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Full Length Research Paper

Optimisation of immobilised cellulase onto carbon nanotubes using response surface methodology

Rasha Mohammed Abd* and Abdurahman Hamid Nour

Faculty of Chemical and Natural Resources Engineering, University Malaysia Pahang, Kuantan, Malaysia.

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Although, the cellulase enzyme can work with high catalysis under mild condition, however, it does not fulfil the industry requirement. In this study, cellulase obtained from the fermentation of sewage treatment plant (STP) sludge by *Trichoderma reesei* RUT C-30 was covalently immobilised on functionalised multi-wall carbon nanotubes (MWCNTs). Statistical optimisation using the Plackett-Burman design (PBD) method was implemented to identify parameters with significant effects on the process of immobilisation. The results obtained from this PBD showed 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 PBD results, these parameters were further optimised using a face-centred central composite design (FCCCD). The resulting optimum conditions for cellulase immobilisation, as determined by FCCCD, were pH 4.5, 30°C and 1 ml of 10 mg/ml EDC. The amount of immobilised cellulase was approximately 98% using these optimum conditions. The resulting MWCNT-cellulase composite was further characterized by Fourier transform infrared spectroscopy (FTIR) technique.

Key words: Multi-wall carbon nanotubes (MWCNTs), immobilisation, sewage treatment plant (STP) enzyme, optimisation, face-centred central composite design (FCCCD).

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 catalysing 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 the production of detergents, food, animal feed, textiles, pulp and paper and pharmaceuticals (Prato et al., 2003; Modibbo et al., 2007). 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 facility-ates enzyme recycling, enables improvements in thermo-stability, and allows for greater control of enzyme activity (Worsfold, 1995; Tu et al., 2006). Immobilised enzymes have been defined as enzymes that are physic-ally con-fined 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 immobilisation of cellulase enzymes for

*Corresponding author. E-mail: rashaam12@gmail.com. Tel: +601116116015.

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). The results indicate improvement in the catalytic activity of the immobilised enzyme corresponding to the free enzyme.

Since the discovery of carbon nanotubes (CNTs) in 1991 (Iijima and Ichihashi 1993), 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., 2003). 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; Mopoung, 2011). 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 (Kim et al., 2006; Lee et al., 2006). Based on these studies, it was found that immobilisation techniques can be influenced by many factors, including temperature, enzyme concentration, coupling reagent and immobilisation time.

In this study, optimisation of immobilised cellulase (obtained from the fermentation of sewage treatment plant (STP) sludge by *T. reesei* RUT C-30) onto CNTs by applying statistical design methods was investigated. Optimisation was carried out in two stages. In the first stage, immobilisation parameters (CNT concentration, enzyme concentration, immobilisation time, pH, temperature and N-ethyl-N-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) coupling reagent concentration) were evaluated by using Plackett-Burman design (PBD). Consequently, the optimal values for each of the significant variables were determined by using FCCCD with response surface methodology (RSM) 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 to 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 STP sludge by *T. reesei* RUT C-30. Hydrophilised polytetrafluoroethylene (PTFE) membranes (0.45 µm) were purchased from Sartrious Stedim, Germany. HNO_3 (65%), H_2SO_4 (97%), EDC and N-hydroxysuccinimide (NHS), were purchased from MERCK, Germany. 0.05 M 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, ~50 Hz, 430 W) at 40°C for 2 h. 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 to 5 times) until the pH was measured to be neutral. Functionalized MWCNTs were then dried in a vacuum oven at 80°C for 48 h, followed by cooling in a desiccator for 24 h (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 was re-suspended in 2.5 ml of distilled water, followed by brief sonication. Then, 1 ml of a 0.1 M buffer solution (pH = 6) and (50 mg/ml) N-hydroxysuccinimide (NHS) solution was added to the suspension and mixed. Under continuous stirring, varying amounts of a 10 mg/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 was rinsed thoroughly 4 to 5 times with 0.1 M buffer solution (pH = 6). In the second step, resulting MWCNTs were re-dispersed in 4.5 ml of 0.1 M buffer solution (pH = 6) and 10 to 20 ml of cellulase enzyme in 1 ml of 0.1 M buffer solution (pH = 6) was added. After shaking the mixture in an incubator shaker at 150 rpms for 1 h, the suspension was centrifuged at 4000 rpms at 4°C for 40 min. Unbound enzyme was removed by rinsing three times with 0.1 M buffer solution (pH = 6). The MWCNT-cellulase composite was left to air dry for 24 h before characterisation and further use.

Analytical methods

Cellulase activity before and after immobilisation was determined using the carboxymethyl cellulose (CMC) assay (Ghose, 1978). 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 min, then, 0.5 ml of substrate solution (CMC) was added and the samples were incubated in a water bath at 50°C for exactly 30 min. 3 ml of DNS reagent was added to all enzyme samples. 0.5 ml of the 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 min and then transferred immediately to an ice bath, and kept on ice until cool. After dilution in 5 ml 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.

Fourier transform infrared spectroscopy (FTIR) was used to analyse the functional groups of as-received MWCNTs and functionalised MWCNTs. FTIR spectra was recorded by using KBr pellets in frequency range (4000 to 400 cm^{-1}) and the optical

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.

properties of the samples were determined using HeNe laser that emits red light wavelength of 633 nm.

Characterisation techniques

The chemical composition of the functionalised MWCNTs and MWCNT-cellulase composite was characterised by Fourier trans-form infrared spectroscopy (FTIR, Bruker, IFS66v/S, Germany). 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:

$$Y = \beta_0 + \sum \beta_i x_i \quad (1)$$

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)

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:

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

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 was acquired for MWCNTs and functionalised MWCNTs in order to confirm MWCNT functionalisation. Figure 1a shows the FTIR spectrum for a MWCNT sample. The peak observed at 1630 cm^{-1} corresponds to C=C stretching associated with side wall

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 < 0.05 indicates that the term is significant; **P < 0.01 indicates that the term is highly significant.

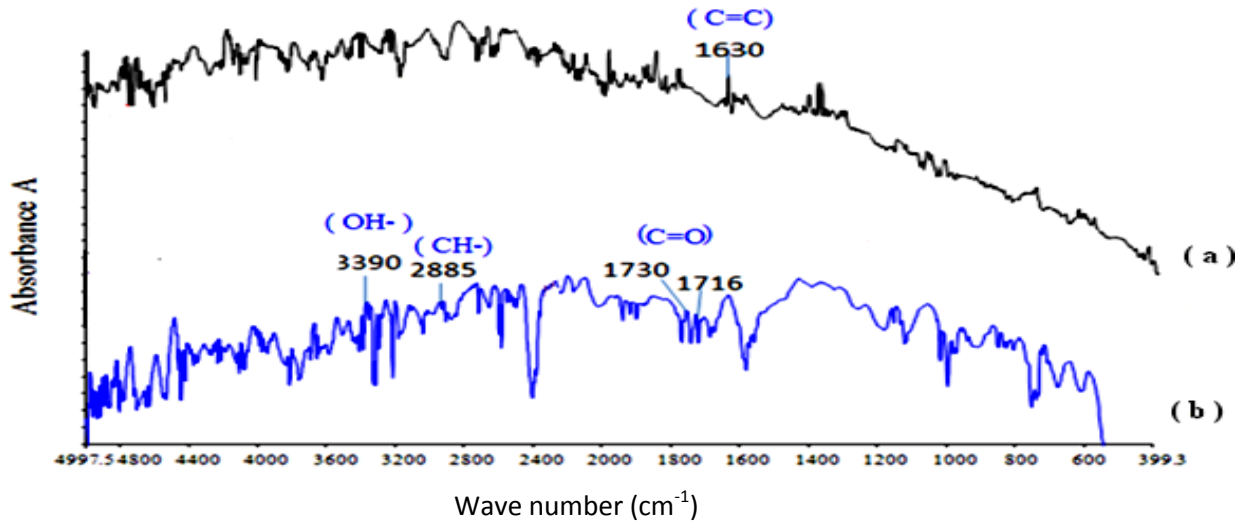

Figure 1. FTIR spectrum of (a) MWCNTs and (b) functionalised MWCNTs.

defects. In contrast, Figure 1b shows the FTIR spectrum for functionalised MWCNTs. Peaks at 2885 and 3390 cm^{-1} most likely correspond to CH groups and –OH groups, respectively. In addition, the small peaks in the 1716 to 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, the acid treatment helps in imparting hydroxyl, carboxyl and carbonyl groups, which facilitate functionalisation of the tubes.

Immobilisation of cellulase enzyme on MWCNTs

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 as 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 (Figure 2). 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.

In the second step of optimisation process, FCCCD

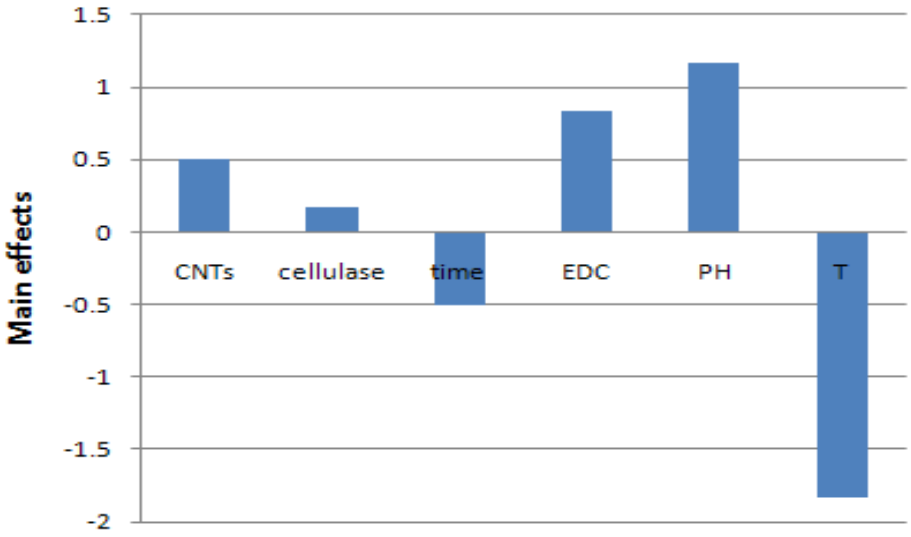

Figure 2. Main effects of parameters on cellulase enzyme immobilization.

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 27 U/ml.

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, 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).

The 'analysis of variance' (ANOVA) test for the response surface quadratic model is as 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 (probability > 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

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.

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.

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

$$Y \text{ (Residual enzyme activity, U/ml)} = +2.8 +1.23A -0.04B +0.9C +0.60\ AB -1.39\ AC +1.33\ BC +1.80\ A^2 -1.15\ B^2 - 0.64\ C^2 \quad (3)$$

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 Figure 3, 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 to 40°C and 1 to 2 ml of 10 mg/ml EDC reagent, respectively.

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 10 mg/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 polyvinyl alcohol (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. In order to investigate whether cellulase enzyme was indeed present on the functionalised MWCNTs, FTIR spectrum was performed. As shown in Figure 4, 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, 1978).

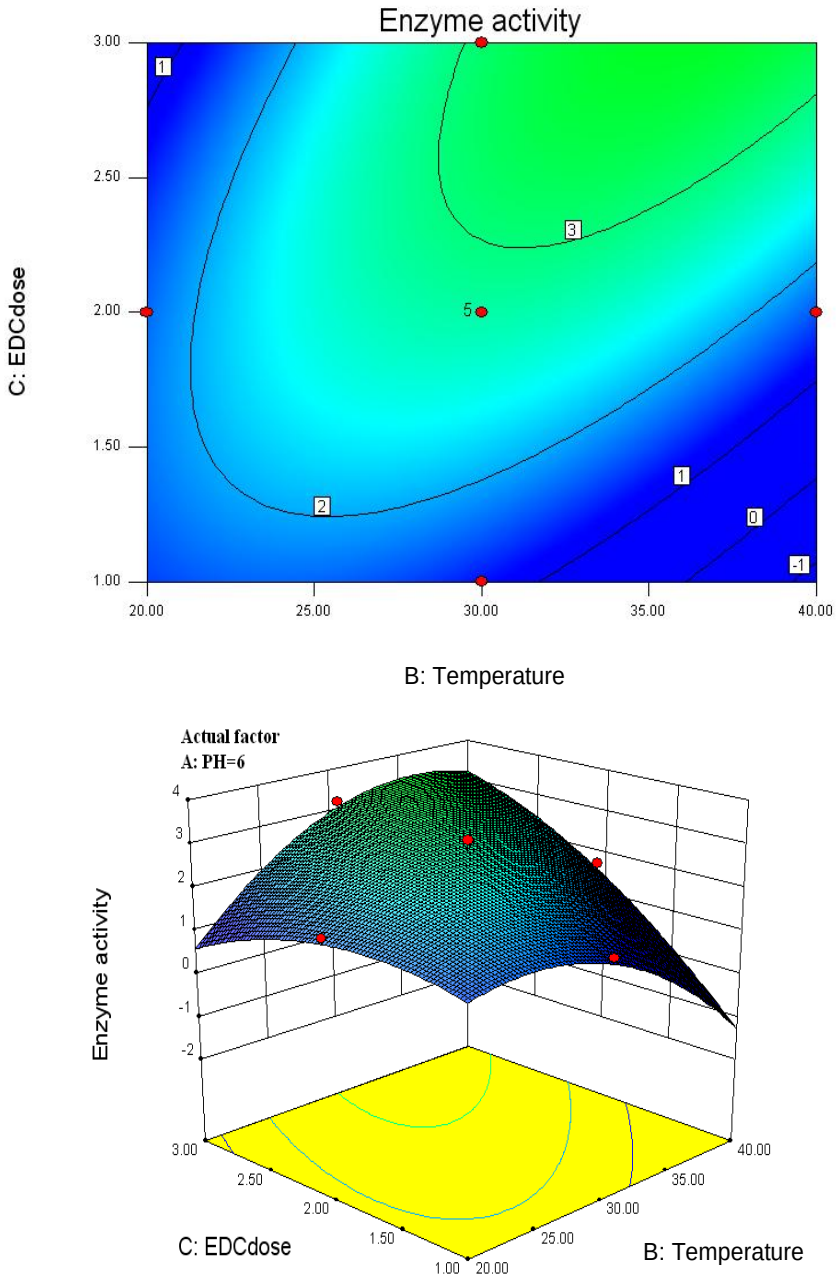

Figure 3. The effect of the interaction between EDC dose and reaction temperature on enzyme activity when the pH is maintained at 6.

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

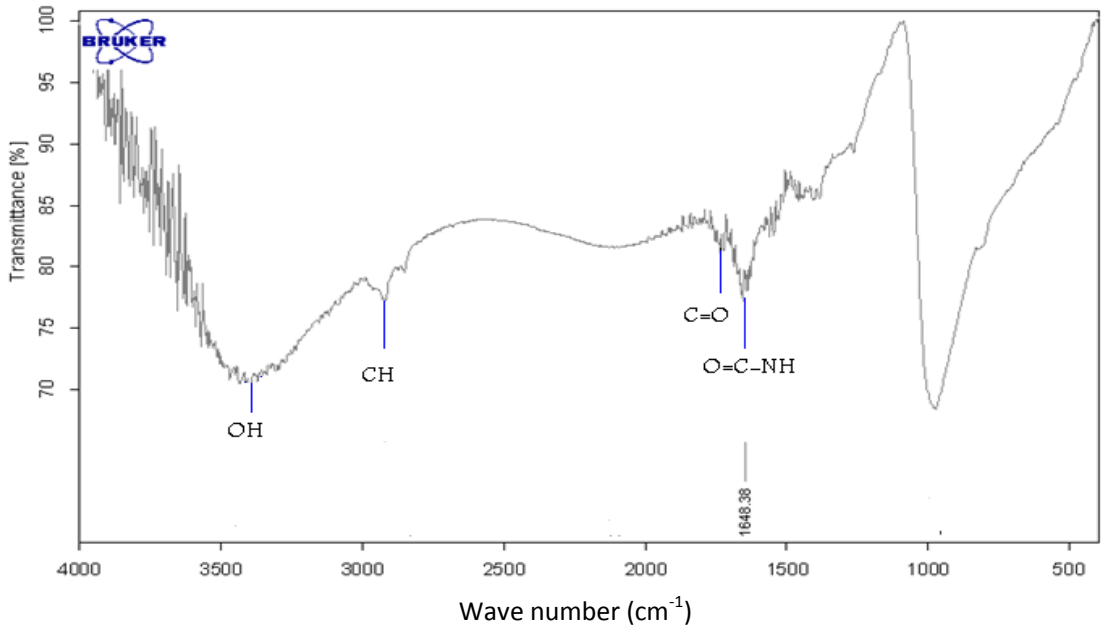

Figure 4. FTIR spectrum of an MWCNT-cellulase composite sample.

Conclusions

Optimisation of immobilised cellulase onto CNTs by applying statistical design methods was conducted to determine the optimal conditions. 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). FTIR technique was used to confirm the functionalisation of the MWCNTs and the presence of immobilised cellulase enzyme on the functionalised MWCNTs.

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