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# Enhancing enzyme activity and enantioselectivity of *Burkholderia cepacia* lipase via immobilization on the modified multi-walled carbon nanotubes

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## Abstract

*Burkholderia cepacia* lipase (BCL) was proved to be a potential catalyst in chiral resolution. However, it is not widely applied in industry because of the low catalysis activity and poor stability of the free lipase. In this study, BCL was immobilized on the modified multi-walled carbon nanotubes to enhance its catalysis performance. The immobilization conditions were further optimized via single factorial experiments and response surface methodology (RSM). Under the optimum conditions, the enzyme activity attained 50,200 U/g, 54 fold that of the free lipase in resolution of 1-phenylethanol, resulting in an immensely shortened reaction time from 30 h of the free lipase to 10 min of the immobilized one. SEM micrographs verified that CNTs were truncated and the closed ends were opened by concentrated $H_2SO_4$. EDS further confirmed the modification and successful immobilization of the lipase. FT-IR analysis demonstrated that improvement of enzyme activity and $ee_s$ was correlated to the alteration of secondary structure. Compared with other immobilized lipases, CNTs-BCL exhibits great advantage and possesses promising potential in industrial application.


**Keywords:** Immobilization, Carbon nanotubes, *Burkholderia cepacia* lipase, Resolution, 1-phenylethanol, Response surface methodology (RSM)

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## 1. Introduction

Lipase (EC3.1.1.3) has been extensively utilized in food, detergent, oil process, biodiesel preparation, and many other biosynthetic industries.[1, 2] Recently, great attention has been paid to the application in preparation of pharmaceutical intermediates and chiral building blocks.[3] Although many studies have been conducted to improve the catalytic performance of lipases, there are still some limitations in their industrial applications, such as poor stability, sensitivity of environment, narrow pH range adaptability, etc. Moreover, it is also very difficult to separate the free lipase from substrates and products. However, immobilized lipases can overcome these above-mentioned limits and have demonstrated as one of the most useful methods to enhance catalytic properties of free enzymes.[4] So far, a variety of materials has been used to immobilize enzymes and can improve catalysis performance to some extent. Among them, a kind of newly emerging material---carbon nanotubes (CNTs) has attracted more and more attention.[5] CNTs are being proved to be wonderful immobilization matrixes owning to their extraordinary mechanical, electrical, and thermal properties as well as biocompatibility.[6, 7] It is reported that CNTs and other nanomaterials exhibit an ability to stabilize protein.[8] Using CNTs as immobilize matrix, Mubarak et al.[9] and Gòmez et al.[10] successfully immobilized cellulase enzyme and β-Glucosidase, and achieved high immobilization efficiency and satisfying enzyme activities. Furthermore, it is important and urgent to explain the probable mechanism for the enhancement of catalysis performance caused by immobilization. On the other hand, though amount of studies have been done in

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immobilizing lipase on CNTs, yet most of them were focused on biosensors and bioelectrochemical applications. There are few reports focused on immobilized lipases via carbon nanotubes (CNTs) being used as biocatalyses.

Therefore, in this work, *Burkholderia cepacia* lipase (BCL) was chosen as the target enzyme for immobilization because it is widely used in biofuel synthesis, biorefinery and a wide variety of reactions in aqueous and non-aqueous phases.[11] The BCL was produced from *B. cenocepacia* which was cloned, expressed and described in our previously work. *B. cenocepacia* consists of lipA and its chaperone gene lipB from a stable and high lipase-producing strain *Burkholderia cepacia* G63 (formerly known as *Pseudomonas cepacia*).[12] Till now, a number of immobilization methods were utilized to improve catalysis of BCL, and many materials were chosen as immobilization matrixes, such as macroporous resin NKA[13], silica-monolith[14], κ-carrageenan[15], etc. However, in term of enantioselective resolution, few methods achieved good effects. Thanks to the good results of other kind of enzymes, herein, CNTs were employed to immobilize BCL.

The reaction of enzymatic kinetic resolution of *(R, S)*-1-phenylethanol was utilized to evaluate the enzyme activity and enantioselectivity ($ee_s$) as many reports on enantioselective transesterification of 1-phenylethanol with vinyl acetate are available in the literature, and may be regarded as a model reaction.[16, 17] It is very easy to compare the catalytic efficiency of the immobilized BCL with other enzyme towards the same substrate and under similar reaction conditions. Moreover, the utilization of 1-phenylethanol as essential building block and synthetic intermediate has been

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applied in cosmetic, chemical industries and many other fields.[18]

Thus, this model resolution reaction was used to evaluate the enzyme activity/enantioselectivity ($ee_s$) and to compare the catalytic efficiency between the free and immobilized lipases in non-aqueous medium so as to explore the BCL-CNTs immobilization strategy. The main purposes of this work were to investigate the BCL-CNTs immobilization strategy, exploit the probable mechanism for the catalysis enhancement of the immobilized lipase, and to make comparison between the immobilized lipases and the free form, and other immobilized lipases in non-aqueous medium for enantioselective resolution.

## 2. Materials and methods

### 2.1 Materials

The lipase of *B. cepacia* G63 was self-produced. *B. cepacia* G63 strain was fermented in 10L bioreactor and purified by using the methods described in our previous study.[12] All the carbon nanotubes used in this work was purchased from Shenzhen Nanotech Port Co., Ltd. *(R, S)*-1-phenylethanol was bought from Sigma-Aldrich Co., Ltd (St. Louis, Missouri, USA). Potassium sulfate, ethanol, and other reagents of analytical grade were obtained commercially from Sinopharm Chemical Reagent Co., Ltd, Shanghai, P. R. China. High-performance liquid chromatography (HPLC) grade organic solvents were purchased from TEDIA (USA).

### 2.2 Modification of CNTs and characterization via SEM and EDS

The CNTs were pretreated by the concentrated $H_2SO_4$ under sonication for 3 h to open

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the closed end and truncate the tubes. The nanotube suspension was then diluted and washed with pure water by filtering through a 0.45 μm polycarbonate membrane. The samples were dried at 65 °C with a dryer. Then, the obtained samples were ground into powder for the future use. Furthermore, the modified CNTs were analyzed by SEM and EDS (Nova Nano SEM 450, FEI Company, and Eindhoven, Netherlands). The samples were coated with gold using a sputter coating system and measured at an acceleration voltage of 5 kV.

**2.3 Preparation of the immobilized lipase and characterization via EDS**

The prepared CNTs were used as the support matrix for immobilization. The properties of CNTs were listed in Table 1. A certain amount of lipase was dissolved in 5 ml buffer solution. The solution was then loaded into a 50 ml tube containing 0.2 g prepared CNTs. The mixture was shaken at 37 °C, 200 rpm for several hours. Then, the suspensions were centrifuged at 4 °C, 12,000 rpm for 5 min to remove the supernatant. The derived immobilized lipase (CNTs-BCL) was dried in the thermostatic vacuum drier and ground into powder for later use. The protein content of the supernatant was determined by the Bradford method using bovine serum albumin (BSA) as standard.[19] The immobilization of BCL was further confirmed by EDS.

During the immobilization procedure, the effects of pH (pH 3.0–10.0), free lipase loading (2–15 %), and immobilized time (1-6 h) on the immobilization efficiency, specific activity and lipase activity were specifically addressed. To study the effect of

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pH, three kinds of buffers were used for pH optimization. They were $Na_2HPO_4$–citrate acid buffer (0.2mol/L, pH 3.0–5.0), phosphate buffer (0.2mol/L, pH 6.0–8.0), and glycine–NaOH buffer (0.2mol/L, pH 9.0–10.0).

**----------Table 1----------**

### 2.4 Lipase activity and protein content measurements

The resolution reaction mentioned above was utilized to measure the enzyme activity. One unit (U) of enzyme activity was defined as the amount of enzyme which produces 1 μmol α-phenylethyl acetate in one minute under the assay conditions. The protein content of the free and immobilized lipase was 0.58 wt% and 0.85 wt% respectively. Immobilization efficiency (%) was estimated via Eq. (1).

$$\text{Immobilization efficiency}(\%) = \frac{\text{immobilized protein}}{\text{total loading protein}} \times 100 \quad (1)$$

### 2.5 Kinetic resolution of (R, S)-1-phenylethanol

The resolution reactions were carried out in 5 mL pure heptane, containing 1 mmol racemic 1-phenylethanol, 4 mmol vinyl acetate and 0.05 g free or immobilized BCL. The reactions were performed in a 50 mL stoppered flask at 50°C, 200 rpm for 10 min. During the resolution of *(R, S)*-1-phenylethanol, effects of substrate molar ratio (vinyl acetate/1-phenylethanol), water content, reaction time, and reaction temperature were examined and optimized. The standard assay conditions (molar ratio, water content, reaction time, temperature) were used except when otherwise stated in

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the text. After the reactions, the free or immobilized lipase was removed by centrifugation. The samples were filtered through a 0.44μm filter and analyzed by HPLC.

**2.6 HPLC analysis and calculation**

As reported in our previously work[20], the samples were analyzed by HPLC (Model 2300-525 SSI. Co., Ltd USA) using a Chiralcel OD-H column (4.6 mm×250 mm, Daicel Chemical, Japan). The mobile phase consisted of hexane/2-propanol alcohol at 95/5 (v/v) with a flow rate of 1.0 ml/min. At 254 nm (Model 525 UV Detector SSI. Co., Ltd USA), the substrate and product were detected. In the above condition, the retention times of *(R)*- and *(S)*- 1-phenylethanol in the Chiralcel OD-H column were 7.28 and 8.23 min, respectively. All samples were run under the same conditions as stated above.

According to method described by Chen et al.,[21] enantioselectivity was expressed as E value and calculated by Eq. (2), substrate enantiomeric excess ($ee_s$) was calculated by Eq. (3), and substrate conversion (C) by Eq. (4).

$$E = \frac{\ln[(1-C)(1-ee_s)]}{\ln[(1-C)(1+ee_s)]} \quad (2)$$

$$ee_s = \frac{S-R}{S+R} \quad (3)$$

$$C = \frac{S_0+R_0-(S+R)}{S_0+R_0} \quad (4)$$

where, $S_0$ and $R_0$ respectively represented the concentrations of the *(S)*- and *(R)*-enantiomers of 1-phenylethanol before reaction, S and R were the concentrations of

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the *(S)*- and *(R)*- enantiomers of 1-phenylethanol after reaction.

### 2.7 Experimental design and statistical analysis

Box–Behnken design (RSM) was employed to further optimize the reaction conditions. Experiments were designed to examine the interaction of three variables (pH, loading lipase of free BCL, and temperature). SAS version9.0 was used for regression analysis and analysis of variances (ANOVAs). The experimental data were analyzed by the response surface regression (RSREG) procedure to fit the following second-order polynomial equation (SAS 9.0).

$$Y_1 = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} x_i x_j \quad (5)$$

where, $Y_1$ was the response ($ee_s$ %), $\beta_0$, $\beta_i$, $\beta_{ij}$ were constant coefficients, and $X_i$ were independent variables.

### 2.8 Characterization of the immobilized BCL via FT-IR spectroscopy

FT- IR spectra were measured at 25 °C with a Vextex 70 FT-IR spectrometer (Bruker, Germany) equipped with a nitrogen-cooled, mercury–cadmium–tellurium (MCT) detector, in the region of 4,000–400 $cm^{-1}$. The spectrum acquisition (all samples were overlaid on a zinc selenide attenuated total reflectance (ATR) accessory) was from IR spectra, and the secondary structure elements based on the information of amide I region and the band assignment were manipulated using software PeakFit version 4.12 as per the method described by Yang et al.[22]

## 3. Results and discussion

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### 3.1 Carrier selection, modification and characterization with SEM and EDS

Four types of CNTs (L-MWNT-2040, L-MWNT-4060, L-MWNT-60100, and MWNT-OH) with different properties (as listed in Table 1) were selected for immobilization carriers. It is reported that the ideal pore diameter of immobilization supports should be at least 4-5 fold of the immobilized protein so that the access restrictions of the enzyme could be prevented.[23] The L-MWNT-60100 with the maximum diameter of the four types achieved the highest enzyme activity and immobilized efficiency (see Fig. 1) after oxidized by the concentrated $H_2SO_4$.

----------**Fig. 1**----------

SEM analysis was utilized to display the effect of oxidation. Compared to the CNTs before oxidation, the ends of CNTs have already been opened and most CNTs were truncated (see Fig. 2). As shown in Figure 2a, CNTs often assembled and closed. However, the port of CNTs was opened after oxidation which could improve the absorption ability of CNTs for lipase protein (Fig. 2b). The characteristic structure of the modified CNTs, such as the surface defect sites and the open port, renders them highly specific absorption property. In addition, there are four absorb positions[24]: inner space, outer surface, channels between adjacent nanotubes and channel formed by single nanotube, which will further enlarge the surface of lipase immobilization.

----------**Fig. 2**----------

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In addition, EDS was used to further confirm the modification. As shown in Figure 3a, only C was observed in pure CNTs, and C, O occurred after oxidation, which indicated that CNTs oxidized by concentrated sulfuric acid led to –COOH, –OH and/or other active group formation at the defect sites in the end and side wall (Fig. 3b).

**----------Fig. 3----------**

### 3.2 Preparation of CNTs-BCL

3.2.1 Effects of immobilization parameters

As it is well known, the immobilization conditions have significant effects on the immobilization efficiency, specific activity and activity recovery of the immobilized lipase. Such conditions with principal effect as pH, lipase loading and immobilization time were examined in this study.

(1) Effect of pH on BCL immobilization

As known, pH value is a critical factor in many reactions as well as in enzyme immobilization. As can be seen in Figure 4a, the highest enzyme activity and immobilization efficiency were achieved when immobilization pH arrived at 7. Actually, immobilization pH had little effect on immobilization efficiency which always maintained at 90%, and the neutral and basic buffer had little effect on the enzyme activity. However, the acidic buffer seemed to reduce the enzyme activity. For the non-polar immobilized carrier, Panzavolta et al. have reported that pH value had

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little influence on immobilization efficiency while affected a lot on the esterification activity.[25] It is because enzymes in different pH are charged differently which contributes to maintain the active conformation. According to the 'pH memory' theory, enzymes maintain the ionization when they turn to organic phase from aqueous. Thus, the difference of conformation was preserved.[26]

(2) Effect of lipase loading

In the range of 0.1 to 1.0g BCL, the enzyme activity and immobilization efficiency were gradually raised with the increase of lipase loading. Beyond 1.0g, the enzyme activity and immobilization efficiency both declined. Meanwhile, though the activity of enzyme changed a lot in different lipase loading, the immobilization efficiency always remained a high level at > 90% (see Fig. 4b).

(3) Effect of immobilization duration

As shown in Figure 4c, the immobilization efficiency and enzyme activity were gradually increased in the first 4h. After that, there is no increment in enzyme activity and also no significant difference. Thus, the optimal immobilization duration was selected as 4h in the following experiments. There is an equilibration process in the hydrophobic absorption between CNTs and biomolecules. The adsorption capacity increases with time lasting before the equilibration and maintains the maximum when the adsorption reaches equilibrium.[27] But if the equilibration attained, with time lasting even longer, proteins often desorbed from the immobilized matrix slightly. Therefore, the immobilization time should be so long enough to reach the equilibrium, but should not be too long.

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DOI: 10.1039/C4RA10517F



**----------Fig. 4----------**

3.2.2 Further confirmation of CNTs-BCL with EDS

Energy Dispersive Spectrometer (EDS) was utilized to further confirm the successful immobilization of BCL. Exactly, as has been predicted, N occurred after immobilization, which indicated that lipase protein had been successfully immobilized on the oxidized CNTs[22], meaning CNTs-BCL has been formed.

**----------Fig. 5----------**

3.2.3 Optimization of the immobilization conditions via RSM

According to the above single factorial experiments, the immobilization pH, lipase loading and immobilization time were statistically significant to transesterification efficiency. Considering the interactions between these parameters, RSM experiments (Box–Behnken) were designed with three factors to further optimize the immobilization conditions. Factors and level value of response surface analysis were presented in Table 2. The fifteen experiments and the results were all listed in Table 3, providing various levels of $ee_s$ under different reaction conditions designed by SAS version 9.0.

**----------Table 2, 3----------**

Based on the designed experimental results in Table 3, the experimental data

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were fitted to a second-order polynomial equation. The best fitting response surface according to SAS software 9.0 for the ees value could be expressed as Eq. (6).

$$Y=52763.65+486.4583X_1+3513.542X_2+1096.458X_3-64.47917X_1^2+53.125X_1X_2 +5X_1X_3-2580.729X_2^2+143.75X_2X_3-78.85417X_3^2 \quad (6)$$

As equation (6) shows, the model indicated that linear terms $X_1$, $X_2$, $X_3$ and quadratic term $X_1X_2$, $X_1X_3$, $X_2X_3$ had positive effects on ees value, while the quadratic term $X_1^2$, $X_2^2$, $X_3^2$ had negative effects on ees value. Analysis of variance of regression mode was shown in Table 4, from which it could be confirmed that the coefficient of linear terms were bigger than the quadratic terms. Thus, it can be inferred that the impact of single factors was stronger than that of binary terms and interact terms.

**----------Table 4----------**

After the simplification and normative analysis of the equation, the response surface curve was obtained and parts of which were shown in Figure 6. The optimum conditions of the three independent factors were immobilization pH 7.96, immobilization time 4.47h, and lipase loading 0.95g. Under the optimal conditions, the predicted enzyme activity was 49,880 U/g. To verify the validation of the model, an experiment was performed in triplicates to test the enzyme activity. The average enzyme activity attained 50,200 U/g, which coincided with the predicted value, and the associated $ee_s$ was 98.5%.

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----------Fig. 6----------

### 3.3 Enzymatic kinetic resolution of (R, S)-1-phenylethanol by CNTs-BCL

During the resolution of *(R, S)*-1-phenylethanol, enantioselectivity was affected by a variety of reaction parameters, and these parameters may interact with one another. Effects of four principal parameters (substrate molar ratio, water content, reaction time, and reaction temperature) were examined by single factorial experiments, and the results were presented in Figure 7. As shown in Figure 7b, the addition of water had a negative effect on conversion rate and $ee_s$, therefore, it is unnecessary to add extra water during the reaction.

Based on the above factorial experiments, three statistically significant variables (substrate molar ratio, reaction time, and reaction temperature) were selected for further optimization using the response surface methodology. According to Box-Behnken design, the optimum parameters of the three independent factors were: substrate molar ratio 6.57:1, reaction time 9.97 min, and reaction temperature 56.40 °C. Under the optimum conditions, the predicted $ee_s$ was 99.35%. To verify the validation of the model, an experiment was conducted in triplicates. The mean $ee_s$ was 99.52%, which coincided with the predicted value, and the corresponding conversion rate was 49.77%.

----------Fig. 7----------

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DOI: 10.1039/C4RA10517F



### 3.4 Characterization of CNTs-BCL via FT-IR

To further explore the probable mechanism for the performance enhancement of the immobilized lipase, FT-IR was utilized to examine the secondary structure alteration of the lipase. Peptide bond in different secondary structure can absorb infrared in different wavelengths.[28] As known, protein has strong absorbance spectrum in the amide I region (1,700–1,600 $cm^{-1}$) because of the C=O bending vibration. In this range, the main absorbance spectra of α-helix, β-sheet, β-turn and random coil are 1,650–1,658 $cm^{-1}$, 1,620–1,640 $cm^{-1}$, 1,670–1,695 $cm^{-1}$, and 1,640–1,650 $cm^{-1}$, respectively.[29] Calculating the peak area, the amount of the four secondary structures was respectively obtained (see Table 5).

As shown in Table 5, CNTs-BCL has an increase in β-sheet and a decrease in α-helix, β-turn and random coil compared with free lipase. The enhancement of enzyme activity was tightly related to the alteration of protein structure.[30] As reported by Barbe et al., [31] BCL has a 'lid' upon its catalytic centre, and the 'lid' mostly consists of α-helix. When the combination between BCL and hydrophobic support occurs, α-helix composition of the 'lid' decreases, leading to its unfolding, this will make the main catalytic area easier to access substrate. The increase of β-sheet is probably related to the increase of lipase activity and flexibility of the protein. The opening of 'lid' may be along with the formation of β-sheet which attributes to the maintenance of this open and obviously increases the lipase activity.[32]

**----------Table 5----------**

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DOI: 10.1039/C4RA10517F



### 3.5 Comparison with other immobilized lipases

Compared with other immobilized lipases, the CNTs-BCL exhibited a much higher catalytic efficiency and a satisfying reaction equilibrium time. Andrade et al.,[33] Wang et al.,[34] and Hara et al.[35] had immobilized BCL on superparamagnetic nanoparticles, zirconia particles, and Sol–gels and cross-linked aggregates, respectively. The best results of their work were listed in Table 6. In addition, in our previous study, Li et al. reported that the reaction equilibrium time was shortened to 0.5h by BCL immobilized on macroporous resin NKA (MPR-NKA).[13] Meanwhile, the BCL was proved to be much better than the commercial lipases of Novozyme 435, Lipozyme RM IM, and Lipozyme TL IM. (See Table 6)

----------**Table 6**----------

However, in this work, the reaction equilibrium could be reached in an even shorter time----within 10 min, and the conversion and $ee_s$ reached 49.77% and 99.52%, respectively. Moreover, the highest catalysis activity attained 50,200 U/g, which was 54 folds that of the free lipase (924.1 U/g). Therefore, CNTs-BCL can greatly improve production efficiency and effectively reduce production cost, exhibiting a promising prospect in industrial application.

## 4. Conclusion

In this study, the modified CNTs were utilized for BCL immobilization, resulting in a significant enhancement on enzyme activity and enantioselectivity of the immobilized

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lipase. Pre-treated with concentrated $H_2SO_4$, CNTs were oxidized and the closed end was opened to improve the absorption efficiency of lipase protein. Under the optimum conditions (pH 7.96, time 4.47h, lipase loading 0.95g), enzyme activity of the CNTs-BCL attained 50,200 U/g, 54 folds that of the free lipase. More importantly, CNTs-BCL immensely shortened reaction time compared with other immobilized BCL, suggesting CNTs-BCL exhibits a better catalytic efficiency and shows great potential for industrial application.

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View Article Online
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View Article Online
DOI: 10.1039/C4RA10517F



Table 1 Properties of CNTs used in the present study

| CNTs | Diameter (nm) | Length (μm) | Purity (%) | Ash content (%) | Specific area ($m^2/g$) |
|---|---|---|---|---|---|
| L-MWNT-2040 | 20-40 | 5-15 | ＞97% | ＜3 wt% | 90-120 |
| L-MWNT-4060 | 40-60 | 5-15 | ＞97% | ＜3 wt% | 40-70 |
| L-MWNT-60100 | 60-100 | 5-15 | ＞97% | ＜3 wt% | 40-70 |
| MWNT-OH | 20-40 | ＜2 | ＞97% | ＜3 wt% | 100-120 |

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DOI: 10.1039/C4RA10517F



Table 2 Factors and level value of response surface analysis

| Independent variables | Symbols | -1 (Low Level) | 0 (Mid-Level) | 1 (High Level) |
|---|---|---|---|---|
| time/ h | $X_1$ | 2 | 4 | 6 |
| Lipase loading/ g | $X_2$ | 0.4 | 0.8 | 1.2 |
| pH | $X_3$ | 5 | 7 | 9 |

View Article Online
DOI: 10.1039/C4RA10517F



Table 3 RSM design and its experiment results

| Run | $X_1$ (time/h) | $X_2$ (lipase loading/g) | $X_3$ (pH) | Y (enzyme activity U/min/g protein) |
|---|---|---|---|---|
| 1 | 2.0 | 0.4 | 7 | 48, 710 |
| 2 | 2.0 | 1.2 | 7 | 49, 200 |
| 3 | 6.0 | 0.4 | 7 | 48, 900 |
| 4 | 6.0 | 1.2 | 7 | 49, 560 |
| 5 | 4.0 | 0.4 | 5 | 48, 700 |
| 6 | 4.0 | 0.4 | 9 | 48, 980 |
| 7 | 4.0 | 1.2 | 5 | 48, 860 |
| 8 | 4.0 | 1.2 | 9 | 49, 600 |
| 9 | 2.0 | 0.8 | 5 | 48, 900 |
| 10 | 6.0 | 0.8 | 5 | 48, 970 |
| 11 | 2.0 | 0.8 | 9 | 49, 370 |
| 12 | 6.0 | 0.8 | 9 | 49, 520 |
| 13 | 4.0 | 0.8 | 7 | 49, 790 |
| 14 | 4.0 | 0.8 | 7 | 49, 770 |
| 15 | 4.0 | 0.8 | 7 | 49, 730 |

View Article Online
DOI: 10.1039/C4RA10517F



Table 4 Analysis of variance of regression mode (α=0.01, confidence coefficient=99%）

| Source | DF | SS | MS | F | Pr>F |
|---|---|---|---|---|---|
| Model | 9 | 2208302 | 245366.9 | 37.64258 | 0.0004 |
| Linear | 3 | 1059925 | 353308.3 | 54.20225 | 0.0003 |
| Quadratic | 3 | 1086652 | 362217.2 | 55.56899 | 0.0003 |
| Cross product | 3 | 61725 | 20575 | 3.15648 | 0.0124 |
| Error | 5 | 32592 | 6518 | | |
| Lack of fit | 3 | 30725 | 10241 | 10.9732 | 0.0847 |
| Pure error | 2 | 1866 | 933 | | |
| $R^2$ =98.55% | | | | | |
| Adj.$R^2$ = 95.93% | | | | | |

View Article Online
DOI: 10.1039/C4RA10517F



Table 5 Quantitative estimation of the secondary structure elements of the free and immobilized lipases

| | α-helix (%) | β-sheet (%) | β-turn (%) | Random coil (%) |
|---|---|---|---|---|
| Free BCL | 28.3±1.09 | 21.4±2.21 | 25.8±0.91 | 24.5±0.21 |
| CNTs-BCL | 20.4±3.21 | 61.5±1.21 | 6.2±0.81 | 11.9±0.71 |

View Article Online
DOI: 10.1039/C4RA10517F



Table 6 Comparison between the CNTs-BCL and other immobilized lipases

| Lipases | Immobilization matrixes | Conversion | $ee_s$ | Reaction time | Authors |
|---|---|---|---|---|---|
| BCL | CNTs | 50 % | 99 % | 10min | This study |
| BCL | Superparamagnetic nanoparticles | 34% | 50% | 48h | Andrade et al. |
| BCL | Zirconia particles | 50% | 99% | 48h | Wang et al. |
| BCL | Sol–gels and cross-linked aggregates | 50% | 99% | 25h | Hara et al. |
| BCL | Macroporous resin NKA | 50% | 99% | 0.5h | Li et al. |
| Novozym 435 | Macroporous acrylic resin | 43.3 % | 75 % | 0.5h | Li et al. |
| Lipozyme RM IM | Macroporous anion exchange resin | 2.6 % | 24 % | 0.5h | Li et al. |
| Lipozyme TL IM | Silica particles | 4.8 % | 15 % | 0.5h | Li et al. |

View Article Online
DOI: 10.1039/C4RA10517F



# Figure captions

Fig.1 Enzyme activity and immobilization efficiency by different CNTs

Fig.2 SEM analysis of CNTs before (a) and after (b) oxidation.

Fig.3 EDS analyses of pure CNTs (a), oxidized CNTs (b).

Fig.4 Effects of immobilization conditions on BCL immobilization efficiency. (a) Effects of pH; (b) Effects of lipase loading; (c) Effects of immobilization time.

Fig.5 EDS analyses of CNTs-lipase.

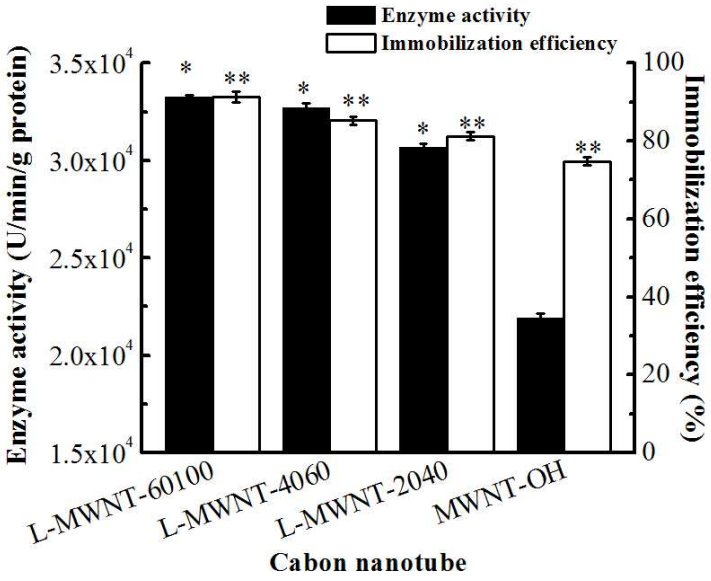
Fig.6 Response surface plot and contour plot of interaction between the three independent factors on the enzyme activity. (a) Response surface plot for the effects of $X_1$(immobilize time) and $X_2$ (lipase loading); (b) Response surface plot for the effects of $X_1$ (immobilize time) and $X_3$(pH); (c) Response surface plot for the effects of $X_2$ (lipase loading) and $X_3$(pH); (d) Response contour plot for the effects of $X_1$ (immobilize time) and $X_2$ (lipase loading); (e) Response contour plot for the effects of $X_1$ (immobilize time) and $X_3$(pH); (f) Response contour plot for the effects of $X_2$ (lipase loading) and $X_3$(pH).

Fig.7 Effects of reaction conditions on enantioselectivity of resolution reaction. (a) Effects of molar ratio; (b) Effects of water content; (c) Effects of reaction time; (d) Effects of reaction temperature.

View Article Online
DOI: 10.1039/C4RA10517F

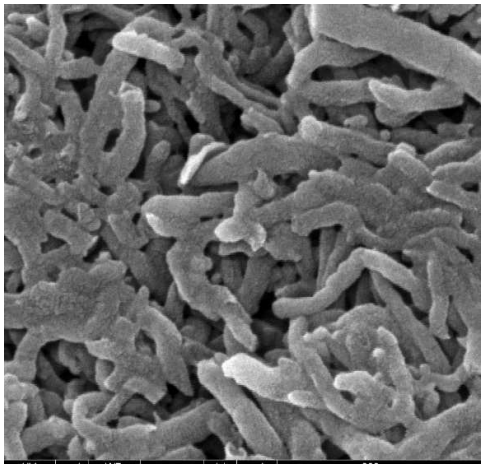


**Fig. 1**

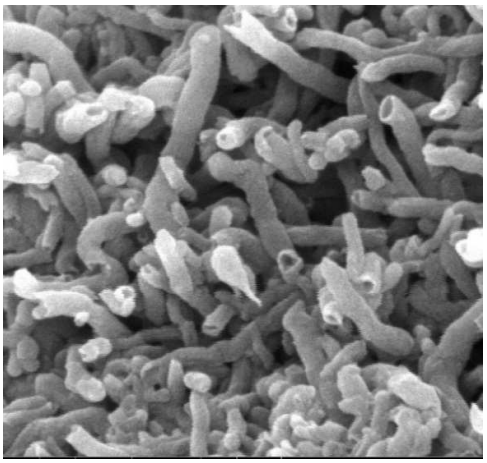


View Article Online
DOI: 10.1039/C4RA10517F

**Fig. 2**



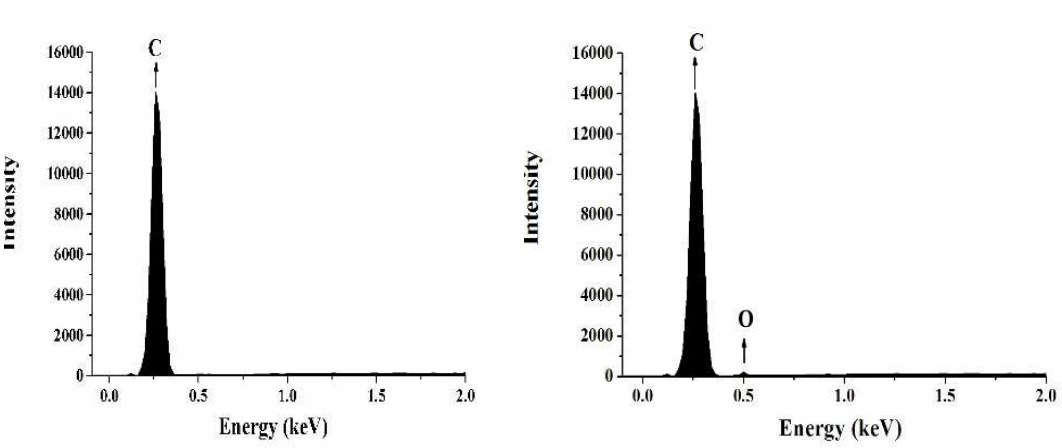
(a) Pure CNTs (Magnification: $3\times10^5$, Accelerating Voltage: 5.00 kV)



(b) Oxidized CNTs (Magnification: $3\times10^5$, Accelerating Voltage: 5.00 kV)

View Article Online
DOI: 10.1039/C4RA10517F

**Fig.3**

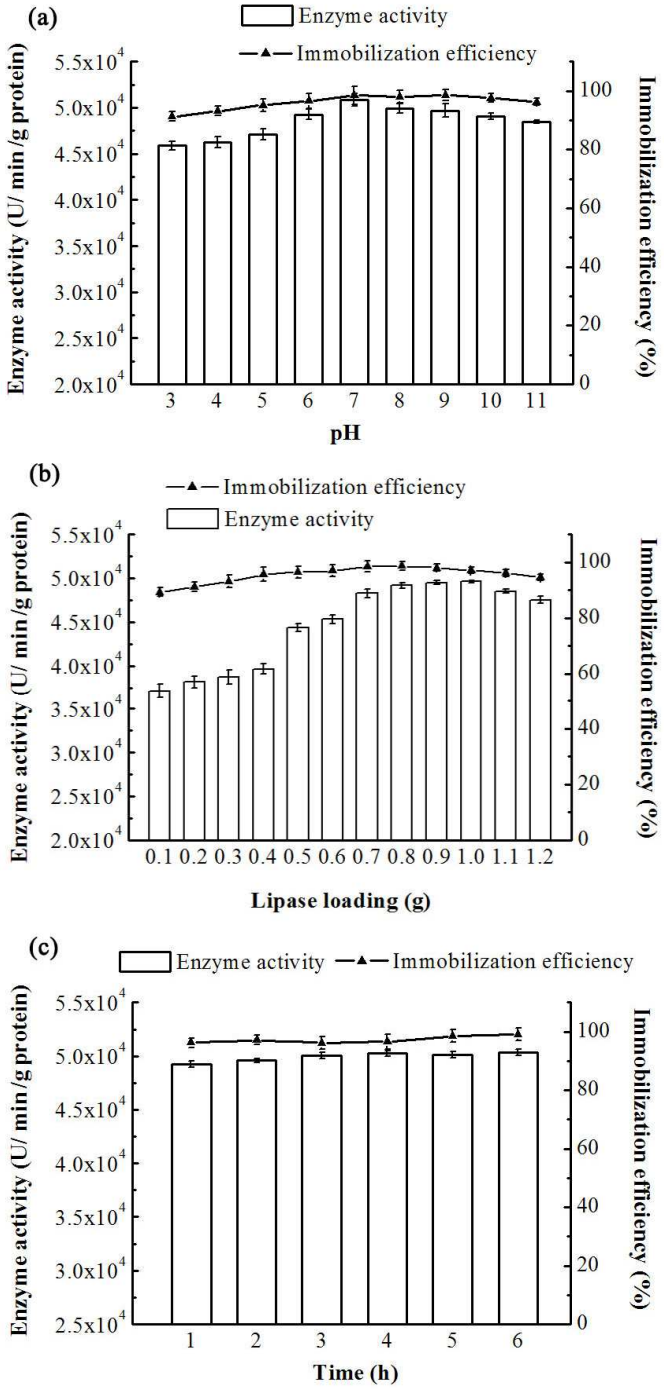



(a) CNTs (Pure) (b) CNTs (Oxidized)

View Article Online
DOI: 10.1039/C4RA10517F



**Fig. 4**



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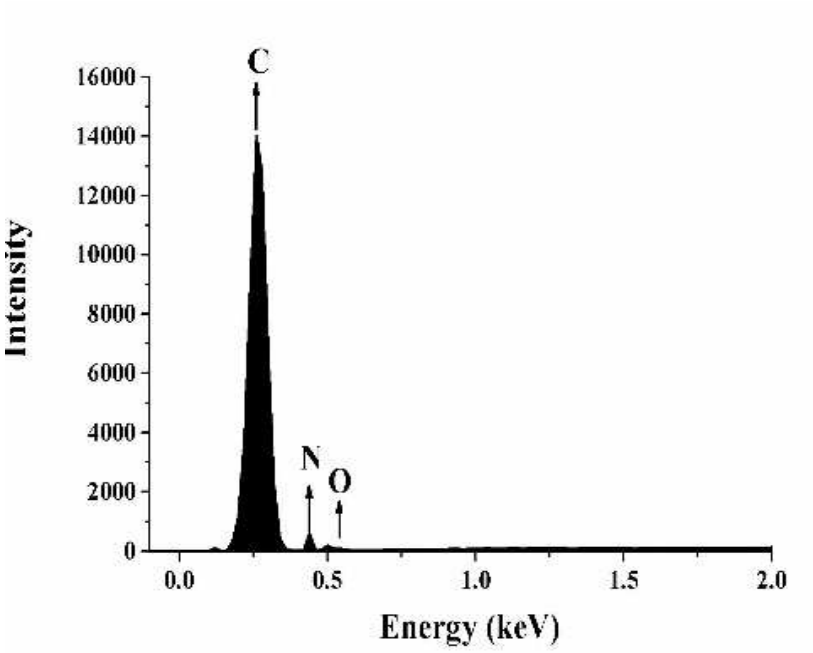
DOI: 10.1039/C4RA10517F

**Fig.5**



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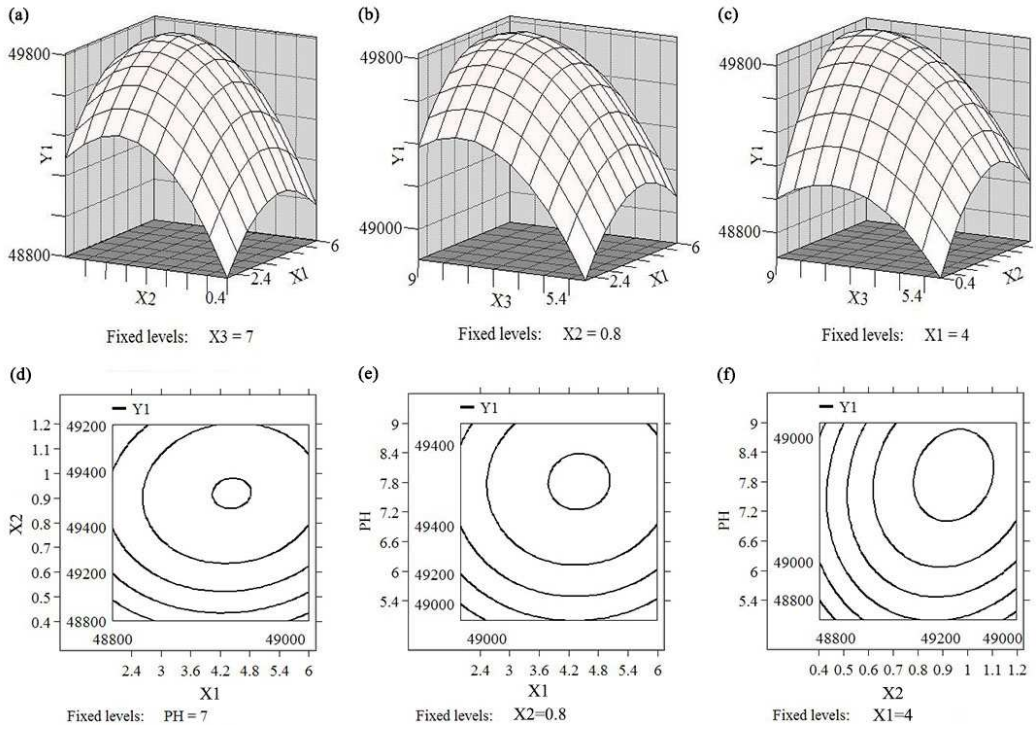
DOI: 10.1039/C4RA10517F



**Fig. 6**



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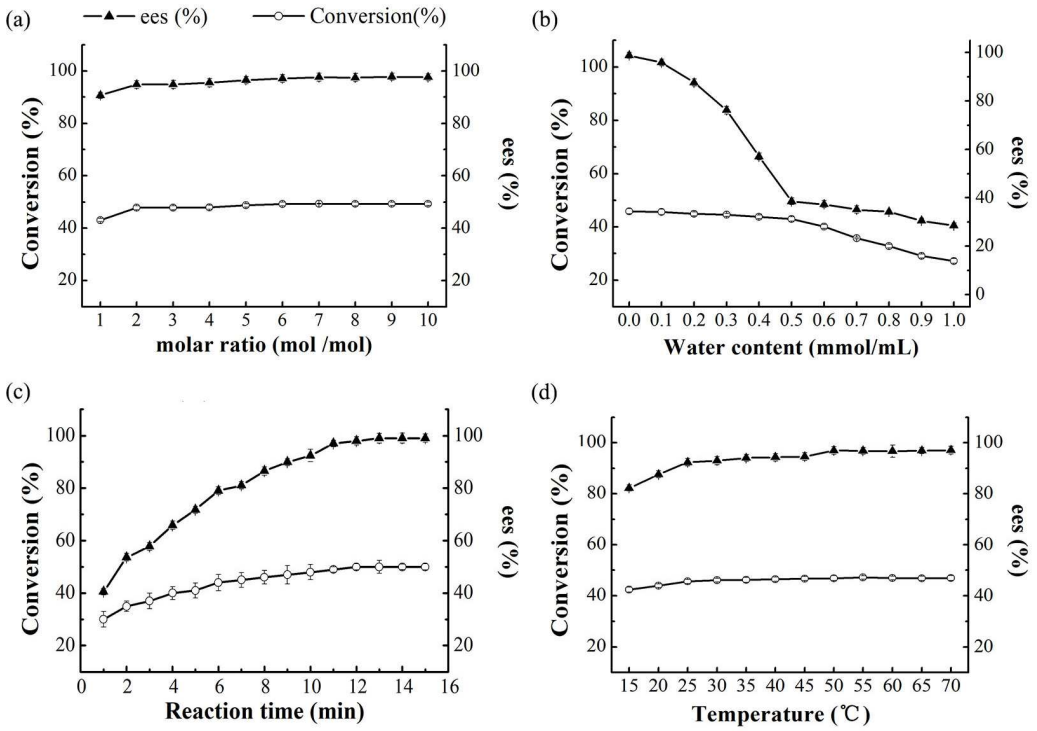
DOI: 10.1039/C4RA10517F



**Fig. 7**



View Article Online
DOI: 10.1039/C4RA10517F



# A table of contents entry

* Colour graphic: No

* Text: *Burkholderia cepacia* lipase immobilized on carbon nanotubes could highly enhanced catalysis performance and immensely shortened reaction time to 10 min.