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Effect of Process Parameters on the Production of Cyclodextrin Using Cyclodextrin Glucanotransferase From *Bacillus licheniformis*

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ABSTRACT
Cyclodextrin (CD) is a non-reducing oligosaccharides constituted by variable number of units of glucose. CD has a doughnut-shaped molecule with hydrophilic outer surface and hydrophobic inner cavity. Such structure features enables CD to form inclusion complexes with guest compound. CD is produced by degradation of starch using cyclodextrin glucanotransferase (CGTase). The process parameters such as types of substrate, reaction time and temperature will affect the production of CD. Most of the studies investigated the effect of process parameters using CGTase from *Bacillus circulans*, *Bacillus subtilis* and *Thermoanaerobacter* sp on the CD production. However, no studies have investigated the effect of pH, temperature and agitation rate on the production of CD using CGTase from *Bacillus licheniformis*. In this present study, the effect of pH (5-9), temperature (20-60°C) and agitation rate (50-250 rpm) of CGTase from *Bacillus licheniformis* on the CD production were studied. The most suitable pH for the production of CD was pH 6 with 4.45 mg/ml of CD. In addition, the best temperature that produced the highest amount of CD was detected at 40°C with 1.473 mg/ml. The optimal agitation rate was found to be 150 rpm with 2.762 mg/ml of CD. The best process parameters proved to be valuable for the highest production of CD. Hence, the high production of CD may be beneficial for the industrial scale purposes.

Keywords: *Cyclodextrin, cyclodextrin glucanotransferase, pH, temperature, agitation rate*

1. INTRODUCTION

Cyclodextrin (CD) also known as cycloamylose, is the non-reducing oligosaccharide consisting mostly of six, seven and eight glucose monomers (α, β and γ-CD). CD is produced by starch degradation using cyclodextrin glucanotransferase (CGTase). Other than starch, the substrates such as amylopectine, glycogen, amylose and dextrin are also used in the CD production [1]. The CD is formed by a conical cylinder with hydrophobic inner cavity and hydrophilic outer surface. This enable CD to remove any unwanted aroma and flavours, to keep the guest molecules from degradation under the light and heat, to stabilize the volatile substances and to decrease the side effects of drug formulations [2]. These features make CD attractive in various applications such as in the food, cosmetic, pharmaceutical, and plastic industries.

CGTase is a unique enzyme produced mainly by microorganisms, usually *Bacillus* species. It is an important industrial enzyme which mostly used in food and pharmaceutical industry [3]. CGTase is member of the α-amylase family, which belong to glycosyl hydrolase family 13 [4]. CGTase act on oligomersor polymers of glucose, simultaneously catalyzing four types of reactions, including hydrolysis and three transferase reactions. The cyclization, in which amylose or starch is cleaved to form CD. Meanwhile, the disproportionation whereas a linear oligosaccharide is cleaved and a portion of the oligosaccharide is transferred to an acceptor. The coupling, in which a CD is cleaved and the resulting linear oligosaccharide is transferred to an acceptor [5]. Among them, the cyclization reaction is the unique reaction of CGTase. Depending on the main CD products produced during the preliminary stage of substrate conversion, CGTase has been classified as α-, β- and γ-CGTase.

The process parameters such as types of substrate, concentration of enzyme, reaction time, pH and temperature will affect the production of CD by using CGTase with the presence of starch. The influence of pH on the production of CD has been examined by Rojas et al. [6]. That study found that pH 6 was the optimum pH on the production of CD using cassava bagasse as a substrate. Moreover, a study conducted by da Natividade Schöffer et al. [7] showed that the highest production of CD was obtained at 90°C and 24 h with 4.93 mg/mL. Meanwhile, a study performed by Szerman et al. [8] reported that 5% (w/v) cassava starch with 15 U of enzyme per gram of substrate were the optimum conditions for the CD production.

To the best of our knowledge, the effect of process

parameters such as pH, temperature and agitation rate of CGTase from *Bacillus licheniformis* on CD production has not been investigated. Therefore, the effect of pH, temperature and agitation rate on the production of CD by using CGTase from *Bacillus licheniformis* was examined in the present study.

2. EXPERIMENTAL

2.1. Materials

Commercial CGTase from *Bacillus licheniformis* (Toruzyme 3.0) was purchased from Novozymes A/S (Bagsvaerd, Denmark). Acetonitrile, methanol, sodium hydroxide, sodium dihydrogen phosphate, glycerine and soluble potato starch were purchased from Chemolab Supplies Sdn Bhd (Selangor, Malaysia).

2.2. Synthesis of Cyclodextrin

The production of CD was carried out in the five different 250 mL of conical flask. About 20 mL of soluble potato starch solution with concentration of 3% (w/v) was prepared by gelatinize in a steam water bath for 10 min and the solution was allowed to cool at room temperature. CGTase with 100 µL was added to the reaction medium and incubated in the incubator shaker for 3 h. The synthesis of CD was analysed by using High Performance Liquid Chromatography [2].

2.3. Effect of Process Parameters of CGTase from *Bacillus licheniformis* on the Production of CD

2.3.1. Effect of pH

The pH used in this study were 5, 6, 7, 8 and 9 [9]. CGTase from *Bacillus licheniformis* reacted with 3% gelatinized potato starch prepared in 50 mM buffers. Different suitable buffers were used including 50 mM sodium acetate (pH 5.0-6.0), 50 mM sodium phosphate (pH 7.0- 8.0) and 50 mM glycine-NaOH buffer (pH 9.0). The temperature and agitation rate used were 30 °C and 100 rpm, respectively. About 0.1 µg/ml of CGTase was added to the sample containing gelatinized soluble potato starch solution with concentration of 3% (w/v) and incubated for 3 h. The sample was analysed using High Performance Liquid Chromatography (HPLC).

2.3.2. Effect of Temperature

The effect of temperature on the synthesis of CD was studied using different temperature of 20°C, 30°C, 40°C, 50°C and 60°C [9]. The pH and agitation rate used were pH 6 and 100 rpm, respectively. Every sample contained 3% (w/v) of gelatinized potato starch. Then, 0.1 µg/ml of CGTase was added to the sample and incubated in the incubator shaker for 3 h. The sample was analysed using High Performance Liquid Chromatography (HPLC).

2.3.3. Effect of Agitation Rate

The effect of agitation rate on the synthesis of CD using CGTase from *Bacillus licheniformis* was conducted at various agitation rate of 50, 100, 150, 200 and 250 rpm [10]. The pH and temperature used were pH 6 and 40 °C, respectively. About 0.1 µg/ml of CGTase was added into the sample that contained 3% (w/v) of gelatinized potato starch and incubated in the incubator shaker for 3 h. The sample was analysed using High Performance Liquid Chromatography (HPLC).

2.4. Analytical Analysis

The concentration of the CD produced was determined by High Performance Liquid Chromatography (HPLC). Agilent Eclipse Plus column was used in this study. The mobile phase is a mixture of acetonitrile: water (60:40) at 1 ml/min and the CD detected by a refractive index detector. The column temperature was controlled 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 [11].

3. RESULTS AND DISCUSSION

3.1. Effect of pH on the CD Production

The pH is one of the important factor that affects the CD production. The effect of the five different pH values (5, 6, 7, 8 and 9) on the production of CD was investigated. Fig. 1 shows the amount of CD produced from different types of pH values. The maximum CD produced was detected at pH 6 (4.45 mg/ml), followed by pH 7 (3.58 mg/ml), pH 8 (3.14 mg/ml), pH 9 (2.89 mg/ml) and finally pH 5 (2.66 mg/ml).

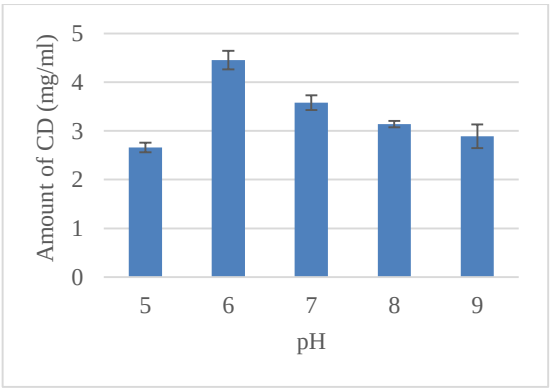

Fig. 1. Effect of pH on CD production. The reaction was conducted for 3 h at 30°C and 100 rpm of agitation rate using soluble potato starch.

Enzyme is a protein that consists of carboxylic acid and amine functional groups. According to Manas et al. [12], during transglycosylation, a pair of carboxylic acids was involved. One of the enzyme residues act as nucleophile. Meanwhile, the other residue plays a dual role as general acid and general base. The dual role requires the enzyme to control the ionization state of the general acid and base residue in the reaction which commonly known as "pKa cycling". A study conducted by Ludwiczek et al. [13] found that it is important to control the pKa of these residues to improve the enzymatic reaction in product formation. Thus, in order to increase the production of CD, adjusting the acidic reaction condition was found to be favourable for the enzyme reaction. Therefore, pH 6 was selected as the optimum pH for the high production of CD in the present study.

In the high alkaline medium, the production of CD was decreased due to the abundant hydroxyl (OH^-) ions in the reaction mixture. Therefore, the OH^- bind to the amino group (NH^{3+}) of the enzyme. Hence, it avoided the substrate to be placed at the right orientation of the enzyme active site [15]. Besides, the high pH would cause deprotonation of tyrosine residue (amino acid) which affected the glycosylation reaction to produce CD.

The results in the present study was similar with the findings by Ibrahim et al. [9], whereas pH 6 was the optimum pH using CGTase from *Bacillus Agaradhaerens* on the production of CD. This is because, the CGTase from *Bacillus Agaradhaerens* requires a near-neutral pH range to perform the reaction and the pH values ranging from 7-9 were not suitable for the enzyme to carry out the cyclization activity. However, a study conducted by Gawande and Patkar [16] showed that the maximum CD production using CGTase from *Klebsiella pneumoniae* was in neutral process parameters which was at pH 7.5 with 5.79 mg/ml of CD.

3.2 Effect of Temperature on CD Production

Temperature also plays an important role in the production of CD. The effect of temperature on the CD production using different temperature of 20, 30, 40, 50 and 60°C is illustrated in Fig. 2. The CD production was improved by increasing the reaction temperature from 20°C to 40°C. The CD production was 1.11 mg/ml at 20°C and 5.93 mg/ml at 40°C. This is because, the kinetic energy increases when the temperature increases from 20 to 40 °C, assisted in a frequent collision between enzyme and starch molecules. Hence, this phenomenon resulted in the high production of CD. However, the production of CD decreased to 1.34 mg/ml at 50°C and 1.25 mg/ml at 60°C. This is due to the denaturation of enzyme at high temperature. Therefore, the substrate molecules unable to bind to the active site of the enzyme and consequently produced low CD [17].

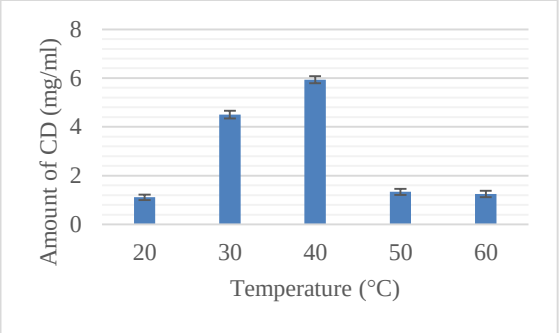

Fig. 2. Effect of temperature on CD production. The reaction was conducted for 3 h at pH 6 and 100 rpm of agitation rate using soluble potato starch.

A study conducted by Ibrahim et al. [9] found that the maximum production of CD using CGTase from *Bacillus agaradhaerens* was at 55°C with the maximum value of 6.9 mg/ml. However, the total CD production was declined at 60ºC. This is because, the enzymatic reaction is enhanced by temperature due to the higher kinetic energy. However, due to the thermostability of the enzyme, higher temperature leads to the enzyme denaturation and loose of function in a short period of time resulting in lower production of the product [18].

There are several findings that depicted greater temperature values than the present study as their optimum temperature. A study conducted by Gawande and Patkar [16]using CGTase from *Klebsiella pneumoniae* found that the maximum CD production was at 45°C with the soluble starch as a substrate with 4.32 mg/ml of CD production. Meanwhile, a study conducted by Sian et al. [19] found that, the optimum temperature on the production of CD using CGTase from *Bacillus* sp. was at 60°C with 3.85 mg/ml.

3.3 Effect of Agitation Rate on the CD production

Agitation rate also has significant effect on the production of CD. The effect of agitation rate on the CD production using different values of 50, 100, 150, 200 and 250 rpm is shown in Fig. 3. The CD production was improved by increasing the agitation rate from 50 to 150 rpm. The CD production was 2.37 mg/ml at 50 rpm and 8.90 mg/ml at 150 rpm. However, the production of CD decreased to 2.59 mg/ml at 200 rpm and 2.51 mg/ml at 250 rpm. At high agitation rate, the probability for the substrate to bind with the active site of the enzyme is high while at low agitation rate, the probability to form the binding between substrate and enzyme could be difficult [10].

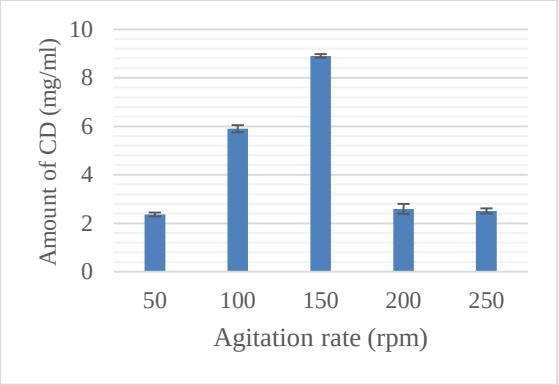

Fig. 3. Effect of agitation rate on CD production. The reaction was conducted for 3 h at pH 6 and 40°C using soluble potato starch.

The optimum agitation rate which was 150 rpm in this present study was almost similar with the finding by Rahim et al. [10] using CGTase from *Bacillus subtilis*. The optimum agitation rate was 120 rpm. It was found that the agitation rate increased the movement of substrate and enzyme molecules for the enzymatic reaction from 40 to 120 rpm and then slightly decreased from 120 to 200 rpm. However, the result from this present study was contradicted with the several other findings. For instance, a study conducted by Kriaa et al. [20] found that the maximum CD production using CGTase from *Bacillus Macarous* detected at agitation rate of 200 rpm with 1.21 mg/ml of CD. This is because, the increased aeration favoured the rapid mixing of starch with CGTase and that the occurrence of catabolic repression as a function of the products increases in the medium. The study also found that, the agitation rate did not showed the significant variation in CGTase production above 200 rpm. Meanwhile, Szerman et al. [8] found that the high CD production occurred at the agitation rate of 100 rpm using CGTase from *Bacillus circulans*. This phenomenon due to the increased aeration resulted in the rapid use of starch. Therefore, it is suggested that the occurrence of catabolic repression as a function of the products increased in the medium.

4. CONCLUSION

The effect of process parameters on the synthesis of CD have been successfully investigated. Among the five different pH tested, pH 6 was the most suitable for the production of CD with 4.45 mg/ml. In addition, the optimum temperature that produced the maximum amount of CD was at 40°C with 5.93 mg/ml. The optimal agitation rate was found to be 150 rpm with 8.90 mg/ml of CD. In conclusion, the best process parameters capable to improve the CD production and consequently give beneficial for the industrial scale purposes.

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