Nano World Journal

https://doi.org/10.17756/nwj.2023-112

Research Article

Open Access

Functionalization of Multiwall Carbon Nanotubes (MWCNTS) using Phosphoric Acid for Immobilization of Cellulase

Hasnol Azahari Natasha Yasmin[1], Thivagar Rao[1], Sandrasekaran Naresh[1], Siew Hoong Shuit[2], and Balakrishnan Kunasundari[1*]

[1]*Fakulti Kejuruteraan dan Teknologi Kimia, Universiti Malaysia Perlis, Kompleks Pusat Pengajian Jejawi 3, Kawasan Perindsutrian Jejawi, Arau, Perlis, Malaysia*

[2]*Department of Chemical Engineering, Lee Kong Chian Faculty of Engineering and Science, Universiti Tunku Abdul Rahman, Sungai Long Campus, Jalan Sungai Long, Bandar Sungai Long, Cheras, Kajang, Selangor, Malaysia*

***Correspondence to:**
Balakrishnan Kunasundari
Fakulti Kejuruteraan dan Teknologi Kimia,
Universiti Malaysia Perlis,
Kompleks Pusat Pengajian Jejawi 3,
Kawasan Perindsutrian Jejawi,
Arau, Perlis, Malaysia
E-mail: kunasundari@unimap.edu.my

Received: November 07, 2022
Accepted: February 20, 2023
Published: February 22, 2023

Citation: Yasmin HAN, Rao T, Naresh S, Shuit SH, Kunasundari B. 2023. Functionalization of Multiwall Carbon Nanotubes (MWCNTS) using Phosphoric Acid for Immobilization of Cellulase. *NanoWorld J* 9(1): 15-22.

Published by United Scientific Group

Abstract

In this paper, functionalization of multiwall carbon nanotubes (MWCNTs) using phosphoric acid (H_3PO_4) was attempted. Based on the One Factor at a Time (OFAT) analysis, 8 M of H_3PO_4, 8 h of reflux time and 80 °C were identified as an ideal condition for the acid oxidation process. Successful functionalization of MWCNTs using H_3PO_4 was revealed from Fourier Transform Infrared Spectroscopy (FTIR) peaks and Scanning Electron Microscope (SEM) images. The resulting f-MWCNTs were immobilized with cellulase via adsorption. The optimum temperature for free cellulase was at 50 °C and for immobilized cellulase was at 60 °C. The optimum pH was at 5 for both free and immobilized cellulase. Higher thermal stability was achieved with immobilized cellulase in which 39% activity still was retained at 80 °C compared to free cellulase. Reusability study of immobilized cellulase depicted 28% of remaining activity after 5th cycle of analysis using model substrate (filter paper Whatman No. 1). These results suggest that H_3PO_4 which is a mild acid is a promising substitution for functionalization of MWCNTs.

Keywords

Multiwall carbon nanotubes, Phosphoric acid, Mild acid, Filter paper, Reusability

Introduction

The extraordinary properties of carbon nanotubes (CNTs) have attracted an overwhelming interest of researchers from various fields [1]. CNTs is a carbon atom in tubular cylindrical form with nanometer diameter and micrometer length [2]. CNTs can be classified as either (i) single wall carbon nanotube (SWCNTs) that comprise of single tube of carbon or (ii) multiwall carbon nanotubes (MWCNTs) which involve multiple carbon atom cylinders. CNTs are acknowledged as a new player in the field of materials science as it possesses exceptional electrical [3], chemical [4], mechanical [5], optical [6] as well as thermal properties which made it beneficial for biological application [7]. The high performance and light weight of CNTs are added advantages of its utilization [8].

Despite of the notable characteristics, dealing with CNTs could be difficult due to its hydrophobic behavior [9] resulting from van der Waals forces within carbon atoms that promote agglomeration [8]. Uniform dispersion of CNTs is required [8] to enhance its material properties that usually attained through functionalization. Functionalization is a process of surface modification particularly CNTs to increase the repelling tendency to improve the dispersion rate.

The process can be achieved through physical and chemical methods [10]. Usually, functionalization involved acid oxidation process in which nitric acid (HNO_3) [11], hydrochloric acid (HCl) [12], hydrogen peroxide (H_2O_2) [13] or mixture of nitric and sulfuric acid (H_2SO_4) [12] will be used. However, the main issue in implementing acid oxidation technique is the disruption of CNTs molecular framework due to breaking and opening thus, shortening the CNTs that affected its mechanical properties [14]. In addition to that, these treatments were reported to cause cytotoxic effect in specific applications involving cell lines [9]. Thus, developing an alternative acid treatment with less toxicity aiming to reduce the damage on CNTs is pivotal [15]. Recently, mild acid such as phosphoric acid (H_3PO_4) has gained much interest as an alternative acid oxidation medium [16, 17] in parallel with the urge for developing an eco-friendly process.

Previously, an attempt on mild acid oxidation using polyphosphoric acid/ phosphorus pentoxide (PPA/P_2O_5) has been reported by Lee et al. [18], revealed that, various functionalities could be introduced on CNTs surface using mild acid medium. Thus, proven the ability of mild acid as substitution in oxidation medium. To achieve an efficient oxidation process, three main parameters, acid molarity, treatment time and temperature were considered to ensure sufficient production of COOH group on CNTs surface [19]. Table 1 represented several attempts using various acids at different parameters for functionalization of CNTs that has been previously published. However, to the best of our knowledge, no further study has been done for immobilization of enzyme on functionalized MWCNTs (f-MWCNTs) treated using mild acid as existing research merely on the characteristic properties of CNTs after oxidation process.

Motivated from existing studies on successful functionalization using mild acid, the present study is focused on the acid oxidation of CNTs using H_3PO_4 by varying three influential parameters (acid concentration, reflux time, and temperature). Subsequently, the resulted f-MWCNTs was subjected for immobilization of cellulase. The efficiency of f-MWCNTs treated with H_3PO_4 was determined via total adsorption of cellulase on it. Subsequently, the f-MWCNTs treated with H_3PO_4 with highest binding of enzyme at optimum condition was selected for stability study by varying pH (3 - 7) and temperature (30 °C - 80 °C) to evaluate its potential as a cellulase carrier. Fourier Transform Infrared Spectroscopy (FTIR) analysis was performed to compare the spectra of f-MWCNTs treated with H_3PO_4 along with common acids (HNO_3 and HNO_3:H_2SO_4) at optimum identical conditions.

Materials and Methods

Cellulase from *Aspergillus Niger* (SIGMA), multiwall carbon nanotubes (MWCNTs) (97% pure, 2 µm in length with a 40 - 60 nm diameter) were purchased from Shenzhen Nanotechnologies Port Co, 69% nitric acid (JT Baker), 85 % ortho-Phosphoric acid (Merck), 95 - 97% sulfuric acid (Merck), 95% ethanol (HmbG), Whatman Filter Paper No.1, 0.05 M sodium phosphate buffer (pH 7), 0.05 M citrate buffer (pH 4.8), deionized (DI) water and 0.1 M 3,5-dinitrosalicyclic acid (DNS) reagent.

Acid oxidation of MWCNTs

A weight of 1 g of MWCNTs was added into 100 ml of H_3PO_4 and subjected for 1 h sonication treatment at room condition prior to reflux process as shown in Figure 1. Subsequently, the mixture solution was filtered using deionized water until the filtrate pH was neutral (pH 7). The washed MWCNTs were dried in an oven at 100 °C for overnight. The dried MWCNTs after the acid oxidation process was denoted as f-MWCNTs. For One Factor at a Time (OFAT) study, acid concentration (4 - 12 M), reflux temperature (40 °C - 120 °C) and time (4 - 12 h) were varied for acid oxidation process according to the previous study [19].

Effect of acid concentration

Acid concentration was varied (4 M, 8 M, and 12 M) meanwhile reflux temperature and time were kept constant at 80 °C and 8 h, respectively [21].

Effect of reflux temperature

Reflux temperature was varied (40 °C, 80 °C, and 120 °C) meanwhile acid concentration and reflux time were kept constant at 8 M and 8 h, respectively.

Effect of reflux time

Reflux time was varied (4 h, 8 h, 12 h, and 24 h) meanwhile acid concentration and reflux temperature were kept constant at 8 M and 80 °C, respectively.

Immobilization of cellulase on f-MWCNTs

Immobilization of cellulase was performed using adsorption technique on f-MWCNTs. A weight of 0.1 g of f-MWCNTs at each condition was added into 5 mg/ml of cellulase in accordance to the previous study [11]. The prepared solution was incubated in an incubator shaker at 200 rpm, 30 °C for 2.5 h [22]. After incubation, the composite mixture was centrifuged at 3800 rpm for 15 min. Then, the supernatant was carefully decanted followed by re-dispersion of f-MWCNTs in phosphate buffer (pH 7) for washing purpose. The sequential process of centrifugation and washing were repeated several times (3 - 4 times) to remove unbound protein. Subsequently, the f-MWCNTs was dried for 24 h at room temperature. The highest binding efficiency of cellulase was calculated and chosen for further analysis. The efficiency of immobilization on each treated f-MWCNTs was evaluated using the following equation:

$$\text{Binding efficiency}\,(\%) = \frac{\text{Enzyme loading}}{\text{Initial activity}} \times 100\% \quad (1)$$

Characterization of MWCNTs, f-MWCNTs and immobilized cellulase on f-MWCNTs

The chemical compositions of MWCNTs, f-MWCNTs, and immobilized cellulase on f-MWCNTs were characterized by FTIR using potassium bromide (KBr) pellets within frequency range (4000 - 400 cm^{-1}). FTIR spectra for f-MWCNTs on each acid treatment (HNO_3, HNO_3:H_2SO_4, and H_3PO_4) at optimum condition were also conducted as for comparison purpose.

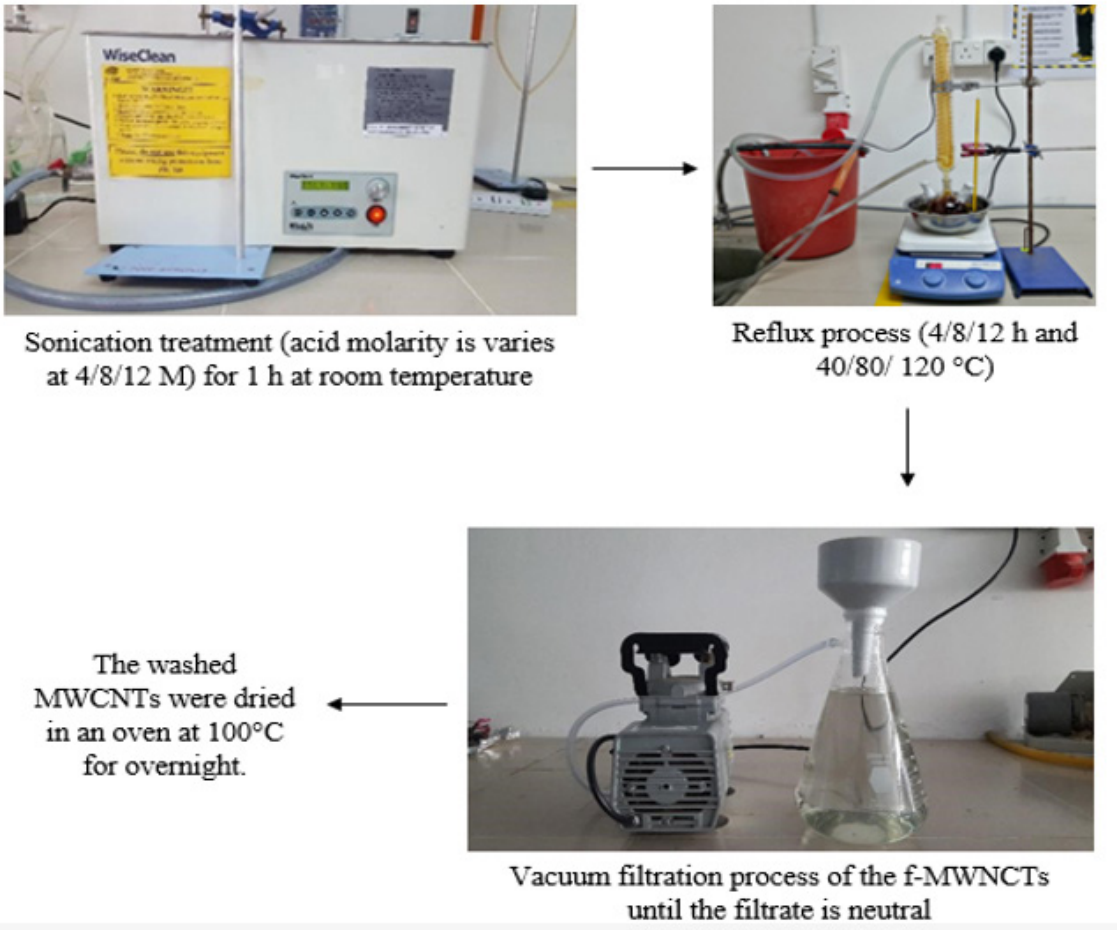

Figure 1: Acid oxidation route of MWCNTs to f-MWCNTs.

For morphological characterization of f-MWCNTs and immobilized cellulase on f-MWCNTs treated using phosphoric acid, a scanning electron microscopy (SEM) was used at a magnification scale of 50,000x.

Determination of cellulase activity

The hydrolytic activity of cellulase was measured using filter paper assay (FPase) as according to Ghose [23]. Cellulase activity was estimated by following equation:

$$\text{Cellulase activity (U/mL)} = \frac{0.37}{[\text{enzyme releasing 2.0 mg of glucose}]} \quad (2)$$

Stability of immobilized cellulase on f-MWCNTs treated using H_3PO_4

Stability study for free and immobilized cellulase was conducted by varying the two parameters (temperature and pH) in accordance to previous study [24, 25]. For effect of temperature on enzyme activity, pH was kept constant at pH 4.5 meanwhile temperatures were varied from 30 °C, 40 °C, 50 °C, 60 °C, 70 °C until 80 °C. For effect of pH on enzyme activity, temperature was kept constant at 50 °C while pH was varied from 3 – 7. Subsequently, the enzyme activity was determined using FPase assay.

Reusability of immobilized cellulase on f-MWCNTs treated using H_3PO_4

The reusability of immobilized cellulase on f-MWCNTs treated using H_3PO_4 was assessed under optimum condition determined from previous stability study. The activity of the immobilized cellulase on the first cycle was used as the control and was assumed to be 100% [26].

Results and Discussion

One-factor-at-a-time on acid oxidation of MWCNTs

Effect of acid concentration

Acid concentration is acknowledged as one of the influential factors in functionalization process. To investigate the effect of acid molarity, three set of acid concentrations were prepared and tested; 4 M, 8 M, and 12 M. As shown in Figure 2, maximum performance was recorded at 8 M in line with previous study conducted by Shamsuddin et al. [19] and Taklimi et al. [8]. Apparently, increase in molarity, showed a minor decrement in the total adsorption of enzyme. The f-MWCNTs treated with 12 M H_3PO_4 experienced 3.6% decrement in total adsorption compared to f-MWCNTs treated with 8 M of acid (98.8%). According to Shamsuddin et al. [19] and Taklimi et al. [8], 8 M was the optimum condition and threshold

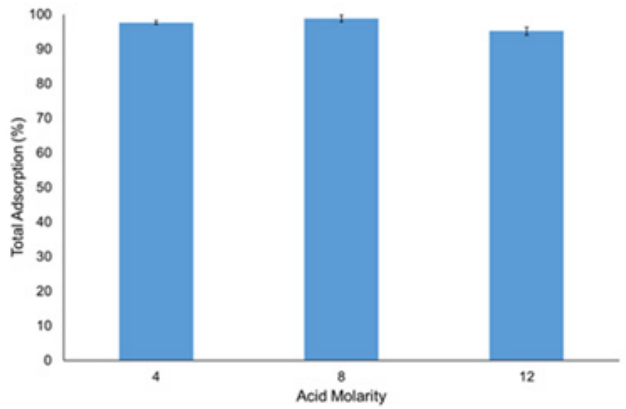

Figure 2: Total adsorption of cellulase on f-MWCNTs treated at various molarities of H_3PO_4 at 80 °C for 8 h.

for reaching maximum level of enzyme binding efficiency and dispersion. Higher molarity of acid could lead to higher number of surface defects during functionalization as it will attack the C=C bond of MWCNTs thus, breaking the aromatic ring structure. Besides that, working with acidic condition could result in loss of MWCNTs weight as reported by Yang et al. [27]. Up to 17% and 100% weight loss of f-MWCNTs were observed when the material was treated with 10 M and 15 M of H_2SO_4:HNO_3 respectively which can be correlated with the strong acidic conditions. Similar finding was experienced by Sezer and Koc [28] upon treatment of MWCNTs with 10 M and 15 M of HNO_3 in which weight loss correspond to 5% and 6.8%, respectively. Thus, 8 M of acid concentration was chosen as an ideal condition for acid oxidation aligned with Aftab et al. [29] finding where 8 M of acid can generate greater oxygenated functional group on f-MWCNTs surface thus lead to higher enzyme binding.

Effect of reflux time

To evaluate the effect of reflux time on f-MWCNTs, 4 set of conditions were chosen (4 h, 8 h, 12 h, and 24 h). Figure 3 represents total adsorption of cellulase on f-MWCNTs treated at various reflux time during acid oxidation. By referring to Figure 3, 8 h reflux time was observed as an ideal condition with 97.7% of total adsorption while slightly lower binding efficiency was recorded at 4 h (94.5%) of treatment

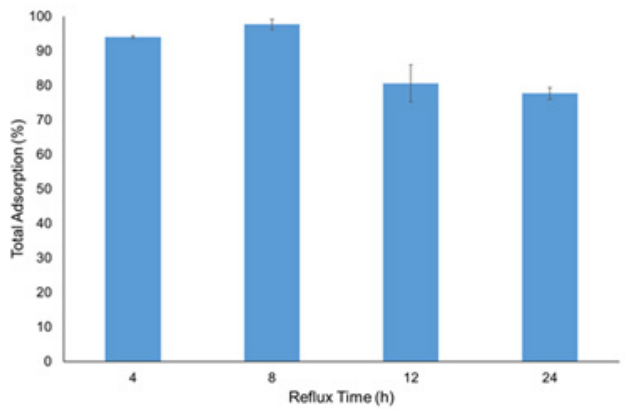

Figure 3: Total adsorption of cellulase on f-MWCNTs treated at various reflux time at 8 M of H_3PO_4 and 80 °C reflux temperature.

time. Further increase in reflux time showed poor adsorption consistent with our presumption as only 77.7% binding efficiency was determined with treatment for 24 h. This outcome was in accordance to Shamsuddin et al. [19] and Taklimi et al. [20] where longer reflux time would expose the MWCNTs towards continuous acid attack resulting in shorter tubes. On the contrary, Thi Hai Moa, [14] documented that longer reflux time, 18 h with lower acid concentration which was 4 M could generate more –COOH groups on f-MWCNTs surface.

Apparently, higher –COOH group could lead to elevated adsorption rate as it allows for higher affinity binding between enzyme molecule and the surface of f-MWCNTs [30]. Another study conducted by Buang et al. [31] also stated that 4 h of treatment time was sufficient in developing high density of oxygenated functional groups on the surface of f-MWCNTs which aid in the dispersion of the material. However, based on the experimental data and previous literature findings, 8 h was chosen as an ideal condition as it provided higher adsorption rate.

Effect of reflux temperature

Reflux temperature was varied from 40 °C, 80 °C, and 120 °C to evaluate the ideal condition for acid oxidation process. Figure 4 shows the total adsorption of enzyme on f-MWCNTs treated with H_3PO_4 at selected temperatures. As shown in Figure 4, higher enzyme adsorption up to 96.3% was obtained with 80 °C while 66.0% of decrement in adsorption rate was determined at 120 °C. According to Likodimos et al. [32] higher temperature process could result in degradation effects, formation of carbonaceous debris and structural defects. However, according to Taklimi et al. [8], 100 °C was an ideal condition as it produced carboxylic group peak that was revealed by FTIR analysis. Another study conducted by Yudianti et al. [33], observed that treatment of f-MWCNTs at both 80 °C and 100 °C showed enhancement in dispersibility and solubility. when the temperature was increased to 120 °C, lower dispersible rate was observed for f-MWCNT. Thus, 80 °C was selected for further experimental purposes.

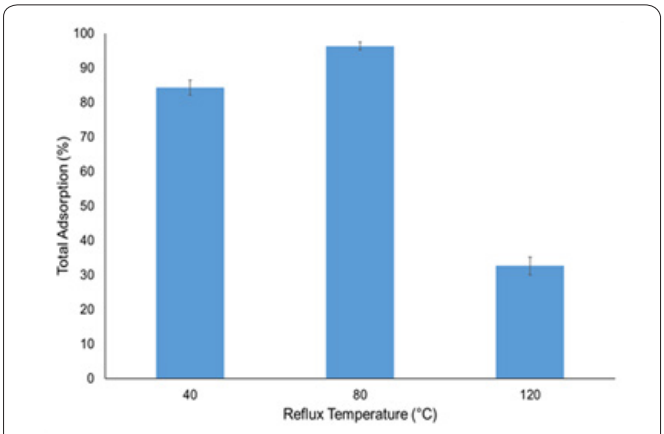

Figure 4: Total adsorption of cellulase on f-MWCNTs treated at various reflux temperatures at 8 M of H_3PO_4 for 8 h.

Characterization of f-MWCNTs treated with H_3PO , HNO_3 and HNO_3:H_2SO_4 at optimum conditions

Based from OFAT study, 8 M of acid molarity, 8 h reflux time and 80 °C of reflux temperature were chosen as an opti-

mum working condition for subsequent acid oxidation process of MWCNTs. To further evaluate the efficiency of H_3PO_4 for functionalization of MWCNTs, commonly used acids such as HNO_3 and the combination of HNO_3:H_2SO_4 were used at identical condition and the recorded FTIR peaks were compared.

Figure 5 displays FTIR spectra for f-MWCNTs treated with H_3PO_4, HNO_3 and HNO_3:H_2SO_4. From Figure 5, it can be concluded that, all f-MWCNTs generated similar peaks pattern. According to Varicca et al. [34], peaks in the range of 3240 – 3400 cm^{-1} attributed to hydroxyl group (-OH). Similar peaks were observed with f-MWCNTs treated with HNO_3 (Figure 5a), H_3PO_4 (Figure 5b), and HNO_3:H_2SO_4 (Figure 5c) at 3241.48 cm^{-1}, 3225.64 cm^{-1}, and 3230 cm^{-1}, respectively. Besides that, the existence of sharp peaks for all acids conditions at 1673 - 1674 cm^{-1} proven the formation of carboxyl group [35] resulting from acid oxidation process. The peaks identified in a range from 1373 to 1634 cm^{-1} indicating the presence of C=C that shows f-MWCNTs preserved its structure even after acid oxidation [36]. Thus, it can be concluded that, H_3PO_4 can be an alternative medium for acid oxidation of MWCNTs.

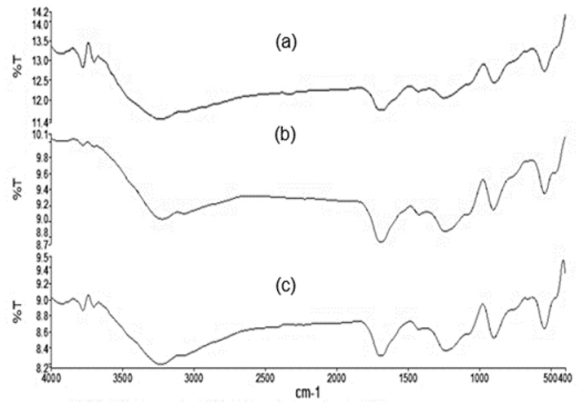

Figure 5: FTIR spectra on f-MWCNTs treated with (**a**) HNO_3, (**b**) H_3PO_4, and (**c**) HNO_3:H_2SO_4.

Characterization of MWCNTs, f-MWCNTs and immobilized cellulase on f-MWCNTs functionalized using H_3PO_4

Subsequently, FTIR analysis were carried out using MWCNTs, f-MWCNTs with H_3PO_4 which was subjected for immobilization of cellulase to differentiate and identify the peaks in each sample in order to evaluate its potential as an enzyme carrier. Figure 6a represent MWCNTs, where C-Br stretching was found at 524.40 cm^{-1} indicated that MWCNTs was generated via CVD method [37]. Comparison between Figure 6b (f-MWCNTs) and Figure 6c (immobilized cellulase on f-MWCNTs) revealed the presence of several additional peaks associated with enzyme binding such as at 1644 cm^{-1} and 2327.64 cm^{-1} indicating the C=N and C≡N linkages which proved that immobilization was successfully done [38].

Figure 7 shows the schematic diagram of MWCNTs mechanism route to f-MWCNTs during acid oxidation using H_3PO_4 followed by immobilization of cellulase on f-MWCNTs. As shown in the schematic diagram, formation of new functional groups were observed at each stage corresponding to the detection of new peaks in the FTIR spectra as in Figure 6.

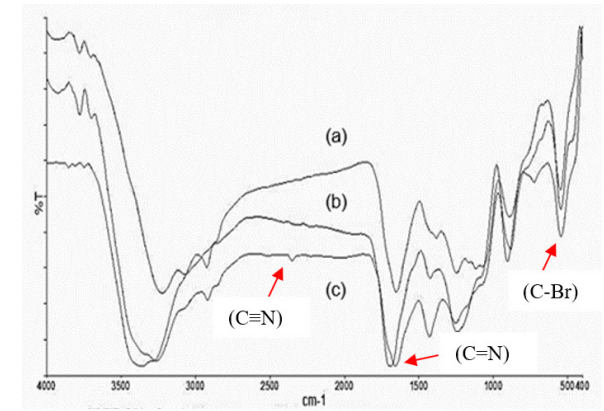

Figure 6: FTIR spectra of (**a**) MWCNTs, (**b**) f-MWCNTs, and (**c**) immobilized cellulase on f-MWCNTs treated via H_3PO_4.

Scanning Electron Microscopy (SEM) Analysis

In general, SEM analysis was performed to reveal the physical morphology of the f-MWCNTs and immobilized cellulase on f-MWCNTs treated using H_3PO_4. In our previous finding, MWCNTs was observed to be a snake-like shape structure [11]. Conversely, after acid oxidation using HNO_3 the f-MWCNTs tubes appeared untangled and distinctive from one to another similar to recent finding of f-MWCNTs treated using phosphoric acid as shown in Figure 8a and 8b provided the structural evidence of cellulase immobilization on f-MWCNTs where agglomeration structure (red circle) was observed compared to f-MWCNTs in Figure 8a which was primarily due to interaction between amine group of cellulase enzyme and carboxylic group of f-MWCNTs surface. Besides that, the existence of several bright spots (red arrow) in Figure 8b compared to f-MWCNTs further supported the successful attachment of cellulase enzyme on f-MWCNTs surface [37].

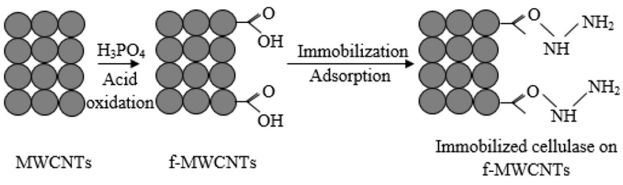

Figure 7: Mechanism route of MWCNTs, f-MWCNTs, and immobilized cellulase on f-MWCNTs treated using H_3PO_4.

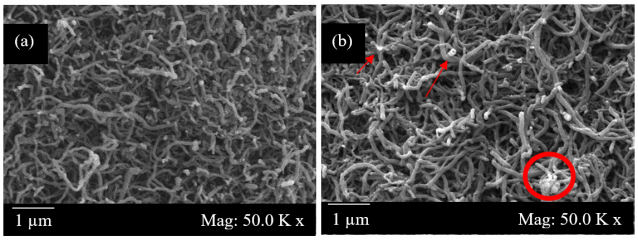

Figure 8: SEM images of (**a**) f-MWCNTs and (**b**) immobilized cellulase on f-MWCNTs treated via H_3PO_4.

Influence of temperature on free and immobilized cellulase performance

The optimum condition for both free and immobilized cellulase were examined by varying the temperature range. Figure 9 summarizes relative activity for both enzymes over temperature. Based on Figure 9, the optimum temperature for free cellulase was recorded at 50 ˚C meanwhile for immobilized cellulase was at 60 ˚C. The shift of the optimum temperature for immobilized cellulase might be due to the conformational changes resulting from immobilization restricting the flexibility of the enzyme on the carrier resulting better temperature tolerance [39]. The immobilized cellulase able to retained 39% of its activity at extreme temperature, 80 ˚C compared to free cellulase. This can be associated with the stability and rigidity of immobilized cellulase backbone which led to increase of temperature working range where denaturation of the catalytic activity structure become less prominent [40]. With the increased in temperature, free cellulase shows major decrement in activity due to denaturation of protein [37]. Similar trend was reported by Abraham et al. [41] where free cellulase from *Trichoderma reesei* showed optima temperature at 50 ˚C compared to 60 ˚C for immobilized cellulase on activated magnetic nanoparticles.

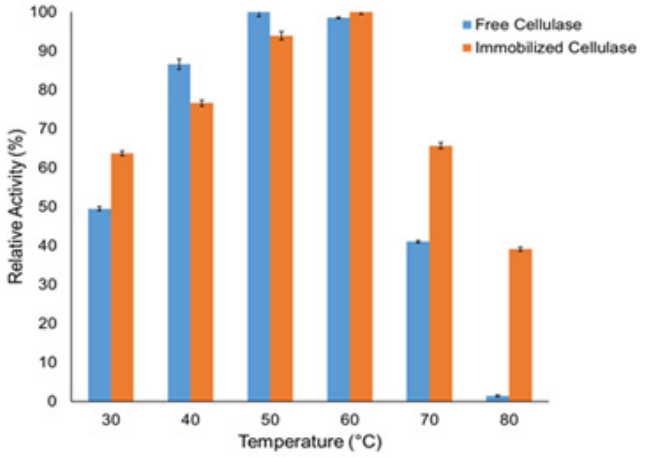

Figure 9: Relative activity of free and immobilized cellulase on f-MWCNTs at different temperatures at pH 4.5.

Influence of pH on free and immobilized cellulase performance

Besides temperature, pH plays a major role in enzyme performance as it would affect net charge and protein dissociation [37]. The optimum condition for both free and immobilized cellulase was recorded at pH 5 (Figure 10). From Figure 10, it can be concluded that immobilized cellulase displayed higher relative activity compared to free cellulase at each pH indicating immobilization allowed for better pH adaptability in aligned with findings by Yasmin et al. [11], Mubarak et al. [37], and Lin et al. [42] findings. Apparently, at alkaline condition, both enzymes demonstrated poor relative activity might be attributed to interaction between the charged group on the carrier surface and charge group present on enzyme molecule consequently modified the structure of both enzymes [39]. However, as shown in Figure 10, immobilized cellulase

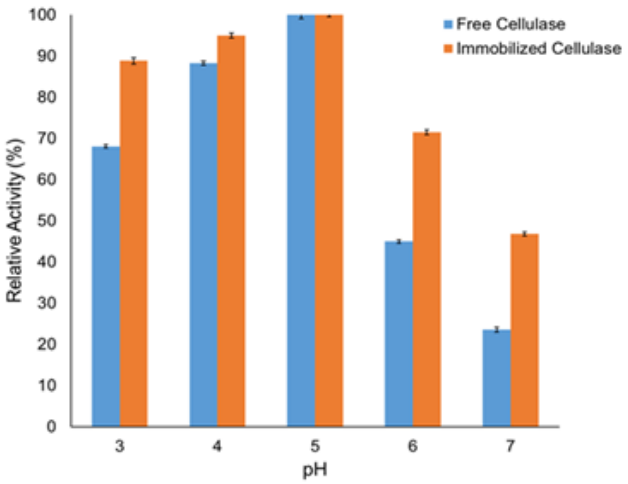

Figure 10: Reusability of immobilized cellulase on m-MWCNTs on pretreated paddy straw.

showed better stability at broader pH range compared to free cellulase despite of having same optima value.

Reusability of immobilized cellulase functionalized via H_3PO_4 using model substrate

In reusability study, the analysis was conducted under optimum working condition (60 ˚C, pH 5) that have been determined from previous section where filter paper Whatman No 1 was served as a substrate. Theoretically, the main aim for immobilization is to recycle the enzyme for several cycles of reactions. Figure 11 depicts the remaining activity (%) over number of reusability where it can be concluded that with multiple use of immobilized cellulase, the relative activity gradually decline. The reduced activity could be justified due to weak linkage of molecules that were lost during the process reaction, as some of the cellulases are not well bound on the surface of f-MWCNTs [43]. Besides, the hydrophilic properties of MWCNTs which resulting to increment of diameter may lead to the decrement of corresponding surface area after several cycles of assays [37]. In addition, Habimana et al. [44] claimed, the decrement on relative activity might be related to inactivation or denaturation of enzyme during recovery process that lead to decrease in enzyme activity.

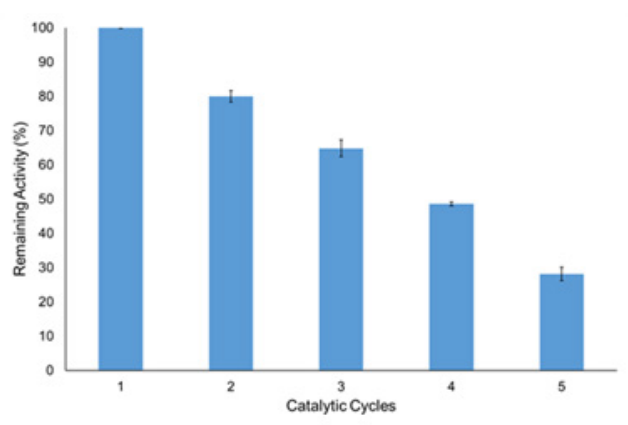

Figure 11: Reusability of immobilized cellulase on model substrate at pH 5 and 60 ˚C.

Previously, Li et al. [43] observed major decrement in immobilized cellulase activity after third cycles of reusability using carboxymethyl cellulose (CMC) (59.5%). However, in our study, the immobilized cellulase retained 64.8% of activity after third cycles using filter paper which is well-known as more complex substrate compared to CMC. Another study involving immobilization of cellulase on chemically synthesized magnetic nanoparticle conducted by Desai and Pawar, [45] experienced 43% loss of activity after fourth cycle of analysis. Abraham et al. [41] reported loss of 53% of activity of immobilized cellulase on nanoparticles after third cycle of analysis. Although the difference in the retained activity not significantly high, this research proved that MWCNTs treated with H_3PO_4 able to produce similar performance in comparison with MWCNTs treated with commonly used acid. Thus, H_3PO_4 could be a perfect substitution alternative for acid oxidation process.

Conclusion

In this work, the process parameters of the acid oxidation treatment of MWCNTs using H_3PO_4 were analyzed by varying the molarity of acid, reflux time, and temperature. Based on the experimental data, 8 M, 8 h, and 80 °C were selected as an ideal acid oxidation condition. The f-MWCNTs derived from the treatment at optimum condition was immobilized with cellulase. Comparison of the FTIR spectra of f-MWCNTs treated with H_3PO_4 and common acids (HNO_3 and HNO_3:H_2SO_4) proved that functionalization has been successfully done. Stability study revealed pH 5 as an optimal condition for both free and immobilized cellulase on f-MWCNTs treated with H_3PO_4. However, immobilized cellulase display better adaptability at broader pH range. Meanwhile ideal temperature was at 50 °C for free cellulase and 60 °C for immobilized cellulase. Reusability of immobilized cellulase at optimal condition using model substrate (filter paper Whatman No. 1) was determined to be 65% of its initial activity after third cycles and 28% after fifth cycle. Even though the reusability cycles were lower, it is important to highlight that H_3PO_4 which is a mild acid could be a perfect substitution in acid oxidation technique where it shows promising outcomes in terms of stability and reusability.

Acknowledgements

This study was supported by Fundamental Research Grant Scheme (FRGS), Grant No: 9003-00889 [FRGS/1/2020/TK0/UNIMAP/03/13] entitled "Ligninolytic Kinetics and Thermodynamic Properties of Laccase from Lignin-degrading Microorganism for Delignification of Agricultural Waste" from the Ministry of Higher Education Malaysia.

References

1. Iijima S. 1991. Helical microtubules of graphitic carbon. *Nature* 354(6): 56-58. https://doi.org/10.1038/354056a0
2. Durkin DP, Gallagher MJ, Frank BP, Knowlton ED, Trulove PC, et al. 2017. Phosphorus-functionalized multi-wall carbon nanotubes as flame-retardant additives for polystyrene and poly (methyl methacrylate). *J Therm Anal Calorim* 130: 735-753. https://doi.org/10.1007/s10973-017-6432-z
3. Gupta S, Murthy CN, Prabha CR. 2018. Recent advances in carbon nanotube based electrochemical biosensors. *Int J Biol Macromol* 108: 687-703. https://doi.org/10.1016/j.ijbiomac.2017.12.038
4. Rajeshwari P. 2016. Kinetic analysis of the non-isothermal degradation of high-density polyethylene filled with multi-wall carbon nanotubes. *J Therm Anal Calorim* 123: 1523-1544. https://doi.org/10.1007/s10973-015-5021-2
5. Zhang W, Johnson L, Silva SRP, Lei MK. 2012. The effect of plasma modification on the sheet resistance of nylon fabrics coated with carbon nanotubes. *Appl Surf Sci* 258(20): 8209-8213. https://doi.org/10.1016/j.apsusc.2012.05.023
6. Cardano F, Frasconi M, Giordani S. 2018. Photo-responsive graphene and carbon nanotubes to control and tackle biological systems. *Front Chem* 6: 102. https://doi.org/10.3389/fchem.2018.00102
7. Teradal NL, Jelinek R. 2017. Carbon nanomaterials in biological studies and biomedicine. *Adv Healthc Mater* 6(17): 1700574. https://doi.org/10.1002/adhm.201770089
8. Taklimi S, Ghazinezami A, Cluff K, Askari D. 2018. Effects of reflux temperature and molarity of acidic solution on chemical functionalization of helical carbon nanotubes. *SAE Int J Mater Manuf* 11(1): 29-36. https://www.jstor.org/stable/26556828
9. Garnica-Gutiérrez RL, Lara-Martínez LA, Palacios E, Masso F, Contreras A, et al. 2018. Effect of functionalized carbon nanotubes and their citric acid polymerization on mesenchymal stem cells *in vitro*. *J Nanomater* 2018: 5206093. https://doi.org/10.1155/2018/5206093
10. Ma PC, Siddiqui NA, Marom G, Kim JK. 2010. Dispersion and functionalization of carbon nanotubes for polymer-based nanocomposites: a review. *Compos Part A Appl Sci Manuf* 41(10): 1345-1367. https://doi.org/10.1016/j.compositesa.2010.07.003
11. Yasmin HAN, Naresh S, Kunasundari B, Shuit SH. 2022. Immobilization of cellulase on magnetized multiwall carbon nanotubes (m-MWCNTs) synthesized via eco-friendly (water-based) method. *Chem Pap* 76: 453-464. https://doi.org/10.1007/s11696-021-01874-7
12. Wulan PP, Ulwani SH, Wulandari H, Purwanto WW, Mulia K. 2018. The effect of hydrochloric acid addition to increase carbon nanotubes dispersibility as drug delivery system by covalent functionalization. *IOP Conf Ser Mater Sci Eng* 316: 012013. https://doi.org/10.1088/1757-899x/316/1/012013
13. Singer G, Siedlaczek P, Sinn G, Rennhofer H, Mičušík M, et al. 2018. Acid free oxidation and simple dispersion method of MWCNT for high-performance CFRP. *Nanomaterials (Basel)* 8(11): 912. https://doi.org/10.3390/nano8110912
14. Hao LTM. 2018. Characterization of multi-walled carbon nanotubes functionalized by a mixture of HNO_3/H_2SO_4. *Diam Relat Mater* 89: 43–51. https://doi.org/10.1016/j.diamond.2018.08.008
15. Gojny FH, Nastalczyk J, Roslaniec Z, Schulte K. 2003. Surface modified multi-walled carbon nanotubes in CNT/epoxy-composites. *Chem Phys Lett* 370(5-6): 820–824. https://doi.org/10.1016/s0009-2614(03)00187-8
16. Li N, Liu H, Zhang X. 2014. Functionalized multiwalled carbon nanotubes in mild polyphosphoric acid/phosphorous pentoxide/phosphoric acid and their composites with epoxy resin. *Polym Compos* 35(7): 1275-1284. https://doi.org/10.1002/pc.22778
17. Zakeri M, Abouzari-lotf E, Miyake M, Mehdipour-Ataei S, Shameli K. 2019. Phosphoric acid functionalized graphene oxide: a highly dispersible carbon-based nanocatalyst for the green synthesis of bio-active pyrazoles. *Arab J Chem* 12(2): 188-197. https://doi.org/10.1016/j.arabjc.2017.11.006
18. Lee HJ, Han SW, Kwon YD, Tan LS, Baek JB. 2008. Functionalization of multi-walled carbon nanotubes with varies 4-substituted benzoic acids in mild polyphosphoric acid/phosphorus pentoxide. *Carbon* 46(14):1850-1859. https://doi.org/10.1016/j.carbon.2008.07.027

19. Shamsuddin SA, Derma MN, Hashim U, Kashif M, Adam T et al. 2016. Nitric acid treated multi-walled carbon nanotubes optimized by Taguchi method. *AIP Conference Proceedings* 1756(1): 09002. https://doi.org/10.1063/1.4958783

20. Taklimi SR, Ghaznine zami A, Askari D. 2019. Chemical functionalization of helical carbon nanotubes: influence of sonication time and concentrations of sulfuric and nitric acids with 3:1 mixing ratio. *J. Nanomater* 2019: 2836372. https://doi.org/10.1155/2019/2836372

21. Shuit SH, Tan SH. 2014. Feasibility study of various sulphonation methods for transforming carbon nanotubes into catalysts for the esterification of palm fatty acid distillate. *Energy Convers Manag* 88: 1283-1289. https://doi.org/10.1016/j.enconman.2014.01.035

22. Khan AK, Mubarak NM, Abdullah EC, Khalid M, Nizamuddin S, et al. 2019. Immobilization of lipase enzyme carbon nanotubes via adsorption. *IOP Conf Ser Mater Sci Eng* 495: 012055. https://doi.org/10.1088/1757-899x/495/1/012055

23. Ghose TK. 1987. Measurement of cellulase activities. *Pure Appl Chem* 59(2): 257-268. https://doi.org/10.1351/pac198759020257

24. Lv P, Feng Q, Wang Q, Li G, Li D, et al. 2016. Biosynthesis of bacterial cellulose/carboxylic multi-walled carbon nanotubes for enzymatic biofuel cell application. *Materials* 9(3): 183. https://doi.org/10.3390/ma9030183

25. Han J, Rong J, Wang Y, Liu Q, Tang X, et al. 2018. Immobilization of cellulase on thermo-sensitive magnetic microspheres: improved stability and reproducibility. *Bioprocess Biosyst Eng* 41: 1051-1060. https://doi.org/10.1007/s00449-018-1934-z

26. Keskin Ş, Ertunga, NS. 2017. Purification, immobilization and characterization of thermostable α-amylase from a thermophilic bacterium *Geobacillus* sp. TF14. *Turkish J Biochem* 42(6): 633-642. https://doi.org/10.1515/tjb-2016-0123

27. Yang J, Wang SC, Zhou XY, Xie J. 2012. Electrochemical behaviors of functionalized carbon nanotubes in LiPF6/EC+ DMC electrolyte. *Int J Electrochem Sci* 7(7): 6118-6126.

28. Sezer N, Koç M. 2019. Oxidative acid treatment of carbon nanotubes. *Surf Interfaces* 14: 1-8. https://doi.org/10.1016/j.surfin.2018.11.001

29. Aftab S, Nawaz H, Siddiq M. 2013. Observing trends in the functionalization of CNTs using same oxidizing acids under two different conditions. *J Chem Soc Pak* 35: 308-313.

30. Manzetti S, Gabriel JCP. 2019. Methods for dispersing carbon nanotubes for nanotechnology applications: liquid nanocrystals, suspensions, polyelectrolytes, colloids and organization control. *Int Nano Lett* 9(1): 31-49. https://doi.org/10.1007/s40089-018-0260-4

31. Buang NA, Fadil F, Majid ZA, Shahir S. 2012. Characteristic of mild acid functionalized multiwalled carbon nanotubes towards high dispersion with low structural defects. *Dig J Nanomater Biostructures* 7(1): 33-39.

32. Likodimos V, Steriotis TA, Papageorgiou SK, Romanos GE, Marque RR, et al. 2014. Controlled surface functionalization of multiwall carbon nanotubes by HNO_3 hydrothermal oxidation. *Carbon* 69: 311-326. https://doi.org/10.1016/j.carbon.2013.12.030

33. Yudianti R, Indrarti L, Onggo H. 2010. Thermal behavior of purified multi walled carbon nanotube. *J Appl Sci* 10(17): 1978-1982. https://doi.org/10.3923/jas.2010.1978.1982

34. Varrica D, Tamburo E, Vultaggio M, Di Carlo I. 2019. ATR–FTIR spectral analysis and soluble components of PM10 and PM2.5 particulate matter over the urban area of Palermo (Italy) during normal days and saharan events. *Int J Environ Res Public Health* 16(14): 2507. https://doi.org/10.3390/ijerph16142507

35. Saxena M, Sharma N, Saxena R. 2020. Highly efficient and rapid removal of a toxic dye: adsorption kinetics, isotherm, and mechanism studies on functionalized multiwalled carbon nanotubes. *Surf Interfaces* 21: 100639. https://doi.org/10.1016/j.surfin.2020.100639

36. Murad FN, Kabbashi NA, Alam MZ, Rashid MAF. 2018. Immobilization of fungal biomass with multi-walled carbon nanotubes as biosorbent. *Malays J Microbiol* 14(3): 113-119. https://doi.org/10.21161/mjm.67318

37. Mubarak NM, Wong JR, Tan KW, Sahu JN, Abdullah EC, et al. 2014. Immobilization of cellulase enzyme on functionalized multiwall carbon nanotubes. *J Mol Catal B Enzym* 107: 124-131. https://doi.org/10.1016/j.molcatb.2014.06.002

38. Zhang D, Hegab HE, Lvov Y, Dale Snow L, Palmer J. 2016. Immobilization of cellulase on a silica gel substrate modified using a 3-APTES self-assembled monolayer. *SpringerPlus* 5: 48. https://doi.org/10.1186/s40064-016-1682-y

39. Kaur P, Taggar MS, Kalia A. 2021. Characterization of magnetic nanoparticle–immobilized cellulases for enzymatic saccharification of rice straw. *Biomass Conv Bioref* 11: 955-969. https://doi.org/10.1007/s13399-020-00628-x

40. Jia J, Zhang W, Yang Z, Yang X, Wang N, et al. 2017. Novel magnetic cross-linked cellulase aggregates with a potential application in lignocellulosic biomass bioconversion. *Molecules* 22(2): 269. https://doi.org/10.3390/molecules22020269

41. Abraham RE, Verma ML, Barrow CJ, Puri M. 2014. Suitability of magnetic nanoparticle immobilised cellulases in enhancing enzymatic saccharification of pretreated hemp biomass. *Biotechnol Biofuels* 7: 90. https://doi.org/10.1186/1754-6834-7-90

42. Lin Y, Liu X, Xing Z, Geng Y, Wilson J, et al. 2017. Preparation and characterization of magnetic Fe_3O_4–chitosan nanoparticles for cellulase immobilization. *Cellulose* 24: 5541-5550. https://doi.org/10.1007/s10570-017-1520-6

43. Li LJ, Xia WJ, Ma GP, Chen YL, Ma YY. 2019. A study on the enzymatic properties and reuse of cellulase immobilized with carbon nanotubes and sodium alginate. *AMB Express* 9(1): 112. https://doi.org/10.1186/s13568-019-0835-0

44. Habimana P, Gao J, Mwizerwa JP, Ndayambaje JB, Liu H, et al. 2021. Improvement of laccase activity via covalent immobilization over mesoporous silica coated magnetic multiwalled carbon nnotubes for the discoloration of synthetic dyes. *ACS Omega* 6(4): 2777-2789. https://doi.org/10.1021/acsomega.0c05081

45. Desai MP, Pawar KD. 2020. Immobilization of cellulase on iron tolerant *Pseudomonas stutzeri* biosynthesized photocatalytically active magnetic nanoparticles for increased thermal stability. *Mater Sci Eng C* 106: 110169. https://doi.org/10.1016/j.msec.2019.110169