EFFECT OF IMMOBILIZATION PARAMETERS ON THE IMMOBILIZATION OF CYCLODEXTRIN GLUCANOTRANFERASE ON HOLLOW FIBER MEMBRANE

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Graphical abstract

Abstract

Cyclodextrin (CD) is a non-reducing maltooligosaccharides which able to form inclusion complexes with many hydrophobic molecules, changing their physical and chemical properties. With these properties, CD has been discovered to have numerous applications in food industries, pharmaceutical, agricultural and environmental engineering. CD is produced by the enzymatic reaction between cyclodextrin glucanotransferase (CGTase) and starch. Various enzyme immobilization techniques such as adsorption, entrapment, encapsulation and cross-linking have been applied to improve the production of CD. Some of the immobilization parameters such as contact time, agitation rate and pH of the immobilization solution play a vital role in enzyme immobilization process, in order to achieve high production of CD. In the present study, the CGTase from Bacillus licheniformis was immobilized on polyvinylidene difluoride (PVDF) hollow fiber membrane via adsorption technique. The efficiency of enzyme immobilization appears to be affected by various factors (immobilization parameters) such as contact time, agitation rate and pH. Therefore, the effect of contact time (6-72 h), agitation rate (50-250 rpm) and pH (3-10) on the immobilization of CGTase on PVDF hollow fiber membrane were investigated in this study. The immobilized CGTase exhibited the highest immobilization yield of 69.37% under the conditions of 24 h contact time, 100 rpm and pH 7.0. Therefore, the influence of the immobilization parameters during the enzyme immobilization process is vital in order to achieve the high production of CD. Hence, high immobilization yield contributed to the high production of CD which in turn may be beneficial for the industrial purposes.

Keywords: Adsorption, CGTase, hollow fiber membrane, immobilization parameter, cyclodextrin
1.0 INTRODUCTION

Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) is one of α-amylase family that catalyzed four related reactions including cyclization, hydrolysis, disproportionation and coupling of starch [1]. The most important application of CGTase is in the production of cyclodextrin (CD) by cyclization process [2]. The sterically arrangement of glucose monomer in the CD results in the shape of a hollow truncated cone with a hydrophilic surface outside and hydrophobic cavity inside [3]. Because of these features, CDs are able to form inclusion complexes with various guest molecules thus making CDs an attractive inclusion agent for numerous applications in diverse field such as food, pharmaceutical and cosmetic [4].

Since CD is growing popular due to their numerous applications in industries, the study and production using CGTase have been a great interest to researchers. However, in commercialization of CGTase for the industrial purposes, the processes are highly challenging due to the instability of the enzyme, sensitivity to the process conditions and high cost of isolation and purification of the enzyme [5, 6]. The instability of enzyme during the reaction process resulted in the low production of CD. Therefore, enzyme immobilization technique is applied to improve the enzyme stability in order to achieve high production of CD.

Enzyme immobilization is process that involve the attachment of the enzyme to or within the support phase by several methods [7]. Enzyme can be immobilized by a variety of techniques which may be broadly classified as physical and chemical techniques. Physical technique involves physical adsorption, entrapment and microencapsulation. Chemical technique occurs when covalent bonds are formed between the support and enzyme [8]. Entrapment of enzyme onto polymer protects the enzyme by preventing the direct contact between the enzyme and the environment. However, its drawbacks of mass transfer limitation and enzyme leakage were found to be in most studies [9].

Immobilization of enzyme via cross-linking has the major disadvantages of expensive and reduce enzyme catalytic activity due to the toxicity of the reagents [10]. Therefore, adsorption method has been chosen in the present study as it remains as the most attractive method due to its simplicity, inexpensive and does not involve pre-treatment with other reagents.

There are numerous studies of enzyme immobilization by using several types of supports such as alginate [11], chitosan [12], chitin [13], silica [3] and hollow fiber membrane [14] that have been successfully observed. Compared to other supports, hollow fiber membrane offers several distinct advantages such as larger surface area to volume ratio, high mechanical strength, operational durability, lack of toxicity, good thermal property and excellent chemical resistance [15-17]. In addition, the hollow fiber membrane is economically attractive since this membrane is readily available and inexpensive [18]. Moreover, membrane-type system is suitable to be employed for scale-up operation due to its simple and easy operation as well as maintenance [16].

In this study, polyvinylidene fluoride (PVDF) hollow fiber membrane was used for immobilization of CGTase. PVDF hollow fiber membrane that act as hydrophobic membrane and possess both positive (hydrogen ion) and negative charged ion (fluoride ion) give major advantages compared to other support [19]. With these properties, immobilization of enzyme on the support surface can be occurred by electrostatic and hydrophobic interaction whereas, other membrane only possesses electrostatic interaction [20]. Therefore, on the adsorption of CGTase on PVDF hollow fiber membrane, enzyme pre-treatment in immobilization process does not required and toxicity of the reagent can be prevented. Results presented here suggest that immobilization of CGTase on the PVDF hollow fiber membrane is a promising technique to improve production of CD.
The immobilization conditions such as enzyme concentration, temperature, reaction time, pH and agitation rate should be properly studied to enhance the immobilization efficiency and stability of the immobilized enzyme. The effect of time on the enzyme immobilization system has shown to be important for enhancing the immobilization efficiency. A study conducted by Martin et al. [21] reported that, the catalytic efficiency of the immobilized CGTase on Eupergit C was increased with the increase of incubation time from 24 h to 72 h. Then, the CGTase catalytic efficiency remain constant as the incubation time increased from 72 to 120 h.

The agitation rate is one of the important factors in providing an adequate time for the attachment of the enzyme to the matrix. Moreover, the external mass transfer and internal diffusion limitation in enzyme immobilization can be minimize by carrying the process at an optimum agitation rate [22]. A study conducted by Rahim et al. [23] showed that the activity of immobilized α-amylase on calcium alginate-clay beads was increased with the increase of agitation rate from 40 to 120 rpm. However, further increase of agitation rate from 120 to 200 rpm results in decrease of the enzyme activity due to the shear stress that could cause the inactivation of enzyme [24].

The pH of the buffer is another factor that might influence the attachment of enzyme to the support. The effect of pH on the immobilization of CGTase on Eupergit C was investigated by Martin et al. [21]. The optimum binding of CGTase on Eupergit C was achieved at pH 7 with highest catalytic efficiency (7.0%) and 71% of immobilization yield (Martin et al., 2003). Decrease in the CGTase catalytic efficiency was observed at lower pH (pH 4) and higher pH (pH 8). The results showed that the activity of the immobilized enzyme was significantly affected by the pH. Therefore, the aim of this study is to determine the best immobilization conditions for immobilization of CGTase on PVDF hollow fiber membrane. The effect of contact time, agitation rate and pH on the immobilization of CGTase were investigated.

2.2 Immobilization of CGTase

The PVDF membranes were cut into 3 cm and transferred into 9.0 ml of 0.05 M sodium phosphate buffer (pH 6.0) containing 1.0 ml of enzyme. The immobilization process was carried out at 25 °C temperature with gentle orbital shaking of 150 rpm for 24 h incubation. After incubation, the immobilized enzyme was collected and rinsed thoroughly with 0.1 M phosphate buffer (pH 6.0) and immediately stored at 4 °C. CGTase activity in the remaining buffer was further analyzed under standard enzyme assay. The immobilization yield was calculated as in (1).

\[ \text{Immobilization yield (\%) } = \frac{A - B}{A} \times 100 \]  

where A is the initial amount of CGTase before immobilization and B is the amount of CGTase in remaining buffer after immobilization.

2.3 Effect of Immobilization Parameters

2.3.1 Effect of Agitation Rate

The effect of agitation rate on the immobilization of CGTase was studied by incubating the solution for 24 h at various speeds of 50, 100, 150, 200, and 250 rpm [20].

2.3.2 Effect of Contact Time

The effect of time on the immobilization of CGTase on the hollow fiber membrane was determined by incubating the solution at 6, 12, 24, 48, and 72 h based on the study conducted by Ibrahim et al. [3].

2.3.3 Effect of pH

The effect of pH on the enzyme immobilization on hollow fiber membrane was examined from pH 3-10 by using various buffers including phosphate-citrate buffer (pH 3-5), sodium phosphate buffer (pH 6-8) and glycine-NaOH buffer (pH 9-10) as described by Li et al. [25].

2.4 CGTase Assay

CGTase activity was performed by methyl orange method according to the previous study [26]. Briefly, 0.1 mL of enzyme was added into 0.9 mL of 3% (w/v) soluble starch in 0.05 M phosphate buffer (pH 6.0), before incubation at 40 °C for 10 min. The reaction was terminated by adding 1.0 mL of 1.0 M HCl and 0.1 mL of 0.01 M methyl orange. After 20 min of incubation at 16 °C, the amount of α-CD was spectrophotometrically at 507 nm. The activity of α-CGTase shows the amount of enzyme that is able to produce 1 mol of α-CD per min [27].
3.0 RESULTS AND DISCUSSION

The efficiency of the enzyme immobilization appears to be affected by various factors such as temperature, enzyme concentration, pH, agitation rate and contact time. These factors can affect the process of the immobilization, the attachment of enzyme on the support and also the production of desired product. Therefore, the influences of the immobilization parameters in the enzyme immobilization process were investigated in order to achieve the desired immobilized enzyme.

3.1 Effect of Contact Time

Contact time is one of the critical variables that would affect the immobilization of CGTase on the hollow fiber membrane. In this study, the immobilization of CGTase was performed at various contact time ranging from 6 to 72 h. The influence of the contact time on the immobilization of CGTase on the hollow fiber membrane is presented in Figure 1. From the result, it showed that at 6 and 12 h of contact time, there were only 4.94% and 30.52% of enzyme that were successfully immobilized on the hollow fiber membrane, respectively. When the time reached 24 h of contact time, the immobilization yield was significantly increased to 60.95%. The increase of contact time had improved the amount of enzyme interacted to the surface of the support and enhancing the immobilization process. A similar result was observed in the study conducted by Li et al. [25], whereby the immobilization yield of pectinase on the activated agar-gel support was increased with the increased of time from 4 h to 24 h of contact time.

However, another study of pectinase immobilized onto amphiphilic block copolymers showed that the optimum immobilization yield was achieved at only 1 h of contact time [28]. This shows that although the immobilization process may be very rapid in some research, multipoint interaction between the enzyme and the support surface is a time dependent process [29]. Immobilization process required correct alignment of groups located on the enzyme and the support surface, otherwise the adsorption will not occur [30]. The results may varies depending on the type of enzyme and support been used.

Therefore, the immobilization of CGTase on the hollow fiber membrane in the present study required 24 h of contact time to achieve a maximum immobilization yield. As shown in Figure 1, as the contact time increased to 72 h, the immobilization yield was significantly decreased to 19.17%. This is due to the detachment of enzyme from the support surface. According to Pang et al. [31], during the physical adsorption, detachment of enzyme from the support can be occurred when it was immersed in the immobilization solution for a long period of time.

3.2 Effect of Agitation Rate

The immobilization of CGTase was carried out at a constant temperature of 25 °C with different agitation rate of 50, 100, 150, 200 and 250 rpm to determine the optimal agitation rate for the immobilization process. Results in Figure 2 illustrated that the low immobilization yield of 45.11% was recorded at 50 rpm compared to 69.74% of immobilization yield at 100 rpm. Low immobilization yield at 50 rpm of agitation rate can be correlated with the non-uniform distribution of the enzyme in the immobilization mixture during the adsorption process [32].

![Figure 1](image1.png)

**Figure 1** Effect of contact time on the CGTase immobilization on PVDF membrane

![Figure 2](image2.png)

**Figure 2** Effect of agitation rate on the immobilization yield of CGTase on PVDF membrane

As the agitation rate increased from 50 to 100 rpm, increased of immobilization yield by 25% was observed. This phenomenon was due to the higher shear values at higher agitation rate. According to Man et al. [20], at high shear values, the enzyme was pulled closer to the support and attributed to the increased of electrostatic and hydrophobic interaction. Moreover, when the mixture was agitated reasonably, more surface area of the
support was exposed to the enzyme and allowing more enzyme to interact with the hollow fiber membrane.

However, too vigorous agitation rate had significantly decreased the CGTase immobilization yield. More than 38% reduction in CGTase immobilization yield was recorded when the agitation rate was increased to 250 rpm (31.01%) and followed by 33.18% at 200 rpm. The reduction of immobilization yield was correlated with the detachment of the immobilized CGTase from the support surface due to the shear forces created by the vigorous shaking. As shown in Table 1, high agitation rate resulted in the increase of the detachment of the enzyme from hollow fiber membrane into the mixture.

Table 1 Detachment of the enzyme at various agitation rates

| Agitation rate (rpm) | Detachment of CGTase (U/ml) |
|----------------------|-----------------------------|
| 50                   | 48.30                       |
| 100                  | 33.60                       |
| 150                  | 55.10                       |
| 200                  | 80.40                       |
| 250                  | 95.44                       |

The highest detachment of enzyme recorded was 95.4 U/ml at 250 rpm and 80.4 U/ml at 200 rpm, which indicated that a vigorous agitation rate would increase the detachment of the enzyme from the support. This phenomenon had also been observed on the study of the immobilized xylanase on activated alginate beads by covalent attachment whereby the reduction of the immobilization yield was observed when increased of agitation rate from 50 rpm (51.4%) to 250 rpm (49.6%) [33]. This study demonstrated that the enzyme tends to detach easily from the support surface due to the high shear stress caused by the increase of the enzyme and support collision at higher agitation rate.

3.3 Effect of pH

Immobilization of CGTase on hollow fiber membrane at different pH values was investigated to determine the optimum pH at which the enzyme-support interaction was favored. The optimum attachment of the CGTase on the hollow fiber membrane was achieved at pH 7.0 with 69.37% of the immobilization yield followed by pH 6.0 with 58.2% of immobilization yield as shown in Figure 3. These results could be explained by the charging phenomenon at the surface of the enzyme which is pH-dependent. The highest immobilization yield was observed at pH 7.0, which is close to the isoelectric point of CGTase (pI 6.5) [34]. This finding was supported by Ajitha & Sugunan [35], whereby the maximum adsorption of α-amylase on mesoporous sieve was occurred at pH 6.0 which is near to the isoelectric point of a-amylase (pI 4.5).

From Figure 3, it is clearly shown that the immobilization efficiency was significantly affected by the changes of pH. The immobilization of CGTase on the hollow fiber membrane showed a decreased in immobilization yield when incubated in more acidic (pH 3.0 – 5.0) and more alkaline (pH 8.0 – 10.0). This reduction of enzyme immobilization yield could be explained by the electrostatic repulsion effects between the same charged groups on the enzyme and the support surface. Generally, hydrophobic interaction should not be affected by the pH of the immobilization system [36]. However, adsorption processes which also govern by the electrostatic interaction are very pH-dependent. The slight changes of the pH of the immobilization system would affect the total net charge of the enzyme and the support that may resulted in the repulsion between the enzyme and the support.

According to Sun et al. [37], the enzyme environmental pH (pH of immobilization mixture) would influence the attachment of the enzyme on the support by altering their charges. Enzymes are amphoteric molecules that contain both basic [OH⁻] and acid [H⁺] groups that are located on their surface and would determine the total net charge of the enzyme. Therefore, as the total net charge of the enzyme was disrupted by the extreme pH value in the immobilization solution, the electrostatic repulsion between enzyme and the support were increased and the immobilization yield would decrease. A similar result was recorded by Wang et al. [38] on the immobilization of glucoamylase on magnetic chelator particles by adsorption technique. In the study, decreased in the enzyme immobilization efficiency was also recorded in the more acidic (pH 6.0-7.0) and more alkaline (pH 3.0-4.0) conditions, which correlated by the electrostatic repulsion between the support and the enzyme. The maximum immobilization efficiency of glucoamylase was recorded at pH 5.5 which was near to the enzyme isoelectric point (pl 4.5).
4.0 CONCLUSION

In conclusion, adsorption which majorly involved physical attachment of enzyme on the support is one of the simple immobilization techniques. The effect of contact time on the immobilization yield can be observed by two phases whereby at first phase, the immobilization yield was increased from 4.94% to 60.95% with the increased of contact time (6-24 h) and in the second phase, the immobilization yield was decreased from 32.9% to 19.17% as the contact time was further increase (48-72 h). This showed that at the second phase, desorption of the enzyme might be occurred. Apart from that, moderate agitation rate of 100 rpm is important to minimize the high shear force at high agitation rate, detachment of the enzyme from the support and also damage of the support. Moreover, physical adsorption usually involved electrostatic interaction that varying due to the immobilization conditions such as pH of the immobilization solution. Therefore, near to the neutral pH (pH 7.0) promoted a better adsorption of CGTase on the hollow fiber membrane due to the effective electrostatic interaction. As the result the interaction between the CGTase and the hollow fiber membrane was enhanced and more enzyme molecules were attracted and absorbed on the support. Thus, this study showed that the efficiency of the enzyme immobilization process is highly related with the immobilization conditions. In addition, immobilization conditions for the enzyme immobilization process are varying depending on the type of enzyme and method used. The results described herein are of considerable interest in the possible application of the immobilized enzyme.

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