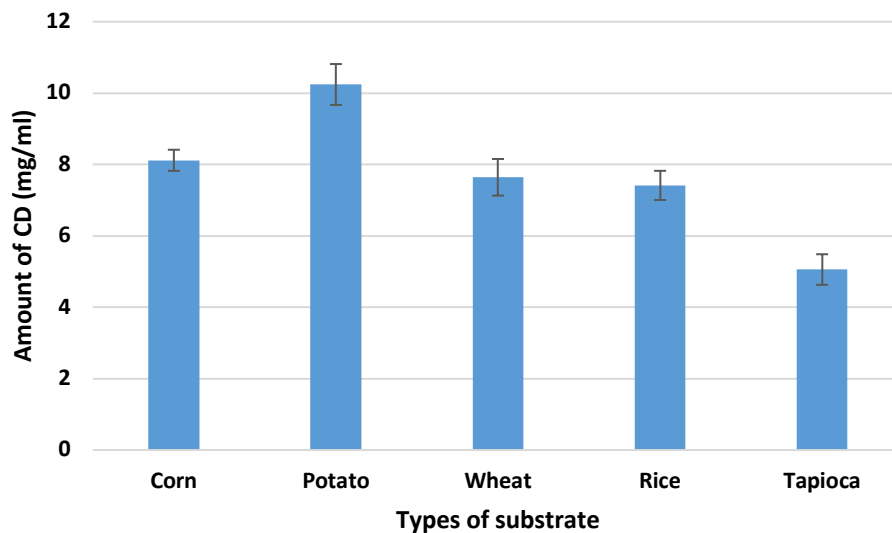


Figure 1. Effect of types of substrate on the synthesis of CD using 0.1 $\mu\text{g/ml}$ of CGTase. About 3% (w/v) of substrate was prepared in 50 mM sodium phosphate buffer (pH 6.0). The conditions for reaction were as follows: 4 hr of reaction time, 100 rpm and 40°C.

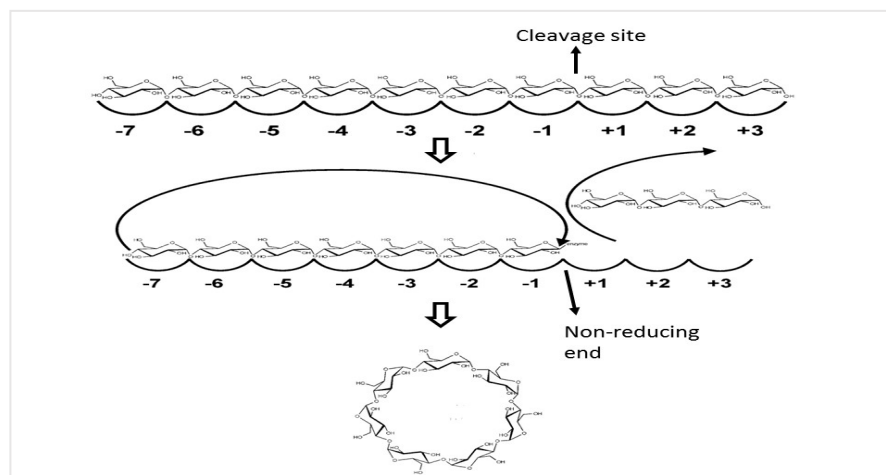


Figure 2. Schematic view of CD formation using CGTase (Source: Leemhuis et al., 2010)

A study investigated by Duan et al. (2013) using CGTase from recombinant *E. coli* also showed that the potato starch that consists of high content of amylopectin produced high amount of CD. The total yield of CD was 84.6 % (w/w) after 24 hr of reaction time. Moreover, the findings in the present study was also similar with the results obtained by Cheirsilp et al. (2010). In this study, potato starch was also the best substrate for the production of CD using CGTase from *Bacillus* sp. C26. It was reported that the high amylopectin content in the potato starch assisted in the production of CD. The amylopectin with highly branched structure allowed the reaction of enzyme to start from various points. Therefore, in this present study, the potato starch was selected as the best substrate to be used for the subsequent study due to the high production of CD.

Effect of substrate concentration (potato starch) on the synthesis of CD

The effect of soluble potato starch on the synthesis of CD was investigated using different concentration of 1, 2, 3, 4, 5, 6, 7, 8, and 9% w/v (Figure 3). The high production of CD (15.16 mg/ml) was obtained when 6% (w/v) of substrate was used. This occurrence because of high content of amylopectin in high concentration of potato starch (Weil et al., 2020). Therefore, the CGTase reacted with the highly branched amylopectin at numerous points and resulted in high production of CD. Further increase the concentration of soluble potato starch above 6% resulted in decrease of CD production. This phenomenon happened due to the saturation of starch binding on the enzyme active site, thus affected the production of CD (Sakinah et al., 2014).

The study conducted by Rakmai et al. (2015) showed that the CD production using CGTase from *Bacillus* sp. C26 increased with an increasing the starch concentration from 1% to 4%. Nevertheless, the CD production was not significantly different when the concentration of starch was further increased up to 8%. The reaction process for production of CD was reversible and the production of CD was prevented if high product concentration was obtained. Moreover, the high starch concentration in the reaction mixture increased the viscosity and this phenomenon obstructed the binding process of enzyme and substrate.

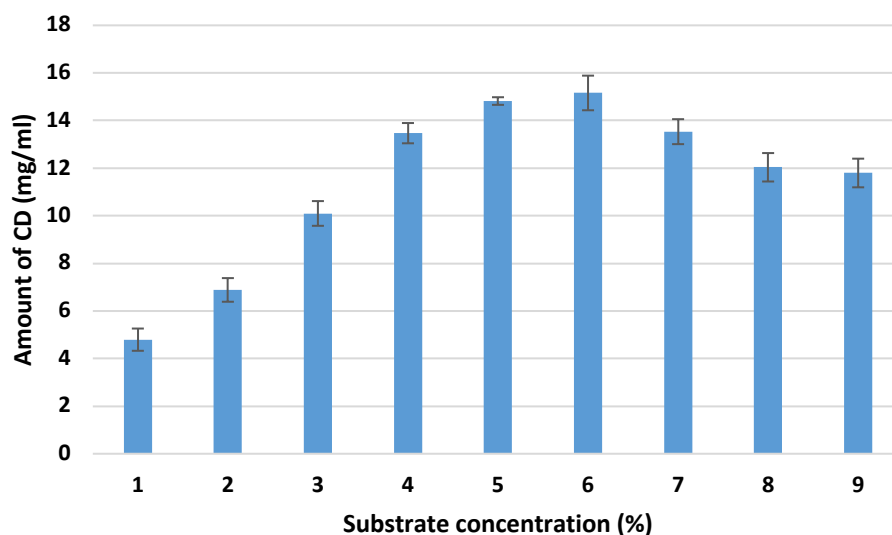


Figure 3. Effect of substrate concentration on the synthesis of CD using 0.1 µg/ml of CGTase. The potato starch was used as substrate and it was prepared in 50 mM sodium phosphate buffer (pH 6.0). The reaction conditions were 4 hr of reaction time, 100 rpm of agitation rate and 40°C of temperature.

A study conducted by Rajput et al. (2016) on the production of CD using CGTase from *Alkaliphile Microbacterium terrae* KNR 9 showed that increased in the potato starch concentration has favourable impact on the production of CD. The CD production was optimum using 150 gm/l of potato starch concentration with 28.22 gm/l of CD. However, further increased the concentration of potato starch caused low production of CD. This occurrence due to the high potato starch concentration lead to the high viscosity in a reaction mixture, thus affecting difficulty in stirring and hinders the substrate binding to the enzyme active site (Schöffner et al. 2013).

As a conclusion, 6% of potato starch concentration was chosen as the optimal concentration in the present study because of high production of CD. The use of low concentration of potato starch could also decrease the production cost of CD.

Effect of reaction time on the synthesis of CD

Figure 4 shows the effect of reaction time on the synthesis of CD by different reaction time (1, 2, 3, 4, and 5 hr). The production of CD was improved significantly by increasing the reaction time from 1 to 4 hr. The CD production was 12.41 mg/ml at 4 hr of reaction time. The CD increment occurred when the reaction time was increased due to the frequent binding between tapioca starch and enzyme, thus improved the reaction process for the production of CD (Ibrahim, 2013). The results from Schöffner et al. (2017) was similar with the findings from the present study on the production of CD using CGTase from *Thermoanaerobacter* sp. Reduction in the concentration of CD was observed when increasing the reaction time for reaction process. It might be due to the usage of CD as a substrate whereby the CGTase is able to catalyze the α (1-4) bonds hydrolysis and disproportionation, besides capable to involve in the transglycosylation and cyclization reactions. Moreover, Terada et al. (1997) reported that the CGTase firstly able to produce larger CD when reacted with starch, then capable to reduce the size of CD after the reaction process.

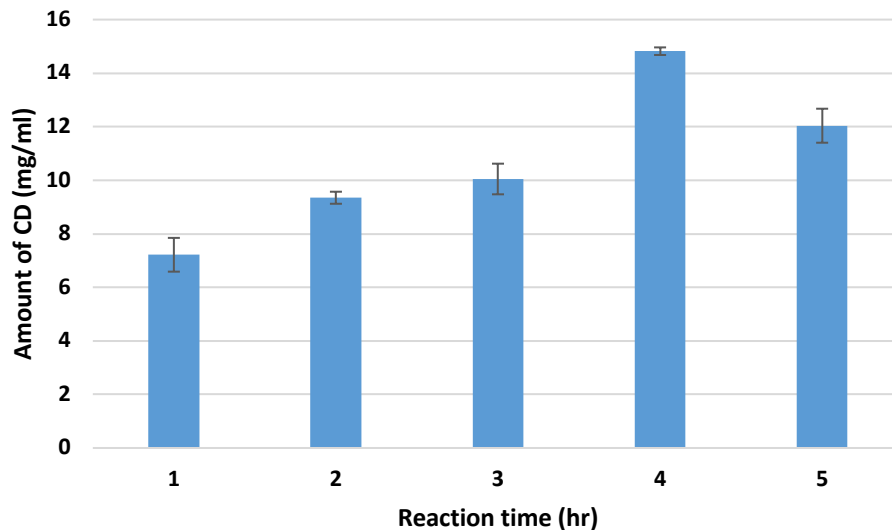


Figure 4. Effect of reaction time on the synthesis of CD using 0.1 $\mu\text{g/ml}$ of CGTase with potato starch was used as a substrate. About 6% (w/v) of substrate was prepared in 50 mM sodium phosphate buffer (pH 6.0). The conditions for reaction were 100 rpm of agitation rate and 40°C of temperature.

Slightly decrease in the CD production was detected when the reaction time was prolonged. The CD was 12.04 mg/ml at 5 hr of reaction time. The inhibition of product occurred when CD accumulated in the reaction mixture. The CD would accumulate on the enzyme active site and avoid the binding process of the substrate to the enzyme active site. Thus, this occurrence affected the production of CD production.

A study carried out by Charoenlap et al. (2004) on CD production using CGTase from *Bacillus circulans* (TISTR 907) with the sago starch as a substrate showed that the CD production was maximum (4.33 mg/ml) at 9 hr of reaction time. However, the CD production at 6 hr and 9 hr of reaction time were not significantly different with 4.05 mg/ml of CD was detected at 6 hr of reaction time. For economic point of view, 6 hr of reaction time was selected as the optimum reaction time for the production of CD.

Therefore, 4 hr of reaction time was selected as the optimal reaction time in this present study as it requires less time to produce maximum amount of CD. Moreover, sufficient time allows the starch to bind more frequently and extensively on the enzyme, thus increased the enzymatic reaction for the production of CD. Shorten time required for the reaction process of CGTase for high CD production gave significant advantage to be used in the industrial scale production.

CONCLUSION

The influence of reaction parameters (starch types, potato starch concentration and reaction time) of CGTase from *Bacillus licheniformis* on CD production have been successfully investigated. This study showed the best type of substrate for the production of CD was the soluble potato starch. In addition, the production of CD was optimum using 6% (w/v) of potato starch and 4 hr of reaction time. In conclusion, the best reaction parameters of commercial CGTase from *Bacillus licheniformis* capable to improve the CD production and could be a promising approach for the application in various industries.

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