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EFFECT OF REACTION CONDITIONS ON THE SYNTHESIS OF CYCLODEXTRIN (CD) BY USING IMMOBILIZED ENZYME

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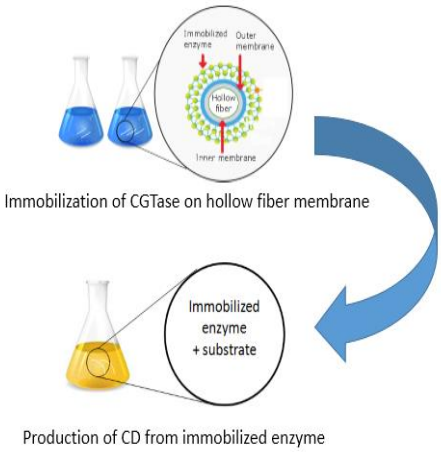
Graphical abstract



Abstract

The production of cyclodextrin (CD) over the years has been increasing due to the numerous applications in industries such as in food, cosmetic, pharmaceutical and agricultural industries. However, cyclodextrin glucanotransferase (CGTase) which involved in the enzymatic reaction on the production of CD is unstable and easily denatured at extreme conditions resulted in low CD production. Hence, the enzyme immobilization technique is introduced to overcome these problems and subsequently increase the production of CD. In the present study, the CGTase was immobilized on hollow fiber membrane to increase the production of CD during the reaction. The effect of reaction conditions (types of starch, concentration of starch, temperature and pH) of the immobilized enzyme on the production of CD were investigated. Among the three types of starch tested, the soluble potato starch was the most suitable substrate for the production of CD with 4.13 mg/mL. In addition, by using 3% (w/v) of the soluble potato starch, the production of CD was 5.22 mg/mL . The optimal reaction temperature and pH were found to be at 40°C and pH 6 with 5.21 mg/mL and 4.62 mg/ml of CD, respectively. The immobilized enzyme exhibited a 1.3-3-fold increase in CD production compared to the free enzyme. Therefore, the hollow fiber membrane is suitable to be used as a support for enzyme immobilization with the high production of CD.

Keywords: Cyclodextrin, cyclodextrin glucanotransferase, enzyme immobilization, hollow fiber membrane

Abstrak

Penghasilan siklodekstrin (CD) dari tahun ke tahun semakin meningkat kerana kepelbagaian aplikasi di dalam industri seperti industri makanan, kosmetik, farmaseutikal dan pertanian. Walau bagaimanapun, siklodekstrin glukanotransferase (CGTase) yang terlibat dalam tindak balas enzim dalam penghasilan CD tidak stabil dan mudah berubah bentuk pada keadaan yang melampau, menyebabkan kurangnya penghasilan CD. Oleh itu, teknik imobilisasi enzim telah diperkenalkan untuk mengatasi masalah-masalah ini dan seterusnya penghasilan CD dapat ditingkatkan. Dalam kajian ini, CGTase diimmobilisasi di atas membran gentian berogga untuk meningkatkan

penghasilan CD. Kesan keadaan reaksi (jenis kanji, kepekatan kanji, suhu dan pH) imobilisasi enzim pada penghasilan CD telah dikaji. Antara tiga jenis kanji yang dikaji, larutan kanji ubi kentang merupakan kanji yang paling sesuai digunakan dalam penghasilan CD iaitu sebanyak 4.13 mg/mL. Di samping itu, dengan menggunakan 3% (w/v) kepekatan larutan kanji ubi kentang, CD yang terhasil adalah sebanyak 5.22 mg/mL. Suhu dan pH optimum adalah pada 40°C dan pH 6 dengan penghasilan CD masing-masing sebanyak 5.21 mg/mL dan 4.62 mg/mL. Enzim yang telah diimmobilisasi menunjukkan peningkatan CD sebanyak *1.3-3-kali ganda* berbanding enzim bebas. Oleh itu, membran gentian berongga sangat sesuai digunakan sebagai penyokong untuk immobilisasi enzim dengan penghasilan CD yang tinggi.

Kata kunci: Siklodekstrin, siklodekstrin glukanotransferase, imobilisasi enzim, membran gentian berogga

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1.0 INTRODUCTION

Cyclodextrins (CDs) or also known as cycloamyloses are the non-reducing oligosaccharide consisting of six (α-cyclodextrin), seven (β-cyclodextrin) and eight (γ-cyclodextrin) units of glucopyranose linked by α-(1,4) bonds. CDs are produced by intramolecular transglycosylation reaction from the degradation of starch by cyclodextrin glucanotransferase (CGTase). Other than starch, substrates like amylose, amylopectin, dextrin and glycogen are widely used in the production of CD [1]. The CD has a unique molecular structure. For example, CD consist of hydrophobic inner cavity which hydrophobic compound can be encapsulated. This enables CD to remove any unwanted flavours and aroma, to protect guest molecules from degradation under the action of light and heat, to reduce side effect of drug formulations and also to stabilize the volatile substances [2].

The improvements of physical and chemical properties of CD over the years have brought many applications in food, cosmetic, pharmaceutical and plastic industries. However, the enzyme used as a biocatalyst is usually unstable and easily denatured which resulted in the low amount of CD produced. In order to overcome these problems, enzyme immobilization technique was performed to increase the production of CD during the reaction. Besides, the immobilized enzyme has the ability to retain high enzymatic activity and can be reused several times for the synthesis of CD [3]. This characteristic definitely brings advantage since no additional cost is needed to separate the enzyme from the reaction mixture. The choice of material as a support for enzyme immobilization is important to increase the production yield.

In the present study, CGTase was immobilized on hollow fiber membrane to increase the production of CD. The porous membrane is capable to act as a support for enzyme immobilization due to the high surface area per unit volume. Other than that, the hollow fiber membrane is also known to have high mechanical strength, high operational stability and lack of toxicity [4]. Most studies for enzyme immobilization on hollow fiber membrane have focused on enzymes such as tyrosinase [5], laccase [3] and lipase [6]. To the best of our knowledge, there have been no studies on CD production by the immobilized CGTase on hollow fiber membrane. Therefore, the effect of reaction conditions such as type of substrate, substrate concentration, reaction temperature and pH on the production of CD by the immobilized enzyme were studied. It is suggested that the enzyme immobilized on hollow fiber membrane is a promising method for the production of CD.

2.0 METHODOLOGY

2.1 Immobilization of CGTase

Hollow fiber membrane was cut into 3 cm length and transferred to 10 mL of enzyme solution in 0.05M sodium phosphate buffer (pH 6.0). Then, the sample was incubated at 25°C with 100 rpm. After 24 h of incubation, the membrane was thoroughly washed with sodium phosphate buffer to eliminate the non-immobilized enzyme. The immobilized CGTase was transferred to 250 mL conical flask for the enzymatic reaction.

2.2 Effect of Reaction Conditions on CD Production

The effect of the optimal reaction conditions (types of substrate, substrate concentration, temperature and pH) by the immobilized CGTase were determined by using one factor at one time (OFAT) method.

i The effect of starch types were investigated using three different types of starch; tapioca, rice and soluble potato starch. The immobilized CGTase was incubated using 3% (w/v) of starch concentration in pH 6 at 40°C for 4 h.
ii The effect of starch concentration was studied using 2, 3, 4, 5 and 6% (w/v) of soluble potato starch. The immobilized CGTase was incubated using soluble potato starch in pH 6 at 40°C for 4

h.

iii The effect of reaction temperature was studied using temperature of 20, 30, 40, 50 and 60°C. The reaction mixture containing immobilized CGTase was incubated in 3% (w/v) of soluble potato starch in pH 6 for 4 h.

iv The effect of pH was studied using pH of 5, 6, 7, 8 and 9. The buffers used were citrate-phosphate buffer (pH 5), sodium-phosphate buffer (pH6-8) and glycine-NaOH buffer (pH 9). The reaction mixture containing 3% (w/v) of soluble potato starch was incubated at 40°C for 4 h.

A free enzyme solution was used as a control in the experiment. The free enzyme undergoes the same reaction conditions as those used for the immobilized enzyme.

2.3 Analysis of CD Production

The concentration of CD was determined by using High Performance Liquid Chromatography (HPLC) as described by Sakinah *et al*. [7]. Agilent Eclipse Plus column was used in this study. The mobile phase was a mixture of acetonitrile: methanol: water (60:30:10) at 1 mL/min and the CD was detected by a reflective index detector. The column temperature was controlled at 30°C. All samples were diluted with 3 volumes of methanol HPLC grade and filtered using Whatman® nylon membrane (before sample injection) [7].

2.4 Field Emission Scanning Electron Microscope (FESEM)

The surface morphology of the immobilized enzyme on the hollow fiber membrane was taken using field emission scanning electronic microscopy (FESEM, JEOL JSM7800F model). The support was immersed in a 2.5% glutaraldehyde solution overnight at 4°C for fixation, completely rinsed with distilled water and dehydrated with 50%, 70%, 80%, 95% and 100% ethanol by submerging the membrane at each concentration for 10 min. The membrane was then dried in cryogenically at the critical point [8]. The dried prior to analysis using FESEM.

3.0 RESULTS AND DISCUSSION

3.1 Immobilization of CGTase on Hollow Fiber Membrane

The surface morphology of hollow fiber membrane was viewed by using field emission scanning electron microscope (FESEM). The type of polymer membrane used in this study was polyvinylidene fluoride (PVDF). Figure 1A shows the surface of hollow fiber membrane before the immobilization of CGTase. Pores with irregular size could be observed on the surface of the membrane. According to Chowdhury *et al*. [9], the porosity of the membrane contributed to the attachment and entrapment of CGTase. Therefore, the high surface area of the hollow fiber membrane could increase the attachment and entrapment of the enzyme to the membrane which subsequently increases the production of CD.

Figure 1B shows that the CGTase have been successfully immobilized on the hollow fiber membrane. This is due to the electrostatic interactions between the enzyme and the surface of the PVDF membrane. The PVDF polymer membrane used in this study consist of hydrogen atoms (positively charged) and fluoride atoms (negatively charged) [10]. Meanwhile, the enzyme contained amino group (positively charged) and carboxyl group (negatively charged) [11]. The electrostatic interactions between the positively charged PVDF with the negatively charged enzyme contributed to the attachment of the enzyme on the hollow fiber membrane.

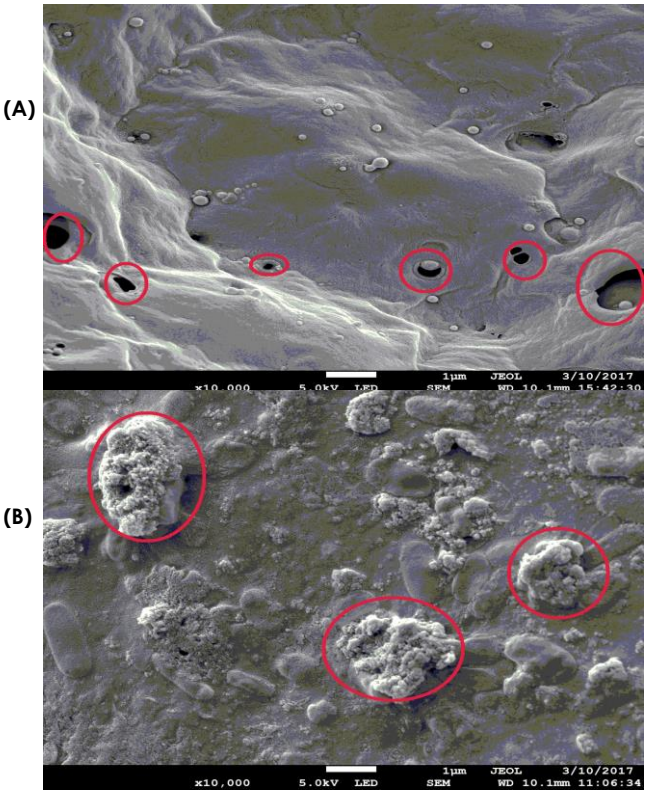

Figure 1 FESEM images of hollow fiber membrane under 10,000x magnification. (A) before enzyme immobilization (B) after enzyme immobilization

3.2 Effect of Starch Types on the CD Production

The effects of starch types on the synthesis of CD by the immobilized enzyme was investigated. Figure 2 shows the amount of CD produced from different types of starch. The highest CD concentration up to 4.13 mg/mL was achieved when the soluble potato starch was used as a substrate. Meanwhile, the lowest concentration of CD was detected when using the rice starch with 2.66 mg/mL of CD. The amount of CD produced depends on the source of starch, whereby the different source of starch contain different content of amylose and amylopectin. However, the starch with high content of amylopectin is preferred due to the branched structure of the amylopectin that allowed the enzyme to start the reaction from various points of the starch [12]. A similar result was obtained from a study conducted by Pishtiyski and Zhekova [7] who proved that the starch that consists of the high content of amylopectin produced the high amount of CD. On the other hand, the result obtained in this study was contradicted with the finding by Egorov *et al*. [14], who used the rice starch as a substrate. The rice starch contained high amount of amylose and less amount of amylopectin. The amylose molecules have a short linear structure which provides a greater surface area for enzymatic reaction, hence assisted in the high production of CD.

Nevertheless, according to Biwer *et al.* [15], the potato starch was preferred in the production of CD even though the percentage of amylopectin in tapioca starch was higher. This is due to the low solubility of the tapioca starch which contained small crystalline structure in the reaction mixture. The crystalline structure does not fully converted into reactive structure hence, leads to low production of CD [16].

The effect of soluble potato starch by the immobilized enzyme on the CD production was compared with the free enzyme. The CD production from the immobilized enzyme was 4.13 mg/mL which was 2.3-fold higher compared to the free enzyme (1.8 mg/mL). This could be due to the high stability of the immobilized enzyme that contributed to the high CD production [17]. A study conducted by Aslan and Tariseven [18] showed that when the β-galactosidase immobilized onto Eupergit beads, the production yield of galactooligosaccharides was higher (24% w/v) compared to the free enzyme. Therefore, it is suggested that the production of the desired product by the immobilized enzyme is preferable than the free enzyme.

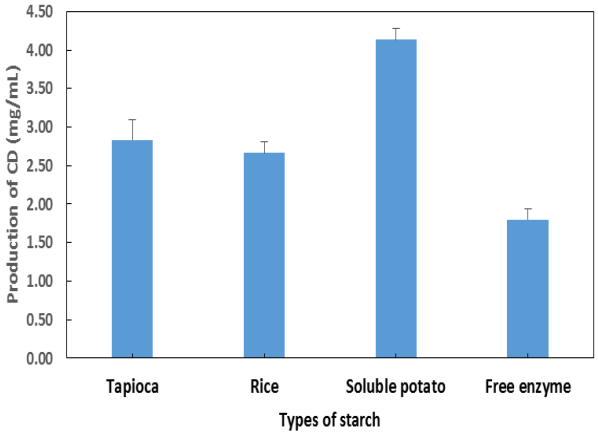

Figure 2 Effect of starch types on the production of CD. The reaction was conducted for 4 h with 100 rpm of agitation

3.3 Effect of Starch Concentration on the CD Production

The effect of substrate concentration on the CD production using different concentrations of soluble potato starch (1, 2, 3, 4 and 5% w/v) is illustrated in Figure 3. As seen in Figure 3, the maximum production of CD (5.22 mg/mL) was obtained when 3% (w/v) substrate was used. However, the CD produced was only 3.25 mg/mL when the starch concentration was 1% (w/v). This phenomenon was due to the high amount of amylopectin contained in the high concentration of soluble potato starch. The high amount of amylopectin molecules reacted with the active site of the enzyme has assist in the high production of CD [6, 7].

Further increased in the soluble potato starch concentration (above 3% w/v) did not resulted in significant increment of the CD production. This occurrence was probably due to the product inhibition whereby, the competition of enzyme active site occurred between the CD and starch molecules during the reaction [19]. Therefore, the production of CD was maintained.

The CD production by the immobilized and free enzyme was compared using the optimal concentration of substrate (3% w/v). The immobilized CGTase recorded higher CD production (5.22 mg/mL) in comparison with the free enzyme (1.8 mg/mL) which was about 2.9-fold increment of CD. As reported by Schöffer *et al*. [20], most of the immobilized enzyme undergo conformational changes which cause alterations in the properties and structure of the enzyme. In the present study, the immobilization of CGTase might attribute to the conformational stability of the enzyme structure. This lead to the reduction of the movement of the CGTase. Hence, preventing the

structural changes and loss of biological activity [21].Therefore, the high production of CD was produced by the immobilized enzyme. A study conducted by Kim *et al*. [22] showed that the production of CD (5.2 mg/mL) by the free enzyme was achieved using 7% (w/v) of starch. However, in the present study, a 57% reduction of starch (3%) was observed when immobilized enzyme was used for the production of CD (5.22 mg/mL). This suggested that the immobilization method is suitable for the production of CD.

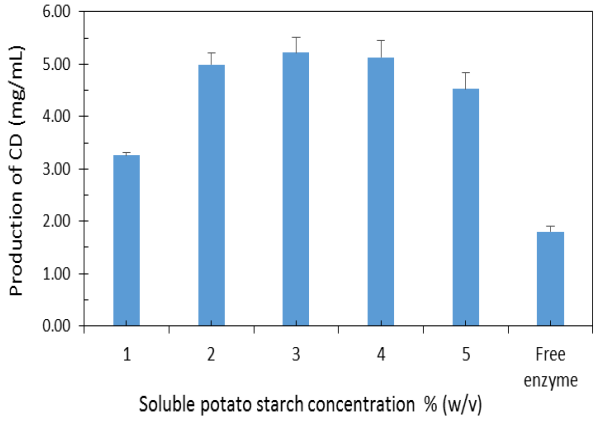

Figure 3 Effect of starch concentration on CD production. The reaction was conducted for 4 h with 100 rpm of agitation using soluble potato starch

3.4 Effect of Temperature on the CD Production

The effect of temperature on the production of CD by the immobilized enzyme by using five different temperatures (20, 30, 40, 50 and 60°C) is presented in Figure 4. The result presented in Figure 4 indicates that the CD production by the immobilized CGTase was improved by increasing the reaction temperature from 20°C to 40°C. The CD production was 3.89 mg/mL at 20°C and 5.21 mg/mL at 40°C. This is because, as the temperature increases, the kinetic energy also increases which resulted in a frequent collision between enzyme and substrate molecules, hence resulted in the high production of CD. However, the production of CD decreased to 4.91 mg/mL at 50°C and 4.72 mg/mL at 60°C. This is due to the enzyme denaturation at high temperature, whereby the substrate molecules unable to bind to the active site of the enzyme which resulted in low CD production [23].

A comparison between immobilized and free enzyme regarding CD production was conducted using the optimal temperature of the immobilized enzyme (40°C). The production of CD by the immobilized enzyme was found to be 5.21 mg/mL which was 1.3-fold higher than the free enzyme (3.97 mg/mL). The difference in CD production between free and immobilized enzyme was probably due to the entrapment of CGTase in the pore of the hollow fiber membrane which created a microenvironment for the immobilized enzyme. This condition protected the immobilized enzyme from thermal denaturation compared to the free enzyme that was exposed directly to the reaction temperature [24]. A study conducted by Charoenlap *et al*. [25] found that the optimum temperature for the production of CD by the free enzyme was 60°C with 1.75 g/L of CD. The optimum temperature was higher compared to the optimum temperature in the present study.

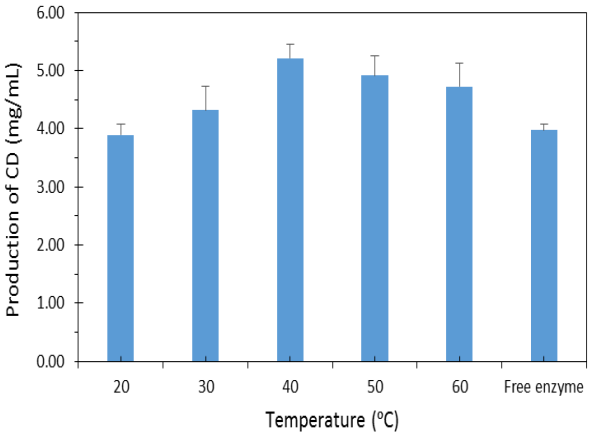

Figure 4 Effect of reaction temperature on CD production. The reaction was conducted for 4 h with 100 rpm of agitation using soluble potato starch

3.5 Effect of pH on the CD Production

The effect of the five different pH on the CD by the immobilized CGTase is shown in Figure 5. The maximum CD produced was detected at pH 6 (4.62 mg/mL), followed by pH 7 (3.66 mg/mL), pH 8 (3.23mg/mL), pH 9 (3.01 mg/mL) and finally pH 5 (2.84 mg/mL). Enzyme is a protein which consists of carboxylic acid and amine functional groups. According to Manas *et al.* [26], during transglycosylation, a pair of carboxylic acids was involved. One of the enzyme residues act as nucleophile while 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 "pK_a cycling". Thus, in order to increase the production of CD, Adjusting the acidic reaction condition was found to be favourable for the enzyme reaction.

In the high alkaline reaction condition, the production of CD was decreased due to the excess of hydroxyl (OH^-) ions in the reaction mixture. The OH^- bind to the amino group (NH_3^+) of the enzyme and prevented the substrate to be placed at the correct orientation of the active site of the enzyme [27]. Besides, according to Gebler *et al.* [28], the high pH would caused deprotonation of amino acid (tyrosine) residue which affected the glycosylation reaction to produce CD. The

optimum pH (pH 6) on the production of CD by the immobilized CGTase was contradicted with the finding by Gawande *et al.* [14]. The study found that the maximum CD production was in neutral reaction condition which was at pH 7.5.

The effect of pH on the CD production by using immobilized enzyme was compared with the free enzyme. As shown in Figure 5, the immobilized enzyme produced 4.62 mg/mL of CD which was 1.7-fold higher compared to the free enzyme (2.71 mg/mL). This was probably due to the attachment of the enzyme to the hollow fiber membrane that prevented the conformation change of the enzyme, hence conserved the active site from denaturation. A study conducted by Ibrahim *et al*. [29] showed that about 8 mg/mL of CD was successfully produced by the free enzyme with the addition of 10 mM calcium chloride ($CaCl_2$) in the reaction mixture. The $CaCl_2$ in the reaction mixture acted as a stabilizer which could enhance the stability of enzyme and increase the production of CD. Interestingly, in the present study, no additional stabilizer was used to enhance the production of CD. By using the immobilization technique, the production of CD was increased.

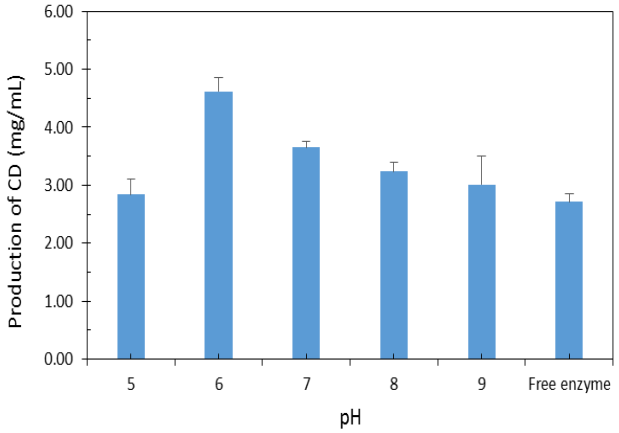

Figure 5 Effect of reaction pH on CD production. The reaction was conducted for 4 h with 100 rpm of agitation using soluble potato starch

4.0 CONCLUSION

The effect of reaction conditions on the synthesis of cyclodextrin (CD) by using immobilized enzyme have been successfully investigated. The results showed that the immobilization of CGTase on the hollow fiber membrane resulted in a the high production of CD with 3-fold higher compared to the free enzyme system. This study also showed that the low concentration of soluble potato starch and high reaction temperature as well as acidic conditions were favourable reaction conditions for the production of CD by the immobilized CGTase.

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