Immobilization of Cellulase from Trichoderma Reesei on Multiwall Carbon Nanotubes (MWCNTs)

Natasha Yasmin Hasnol Azahari, Naresh Sandrakesaran, Cheah Chin Gaik Connie, Kunasundari Balakrishnan

Department of Chemical Engineering Technology, Faculty of Engineering Technology, Universiti Malaysia Perlis, P.O Box 77, D/A Pejabat Pos Besar Kangar, Perlis, 01000, Malaysia

Email: kunasundari@unimap.edu.my

Abstract. Cellulase is an enzyme commonly used to break down cellulose to beta-glucose. The demand of cellulase has been arisen due to its extensive range of applications in the industries such as detergents, foods and biofuel production. In present study, cellulase from Trichoderma reesei was immobilized on a functionalized multiwall carbon nanotube (MWCNTs) by physical adsorption method. Fourier Transform Infrared (FTIR) was used to confirm the successful functionalization of MWCNTs and immobilization of cellulase. In this approach, effects of pH and temperature for both free and immobilized cellulase were evaluated. Practically, the immobilized cellulase showed an improvement in pH and thermal stability compared to free cellulase at optimum condition of 50°C and pH 5 with 12% and 8% of increments respectively. For reusability study on CMC analysis, the bionanoconjugate retained 60% of its initial activity even after 3rd cycle of analysis. This feature is beneficial to the industrial applications because of its potential to be easily separated from the end product at the end of the reaction, reuse for several times and helps in development of multiple enzyme reaction systems.

1. Introduction
For past decades, immobilization of enzyme has been extensively studied particularly for industrial enzyme (example: cellulase, proteases and amylases) as an attempt to improve the stability and performance of enzyme. Among all industrial enzyme, cellulase hold up a record as a major contributor in worldwide enzyme market with 39.57% in 2016 and expected to grow every year [1]. Cellulase mainly produced by cellulolytic microorganisms [2] where Trichoderma reesei from fungi family been recognized as a chief producer for industrial use. Despite of the continuous attempt in establishing a stable enzyme system along with economical factor in consideration, the existing cellulase still do not fulfill the market demand due to the high cost of enzyme. Based on the previous data reported by [3], cellulase enzyme cost range from $0.1 to $0.4/gal of ethanol while others stated the cost is still high as up to $0.69/gal ethanol [4] or even $1.47/gal ethanol [5] in which the values are derived based on cellulases used in animal feed, detergent and textiles [6].

Immobilization successful rate highly relies on carrier selection with several criteria’s such as it is economically viable, stable, exhibited regenerability and can overcome microbial contamination, reduce product inhibition as well as ability to maximize enzyme activity [7]. In recent study, polymers are most likely to be chosen due to reproducibility, easy processing and low cost [8]. Since the discovery of
carbon nanotubes (CNTs) by [9], it has gained an outrageous attention of researchers from various fields due to their excellent mechanical properties and astonishing structure [10]. From the enzyme immobilization perspective, CNT is applied due to the higher enzyme loading capacity, good bio catalytic potential and large surface area to volume [11].

In this research, cellulase from *Trichoderma reesei* was immobilized onto MWCNTs through adsorption method. The confirmation of functionalized and immobilized MWCNTs with cellulase was established by comparing the spectrums from Fourier Transform Infrared (FTIR) analysis. Next, the cellulase activities before and after immobilization were determined using CMC assay [12, 13]. The effect of temperature and pH on the activity of free and immobilized cellulase were evaluated in an attempt to optimize the most favorable condition for the enzyme. In addition, a reusability study on the immobilized cellulase using CMC was carried out to evaluate the potential of bio-nanoconjugate to retain its activity upon application.

2. Experimental

2.1 Material

Cellulase (from *T. reesei* purchased from SIGMA), multiwall carbon nanotubes (MWCNTs) (97% pure, 2 μm in length with a 40-60 nm diameter), 69% nitric acid (JT Baker), Iron Oxide, (Fe₃O₄) nanoparticle, 95% ethanol, 2% (w/v) carboxymethyl cellulose (CMC) solution, 0.05 M sodium phosphate buffer (pH=7), 0.05 M citrate buffer (pH=4.8), 0.1 M 3,5-Dinitrosalicylic acid (DNS) reagent, , acetone, filter paper, filter funnel, distilled water, deionize water, vacuum pump, UV-vis spectrophotometer (Shimadzu UV-1800), FTIR (Perkin Elmer), sonicator (WiseClean), oven (Memmert UF110) and water bath (Julabo TW12).

a. Functionalization of MWCNTs

Briefly, 1 g of pristine MWCNTs and 100 mL of 65 % concentrated nitric acid, HNO₃ were mixed in three-neck round-bottom flask. Then, the mixture of acid with pristine MWCNTs was subjected to ultrasonication treatment (53 kHz frequency) for 1 h in order for functionalization to take place. The functionalized MWCNTs were heated to 80°C for 8 h using reflux method in oil bath. Then, the treated MWCNTs were filtered followed by washing with distilled water until the pH of the filtrate was equal to the pH of the distilled water. The pH of filtrate was tested using litmus paper. The purified MWCNTs were dried at 120°C for 12 h [14].

b. Immobilization of Cellulase Enzyme on Functionalized MWCNTs

A total of 0.1 g of functionalized MWCNTs and 0.02 g of cellulase were added into 4.5 mL of 0.05 M phosphate buffer (pH = 7.0) solution. The prepared solutions were mixed in a container, followed by incubation in an incubator shaker at 30°C, 200 rpm for 2.5 hours. After incubation, the composite mixture was centrifuged at 3800 rpm for 12 min to settle the MWCNT–cellulase conjugate and binding efficiency of the enzyme on functionalized MWCNTs were determined [13].

c. FTIR Analysis

FTIR spectra was recorded using KBr pellets in the frequency range (4000-400 cm⁻¹) and the optical properties of the samples were identified using He–Ne (helium–neon) laser that emits red light with wavelength of 633 nm [15]. Three types of samples analyzed with FTIR, which were pristine MWCNTs, functionalized MWCNTs and immobilized cellulase on MWCNTs.

d. Determination of Cellulase Activity

i. Preparation of DNS reagent

DNS reagent was prepared by mixing 10 g of 3, 5-dinitrosalicylic acid, 0.5 g of sodium sulfide, 2 g of phenol and 10 g of Sodium Hydroxide into a beaker containing 500ml of distilled water. The weight of
200 g of potassium sodium tartrate was prepared separately prior addition into the mixtures. The mixture was topped up to 1 L.

ii. Carboxymethyl Cellulose (CMC) Assay
The cellulase activity was determined via CMC analysis where 2% of CMC served as substrate. To determine the cellulase activity, at least two dilutions were prepared for each enzyme sample by diluting with 0.05M citrate buffer (pH 4.8) where one dilution releasing slightly more than 0.5 mg of glucose and one dilution releasing slightly less than 0.5 mg of glucose in the reaction process.

Firstly, 0.5 mL of all samples in test tubes (enzyme sample including diluted enzyme and spectrophotometer blank) were pre-warmed at 50°C for 5 min, followed by the addition of 0.5 mL of CMC solution into the samples, and then incubated in a water bath at 50°C for 30 min. Next, 3 mL of DNS reagent was added to each enzyme sample and mixed thoroughly. All samples were boiled vigorously in a boiling water bath at 95°C for exactly 5 minutes. After boiling, all samples were immediately transferred to an ice bath for cooling for 2 minutes. An aliquot of 0.2 mL of each sample was diluted in 2.5 mL of distilled water and mixed completely by inverting the tube several times [12].

After the dilution of the sample, color formations for all samples were determined by measuring absorbance against spectrophotometer blank at a wavelength of 540 nm via UV–vis spectroscopy. The resulted absorbance values, were translated into amount of glucose liberated during reaction process by using glucose standard curve. The activity of cellulase was evaluated according to the following equation:

\[
\text{Cellulase activity (U/mL)} = \frac{0.185}{\text{critical enzyme concentration to release 0.5mg of glucose}}
\]  

(1)

e. Screening for Parameters Affecting Cellulase Activity

i. pH
The effect of pH on the cellulase enzyme activity on both free enzyme and immobilized enzyme were examined by replacing citrate buffer (pH = 4.8) used in the CMC assay to pH 3.0, 4.0, 5.0, 6.0 and 7.0.

ii. Temperature
The effect of temperatures on activities of both free enzyme and immobilized enzyme were examined from 30°C, 40°C, 50°C, 60°C, 70°C and 80°C using CMC assay.

f. Reusability Study
Reusability study of immobilized cellulases on MWCNTs using CMC was assessed under optimized process conditions which have been determined from Section 2.6.1 and 2.6.2. After each cycle, the immobilized cellulase enzyme was removed through centrifugation at 3800 rpm for 30 min. The immobilized cellulase was then collected and washed with phosphate buffer (pH 7.0). For the subsequent second cycle, the immobilized enzyme was re-suspended with fresh phosphate buffer prior to analysis as described in section 2.6. The activity of the immobilized enzyme after first cycle was defined as the control and recognized as a relative activity of 100%. The immobilized cellulase enzyme re-used until the absorbance readings showed the enzyme activity was reduced to the maximum [13].

3. Results and Discussions

3.1 FTIR Analysis of Pristine MWCNTs, Functionalized MWCNTs and Functionalized MWCNTs – cellulase composite
A comparison of FTIR spectrum of pristine MWCNTs, functionalized MWCNTs and functionalized MWCNTs – cellulase composite were conducted as shown in figure 1. Based on the FTIR spectrum of pristine MWCNTs, the peak at 1446.30 cm⁻¹ indicates the graphite structure of alkene, (C=) bending
group. The strong and sharp peak at 2762.05 cm\(^{-1}\) shown the (C – H) stretching bond present in the structure of pristine MWCNTs. On the contrary, (C – O) bond stretching at 1198.10 cm\(^{-1}\), hydroxyl group (–OH ) at 3384.32 cm\(^{-1}\) and (C – Br) stretching at 594.15 cm\(^{-1}\) shows that MWCNTs used were generated through Chemical Vapor Deposition (CVD) method [16].

Based on the FTIR spectrum of functionalized MWCNTs, the peak at 1782.30 cm\(^{-1}\) indicates the (C=O) stretching bond of carboxylic group, as a result of acid treatment during functionalization process of the carrier. It can be considered that they are more ubiquitous at the termini of MWCNTs. The peak at 2940.54 cm\(^{-1}\) shows the (C – H) stretching bond present in the structure of functionalized MWCNTs. Besides, the peak at 3574.10 cm\(^{-1}\) and 1132.34 cm\(^{-1}\) corresponding to the (O – H) and (C – O) functional group stretching in the compound where the presence of these peaks are used to support the presence of the carboxyl groups on MWCNT. The (C=C) bond stretches at the peak of 1610.44 cm\(^{-1}\) which emphasized that the MWCNTs retained its structure after the process of functionalization [13].

Based on the FTIR spectrum of functionalized MWCNTs – cellulase composite, the peak at 3316.11 cm\(^{-1}\) and 3214.27 cm\(^{-1}\) s indicated the combination of stretching vibration of (N – H) bond and (O – H) bond. These bonds stretching in the composite sample proposed the presence of cellulases in the functionalized MWCNTs. Besides, the presence of nitriles (C≡N) at 2226.33 cm\(^{-1}\), amide group (O=C-NH) at 2038.76 cm\(^{-1}\) and aliphatic amide bond (C-N) at 1066.55 cm\(^{-1}\) represents the amidination reaction and the immobilization of cellulase are both occurred at the membrane of functionalized MWCNTs. Based on the information from the FTIR spectra, it can be deduced that the immobilization of cellulase onto functionalized MWCNTs by physical absorption method is successful [13].

Besides, the presence of halo compound (C – Br) at 586.87 cm\(^{-1}\) and (C – H) alkene stretching at 958.15 cm\(^{-1}\) and (C – O) ester stretching bond at 1254.98 cm\(^{-1}\) are because of side reaction occurs between the functionalized group present in MWCNTs with phosphate buffer (pH 7.0) solution and reaction between cellulase enzyme with the buffer. In addition, the peak at 1342.91 cm\(^{-1}\) indicates the (C=C) stretching aromatic mode in MWCNTs, which revealed it still retained the structure after immobilization [13].

![Figure 1. RCO% Comparison of FTIR spectrum for pristine MWCNTs, functionalized MWCNTs and functionalized MWCNT-cellulase composite.](image-url)
3.2. Parameters Affecting Cellulase Activity

i. **pH**

Figure 2 represents enzyme activity of both free and immobilized cellulase at different pH range which indicated that the bionanoconjugate showed better stability at wider pH range compared to free cellulase highlighting the adaptability. The optimum pH for both free and bio-nanoconjugate were recorded at 5 with 56.0 U/mL and 61.0 U/mL of enzyme activity suggesting 8% improvement of bio-nanoconjugate performance compared to native cellulase. Theoretically, the stability of bionanoconjugate can be explained by multi point attachment of enzyme molecules on functionalized CNTs surface which resulted in rigidity of the structure thus restricting conformational changes [17]. Meanwhile, in contrast to immobilized cellulase, free enzyme structure’s expected to be more prone for disruption as higher pH values producing strong electrostatic repulsion resulting in deactivation of enzyme active site thus decreasing its performance efficiency. This finding in accordance to the previous research done by [13] and [18].

![Figure 2. Effect of pH on the cellulase activity of free and immobilized cellulases.](image)

ii. **Temperature**

Thermal stability for free and immobilized cellulase is shown in figure 3 where it can be concluded that the performance of both enzymes were reduced with increased in temperatures. However, at extreme temperature condition, immobilized cellulosases portrayed better stability compared to free cellulase as it able to retained at least 30% of its initial activity. It can be deduced from this temperature profile that free cellulase is not fit for industrial use as most of the operations usually take place at higher temperatures. At lower temperature condition (30°C-40°C), free cellulase permits higher activity compared to immobilized cellulase aligned to finding reported by [13] in which the general working range for cellulases is within 30°C-40°C. In contrast, as temperature increase up to 80°C, bio-nanoconjugate exhibit better stability and performance due to rigid external backbone that holds the enzyme molecule in fix position thus, any environmental changes caused less effect. For free cellulase, increase in temperature could affect the enzyme linkage which responsible for catalytic structure. Thus, changes of globular protein became significant led to lower enzyme activity with poor efficiency at higher temperature [19]. In this study, both free and immobilized cellulase recorded highest activity of 56.92 U/mL and 64.91 U/mL at 50°C respectively which concluded to be optimum condition [13]. Free cellulosases observed to be deactivated at temperature above 50°C.
3.3. Reusability of Immobilized Cellulase on CMC

In this section, 50°C and pH 5 were chosen as a set of reaction conditions based on results from previous experiments. The greatest advantage of practicing immobilization technique is making the enzyme feasible to be reused after several cycles of reaction. It is evident from figure 4, where bionanoconjugate showed reusability rate up to 7th cycles of reaction. Practically, immobilized cellulase activity retained up to 63% after third cycle of reaction. As the number of cycles increase to 7, the retained cellulase activity reduced to 18%. Thus, it can be concluded that the efficiency of bio-nanoconjugate decrease as the number of cycles increase which is in accordance to previous research done by [13] and [19]. This condition can be further explained by two possible conditions. One of the reasons is the weak bonded molecules were lost during the measurement process, as some of the enzyme molecules are not efficiently attached on the wall of functionalized MWCNTs. Other than that, the increased in diameters of MWCNTs leads to the decrease of the corresponding surface area after several assays due to hydrophilic properties of functionalized MWCNTs [13].
4. Conclusions
The overall objectives of this project had been achieved in which the functionalization of MWCNTs was attained via acid oxidation method and the functionalized MWCNTs were characterized with FTIR. Besides, the cellulase was successfully immobilized onto functionalized MWCNTs via physical absorption. In this project, the optimal pH and temperature of free enzyme and immobilized cellulase were determined to be 5 and 50°C respectively. From the analysis results, it was shown that the activity of immobilized cellulase is higher than free enzymes at their optimal pH and temperature conditions. The reusability test of the immobilized cellulase at optimal pH and temperature condition was revealed that 18% of its activity was retained after 7th cycles of the consecutive hydrolysis reaction with CMC substrate. The reusable properties of enzyme is important to reduce the requirement of purchasing large quantities of cellulase enzyme to minimize the production quantity of cellulase enzyme from its raw materials which indirectly reduce the environmental problem.

References
[1] Cellulase (CAS 9012-54-8) Market 2020 Global Industry Brief Analysis by Top Countries Data, Market Size, Definition, Industry Trends, News and significant Growth With Regional Trends By Forecast 2024 [Online]
[2] Sadhu S and Maiti T K 2013 British Microbiology Research Journal 3 235-258
[3] Sassner P, Galbe M and Zacchi G 2008 Biomass Bioenergy 32 422–430
[4] Kazi F K, Fortman J, Anx R, Kothandaraman G, Hsu D, Aden A and Dutta A 2010 National Renewable Energy Laboratory, Golden
[5] Klein-Marcuschamer D, Oleskowicz-Popiel P, Simmons B A and Blanch H W 2012 Biotechnol. Bioeng. 109 1083–1087
[6] Phitsuwan P, Laohakunjit N, Kerdchoechuen O, Kyu K L and Rata-nakanokchai K 2013 FoliaMicrobiol. 58 63–176
[7] Datta S, Christena L R and Rajaram Y R 2013 3 Biotech. 3 1-9
[8] Mittal G, Dhand V, Rhee K Y, Park S J and Lee W R 2015 Journal of Industrial and Engineering Chemistry 21 p 11-25
[9] Iijima S, Ichihashi I and Ando Y 1992 Nature 356 776
[10] Sodtipinta J, Weeraphat P O, Waret V, Siwaporn M S and Pasit P 2013 Materials Research Bulletin 48 p 1204-12
[11] Verma M L, Naeeb M, Barrow C J and Puri M 2013 PLOS ONE 8
[12] Ghose T K 1987 Pure & Appl. Chem. 59 257-268
[13] Mubarak N M, Jing R W, Khang W T, Jaya N S, Ezzat C A, Jayakumar N and Poobalan G 2014 Journal of Molecular Catalysis B Enzymatic 107
[14] Shuit S H and Tan S H 2014 Energy Conversion and Management 88 1283-89
[15] Gokhalea A A, Lu J and Ilsoon I 2013 J. Mol. Catal. B: Enzym. 90 76-86
[16] Noorhana Y, Bojan O B and Krzysztof K 2010 Carbon and Oxide Nanostructures
[17] Ahmad R and Khare S K 2018 Bioresour Technol. 252 72-75
[18] Zhang S, Shang W, Yao X, Zhang S, Zhang X and Chen J 2013 Bull. Korean Chem. Soc. 34 2741-47
[19] Neerupa N, Rajvinder S and Jagdeep K 2006 Electron. J. Biotechnol. 5 1-5