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Bleached kenaf microfiber as a support matrix for cyclodextrin glucanotransferase immobilization *via* covalent binding by different coupling agents

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ABSTRACT

Enzyme immobilization *via* covalent binding provides a strong interaction between enzyme and support material. In this study, the effect of different coupling agents (spacer arms and ligands) in cyclodextrin glucanotransferase (CGTase) immobilization on bleached kenaf microfiber as a support matrix was investigated. The immobilized CGTase properties such as storage stability, thermal stability and reusability were evaluated. Immobilized CGTases on microfiber resulted in 0.162–0.24 U/mg-fiber when 55.6 U/mL of CGTase activity was initially added during the immobilization. The highest storage stability (60 °C) was shown by CGTase that was immobilized with ethylenediamine and o-phthalaldehyde, whereby 60% of its activity remained after 15 days. Its high stability was also confirmed by the lowest deactivation constant, k_d that was obtained at 25 °C (0.0161 day^{-1}) and 60 °C (0.0361 day^{-1}). The CGTase immobilized using ethylenediamine and glutaraldehyde has shown the best retention of enzyme activity up to 72.72% after 12 cycles of batch reaction. The results indicate that kenaf microfiber has potential to be applied as a support for enzyme immobilization and its enzymatic properties were affected by the coupling agents.

1. Introduction

Enzymes are biocatalysts that boost up the rate of chemical reactions by reacting with substrates at their active site. Cyclodextrin glucanotransferase, CGTase (E.C.2.4.1.19) is an important enzyme in converting long complex chain of starch into various kinds of cyclodextrins (CDs) *via* cyclization reaction, which is also called as intramolecular transglycosylation reaction [1,2]. In cyclization reaction, a linear oligosaccharide will attach to the CGTase active site pocket. This oligosaccharide consisted of glucose units, linked by α-(1,4) glycosidic bond. The enzyme will react on non-reducing end glucose molecule on the oligosaccharide, to form a cyclic oligosaccharide, which is also called CDs [2,3]. Besides cyclization, CGTase can also catalyze another 3 reactions which are disproportionation, hydrolysis and coupling [4,5]. CGTases are produced by several types of bacteria but mainly by the bacteria of the genus *Bacillus* with the parameters that can affect its production the most are pH and temperature [6].

CGTase produces 3 main types of CD, which are α-, β-, and γ-CD with a trace amount of larger CDs [7]. α-CD is a cyclic-maltooligosaccharide that consists of 6 D-glucose units that are linked by α-(1,4) glycosidic bonds, whereas β- and γ- CD consist of 7 and 8 D-glucose units, respectively [2,8,9]. The CDs produced have a shape of a hollow truncated cone whose outer layer shows good hydrophilicity, whereas the internal cavity being hydrophobic. This allows CDs to form inclusion complexes with many kinds of guest molecule, which possess hydrophobic nature [10]. Due to this special characteristic, CDs have been widely used and applied in many fields such as in pharmaceuticals, instrumental analysis and cosmetics [11–13].

In comparison with free CGTase, the immobilized CGTase allows continuous and repeated operation [14]. In addition, enzyme immobilization also simplifies the handling of the enzyme as it is mostly used as solid rather than in solution form. Besides that, the immobilization also enables easier and better separation of the enzyme from the products and thus preventing contamination of the product by the enzyme [15]. Other than these, the physical and chemical properties of the enzymes such as 3D conformational structure, enzyme stabilities, kinetic properties, specificity, selectivity and resistance to inhibition are also improved usually upon immobilization [16].

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There are several immobilization methods available [17] and one of the popular methods is by covalent binding, which it provides rigid bonds between enzyme and support, as well as it minimizes enzyme leaching after repeated use, compared to other immobilization methods [18]. The immobilized enzymes are very likely to retain its structure and have better performance than free enzymes under drastic conditions. For example, higher optimal temperature and some distorting reagent may significantly decrease free enzyme activity while inducing a lesser effect on the activity of immobilized enzymes [19]. Therefore, the use of stabilizing agent to prevent bond breakage after immobilization is often not required [20].

Different immobilization strategies are used to serve certain purposes and functions such as enzyme rigidification, prevention of enzyme aggregation and prevention of diffusional problems. Generally in covalent binding, most of the strategy involves is activation of the functional group that presents on the support matrix [21,22]. The activated functional group will then react with the functional group presents on the enzyme (*e.g.* amine group) [22]. Selection of proper coupling agents during immobilization is of utmost importance as the binding efficiency and the properties of the immobilized enzyme are also affected by distance between enzyme and support [23]. When compared to direct immobilization of enzyme on any support (*e.g.* adsorption), the use of coupling agents that act as spacer arms and ligands often provide better performance of the immobilized enzyme such as specific activity and recovered activity [24,25]. This is because spacer arm increases the distance between support and enzyme molecule and subsequently minimize the effect of steric hindrance and diffusional resistance [23]. Meanwhile, the use of ligands can help increase the efficiency of enzyme molecules to attach on end-terminal of ligand-spacer arm-support interaction. Several previous studies proved that suitable selection of chemical coupling would effect to the properties of immobilized enzyme especially in enzyme activity, stability and reusability [26–28]. In facts, covalent immobilization of enzyme could form an intense multipoint covalent binding on support, which provide a rigidity toward immobilized enzyme structure and enhance the stability, compared to the single point binding [23].

Selecting suitable support materials is also importance for enzyme immobilization purpose. Parameters such as mechanical resistance, pore diameter and specific surface area are need to be considered as a suitable support material [29]. All these parameters can affect the properties of the immobilized enzymes. Previous studies were reported for support matrix in CGTase immobilization such as Eupergit C [30], PVC [31], silica microspheres [32], Accurel MP 1000 [33], divinyl sulphone-agarose [34], core-shell polymeric support [35], poly-styrene-divinylbenzene matrices [36] and glyoxal-agarose beads [37]. Lignocellulosic fiber is expected to have high potential to be used as support for enzyme immobilization. For example, it has been studied by Souza et al. [38], where cashew apple bagasse fiber was used as support material in the immobilization of lipase B from *Candida Antarctica,* resulting an excellent performance in storage stability and reusability of the enzyme.

In this study, bleached kenaf bast microfiber extracted from kenaf bast was used in covalent immobilization. The selection of bleached kenaf bast microfiber is due to its high potential for future applications in industrial biotechnology [22,39]. CGTase from *Bacillus macerans* that mainly produces α-CD [40–42] was immobilized on the bleached kenaf microfiber by using various coupling agents such as hexamethylenediamine (HMDA) and ethylenediamine (EDA) as spacer arms, whereas glutaraldehyde (GA) and o-phthalaldehyde (OPA) as ligands. The properties of the immobilized CGTase with different coupling agents such as reusability and storage stability were then investigated in this study.

2. Materials and methods

2.1. Materials

Kenaf bast fiber was supplied by the Lembaga Kenaf dan Tembakau Negara, Malaysia. CGTase from *Bacillus macerans* with activity of ~600 U/mL (~4 mg protein/mL) was purchased from Amano Enzyme Inc. (Japan). HMDA, EDA and OPA were bought from Sigma Aldrich (Malaysia). Water soluble potato starch powder was bought from Fluka (Switzerland). GA 25% was bought from Ajax Finechem (Australia) while sodium chlorite and α-CD were bought from Acros Organics (USA). Other chemicals used in this research were reagent grade.

2.2. Bleaching of kenaf bast fiber

Kenaf bast fiber was firstly cut into approximately 5 cm in length. Then, the fiber was bleached and mercerized according to the method described by Tee et al. [43]. 20 g fiber was immersed in 640 mL of heated water (70 °C) and then 4 mL of CH_3COOH and 8 g of $NaClO_2$ were added. In each subsequent hour, the same amount of CH_3COOH and $NaClO_2$ were added to the solution, stirred and left to bleach the fiber in the beaker. This was repeated for 5 h. The delignified holocellulose was then washed and filtered with distilled water until the filtrate become colorless.

The obtained holocellulose was then immersed in 500 mL of 5% w/v NaOH solution at room temperature for 2 h. After that, the brown solution formed was filtered and 500 mL of diluted CH_3COOH (1.47% w/v) was added to neutralize the fiber. The mixture was left to settle for 5 mins. This was followed by rinsing the fiber with distilled water and then filtration. This washing process was repeated until the pH become neutralized (pH 7.0). The bleached kenaf fiber was finally dried in the conventional oven at 105 °C for 24 h.

2.3. Support activation

Two types of spacer arm were used in this study; HMDA and EDA. The coupling of bleached kenaf microfiber with HMDA was done according to the method described by Chang and Shaw [44]. Similar method was repeated when EDA was used. Then, the fiber that had already been coupled with the spacer arm was activated by ligands, which were GA and OPA. To activate the fiber with GA, 1 g of the fiber-spacer arm was immersed into 20 mL of GA (0.05 M) in phosphate buffer (0.1 M, pH 8.0). The mixture was stirred at 4 °C for 12 h. After that, the activated support was washed with distilled water and stored at 4 °C before further use. For OPA activation, 1 g of the fiber-spacer arm was immersed into 20 mL of OPA (0.05 M) in ethanol and the mixture was stirred for 6 h at room temperature.

2.4. Surface morphology characterization

The morphological changes of kenaf microfiber before and after covalent coupling with spacer and ligand were observed by Hitachi S3400N SEM at 5 kV accelerated voltage. Carbon coating was applied to each sample prior to the observation under SEM.

2.5. CGTase immobilization

CGTase with different activities, 13.9, 27.8, 41.7 and 55.6 U/mL (with protein concentration of 0.18 ± 0.02, 0.36 ± 0.034, 0.54 ± 0.06, 0.72 ± 0.08 mg/mL, respectively) were prepared separately by dilution of enzyme stock solution with phosphate

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