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Study on the Preparation of Cellulose Nanofibre (CNF) from Kenaf Bast Fibre for Enzyme Immobilization Application

(Kajian terhadap Penyediaan Nano-serabut Selulosa (CNF) daripada Serabut Kulit Kenaf untuk Aplikasi Pemegunan Enzim)

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ABSTRACT

This paper discussed on the preparation of natural CNF from kenaf bast fibre for the application as a support structure in enzyme immobilization. The treatments involved for this preparation were delignification, bleaching and high-intensity ultra-sonication process to obtain nanofibre with high cellulose content and less than 100 nm diameter. Chemical composition analysis showed the influence of each process treatment on cellulose content of raw bast fibre, bleached pulp fibre and CNF(63.67, 81.12 and 91.97%, respectively). By increasing the cellulose content and decreasing the size of cellulose fibre, it resulted in a greater number of –OH functional group on its surface that plays as important role in enzyme immobilization. FTIR spectroscopy confirms that the removal of lignin and hemicellulose from the fibre after the treatments, as well as its interaction with coupling agents and CGTase enzyme. About 62.10% of enzyme loading and 45.62% of its activity yield were obtained after immobilization. Enzymatic reaction of immobilized CGTase on CNF indicates about more than 60% relative production yield of α-CD was achieved and its reusability was able to retain about 67.0% from its initial activity after 8 cycles of reaction. Therefore, the CNF is a good potential as a support for enzyme immobilization.

Keywords: Cellulose nanofibre (CNF); covalent immobilization; cyclodextringlucanotransferase (CGTase); kenaf

ABSTRAK

Kertas ini membincangkan penyediaan CNF semula jadi daripada serabut kulit kenaf untuk aplikasi sebagai struktur sokongan dalam pemegunan enzim. Rawatan yang terlibat dalam penyediaan ini ialah delignasi, pelunturan dan proses ultrasonikasi berkeamatan tinggi untuk memperoleh nano-serabut dengan kandungan selulosa yang tinggi dan berdiameter kurang daripada 100 nm. Analisis komposisi kimia menunjukkan kesan akibat daripada proses rawatan terhadap kandungan selulosa pada serabut kulit mentah, serabut pulpa terluntur dan CNF (masing-masing adalah 63.67, 81.12 dan 91.97%). Dengan peningkatan kandungan selulosa dan pengurangan saiz serabut selulosa, ia menghasilkan lebih banyak kumpulan berfungsi –OH pada permukaannya yang memainkan peranan penting dalam pemegunan enzim. FTIR spektroskopi mengesahkan penyingkiran lignin dan hemiselulosa daripada serabut selepas proses rawatan tersebut serta interaksinya dengan agen perhubungan dan enzim CGTase. Sebanyak 62.10% muatan enzim dan 45.62% hasilan aktiviti diperoleh selepas pemegunan. Tindak balas enzim CGTase terpegun pada CNF menunjukkan lebih daripada 60% hasil pengeluaran relatif α-CD dicapai dan penggunaan semulanya dapat mengekalkan sebanyak 67.0% daripada aktiviti awal selepas 8 kitaran tindak balas. Oleh itu, CNF berpotensi baik sebagai penyokong untuk pemegunan enzim.

Kata kunci: Kenaf; nano-serabut selulosa (CNF); pemegunan kovalen; siklodekrin glukanotransferase (CGTase)

INTRODUCTION

Kenaf (*Hibiscus cannabinus* L.*)* is one of the non-wood lignocellulosic fibres, which presents numerous advantages such as low specific gravity, low cost, good mechanical properties, better chemical characteristic, abrasiveness, availability and biodegradability (Li et al. 2000). Lignin, hemicellulose and cellulose are major components in lignocellulosic fibres. Cellulose is a linear polymer that consists of both crystalline and amorphous region, while hemicellulose and lignin display a complete amorphous structure (Brinchi et al. 2013; Jonoobi et al. 2009). Cellulose fibre exhibits a unique structural hierarchy which composed of individual nano-sized fibre (Siró & Plackett 2010). Immobilization of enzyme is one of the effective methods to improve the operational stability and reusability of enzymes. The stripping down from macro- to nano-sized fibre using a combination of chemical-mechanical treatments can improve the surface morphology of support, which contribute to a better enzymatic performance of immobilized enzyme, since enzyme is already in nanomolecules size. The presence of CNF support in enzyme immobilization can improve the enzyme loading due to large surface area per volume ratio (Kim at al. 2006). The covalent immobilization of CGTase

enzyme onto CNF support has a potential to form the most stable binding and able to minimize any conformation due to multipoint interaction (Sulaiman et al. 2014). The objective of this work was to study the potential of CNF support in the covalent immobilization of enzyme.

MATERIALS AND METHODS

RAW MATERIAL AND CHEMICALS

Kenaf raw bast fibre was obtained from Lembaga Kenaf dan Tembakau Negara (LKTN), Malaysia. All chemicals with technical grade such as sodium hydroxide (NaOH), sodium chlorite ($NaClO_2$), acetic acid (CH_3COOH), aqueous hydrogen peroxide (H_2O_2), anthraquinone (AQ), Bradford reagent (coomasive blue), glutaraldehyde (GA) and 1,12-diaminododecanewere were purchased from Merck Chemicals and R&M Chemicals, Malaysia. Cyclodextrin Glucanotransferase (CGTase, EC 2.4.1.19) was purchased from Amano Enzyme Inc., Japan meanwhile water-soluble potato starch and α-cyclodextrin (α-CD) standard were purchased from Sigma, Malaysia.

PREPARATION OF CNF SUPPORT

Dried raw bast fibre was ground and cut into length of 1-2 cm. In delignification process, approximately 20 g of dried short raw bast fibre was cooked under pressure in aqueous solution (15 wt. % of NaOH and 0.1 wt. % of AQ) using non stirred pressure vessel (MOC: SS316, equipment no.: 2311, AMAR Equipments Pvt. Ltd., Mumbai, India). The ratio of fibre to liquor was 1:7 and the process was carried out at 160°C (30 ± 3 bar) for 30 min. The obtained pulp was then rinsed thoroughly with distilled water. The bleaching process was performed with three stages of different chemical processes ($NaClO_2$, CH_3COOH, NaOH and H_2O_2) as described by Jonoobi et al. (2009). After that, the bleached pulp fibre was filtered and rinsed with distilled water until it becomes neutralized (pH~7). The bleached pulp fibre was then kept in a water-swollen state.

The bleached pulp fibre was soaked in distilled water at approximately 0.5 wt. % and ultrasonicated by high-intensity ultrasonic dismembrator (FB705, Fisher Scientific(M) Sdn. Bhd., Malaysia) at 20 kHz of operating frequency, which equipped with 25.4 mm in diameter size of cylindrical titanium alloy probe. Ultrasonication process was run several times and stirred to increase the nano-order CNF yield. Ice was maintained throughout ultrasonication process to avoid any overheated (Chen et al. 2011a).

SCANNING ELECTRON MICROSCOPY (SEM)

The SEM (S-3400N, Hitachi, Japan) analysis was performed using an accelerating voltage of 5-20 kV. Prior to analysis, all samples were sputter-coated with gold under vacuum to avoid any charging effect during observation. Approximately, 200 measurement of fibre dimension on obtained micrograph was done using the Bethesda Image Analyser software. The results were reported as an average values for each set of measurement to obtain a fibre diameter distribution.

CHEMICAL COMPOSITION

Cellulose, hemicellulose and lignin contents in fibre were determined by using Fibertec™ 2010 Auto Fibre Analysis System (FOSS Analytical AB, Sweden). The analysis was carried out according to its standard procedure (FOSS manufacturer's manual) based on analysis of acid detergent fibre (ADF), neutral detergent fibre (NDF) and acid detergent lignin (ADL). Since NDF consists of hemicellulose, cellulose and lignin, ADF consists of cellulose and lignin while ADL consist of only lignin, hemicellulose content can be determined by subtracting value of NDF with ADF. Meanwhile, cellulose content was determined by subtracting value of ADF with value of ADL (van Soest et al. 1991).

FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY

The analysis was performed using Perkin-Elmer spectrometer 100 series, equipped with universal attenuated total reflectance (UATR). The samples were prepared in water-swollen state for the fibre and dried state for immobilized enzyme-fibre. Transmittance mode for the spectra was within the range of 4000-650 cm^{-1}, with 32 scans and scan resolution of 4 cm^{-1}.

CGTASE IMMOBILIZATION VIA COVALENT BOND

Dried CNF support, approximately 0.1 g was mixed with 25 mL of methanol, 5 g of sodium methoxide and 1 g of 1,12-dodecanediamine in a round bottom flask, subsequently. The reaction mixture was refluxed in a water bath at 80°C for 6 h. Then, it was filtered after cooling and washed thoroughly with distilled water. CNF–1,12-dodecanediamine derivative and GA were prepared according to the method of Abdel-Naby et al. (1999). A total of 0.1 g CNF–1,12-dodecanediamine was stirred in 25 mL of 0.1 M potassium phosphate buffer (pH8.0), containing 2.5% (v/v) of GA for 2 h at 25°C. The precipitate was collected and washed with phosphate buffer. The wet activated support (CNF–1,12-dodecanediamine-GA) was shaken with 2.5 mL of the enzyme solution (CGTase activity-36 U, 0.1 M potassium phosphate buffer (pH6.0)) for 12 h at 4°C. An unbound enzyme was removed by washing with phosphate buffer until enzyme activity could not be detected in the filtrates. The preparation of enzyme immobilization was repeated by using raw bast fibre and bleached pulp fibre.

PROTEIN ESTIMATION

The amount of protein loaded onto the support was determined by using Bradford method. Immobilized protein quantity was measured after each washing step at

595 nm using UV-VIS spectrophotometer (ultrospec 3100 Pro, Amersham). Bovine serum albumin (BSA) was used as a standard protein. The protein content of the immobilized enzyme was calculated by subtracting the amount of unbound protein including the amount of protein recovered in the washing filtrates.

ASSAY FOR CGTASE ACTIVITY

The residual activity of free and immobilized CGTase were measured at different temperatures (40, 50, 60, 70 and 80°C) in 10 mL phosphate buffer (0.1 M, pH6.0), containing 5 w/v % soluble starch. For each experiment, 0.1 g of immobilized CGTase-support was used. The enzymatic reaction was run for 5 min and then stopped by placing the samples in a boiling water bath for 15 min. The supernatant was collected by centrifugation (10000×***g***), passed through a Millipore filter 0.22 μm and the α-CD product was analysed by Shimadzu HPLC system (LC-10AT with RID 6A), equipped with a Zorbax Carbohydrate column(L: 150 mm, I.D: 4.6 mm,YMC Co. Ltd., Japan). The sample of α-CD (injection volume: 20 μL) was eluted isocratically with acetonitrile-water (75:25) at a flow rate of 1.0 mL min^{-1}. One enzyme activity unit (U) was defined as the enzyme that liberates 1 μmol of α-CD per min under the assay conditions.

The α-CD production profile was carried out for 120 min. 0.1 g of support with immobilized CGTase was incubated at 70°C, whereas the free enzyme at 60°C (based on the optimum temperature) in 10 mL of 0.1 M phosphate buffer, containing 5w/v % of water-soluble starch. The samples were taken (20 min interval) and instantly the enzymatic reaction was terminated and then the reaction product in supernatant was analyzed using the same method as described earlier.

The operational stability of the immobilized CGTase was run for 8 cycles. The reactant containing 5w/v % water-soluble starch solution in 10 mL was incubated for 1 h at 70°C with immobilized CGTase. At the end of each reaction, the supernatant was collected by centrifugation at 10000×g for 5 min, and the precipitate that contain the immobilized enzyme was washed with phosphate buffer (0.1 M, pH6.0). Then, the precipitate was re-suspended in 10 mL of freshly prepared substrate to start a new cycle. The reaction product in supernatant was analysed as described earlier.

RESULTS AND DISCUSSION

MORPHOLOGICAL ANALYSIS

The surface morphological of the raw bast fibre, bleached pulp fibre and CNF were visualized by SEM as depicted in Figure 1. This figure illustrates on how the fibre morphology changed from the micro to nano scale by the applied procedures. This study showed that the surface morphology of untreated fibres was different from treated fibre, particularly in terms of their level of smoothness and roughness. The diameter distribution of the raw bast fibre, bleached pulp fibre and CNF were also presented in Figure 1, which indicates the reduction of diameter distribution was positively affected by chemical-mechanical treatment. Under the SEM image, the surface of raw bast fibre, which is in brown colour composed of individual micro fibres linked together by lignin, hemicellulose and other impurities such as pectin and waxy substances. The average diameter in size of raw bast fibre is in range of 30-80 μm, which almost 80% of fibre a diameter within 50-70 μm range. The surface of bleached pulp fibre as shown in Figure 1(b), which turned to reddish colour is smoother than raw bast fibre and it was reported to be composed of strong hydrogen bonding between cellulose fibril (Abe & Yano 2009). The strong hydrogen bonding would form a strong interaction among cellulose fibril, which may agglomerate to form cellulose bundle. The prevention of this interaction will help to maintain its diameter size. The removal of lignin and hemicellulose by these processes can decrease the diameter of fibres, as well as disintegrated the single sized into individual micro-sized cellulose fibres with average diameter size of 3-20 μm. The diameter distribution of bleached pulp fibres showed 68% of fibre size in range of 10-15 μm. Figure 1(c) demonstrates the CNF suspension with white colour and homogenously dispersed in water after ultrasonication process; it represents the nano-sized fibre with sufficiently dispersed and converted to the highly viscous suspensions (Chen et al. 2011b). Based on SEM image, CNF displays as a spider web-like network structure with long entangled cellulose filaments, indicating that the individual micro-sized cellulose fibres from bleached pulp fibre was completely stripped down into nano-sized cellulose fibres. The average diameter of CNF was obtained is less than 100 nm, which is more than 60% of CNF that have a diameter distribution in range of 50-80 nm after ultrasonication process. According to Zhao et al. (2007), the ultrasonic treatment confirmed that it can be used to gradually disintegrate the micro-sized cellulose fibre into nano fibre. Therefore, it clearly shows that the size of fibres was decreased after having a series of chemical and mechanical treatment.

CHEMICAL COMPOSITIONS

The percentage of chemical composition of raw bast fibre, bleached pulp fibre and CNF after chemical and ultrasonication process are presented in Table 1. Raw bast fibre consists of around 63.67, 16.35 and 11.87% of cellulose, hemicellulose and lignin, respectively. When raw bast fibre was subjected to delignification and bleaching processes, the cellulose content increased, whereas hemicellulose and lignin contents decreased. NaOH solution in delignification process was found very efficient in removal of most of lignin in raw bast fibre (Jonoobi et al. 2009). This process was helped by using non stirred pressure vessel that may contribute to improve in loosening the lignin structure. Meanwhile, bleaching steps using $NaClO_2$ solutions was found very efficient in removing the

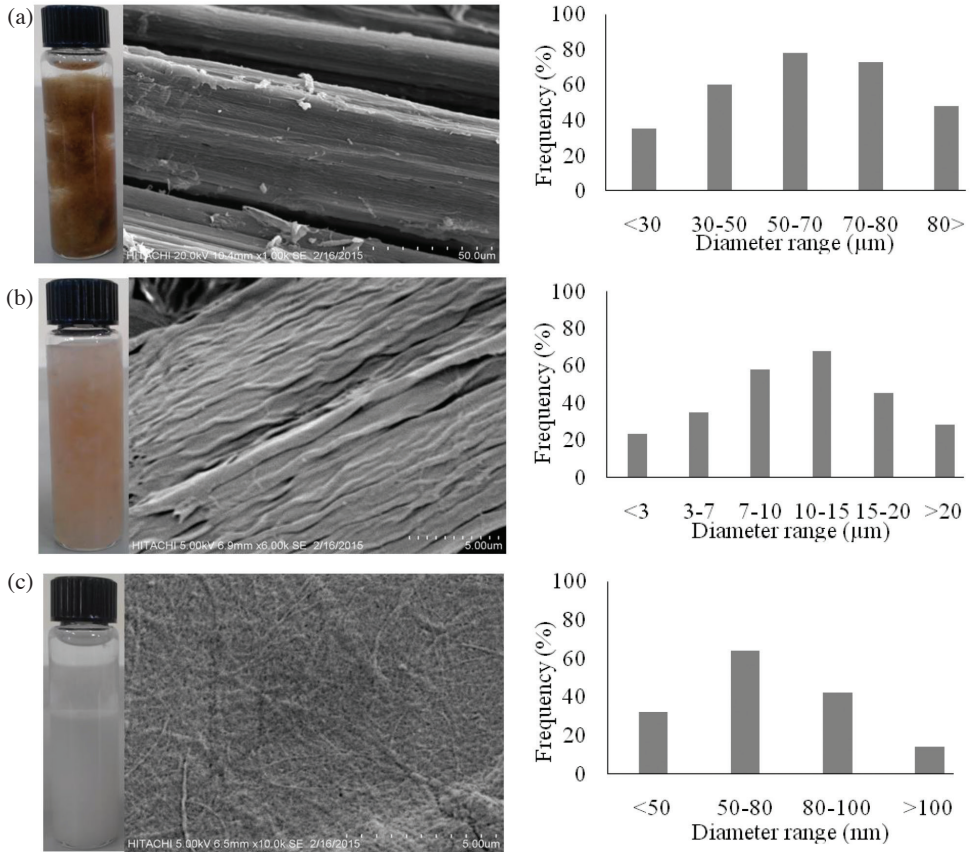

FIGURE 1. Dispersion states and SEM micrographs of surface structure (left) and diameter distribution (right) of the (a) raw bast fibre, (b) bleached pulp fibre and (c) CNF

hemicellulose and residual lignin, as well as to increase the percentage of cellulose content, which was also found by Joonobi et al. (2010). In fact, hemicellulose and lignin formed strong matrix surrounding cellulose structure. Therefore, the removal of hemicellulose and lignin are essential to isolate the high cellulose content of fibre.

The cellulose content in CNF increased up to 91.97% after the treatment using ultrasonication. High-intensity of ultrasonication process was found very efficient as a mechanical treatment due to vibration of water molecules to provide a dispersion of nanofibre bundle into individual nano-sized cellulose fibre. Theoretically, ultrasound energy is transferred to the cellulose chains and process called acoustic cavitation (referred to the formation, expansion and implosion of micro bubbles in aqueous solution), which capable to create energy approximately to hydrogen bond energy scale (10-100 kJ/mol) (Chen et al. 2011a). In detail, the sonification process converted electrical energy to acoustical energy by the transducer, in which the energy exhibited as small intensive bubbles throughout the liquid medium that rapidly burst to produce effects that are similar to a small bomb; resulting in mechanical shear stress and volatility (Wang et al. 2015). Acoustic cavitation introduces an extreme physical environment to CNF preparation by inducing microjets and shock waves on the surfaces of the CNF, causing an erosion of the surface fibres to split along the axial direction (Chen et al. 2011a). Ultrasonication process can break the relative weak interfaces among fibres which mainly bonded by hydrogen bond and reduction of molecular weight can be achieved (Wang et al. 2015). Thus, the ultrasonication impact can gradually disintegrate the micron-sized cellulose fibers into nanofibers, which also help to remove the non-cellulosic contents in CNF especially the remaining hemicelluloses and lignin. Nano-order is not only to attribute the increasing of cellulose content in CNF, but also to provide it with large surface area per volume ratio.

FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY

FTIR spectra among raw bast fibre, bleached pulp fibre and CNF are shown in Figure 2(a) to 2(c). All spectra show broad bands around absorbance peak at 3394cm^{-1}, corresponding to the –OH functional group on the surface of cellulose fibre. This region also represents to the intra- and inter-molecular hydrogen bonding, which can be a potential target functional group for the interaction via covalent immobilization of enzyme (Sulaiman et al. 2014). The spectra of all samples exhibit to the –CH stretching absorption at 2905 cm^{-1} (Khalil et al. 2001). The spectra in region at 1650 cm^{-1} indicates that the cellulose adsorbed water (–OH) (Nacos et al. 2006). The region

TABLE 1. Chemical composition of kenaf bast fibre via chemical and mechanical process treatment

Materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Raw bast fibre	63.67±1.3	16.35±0.2	11.87±1.0
Bleached pulp fibre	81.12±0.5	12.71±1.4	2.34±0.7
CNF	91.97±0.6	5.35±0.4	1.13±0.1

of absorbance peak at 1477 cm^{-1} indicates the group of $–CH_2$ symmetric bending and/or –C=C stretching in aromatic group of lignin and hemicellulose (Jonoobi et al. 2009). Bending vibrations of –CH and –CO groups of aromatic rings in polysaccharide were detected at 1368 and 1314 cm^{-1}, respectively. The absorption peak at 1227 cm^{-1} represents–CO stretching of the aryl group in lignin (Le Troedec et al. 2008). Disappearance of this peak from the spectra was attributed by the removal of lignin after the delignification process. The absorbance at 1160 cm^{-1} indicates the stretching of –COC group due to anti-symmetrical deformation in cellulose (Silva et al. 2008). The stretching of –C=O at absorbance spectra at 1059 cm^{-1} indicate the aromatic ring of surface cellulose fibre (Karimi et al. 2014). Interestingly, there was no significant difference found among the spectra of raw bast fibre, bleached pulp fibre and CNF. The only differences are the stretching vibration and shifted of the absorbance peak on surface functional group after the chemical and ultrasonication process treatment. This fact indicates that the amount of hemicelluloses and lignin were removed during the chemical process and the original molecular structure of cellulose was almost maintained even after the treatments. Thus, this spectra hierarchy of sample clearly indicated that the effectiveness of the applied treatments to obtain high cellulose content fibre.

The comparison FTIR spectra between free fibre and immobilized CGTase onto fibres (raw bast fibre, bleached pulp fibre and CNF) are presented in Figure 2(d) to 2(f). The absorbance peak at 2853 cm^{-1} indicates the stretching vibration of –NH group, which confirmed the presence of 1,12-dodecanediamine (as a spacer arm) on the surface support (Mubarak et al. 2014). The appearance of this absorption peak indicates the interaction of chemical coupling agent (spacer arm) onto –OH group of the surface support, which exhibits a wide range of the combination of stretching spectrum compared to the support itself. Besides, the presence of amines –C=N at 2305 cm^{-1}, amide group –O=C–NH at 1583 cm^{-1} and aliphatic amide bond –CN at 1130 cm^{-1}, indicated the chemical coupling reaction and immobilization both occurred on the surface support. These results showed that CGTase enzyme was successfully immobilized onto the support via covalent binding (Mubarak et al. 2014). The absorbance peak at 1757 cm^{-1} shows the stretching of –C=O of the aldehydegroup in the support, which attributed to the presence of GA (as a ligand) as one of chemical coupling agent. Furthermore, –C=O group also corresponding to the –C=N group, indicating

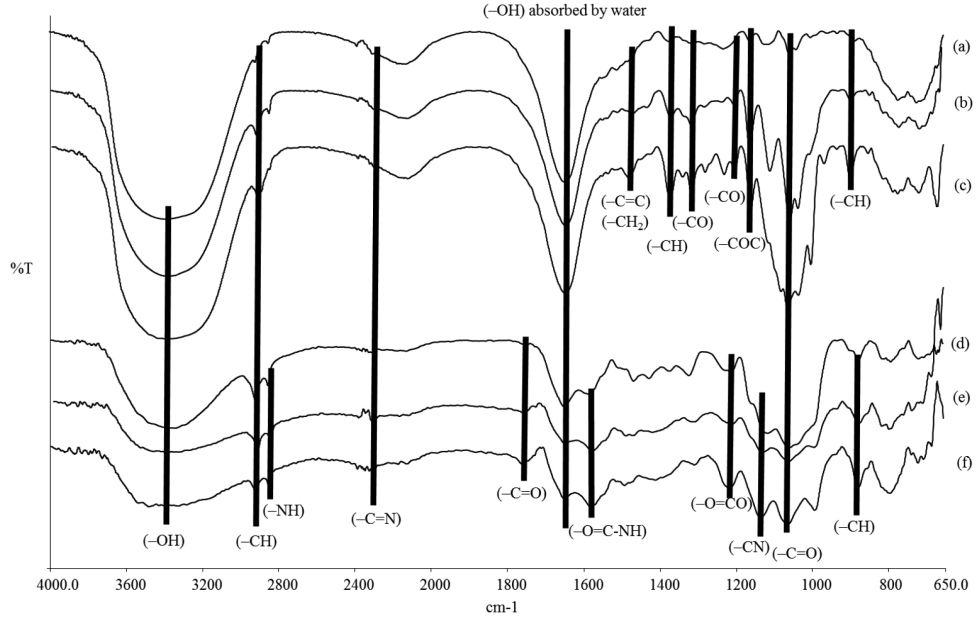

FIGURE 2. Comparison of FTIR spectra between support itself: (a) raw bast fibre, (b) bleached pulp fibre, (c) CNF; and Immobilized CGTase onto support: (d) raw bast fibre, (e) bleached pulp fibre, and (f) CNF

the interaction of terminal point between ligand and spacer arm (Sulaiman et al. 2014). The spectra presence of –CH alkenes stretching at 873 and 988 cm^{-1}, –CO ester stretching bond at 1212 cm^{-1} and –C=O aromatic stretching at 1060 cm^{-1} are mainly due to the side reaction occurred between the functionalized group of support and the phosphate buffer solution and/or reaction between the CGTase enzyme and the buffer (Mubarak et al. 2014). When comparing to Figure 2(a) to 2(c), absorbance peak of –OH, –CO and –CH in the immobilized CGTase onto CNF are broader than them. In detail, each of compound contains a large quantity of individual molecules and each of these molecules may have hydrogen bonded to a slightly different extent (Mubarak et al. 2014). Thus, this is the reason FTIR spectra could occur at various wavelengths for each of these bonds, which leads to the broaden absorption peak.

CGTASE IMMOBILIZATION

CGTase enzyme was covalently immobilized on chemical coupling agents, 1,12-dodecanediamine (acts as spacer arm) and GA (acts as ligand), which already interacted to the functional group on the surface support. According to the previous research studies, cellulose fibre consists of mainly –OH group on its surface (Chen et al. 2011a, 2011b; Jonoobi et al. 2009; Karimi et al. 2014). The presence of chemical coupling agents act as 'smart' linker that represents as specific linkage or anchor from one side of functional group to other side of functional group, represent as (CGTase enzyme-(ligand-spacer arm)-support), which guarantee the flexibility and accessibility to the target enzyme molecules (Sulaiman et al. 2014).

The chemical coupling agents in covalent immobilization of enzyme permit very strong binding between target enzyme molecule and support at a specific point of reactive group which acts as a platform for the interaction between two end-terminals of the chemical coupling. Thus, this interaction showed a more robust way to create bio-functionalized linkage with the amino group of enzyme, as well as to improve the binding efficiency, provide a greater mobility and minimize steric hindrance (Redeker et al. 2013; Sulaiman et al. 2014). The percentage of immobilization yield correlates to the interaction of chemical coupling agent onto surface CNF. In Table 2, the immobilized enzyme yield onto CNF support shows the highest immobilized activity (45.62%, with 115.48 U/g-support) and the highest protein loading (62.10%), compared to immobilized enzyme onto bleached pulp fibre and raw bast fibre.

The observed increasing in the percentage of immobilization activity and binding yield are associated to the successfully of covalent interaction among CGTase molecules-chemical coupling agents–(–OH) group on the surface of support. In other word, by increasing the interaction of chemical coupling, it will increase the percentage of enzyme loading. This explanation is supported by the fact of the effect of large surface area, resulting in increasing number of –OH group for the interaction to the spacer arm and followed by ligand interaction (Abdel-Naby 1999). High percentage of the cellulose content also indicated to the number of exposed –OH group on the surface of CNF support (Chen et al. 2011a). Immobilized enzyme showed higher result in immobilization binding yield, but lower in activity compared to native enzyme. This was due to the inactivation of enzyme active site and/or misdirection of enzyme orientation which affected from the reaction of chemical coupling agents (Cao 2006).

The immobilized CGTase onto CNF showed a specific activity of 18.59 U/mg protein; therefore the enzyme immobilization activity was around 0.52-fold, compared to the bleached pulp fibre and raw bast fibre which are 0.16-fold and 0.05-fold, respectively. The reduction of CGTase specific activity may attribute to the protein crowding which may impair or prevent the proper conformational changes required for the enzymatic reaction. An excessive of protein also lead to the steric hindrance around the enzyme active site (Abdel-Naby 1999).

Martin et al. (2003) reported the limitation in diffusion of enzyme-product and microenvironment factor probably affected by the interaction of immobilized enzyme-chemical coupling toward the surface support. Thus, minimize of these factors would increase the specific activity of protein, as well as increase the efficiency of immobilized enzyme activity. Table 3 shows a similar finding in the previous studies in term of immobilized CGTase onto different types of support. Based on this result, it shows that immobilized CGTase onto CNF showed a good result in enzyme activity as long as the result was by polymer-grafted material.

EFFECT OF CGTASE ACTIVITY AT DIFFERENT TEMPERATURES

The effect of temperature on the enzyme activity of the free and immobilized CGTase onto support is shown in Figure 3(a). It was found that the optimal reaction temperature was shifted from 60°C for free CGTase to 70°C for immobilized CGTase onto support (raw bast fibre, bleached pulp fibre and CNF). The effect of temperature for immobilized CGTase onto CNF indicates higher in relative activity of enzyme compared to bleached pulp fibre and raw bast fibre. This is because of the effect on the interaction of covalent binding, which have the same explanation as described previously. In addition, the immobilized enzyme shows higher relative activity than free enzyme, particularly at high temperature (70-80°C). This higher value of the optimal reaction temperature for the immobilized enzyme contributes to the great stability of applied covalent immobilization procedure, as well as improves in enzyme rigidity (Ortega et al. 2009). Similar results have been previously reported based on covalently immobilized CGTase (Ferrarotti et al. 2006).

TABLE 2. Covalent immobilization of CGTase enzyme

Support	Enzyme added		Unbound enzyme		Immobilized enzyme		Specific activity	Immobilization yield	
	Protein, Po (mg/g-support)	Activity, A (U/g-support)	Protein (mg/g-support)	Activity, I_u (U/g-support)	Protein, P (mg/g-support)	Activity, I_i (U/g-support)	(U/mg protein)	Binding (%) (P/Po) x100	Activity (%) $I_i/(A-I_u)$ x 100
Raw bast fibre	10.0	360.0	6.62	297.67	3.38	6.73	1.99	33.80	10.80
Bleached pulp fibre	10.0	360.0	4.65	246.88	5.35	32.59	6.09	53.50	28.81
CNF	10.0	360.0	3.79	106.89	6.21	115.48	18.59	62.10	45.62

TABLE 3. Covalent immobilization of CGTase reported in the literature

Support	Chemical coupling agents	Immobilized enzyme		Specific activity	Immobilization yield		Ref
		Protein (mg/g-support)	Activity (U/g-support)	(U/mg-protein)	Binding (%)	Activity (%)	
Polyvinylchloride (PVC)	1,6-Diaminohexane Glutaraldehyde (GA)	9.12	63.0	6.90	91.2	45	(Abdel-Naby 1999)
Eupergit C	n.r	8.1	147	18	80	13	(Martı´n et al. 2003)
Eupergit C 250L	n.r	7.2	67	9.3	72	6.7	(Martı´n et al. 2003)
*Chitosan spheres	GA	20	252.7	12.6	95.8	6.1	(Schöffer et al. 2013)
Silica microspheresilanized	3-aminopropyltrimethoxysilane (APTMS) GA	27	117	4.3	83	73	(Matte et al. 2012)
cellulose-coated magnetite microparticles (CCMM)	Periodic acid	1.10	397	360.9	73	37	(Ivanova 2010)
Silanized magnetic nanoparticles modified with PEI (Mag-PEI)	3-Aminopropyltriethoxysilane (APTS) (3-chloropropyl)-trimethoxysilane GA	0.95	416	437.9	63	45	(Ivanova 2010)
Polyethyleneimine-silica (PEI-silica)	GA	0.81	198	244.4	54	25	(Ivanova 2010)
Eupergit C	APTS GA n-dodecyl-(1,4)-maltopyranoside ($C_{12}G_2\beta$)	11	80.3	7.3	98	33	(Svensson &Adlercreutz 2011)
*Alumina	APTS GA	2.41	14	5.82	32	74.4	(Prousoontorn & Pantatan 2007)
Glyoxyl-agarose 6BCL	Glyoxal	n.r	2.1	n.r	>80	70	(Ferrarotti et al. 2006)

n.r: not reported
*Analysis was measured based on β-CD production

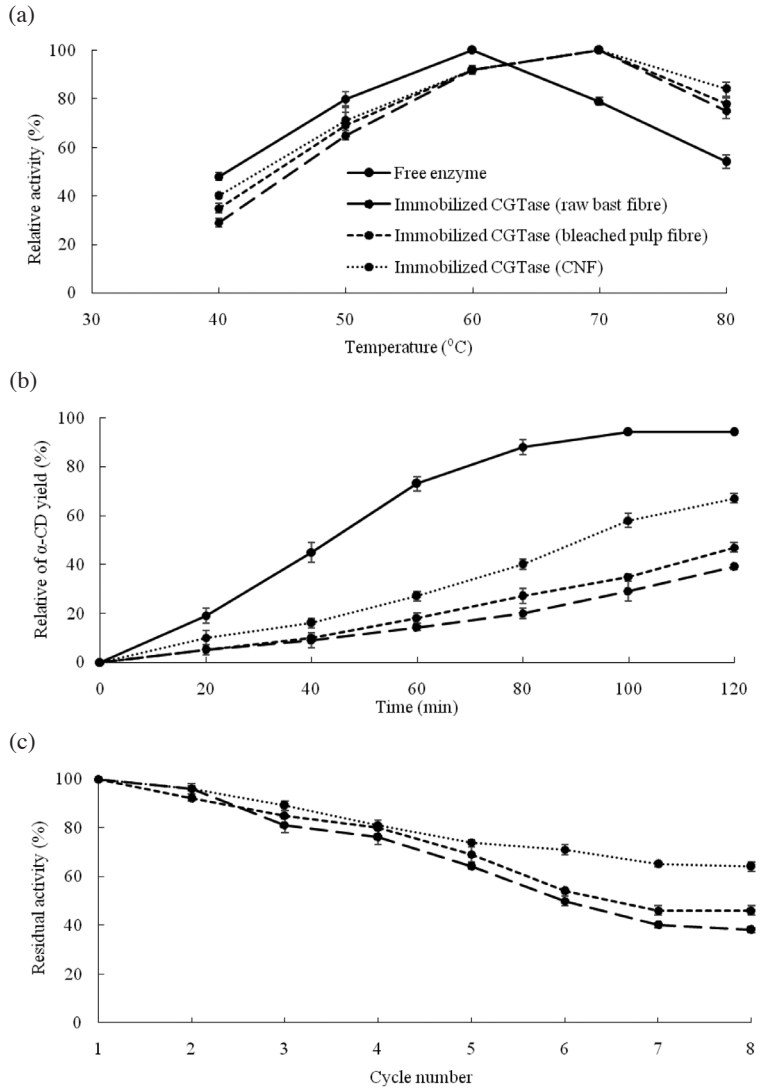

FIGURE 3. Activity of free and immobilized CGTase: (a) effect of temperature, (b) α-CD production and (c) their residual activity at different cycles

ENZYMATIC PRODUCTION OF α-CD

Figure 3(b) shows the profile of α-CD forming for both the free enzyme and immobilized CGTase. The percentage of α-CD production for free enzyme increased up to its maximum production at 100 min of reaction time. Substrate limitation and product inhibition may affect the reaction process of CGTase, indicating stationary line when the reaction process time was extended (Shahrazi et al. 2013). The percentage of α-CD production by immobilized CGTase gradually increased even though at 120 min of reaction time. In addition, immobilized CGTase onto CNF showed higher α-CD production, compared to the immobilized CGTase onto bleached pulp fibre and raw bast fibre. A similar result regarding the α-CD production of immobilized CGTase was obtained by Ivanova (2010) in covalent binding onto cellulose-coated magnetite microparticles (CCMM).

IMMOBILIZED ENZYME REUSABILITY

The reusability of the immobilized CGTase was evaluated in a repeated batch process, as presented in Figure 3(c). It shows that there is an increment of the loss of residual activity of the immobilized CGTase as the recycle number of reaction increases which also mentioned by Mubarak et al. (2014). The immobilized CGTase onto CNF shows the highest relative activity (67.0%) after 8 cycles of reaction, compared to the immobilized CGTase onto bleached pulp and raw bast fibre. The reusability study showed one of the benefits upon enzyme immobilization is the enzyme feasible to be reused since it can be easily recovered or separated from the reaction medium. The reusability of immobilized CGTase onto CNF that present in this work also indicates the applicability of the immobilized CGTase for continuous production of cyclodextrin.

CONCLUSION

The isolated CNF from kenaf bast fibres was successfully obtained with the combination of chemical and mechanical process treatments and it was successfully immobilized with CGTase via covalent binding. SEM micrograph shows the surface morphology with different structure and size distribution, attributed to the significant effect of chemical and mechanical treatments. Chemical analysis showed the increasing of cellulose content, whereas decreasing in the lignin and hemicellulose contents due to the treatments. The changes of spectra between CNF and immobilized enzyme onto CNF also describe the interaction of chemical coupling agents to the hydroxyl group of surface CNF and the successful immobilization of CGTase enzyme. There is no significant damages on the surface morphology of CNF after it went through the chemical coupling reaction and CGTase immobilization, as confirmed by the FTIR spectra. Immobilized CGTase promotes high α–CD production and reusability of enzyme. Thus, CNF support is highly able to be applied in enzyme immobilization due to large surface area as well as to provide high cellulose content.

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