Statistical Modelling Optimisation of Cellulase Enzyme Immobilisation on Functionalised Multi-Walled Carbon Nanotubes for Empty Fruit Bunches Degradation

Ma'an F. Alkhatib, M.d. Zahangir Alam, Rasha Mohammed

Nanoscience and Nanotechnology Research Group (NANORG) Biotechnology Engineering Department Faculty of Engineering International Islamic University Malaysia Gombak Kuala Lumpur, Selangor, Malaysia.

Abstract: Cellulase obtained from the fermentation of sewage treatment plant sludge (STP) by *Trichoderma-reesei* RUT C-30 was covalently immobilised on functionalised multi-wall carbon nanotubes. Statistical optimisation using the Plackett–Burman design method was implemented to identify parameters with significant effects on the process of immobilisation. The results obtained from this Plackett–Burman design show that three parameters have a significant effect on immobilisation: pH, temperature, and N-ethyl-N-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) concentration. Based on our Plackett-Burman design results, these parameters were further optimised using a face-centred central composite design. The resulting optimum conditions for cellulase immobilisation, as determined by face-centred central composite design, were pH 4.5, 30°C, and 1 mL of 10mg/mL EDC. The amount of immobilised cellulase was approximately 98% using these optimum conditions. The resulting MWCNT-cellulase composite was further characterized by FTIR and SEM. The FTIR spectrum of MWCNT-cellulase composite showed an amide group peak (O = C-NH) corresponding to cellulase enzyme, which confirms that immobilisation took place.

Key words: MWCNTs; Immobilisation; STP Enzyme; Optimisation; FCCCD; EFB.

INTRODUCTION

The catalytic properties of enzymes have enhanced the application of Nature's biocatalysts for numerous industrial products and processes for sustainable development (Guisan, 2008). Cellulase is an industrial enzyme, which is mainly produced by fungi and bacteria. It is responsible for cellulose degradation by catalyzing the hydrolysis of β-1, 4 glycosidic bonds in cellulosic materials, to produce short cellulo-oligosaccharides and glucose (Afsahi *et al.*, 2007; Xu, *et al.*, 2007). Among cellulase enzymes produced by different microorganisms, cellulase from *Trichoderma reesei* has been widely studied, and is among the best characterised. This cellulase displays both high stability and resistance to chemical inhibitors under industrial enzymatic hydrolysis conditions (Mansfield and Saddler, 2003; Taherzadeh and Karimi, 2007). Cellulase enzymes have many industrial applications, including in the production of detergents, food, animal feed, textiles, pulp and paper, and pharmaceuticals (Prato *et al.*, 2003). However, because cellulase enzymes are water soluble, easily undergo inactivation, poorly adhere to non-natural substrates, and are difficult to separate from end value products; cellulase is not yet an attractive option for all potential industrial applications (Guisan, 2008). These problems, associated with free cellulase in solution, may be overcome through the use of immobilised cellulase.

Immobilisation of enzymes on a carrier offers significant cost benefits for industrial processes, because it facilitates enzyme recycling, enables improvements in thermo-stability (thereby reducing enzyme inactivation), and allows for greater control of enzyme activity (Worsfold, 1995; Tu *et al.*, 2008). Immobilised enzymes have been defined as enzymes that are physically confined or localised, while retaining their catalytic activity, and which can be used repeatedly and continuously (Worsfold, 1995). Recently, several substrates have been tested for use in the immobilisation of cellulase enzymes for industrial applications, such as: polyurethane foam (Chakrabarti and Storey ,1988), acrylamide grafted membranes (Yuan *et al.*, 1999), polyvinyl alcohol nanofibers (Wu *et al.*, 2005), and ultrafine silica particles (Afsahi *et al.*, 2007).

Since the discovery of carbon nanotubes (CNTs) in 1991 (Iijima and Ichihashi, 1991), CNTs have attracted the attention of researchers from the fields of physics, chemistry and materials science, because of their astonishing structural and mechanical properties (Kirk *et al.*, 2002). In addition, the size of CNTs, and in particular their very high specific surface area and extremely large length-to-diameter ratio, make them accessible to both electrochemistry and the immobilisation of biomolecules (Lee *et al.*, 2006; Yang *et al.*, 2007). Immobilisation of biomolecules onto CNTs has been successful using several mechanisms. CNTs have been used as an adsorptive support material, for example, in the immobilisation of β- glucosidase (Gomez *et al.*, 2005), whereas immobilisation of avidin and horseradish peroxidase was achieved through covalent linkage

Corresponding Author: Ma'an F. Alkhatib, Nanoscience and Nanotechnology Research Group (NANORG) Biotechnology Engineering Department Faculty of Engineering International Islamic University Malaysia Gombak Kuala Lumpur, Selangor, Malaysia. Tel.: +60361964441, Fax: +60361964442
E-mail: maan@iium.edu.my

(Lee *et al.*, 2006; Kim *et al.*, 2006). However, to our knowledge no work is done on immobilization of cellulase (endo-glucabase) enzyme on CNTs.

Based on these studies, it was found that immobilisation techniques can be influenced by many factors, including temperature, enzyme concentration, coupling reagent, pH, substrate amount and immobilisation time. If no proper experimental design is followed, studying the effects of all these factors will result with large number of experiments, which consequently engage more time with a higher cost. For an efficient experimental design, screening of all these factors, using for example Plackett–Burman design (PBD), is essential to find the most significant ones on the immobilization process. Subsequently, response surface method (RSM) which is found to be a useful tool to study the interactions of two or more factors (Ahmad *et al.*, 2009) can be applied to obtain the optimal values of the significant factors under study.

In this study the immobilisation of cellulose, endo-glucabase, (obtained from the fermentation of sewage treatment plant (STP) sludge) by *Trichoderma-reesei* (RUT C-30) on CNTs was done by applying statistical design methods. Optimisation was carried out in two stages. In the first stage, Plackett–Burman design (PBD) was used to evaluate immobilisation parameters (CNT concentration, enzyme concentration, immobilisation time, pH, temperature, and EDC coupling reagent concentration). Consequently, face-centred central composite design (FCCCD) of response surface methodology (RSM) was used to determine optimal values for each of the significant variables to obtain a high level of enzyme loading.

MATERIALS AND METHODS

Supplies and Chemicals:

Multi-Walled Carbon Nanotubes (MWCNTs) (> 95% pure, 30 μm in length, and with a 10-20 nm outer diameter) were purchased from the Chinese Science Academy and used without further modification. Cellulase enzyme (27 U/mL activity) was obtained by fermentation of sewage treatment plant (STP) sludge by *T. reesei* RUT C-30, at the Department of Biotechnology Engineering, International Islamic University of Malaysia (IIUM). Hydrophilised PTFE membranes (0.45μm) were purchased from Sartrious Stedim, Germany. (65%) HNO_3, (97%) H_2SO_4, N-ethyl-N-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC), and N-hydroxysuccinimide (NHS), were purchased from MERCK, Germany. pH adjustments were made using 0.1M hydrochloric acid and/or 0.1 M sodium hydroxide. 0.05M citrate buffer (pH=4.8), dinitrosalicyclic acid (DNS) reagent, and 500 mL of 2% (w/v) Carboxymethyl Cellulose (CMC) solution were prepared for use as reagents for enzymatic assays.

Functionalisation of MWCNTS:

Functionalisation of MWCNTs with carboxylic acid groups was carried out by sonicating 200 mg of MWCNTs in 10 mL of a concentrated acid mixture of HNO_3 and H_2SO_4 at a 1:3 ratio (v/v) using bath sonication (230 V, ~50Hz, 430W) at 40°C for 2 hrs. The resulting sample was then diluted with distilled water and filtered through a 0.45 μm PTFE membrane using a vacuum filtration system. The sample was rinsed with distilled water (4-5 times) until the pH was measured to be neutral. Functionalized MWCNTs were then dried in a vacuum oven at 80°C for 48 hrs, followed by cooling in a desiccator for 24 hrs (Al-khatib *et al.*, 2009).

Immobilisation of Cellulase Enzyme on Functionalised MWCNTs:

Covalent bond formation, between carboxylic acid groups on the functionalised MWCNTs and amide groups on the cellulase enzyme, was achieved with the aid of a chemical coupling agent (Jiang *et al.*, 2004). 1 mg of functionalised MWCNTs were re-suspended in 2.5 mLs of distilled water, followed by brief sonication. Then, 1 mL of a (0.1M) buffer solution (pH = 6) and (50mg/mL) N-hydroxysuccinimide (NHS) solution was added to the suspension and mixed. Under continuous stirring, varying amounts of a 10mg/mL EDC solution were added and the resulting mixture was stirred at room temperature (30 ± 2°C) for 30 min. This mixture was then filtered through a 0.45 μm PTFE membrane and rinsed thoroughly 4-5 times with (0.1M) buffer solution (pH = 6). In the second step, resulting MWCNTs were re-dispersed in 4.5 mLs of (0.1M) buffer solution (pH = 6) and (10-20 mLs) of cellulase enzyme in 1 mL of (0.1M) buffer solution (pH = 6) was added. After shaking the mixture in an incubator shaker at 150 rpms for 1 hr, the suspension was centrifuged at 4000 rpms at 4°C for 40 min. Unbound enzyme was removed by rinsing three times with (0.1M) buffer solution (pH=6). The MWCNT-cellulase composite was left to air dry for 24 hrs before characterisation and further use.

Analytical Methods:

Cellulase activity before and after immobilisation was determined using the Carboxymethyl cellulose (CMC) assay (Ghose, 1987). CMC was used as a substrate to measure endo-β-1, 4-glucanase activity. One activity unit (U) of cellulase is defined as the amount of enzyme that catalyses CMC hydrolysis to generate 1 mg equivalent of glucose per minute under assay conditions. To determine the activity of the enzyme, 0.5 mL of enzyme sample was diluted with 0.05 M citrate buffer (pH = 4.8). Two dilutions were prepared for each sample

investigated. The diluted enzyme samples were pre-warmed at 50°C for 5 minutes, then, 0.5 mLs of substrate solution (CMC) was added and the samples were incubated in a water bath at 50°C for exactly 30 minutes. 3 mLs of DNS reagent was added to all enzyme samples. 0.5 mL of enzyme was added to the enzyme blank and 0.5 mL of citrate buffer was added to the spectrophotometer blank. Prior to spectrophotometer analysis, samples containing the enzyme blanks, glucose standards and the spectrophotometer blank were boiled in a water bath at 95°C for exactly 5 minutes and then transferred immediately to an ice bath, and kept on ice until cool. After dilution in 5 mLs of distilled water, colour formation in all samples was measured using a spectrophotometer against the spectrophotometer blank at a wavelength of 540 nm. Absorbance values were used to determine the amount of glucose produced using a glucose standard curve.

Characterisation of The Immobilised Cellulase Enzyme:

The chemical composition of the functionalised MWCNTs and MWCNT-cellulase composite was characterised by Fourier Transform Infrared Spectroscopy (FTIR, Bruker, IFS66v/S, Germany). Visualisation of the physical structure of the MWCNTs and confirmation of immobilisation were achieved using Field Emission Scanning Electron Microscopy (FEI Quanta 200 FESEM, Japan). The absorbance of free and immobilised cellulase enzyme was determined using a Spectrophotometer (Sp- 300- Plus- OPTIMA).

Experimental Design And Statistical Modelling:

The statistical software, DESIGN-EXPERT 6.0.8 (Stat Ease Inc., Minneapolis, USA), was used to create the experimental design and analysis, as well as to predict optimal conditions through the development of an appropriate model. Residual cellulase activity in the supernatant was defined as the 'response' for this study.

Plackett–Burman Design (PBD):

PBD was used for preliminary screening and to identify significant parameters that affect the immobilisation of cellulase on MWCNTs. PBD is a very useful, widely employed statistical technique for identifying the major independent variables or factors that have a significant effect on a particular response (Plackett and Burman, 1964). The design is suitable for up to 31 factors, with each factor varying over 2 levels, and is practical for preliminary screening where the goal is to find out if there is little or no effect on a response (in this case residual enzyme activity) due to any of the factors. The design output consisted of 12 experimental runs of 6 variables each: concentration of MWCNTs, enzyme concentration, immobilisation time, pH, reaction temperature, and coupling reagent (EDC) dose. The design contained two levels (high and low values), one block, three replications and one centre point. Residual enzyme activity was defined as the response (Table 1). The design was based on a first order model as follows:

$$Y = \beta_0 + \sum \beta_i x_i \tag{1}$$

Where: Y is the estimated response (residual enzyme activity), β_0 is the model intercept, β_i is the linear regression coefficient, and x_i is the independent variable (Levien *et al.*, 2005).

Faced Centred Central Composite Design (FCCCD):

The faced centred central composite design (FCCCD) method was implemented in order to develop a second order model using the parameters identified as significant by the PBD analysis. This design method consisted of 15 sets of experiments (5 centre points), with three variables: pH, temperature, and EDC dose, based on the results obtained from PBD. Factors were examined at high, centre, and low levels as shown in Table 2. A second order model was selected for predicting optimal points and is expressed as:

$$Y = \beta_0 + \beta_1 A_1 + \beta_2 B_2 + \beta_3 C_3 + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \tag{2}$$

Where: Y represent response variables (residual enzyme activity), β_0 is the interaction coefficient, β_1 and β_2 are linear terms, β_{11}, β_{22}, and β_{33} are quadratic terms and A, B, and C are independent variables (pH, temperature, and EDC concentration). The other parameters: CNT concentration, enzyme concentration, and reaction time, were fixed according to the results of the PBD analysis.

The resulting model was analysed using 'analysis of variance' (ANOVA), *p*- and *F*- values, as well as regression coefficient values. The 'goodness-of-fit' of the quadratic model equation was expressed as the determination coefficient (R^2).

RESULTS AND DISCUSSION

Functionalisation:

FTIR spectrum and FESEM images were acquired for MWCNTs and functionalised MWCNTs in order to confirm MWCNT functionalisation. Fig. 1(a) shows the FTIR spectrum for an MWCNT sample. The peak observed at 1630 cm^{-1} corresponds to C = C stretching associated with side wall defects. In contrast, Fig. 1(b) shows the FTIR spectrum for functionalised MWCNTs. Peaks at 2885 cm^{-1} and 3390 cm^{-1} most likely correspond to CH groups and - OH groups, respectively. In addition, the small peaks in the 1716-1730 cm^{-1} range correspond to C = O stretching resulting from the acid treatment, and indicate that the C = O groups might be more prevalent at the termini of the MWCNTs, thus facilitating functionalisation of the tubes.
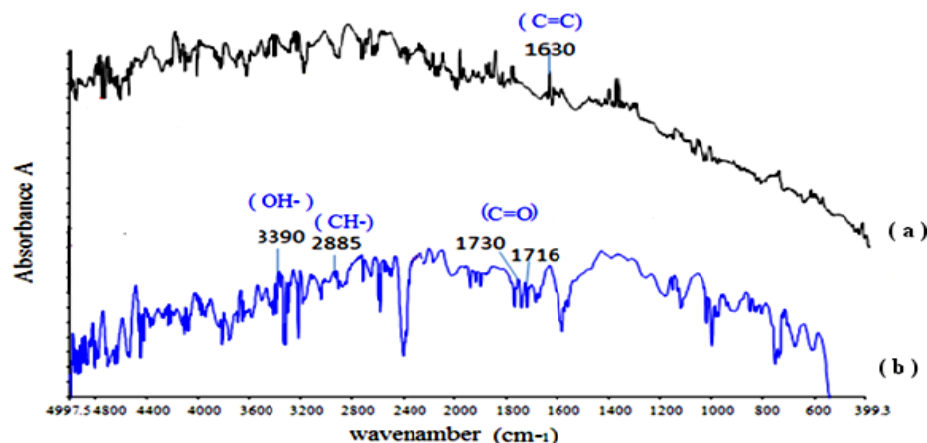

Fig.1: FTIR spectrum of a) MWCNTs b) functionalised MWCNTs.

FESEM images of MWCNTs and functionalised MWCNTs at 100,000X showed that the bundles of nanotubes were tangled, with typical tube lengths on the order of microns, and some impurities present on the surface (Fig. 2a). Upon sonication in the acid mixture, the MWCNTs appeared shorter and untangled, resulting in more free termini. In addition, acid treated MWCNTs appear to have a rough surface and the tubes appear joined together with fewer surface impurities (Fig. 2b), in agreement with previously reported results (Kim *et al.*, 2006).
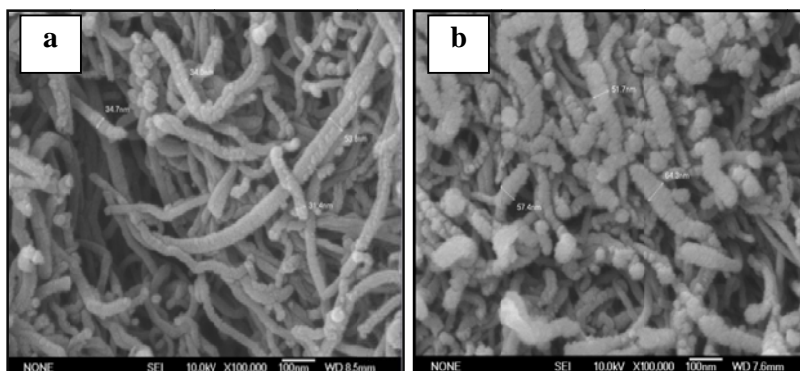

Fig. 2: FESEM images of a) MWCNTs (as received) and b) functionalised MWCNTs.

Immobilisation of Cellulase Enzyme on MWCNTs:

Preliminary Screening of Parameters For Cellulase Enzyme Immobilisation Using PBD:

As a first step in the optimisation process, PBD was used to evaluate the significance of each parameter for optimum conditions. This design, consisting of the variables and response measurement (residual enzyme activity), is shown in Table 1. The design assumes no interaction among the variables, and effects can be calculated by simply observing differences between the average of measurements made at high levels (+1) and the average of measurements made at low levels (-1). By studying these main effects, we observed that pH, EDC concentration, CNT concentration, and enzyme concentration had positive effects on enzyme residual

activity (response), while temperature and time had negative effects (Fig. 3). An 'Analysis of variables' (ANOVA) test (Table 2) for the model resulted in a *p*-value of 0.018, implying that the model is significant. Among the variables tested, pH and EDC concentration had significant positive effects on the response, whereas temperature had a significant negative effect. The remaining variables did not have any significant effect on the response. The determination coefficient (R^2) was 0.94, indicating that the model is credible. As a result, temperature, pH and EDC concentration were selected for further investigation where the interaction among these parameters was considered.

Table 1: (PBD) Screening for immobilization parameters and measurements of response.

Run	A (mg)	B (ml)	C (min)	D (ml)	E	F (°C)	Residual cellulase activity (U/ml)
1	3(+)	10(-)	90(+)	1(-)	4(-)	30(-)	20
2	3(+)	20(+)	30(-)	2(+)	6(+)	30(-)	22
3	3(+)	20(+)	30(-)	2(+)	4(-)	30(-)	21
4	1(-)	10(-)	30(-)	2(+)	6(+)	50(+)	20
5	1(-)	10(-)	90(+)	2(+)	6(+)	30(-)	21
6	1(-)	20(+)	90(+)	2(+)	4(-)	50(+)	18
7	3(+)	10(-)	30(-)	1(-)	6(+)	50(+)	19
8	1(-)	20(+)	30(-)	1(-)	4(-)	50(+)	18
9	3(+)	10(-)	90(+)	2(+)	4(-)	50(+)	18
10	3(+)	20(+)	90(+)	1(-)	6(+)	50(+)	19
11	1(-)	20(+)	90(+)	1(-)	6(+)	30(-)	20
12	1(-)	10(-)	30(-)	1(-)	4(-)	30(-)	19

*A, CNT amount; B, enzyme dose; C, time; D, EDC dose; E, pH; F, temperature; Response, residual enzyme activity. The (-) indicates the low level, the (+) indicates the high level.
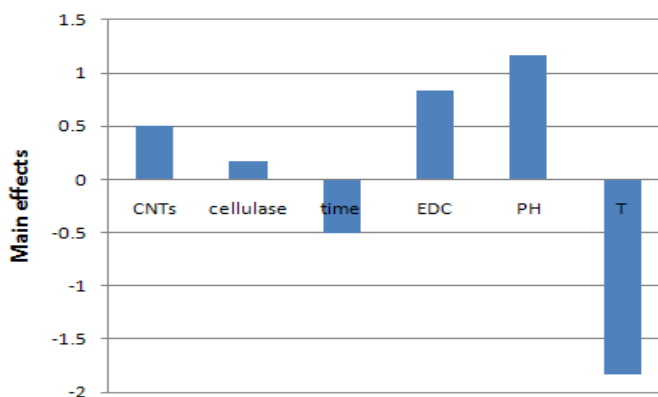

Fig. 3: Main effects of parameters on cellulase enzyme immobilization.

Table 2: Analysis of variance by the ANOVA test for the selected factorial model (PBD).

Source	Sum of square	F value	p-value> F	
Model	17.77	11.10	0.018	Significant
CNTs amount	0.54	2.04	0.226	
Enzyme dose	0.10	0.38	0.573	
Immobilization time	0.75	2.81	0.169	
EDC dose	1.60	6	0.051*	
pH	4.08	15.31	0.017**	
Temperature	8.71	32.67	0.005**	
R-squared	0.943			

*p<0.05indicates that the term is significant
**p<0.01indicates that the term is highly significant

Statistical Optimisation of Immobilisation Conditions by FCCCD Using Response Surface Methodology (RSM):

FCCCD with RSM was used to evaluate the nature of the response surface in the experimental region and to identify optimal values for the most significant variables. In this step of optimisation, three parameters: pH, temperature, and EDC concentration, were selected as independent variables (Table 3) in an effort to minimise the residual enzyme activity in the supernatant and, consequently, maximise cellulase enzyme loading onto MWCNTs during immobilisation. The regression equation was developed using RSM, allowing for analysis of interacting factors by identifying which significant factors contribute to the regression model, and determining the optimal values of the most significant independent variables (Rashid *et al.*, 2009).

Table 3: FCCCD showing coded and actual factors with experimental response values.

Run	A	B (°C)	C (ml)	Residual cellulase activity (U/ml)
1	6(0)	20(-)	2(0)	1.83
2	6(0)	30(0)	1(-)	1.4
3	3(-)	40(+)	3(+)	4.5
4	9(+)	40(+)	1(-)	3.7
5	9(+)	20(-)	3(+)	1.6
6	6(0)	40(+)	2(0)	1.74
7	3(-)	20(-)	1(-)	1.2
8	6(0)	30(0)	2(0)	2.8
9	6(0)	30(0)	2(0)	2.8
10	3(-)	30(0)	2(0)	3.5
11	6(0)	30(0)	2(0)	2.6
12	6(0)	30(0)	2(0)	3.1
13	6(0)	30(0)	3(+)	3.2
14	6(0)	30(0)	2(0)	2.2
15	9(+)	30(0)	2(0)	5.97

A, pH; B, temperature; C, EDC dose; the (-) indicates the low level, the (+) indicates the high level, and the (0) indicates the centre level.
Initial enzyme activity 27U/mL.

The 'analysis of variance' (ANOVA) test (testing the significance of the model) for the response surface quadratic model is shown in Table 4. The resulting model F-value of 20.11 implies that the model is significant, with only a 0.21% chance that this F-value is due to noise. This very low probability value (prob.>F = 0.0021) means that the model and its terms are highly significant. Moreover, the design shows an insignificant 'lack of fit' (F-value =1.60), which is desirable and related to the pure error. This means that there is only a 27.45% chance that the calculated 'lack of fit' is due to noise. In addition, the determination coefficient (R^2) was calculated to be 0.9731, indicating that 97.31% of variables fit the response. A determination coefficient R^2 value close to 1 indicates that the model describes and represents the experimental data well.

Table 4: Analysis of variance by the ANOVA test for the response surface model (FCCCD).

Source	Sum of squares	F-Value	P-value (Prob.>F)
Model	22.30	20.11	0.002　　　significant
pH, A	3.05	24.76	0.004**
Temperature, B	4.05	0.033	0.863
EDC dose, C	1.62	13.15	0.015*
AB	0.48	3.90	0.105
AC	2.59	21.06	0.006**
BC	2.38	19.29	0.007**
A^2	8.46	68.66	0.0004**
B^2	3.47	28.15	0.003**
C^2	1.06	8.60	0.033*
Lack of fit	0.18	1.60	0.275 not significant
R-squared	0.973		

$p<0.05$ indicates that the term is significant.
**$p<0.01$ indicates that the term is highly significant.*

The effects of variables on the response were predicted by the Design Expert software and the resulting regression equation was as follows:

Y(Residual enzyme activity, U/mL) = + 2.8 + 1.23A - 0.04B + 0.9C + 0.60 AB - 1.39 AC + 1.33 BC + 1.80 A^2- 1.15 B^2 - 0.64 C^2　　　　　　　　(3)

where, the amount of residual enzyme activity (Y) is a function of pH (A), temperature (B), and EDC concentration (C).

Graphical representations of the model using Equation 3 facilitated an examination of the effects of the experimental factors on the response. The resulting 3D response surface and 2D contour plots are representations of the fitted response function. To the best of our knowledge, no other study has been reported in the literature detailing the optimisation of cellulase enzyme immobilisation on MWCNTs that can be referred to for comparison with this study.

The effects of the interaction of reaction temperature and EDC dose on residual enzyme activity are presented in Fig. 4, where pH 6 was selected as the centre point. It can be seen that the maximum residual activity (3.1 U/mL) and the minimum residual activity (0.3 U/mL) occur at reaction temperatures of 30-40°C and 1-2 mLs of (10mg/mL) EDC reagent, respectively.
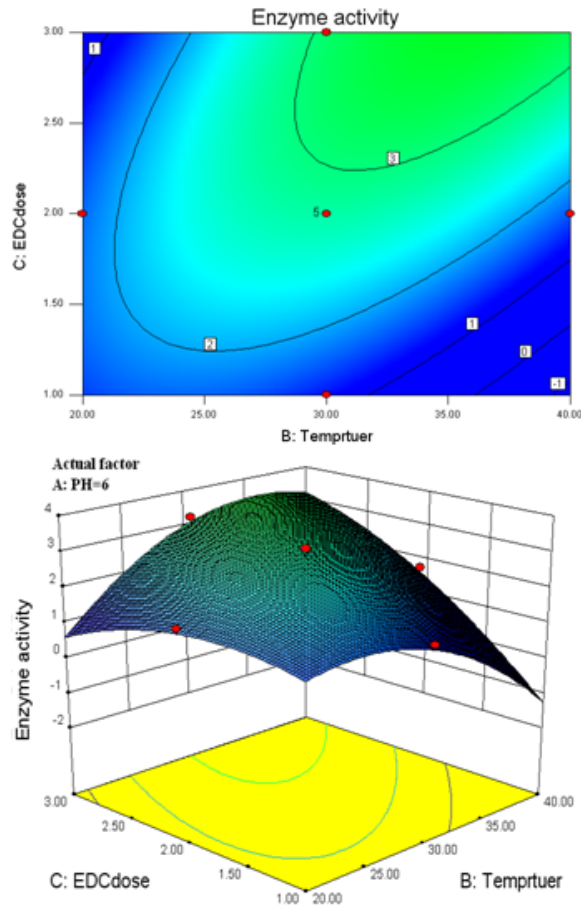
Fig. 4: The effect of the interaction between EDC dose and reaction temperature on enzyme activity when the pH is maintained at 6.

Based on the proposed model, numerical optimisation (using the Design–Expert software) determined that the optimum conditions for STP cellulase enzyme immobilisation are as follows: pH ~ 4.5, 30°C and 1 mL of 10mg/mL EDC. According to results obtained after solving the regression model equation, the lowest predicted residual enzyme activity in the supernatant is 0.4 U/mL, indicating that the highest obtainable enzyme loading on MWCNTs is 26.6 U/mL, representing 98% of the initial activity. Relative activity is defined as the ratio of specific activity of the immobilised enzyme to that of the free enzyme under the same conditions. Previous studies have reported that the maximum relative activity of immobilised cellulase enzyme in nanofibrous PVA membranes (by electrospinning) was greater than 65%, higher than that observed for other forms of immobilisation (Wu *et al.*, 2005). In contrast, the relative activity of immobilised cellulase on non-porous ultrafine silica particles reached only 30% of the free enzyme activity (Afsahi *et al.*, 2007).

In order to verify the model developed in this study, three replicates of three experiments were performed under the optimum conditions (Table 5). A comparison was then made between the experimental results and results predicted by the model. It can be seen that residual enzyme activity from the experiment (0.39 U/ml) was slightly less than that predicted by the model (0.41 U/mL). Accordingly, enzyme loading on the MWCNTs was approximately 26.61 U/mL using the optimum conditions.

Table 5: Optimum immobilisation conditions and validation of the developed model.

Run	A: PH	B: Temperature °C	C: EDC dose mL	Predicted (Residual cellulase)	Experiment (Residual cellulase)
1	4.5	30	1	0.41	0.39
2	5	35	1.2	0.15	0.19
3	6	25	1.5	1.57	1.11

In order to investigate whether cellulase enzyme was indeed present on the functionalised MWCNTs, FTIR spectrum and FESEM images were performed. As shown in Fig. 5, FTIR peaks from CH, -OH, and C=O groups are broader in comparison with the same peaks recorded for functionalised MWCNTs. Moreover, an amide group peak (O=C-NH) corresponding to cellulase enzyme at 1648.38 cm^{-1} was observed in the MWCNT-cellulase composite sample, in agreement with a previous study quantitating the amount of cellulase using a peak at 1652 cm^{-1} (Ghose, 1987).
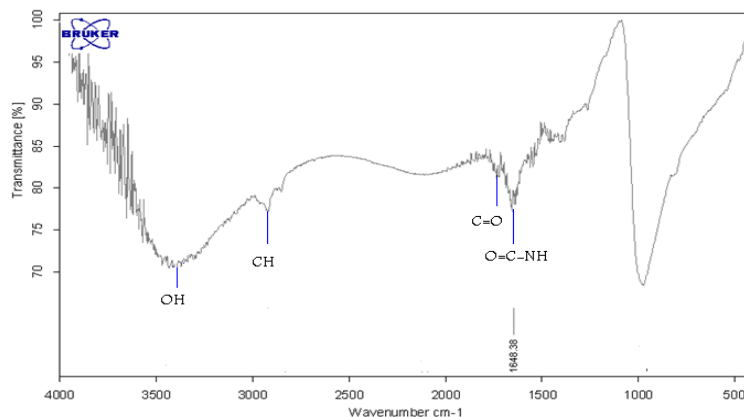
Fig. 5: FTIR spectrum of an MWCNT-cellulose composite sample.

These observations from FTIR were supported by FESEM imaging. Fig. 6 shows an FESEM image of the MWCNTs-cellulase composite sample at 100,000X. It can be seen that the reactivity of MWCNTs was increased resulting in a smooth surface with less bright spots. This could be due to the formation of amide bonds between carboxylic acid groups on the functionalised MWCNTs, and amine groups from the cellulase enzyme (catalysed by activation of carboxyl groups by the EDC coupling reagent), resulting in successful enzyme immobilisation.
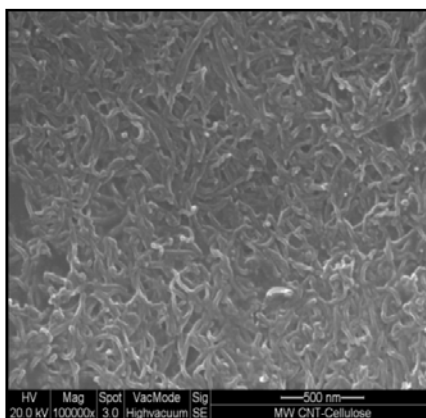

Fig. 6: FESEM image of an MWCNT-cellulose composite sample at 100,000X.

Conclusions:

The study presented here was conducted to determine the optimal conditions for the immobilisation of cellulase enzyme on MWCNTs. Preliminary screening of six parameters was conducted using the PBD design method, which yielded that pH, reaction temperature, and EDC concentration are significant variables. In a second round of optimisation using FCCCD under RSM, it was found that cellulase enzyme loading on MWCNTs was approximately 26.61 U/mL (constituting 98% of the initial activity) under the optimum conditions (pH ~4.5, temperature ~30°C, and ~ 1 mL of 10 mg/mL EDC). Functionalisation of the MWCNTs and the presence of immobilised cellulase enzyme on the MWCNTs were confirmed by FTIR and FESEM techniques.

REFERENCES

Afsahi, B., A. Kazemi, A. Kheirolomoom, S. Nejati, 2007. Immobilization of cellulase on non-porous ultrafine silica particles, Sci. Iran, 14(4): 379-383.

Ahmad, A.A., B.H. Hameed, A.L. Ahmad, 2009. Removal of disperse dye from aqueous solution using waste-derived activated carbon: Optimization study. J. Hazard. Mater., 170: 612-619.

Al-khatib, M.F., M.Z. Alam, R. Mohammed, 2009. Effect of acid treatment on carbon nanotubes for immobilization of Cellulase enzyme. In ATCi, Conference of international advanced technology congress. 3-5 November. Kuala Lumpur.

Chakrabarti, A.C., K.B. Storey, 1988. Immobilization of cellulose using polyurethane foam. Appl. Biochem. Biotechnol., 19(2): 189-207.

Ghose, T.K., (1978). Measurement of cellulase activities. Pure Appl. Chem., 59: 257-268.

Gomez, J.M., M.D. Romro, T.M. Fernandez, 2005. Immobilization of β-Glucosidase on carbon nanotubes. Catal. lett., 101(3-4): 275-278.

Guisan, J., 2008. Improvement of enzyme properties via immobilization technique (14 August 2008) <http://www.scitopics.com/improvement_of_enzyme_properties_viaimmobilization_techniques.html>.

Iijima, S., T. Ichihashi, 1993. Single-shell carbon nanotubes of 1-nm diameter. Nature., 363: 603-605.

Jiang, K., L.S. Schadler, R.W. Siegel, X. Zhang, H. Zhang, M. Terrones, 2004. Protein immobilization on carbon nanotubes via a two-step process of diimide-activated amidation. J. Mater. Chem., 14: 37-39.

Kim, Y-S., J-H. Cho, S.G. Ansari, H-I. Kim, M.A. Dar, H-K. Seo, G-S. Kim, D-S. Lee, G. Khang, H-S. Shin, 2006. Immobilization of Avidin on the functionalized carbon nanotubes. Synth. Met., 156: 938-943.

Kirk, O., T.V. Borchert, C.C. Fuglsang, 2002. Industrial enzyme applications. Curr. Opin. Biotech., 13: 345-351.

Lee, Y-M., O-Y. Kwon, Y-J. Yoon, K. Ryu, 2006. Immobilization of horseradish peroxidase on multi-wall carbon nanotubes and its electrochemical properties. Biotech Lett., 28: 39-43.

Levien, L., F. Forchiassin, A. Viale, 2005. Ligninolytic enzyme production and dye decloolorization by Trametes trogii: application of Placket-Burman experimental design to evaluate nutritional requirements. Proc. Biochem., 40: 1381-1387.

Mansfield, S.D., J.N. Saddler, 2003. Application of Enzymes to Lignocellulosics. The American Chemical Society, Oxford University Press.

Plackett, R.L., J.P. Burman, 1964. The design of optimum multifactorial experiments. Biometrica, 33: 305-325.

Prato, M., D. Tasis, N. Tagmatarchis, V. Georgakilas, 2003. Introducing functional groups onto the surface of carbon nanotubes helps to immobilize these useful molecules and facilitates their study. Chem. Eur. J. 9, 4000-4008.

Rashid, S.S., M.Z. Alam, M.I.A. Karim, M.H. Salleh, 2009. Optimization of the nutrient suppliants for cellulase production with the basal medium palm oil mill effluent. World Acad. Sci. Eng. Techn., 60: 809-815.

Taherzadeh, M.J., K. Karimi, 2007. Enzyme-based hydrolysis processes for ethanol from lingocellulosic materials: a review, Bioresourc., 2(4): 707-738.

Tu, M., X. Zahang, A. Kurabil, N. Gilkes, W. Mabeel, J. Saddlermaobing, 2006. Immobilization of β-glucosidase on Eupergit C for lignocellulose hydrolysis. Biotechnol. Lett., 28(3): 151-156.

Worsfold, P.J., 1995. Classification and chemical characteristics of immobilized enzyme. Pure Appl. Chem., 67(4): 597-600.

Wu, L., X. Yuan, J. Sheng, 2005. Immobilization of cellulase in nanofibrouse PVA membranes by electrospinning. J. Membr. Sci., 250: 167-173.

Xu, Q., W.S. Adney, S-Y. Ding, M.E. Himmel, 2007. Cellulase for Biomass Conversion. Industrial Enzymes, 1st.ed., National Bioenergy Center, National Renwable Energy Laboratory, USA.

Yang, W., P. Throdarson, J.J. Gooding, S.P. Ringer, F. Braet, 2007. Carbon nanotubes for biological and biomedical applications. Nanotech., 18(41): 2001-2012.

Yuan, X-Y., N-X. Shen, J. Sheng, X. Wei, 1999. Immobilization of cellulase using acrylamide grafted acrylonitrile copolymer membranes. J. Membr. Sci., 155(1): 101-106.