Stability and reusability of cyclodextrin glucanotransferase immobilized on hollow fiber membrane

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Abstract. Cyclodextrin glucanotransferase (CGTase) is a starch degrading enzyme to produce cyclodextrin (CD), which can encapsulate compounds to remove flavors and aroma. However, the use of the enzyme is often limited due to the instability of the enzyme resulting in low production of CD. Immobilization technique has greatly improved the characteristics of the enzyme during the reaction. In this study, CGTase was immobilized on polyvinylidene difluoride (PVDF) hollow fiber membrane by adsorption method. The stability and reusability of the immobilized CGTase were studied and compared with free form of the CGTase. Thermal stability of the immobilized CGTase able to retain 50% of the initial residual activity at temperature up to 70°C. While there was an improvement in pH stability with a wider pH range from pH 5 to pH 8. The reusability of the immobilized CGTase was able to retain up to 40% of the initial CD production after repeatedly used for 10 cycles. Hence, the immobilization of CGTase shows a good physical and chemical resistance, which may be advantageous to be applied in industry.

1. Introduction
Cyclodextrin glucanotransferase is a type of enzyme that belongs to amylase family [1]. The CGTase has the capability to catalyze glycosidic bonds and due to the amylolytic in nature, the CGTase is able to convert starch into cyclodextrin (CD) [2-3]. The CD can be divided into three types: α, β and γ-CD that contain six, seven and eight glucose residues, respectively. The CD is a cyclic oligosaccharide, which consists of hydrophilic outer surface and hydrophobic inner surface. With this properties, the CD can form an inclusion complex with guest molecules to minimize the side effect of drug formulation and to stabilize volatile substances [4]. Apart from food and pharmaceutical industry, the CD also has been widely used in cosmetic, chemical and agricultural industry [5-7]. Due to this, the demand of the CD has increased about 20 to 30% per annum [8].

In industrial application, the CD is generally produced in batch system with free CGTase used directly in the reaction mixture. This often leads to problem such as low stability of the enzyme during the enzymatic reaction [9]. Besides, long time process and high cost of separation between the enzyme and product are the main drawback in the production of CD [10]. Hence, the enzyme immobilization technique is introduced as an alternative to improve the enzymatic properties. This strategy can enhance the enzyme stability and allows the enzyme to be easily recovered and repeatedly used which
makes the process more economically feasible. The enzyme has been immobilized by using various
 technique such as adsorption [11], entrapment [12], covalent binding [13] and cross-linking [14].
 Among the immobilization technique proposed, adsorption is widely used due to the useful physical
 interactions created between the enzyme and support that does not require chemical alteration. Other
 than that, this technique is relatively simple and inexpensive [15-16].

 Different support offers different physical and chemical properties that can affect the enzyme
 immobilization and its catalytic properties. Thus, the characteristic of the support is important in
determining the performance of the enzyme immobilization. For example, immobilized enzyme on
 nanoporous materials showed remarkably high enzyme loading and enzyme recovery [17]. However,
the nonporous materials usually limit the substrate diffusion due to the entrapment of the enzyme in
the porous support [18]. Other than that, the used of carrageenan as a support in enzyme
immobilization was proven to be ineffective due to weak mechanical stability resulting in enzyme
leakage even though the support is inexpensive [19].

 In the present study, the polyvinylidene difluoride (PVDF) membrane was employed for the
CGTase immobilization. The hollow fiber membrane has a high surface to volume ratio, high
mechanical strength and high operational durability [20]. Besides, the hollow fiber membrane shows a
great chemical resistance, lack of toxicity and also biodegradable [21]. Most studies for the
immobilization of CGTase thus far have focused on the attachment to commercial resin [22];
entrapment on the sodium alginate beads and agarose [22-23] or covalent binding on magnetic carrier
and Eupergit C [24-25]. To this date, the immobilized CGTase on hollow fiber membrane by
adsorption has not been studied. Therefore, the stability and reusability of the immobilized CGTase to
enhance the production of CD was examined. The results presented here suggested that the
immobilization method is a promising approach for CD production with high CGTase stability.

2. Materials and method

2.1. Materials
Polyninylidene difluoride (PVDF) hollow fiber membrane was synthesized in Faculty of Chemical and
Natural Resources Engineering laboratory, Universiti Malaysia Pahang. Commercial CGTase from
Bacillus licheniformis (Toruzyme 3.0) was purchased from Novozymes A/S (Bagsvaerd, Denmark).
Standard α-cyclodextrin (98%) was purchased from Next Gene Scientific Sdn Bhd (Selangor,
Malaysia). Glycine and soluble potato starch were purchased from Chemolab Supplies Sdn Bhd
(Selangor, Malaysia). Methyl orange, hydrochloric acid (37%) and acetonitrile HPLC grade were
purchased from Merck Sdn Bhd (Selangor, Malaysia).

2.2. Immobilization of CGTase
PVDF membrane was cut into 3 cm and transferred into solution containing 1 mL of enzyme and 10
mL of 0.05 M of sodium phosphate buffer (pH6). The sample was incubated at 25°C for 24 h with
agitation of 100 rpm. Then, the membrane was collected and washed thoroughly with sodium
phosphate buffer to eliminate the non-immobilized enzyme.

2.3. Thermal stability of the immobilized and free CGTase
The thermal stability of the immobilized CGTase was determined at various temperatures of 30, 40,
50, 60, 70 and 80°C. The immobilized CGTase was transferred into two different test tube containing
0.05M sodium phosphate buffer (pH 6). The test tubes containing immobilized CGTase was immersed
in water bath for 30 min. Then, CGTase assay was conducted to determine the residual activity of the
enzyme.

Thermal stability for free enzyme was compared with the immobilized enzyme. About 0.3 mL of
free enzyme undergoes similar condition applied in the immobilized enzyme.

2.4. pH stability of the immobilized and free CGTase
The pH stability of the immobilized and free CGTase was investigated at pH 4, 5, 6, 7, 8 and 9. The buffer solutions used were citrate phosphate (pH 4 and pH 5), sodium phosphate (pH 6 and pH 7) and glycine-NaOH (pH 8 and pH 9). The test tube containing immobilized CGTase was immersed in water bath (40°C) for 30 min. Then, CGTase assay was conducted to determine the residual activity of the enzyme.

pH stability for free enzyme was compared with the immobilized enzyme. About 0.3 mL of the free enzyme undergoes the similar condition applied in the immobilized enzyme.

2.5. Reusability of the immobilized CGTase

The reusability of the immobilized CGTase was evaluated using different cycles. The immobilized enzyme was incubated in 20 mL of 3% w/v of soluble potato starch with 0.05M sodium phosphate buffer (pH 6). The reaction was performed at temperature of 40°C with 150 rpm of agitation rate for 1h. After 1 h, the immobilized enzyme was separated from the reaction mixture and rinsed with 0.05M sodium phosphate buffer to eliminate any unwanted substrate or product remained on the hollow fiber membrane. Then, the immobilized CGTase was re-suspended again in a newly prepared reaction mixture for the next new cycle. The amount of CD produced were determined after every cycle. The percentage of reduction was determined by equation (1).

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\text{Retained activity (\%) = } \frac{C_o}{C_i} \times 100\%
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whereby \(C_o\) is the concentration of CD after each cycle and \(C_i\) is the highest concentration of CD. All experiments were conducted in triplicate.

2.6. Analytical analysis

2.6.1. CGTase assay. A 0.1mL of enzyme was added into 0.9mL of 3% (w/v) soluble potato starch in 0.05M of sodium phosphate buffer solution (pH 6). The reaction mixture was set at 40°C for 10 min. The reaction was terminated immediately by addition of 1mL of HCl (1.0M) and 0.1mL of methyl orange (0.01M). The mixture was then transferred into water bath at 16°C for 20 min. The absorbance was determined using UV vis spectroscopy at 505nm. Single unit of enzyme was described as the amount enzyme that produced 1 micromol of α-CD per min under standard conditions [26].

2.6.2. High Performance Liquid Chromatography (HPLC). High Performance Liquid Chromatography was used to determine the production of CD as described by Sakinah et al. [27] with some modification. The column used in this study was Agilent Eclipse Plus C18 and a mixture of acetonitrile: water (60:40) was used as the mobile phase with flowrate of 1 mL/min. Reflective index detector (RID) was used to detect the CD. Samples were centrifuged for 10 min at 5000 rpm. The sample was then filtered using Whatman® nylon membrane.

3. Results and discussion

3.1. Thermal stability of immobilized and free CGTase

High thermal stability is one of the most important criteria for the immobilized enzyme. In the present study, the thermal stability of free and immobilized CGTase were conducted by varying the incubation temperature ranging from 40°C to 90°C. Figure 1 reveals that both free and immobilized CGTase were positively stable at temperature up to 60°C. Nevertheless, the immobilized CGTase was significantly more resistant to heat treatment at higher temperature (70°C to 90°C), in comparison with the free CGTase. A significant different of heat resistance by the immobilized CGTase was clearly showed at temperature 70°C. The immobilized CGTase was able to remain active with 50% of the residual activity at 70°C while the free CGTase was only about 30%. Meanwhile, at temperature 80°C and 90°C the immobilized CGTase managed to retain 15% and 8% of activity, respectively.
The result in this study indicated that the thermal stability of the CGTase was enhanced by the immobilization method. This could be due to the rigidity of the enzyme structure and preservation of the active site from denaturation upon immobilization. Thus, the binding of the CGTase on the membrane would limit the movement of the enzyme molecules at various temperatures. However, for the free CGTase system, the enzyme molecules would gain more kinetic energy resulting in rigorous movement and eventually leads to enzyme inactivation [28]. A research performed by Chiou et al. [29] found that lipase from *Candida rugose* immobilized on chitosan was thermally stable up to 40°C, while the free lipase was stable at 30°C.

![Figure 1](image1.png)

**Figure 1.** Comparison of thermal stability of free and immobilized CGTase. The residual activity was measured after incubated at different temperature for 30 min.

A similar finding was reported previously in the immobilization of CGTase on magnetic carrier whereby, there was a significant loss recorded by the free CGTase (95%) compared to the immobilized CGTase (50%) at 80°C [30]. Therefore, the improvement of the thermal stability of the immobilized CGTase might be advantageous in the industrial application, which required high reaction temperature [26].

### 3.2. pH stability of immobilized and free CGTase

pH stability also should be taken into consideration in order to determine the enzymatic properties of the immobilized CGTase. The pH stability of the enzyme in this study was investigated by varying the pH ranging from pH 4 to 10. As shown in Figure 2, the maximum stability achieved by the free CGTase was from pH 6 to pH 8. Interestingly, the immobilized CGTase showed a wider pH stability which was from pH 5 to pH 8. The different of the enzyme activity between the free and immobilized CGTase was significant especially at pH 5. The free enzyme has lost about 60% of the initial activity while the immobilized enzyme has loss only 20% of the initial activity. The present study showed that the immobilized CGTase has a higher stability in wider pH range which able to produce higher amount of CD in comparison with the free CGTase.

The immobilized enzyme has a higher stability in different pH value was due to the ability to exist in stable conformation and preservation of the enzyme structure from inactivation. According to Manas *et al.* [31], acidic reaction condition was found to be favourable for the enzymatic reaction. Thus, the ability of the immobilized CGTase to remain active in acidic condition would be beneficial in order to improve the production of CD.
Figure 2. Comparison of pH stability for free and immobilized CGTase. The residual activity for was measured after incubated at different pH value for 30 min.

The finding in the present study was consistent with a research done by Abdel-Naby et al. [32] whereby the immobilized CGTase on Duolite XAD 761 resin managed to retain high activity at wider acidic range (pH 4 to 6.8) compared to the free enzyme (pH 5.5 to 6.5). This was due to the cationic characteristic of the resin as a support which resulted in the stabilization of the immobilized enzyme in acidic medium. However, the result showed in the present study was in contrast with a study conducted by Kim et al. [22], whereby there was no significant changes in pH stability (pH 4 to pH 8) between the free and immobilized CGTase on Amberlite IRA-900. The insignificant changes in pH stability after the immobilization process was probably due to the support which unsuccessfully change the microenvironment of the enzyme, subsequently resulted in enzyme denaturation [33].

3.3. Reusability of the immobilized CGTase

For industrial application, the cost of enzyme and downstream process can be reduced greatly if the enzyme can be reused for several times. To ensure a cost-effective production, the reusability of immobilized CGTase was studied by determining the amount of CD produced. The amount of CD in the first batch was taken as a reference (100 percentage of reduction). Based on Figure 3, the immobilized CGTase in the present study was capable to retain up to 50% and 40% of the initial CD production when repeatedly used for fifth and tenth successive batch reactions, respectively. A total of 26 mg/mL of CD was successfully produced by the immobilized CGTase in the 10 cycles.

Figure 3. Reusability of immobilized CGTase on hollow fiber membrane in repeated batch reaction. CD production was measured for every cycle (1 h reaction time) until 10 cycles.
In addition, as the number of cycle increases, the reduction percentage of the immobilized CGTase also decreases. This phenomenon was due to the desorption of the immobilized enzyme upon washing in each cycle [34]. The finding in the present study was consistent with the findings by Ivanova [31] who investigated the reusability of immobilized CGTase on magnetic carrier treated with polyethyleneimine (PEI). In the study, the immobilized CGTase undergoes repeated batch reaction for 7 cycles. Based on the result, the production of CD decreased as the number of cycle increased. Similar finding was also portrayed by different enzyme such as a study conducted by Hung et al. [35] who studied on the reusability of immobilized lipase on chitosan for the production of p-nitrophenol. The results clearly showed decrement of p-nitrophenol in the 10 cycles which concluded that the immobilized enzyme often contributed to the decrease in enzymatic activity after repeatedly used for several times.

The ability of the immobilized CGTase to retain up to 40% of the reduction suggested that the CGTase was stable and remains active throughout the 10 cycles. Interestingly, the present study recorded relatively higher stability compared to the CGTase immobilized on nanofiber activated with dodecanediamine derivative, where 40% of initial activity was maintained after only 7 cycles [36]. Therefore, the reusability of the immobilized CGTase presented in the present study might applicable for industrial scale since it can be reused with high enzyme stability.

4. Conclusion
The immobilization of CGTase on hollow fiber membrane has been successfully conducted via adsorption method without any chemical alteration. The results showed an improvement in resistance against thermal and pH denaturation compared to the free CGTase. Besides, the immobilized CGTase can be used repeatedly with high enzyme stability for the production of CD. This indicated that the enzyme immobilization using PVDF hollow fiber membrane as a support is a promising strategy and is capable to be applied in scale-up process.

Acknowledgment
This research was funded by Universiti Malaysia Pahang (Grant No: PGRS 170396). The authors also would like to acknowledge Faculty of Chemical and Natural Resources Engineering for the facilities.

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