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# Preparation and characterization of oxidized Multi-walled Carbon Nanotubes-Immobilized *Aspergillus sp.* Laccase Hybrid Materials

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**Abstract:** This work deals with preparation and characterization of immobilized laccase (*Aspergillus sp.)* over oxidized multi-walled carbon nanotubes (ox-MWCNTs) *via* simple mixing technique. The resulting materials were characterized by Fourier transform infrared spectroscopy (FTIR), thermogravimetric analysis (TGA), transmission electron microscope (TEM) and particle size distribution analysis using dynamic light scattering technique (DLS). The results showed that the TEM images exhibited more separate individual carbon bundles with particle size around of 396 nm after enzyme immobilization rather than the spaghetti-like tubes with size about 180 nm in the case of ox-MWCNTs. Also, the lowering in the zeta potential negative value (-5 mv) proved that the free carboxyl groups at ox-MWCNT surface were decreased after enzyme immobilization. Moreover, the thermal stability was decreased after enzyme immobilization using TGA. These results confirmed that the laccase could be reacted at the side walls of the ox-MWCNTs without structure damage. The biocatalytic effect of the immobilized laccase was investigated after its incubation with silver nitrate solution for 1 and 24 h. It can be concluded that the biocatalytic efficiency of the immobilized laccase could be enhanced after its incubation with silver nitrate solution for 24 h at room temperature relative to the free form. On the other hand, the enzyme stability was improved after immobilization up to 50ºC and at pH 3.0, while no remarkable differences on the activity values were observed for immobilized and free laccases at acidic pH range (4-6).

**Keywords:** Multi-walled carbon nanotubes, Functionalization, Biocatalysts, Enzyme immobilization, Laccase enzyme, Enzyme stability.

## 1. Introduction

Carbon nanotubes (CNTs) have been considered as an ideal nano-carrier in the field of nanomedicine, which is an applicative field that uses concepts of nanotechnology, biology and medicine. The various applications of these include: controlled drug delivery; targeted delivery of drug molecules to a specific site, in addition to delivery of bio-nanotechnology products; as an additive to improve the solubility of the slight water-soluble drugs; hormone and enzyme [1-4]. Using of CNTs as carriers for enzyme immobilization is recently reported for several applications [5-10]. Functionalization of CNTs with organic, polymeric and biological molecules can provide biocompatible nanotube composites with specific groups on their surface. Furthermore, Haroun *et al.* [10-18] reported that novel functionalized MWCNTs-based nanocomposites could be prepared for several applications. Besides, *Aspergillus flavus*, levansucrase and L-asparaginase enzymes were successfully immobilized onto oxidized MWCNTs [19-22]. Enzyme immobilization is advantageous due to enzyme recovery from the product can be easier, also enzyme reuse and process scaling up. Moreover, immobilized enzymes sometimes have higher activity, stability, selectivity and resistance to inhibitors [23-25]. On the other hand, the free enzymes sever from many disadvantageous such as their deactivation under drastic conditions [26]. Consequently, the enzymes immobilization on CNTs is considered an effective strategy for improving the long-term stability and reusability [27-42]. Generally, many techniques for enzyme loading on carriers including adsorption, covalent bonding, entrapment or encapsulation, have been previously reported [43]. However, during the catalytic reaction, the easily enzyme leaching may be limited its utilization at industrial scale. Thus, the interaction between the enzyme and the carrier is gained more attention for development of highly active and stable biocatalyst. Besides, CNTs have been preferred as carriers for enzyme immobilization, because they may provide high surface area and they can easily functionalized to get the targeting properties

for specific application. Laccase is most widely distributed in a wide range of higher plants, fungi and bacteria [44]. It's secreted out in the medium extracellularly by several fungi during the secondary metabolism but not all fungal species produce laccase [45]. It belongs to the family of the blue multi-copper oxidase, which catalyze the one electron oxidation of four reducing-substrate molecules concomitant with the four-electron reduction of molecular oxygen to water. It is also oxidative enzyme (benzendiol: oxygen oxidoreductase, EC 1.10.3.2), which has been used in various biotechnological processes. This study aims at immobilization of laccase (*Aspergillus sp.*) onto oxidized MWCNTs using simple mixing technique. The immobilized enzyme was characterized using FTIR, TGA, TEM and particle size distribution analysis. The stability of the immobilized enzyme at different temperatures and pH was also investigated relative to the free enzyme. The catalytic activity of the immobilized enzyme in the presence of silver nitrate aqueous solution was carried out after incubation for 1 and 24 h at room temperature in comparison with the free one.

## 2. Experimental Details

### 2.1. Materials and Methods

#### 2.1.1. Materials

Multi-walled carbon nanotubes (MWCNTs), carbon 95%, O.D L 6-9 nm 5 µm, pure commercial laccase from *Aspergillus sp.* and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) substrate for laccase assay were obtained by Sigma-Aldrich. All other chemicals and reagents are used without further purification.

#### 2.1.2. Purification and oxidation of MWCNTs

Pristine MWCNTs (3.0 g) was dispersed in mixed concentrated sulphuric and nitric acids (3:1, v/v) at ratio of 50 mL acid mixture per 10 mg of MWCNTs [46] and then refluxed at 110°C with continuous stirring to produce oxidized carbon nanotubes (MWCNTs-COOH). The mixture was washed with ultrapure water until the filtrate is neutral (pH 7.0). The collected material was resuspended and centrifuged at 8000 rpm for 10 min. Finally, the obtained solid powder was dried in a vacuum oven at 70°C for 12 h and kept for further investigation.

#### 2.1.3. Enzyme immobilization

The commercial laccase (12 mg/mL, 75 U/mL) was added to 10 mg/mL of oxidized MWCNTs and then ultrasonicated for 1 h. The resulting mixture was incubated at 40ºC with a shaking speed of 170 rpm for 1 h to reach the equilibrium state. After that the immobilized laccase was separated using cooling centrifugation (Sigma, 6000 rpm for 5 min), washed with HEPES buffer, freeze-dried and stored at -4ºC for further analysis.

#### 2.1.4. Enzymatic activity assay

The quantitative analysis of enzyme activity was determined after incubating 1.8 mL of the 3 mM ABTS solution (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) prepared in a 0.2 M acetate buffer solution (ABS, pH 4.0) with 0.2 mL of the free enzyme mixture at 25ºC for about 5 min. The absorbance was examined spectrophotometrically at 420 nm. On the other hand, 20 mg of the prepared hybrid material (immobilized laccase onto ox-MWCNTs), 1.3 mL of a sodium acetate buffer (0.2 M, pH 4.0) and 0.7 mL of ABTS (3 mM) were mixed and incubated for 5 min. After that the sample was filtered and the absorbance was determined at 420 nm. Relative activities were adapted as the percentage of the initial activity fixed as 100%. All experiments were performed three times separately [47].

### 2.2. Characterization

#### 2.2.1. Physico-chemical characterization

The samples were examined using Perkin-Elmer (FTIR) spectroscopy under certain condition such as: scan resolution: 4 $cm^{-1}$, scan rate: 2 mm $sec^{-1}$, range: 600-4000 $cm^{-1}$ and mode: transmission. The TGA was carried out on Perkin-Elmer thermogravimetric analyzer TGA 7: The dry samples were heated from room temperature to 400°C at 5°C/min. The morphologies and the particle size were carried out with a JEOL transmission electron microscope (TEM) and the particle size and zeta potential were carried out using DLS Malvern instrument.

#### 2.2.2. Biochemical characterization of the immobilized and free laccase

The influence of pH values on laccase activity was investigated. The immobilized and free laccases were incubated separately for 60 min for each sample in various buffers with pHs ranging from 3.0 to 8.0 at 40ºC and 3 mM ABTS as the substrate. Also, the influence of temperature on laccase activity was carried out. The immobilized and free laccases were incubated for 60 min at temperatures ranging from 30 to 70ºC using a buffer with pH 3.0 and 3.0 mM ABTS as the

substrate, then, the remaining activities were determined at 25ºC after cooling.

The thermal stability at 50 and 70ºC against laccase activities was also determined as follows: the immobilized and free laccases were incubated at temperatures 50 and 70ºC using a buffer with pH 3.0 for 60 min. The remaining activities were determined at 25ºC. Aliquots of samples of 3 mL were taken at each time interval (0, 15, 30, 45 and 60 min), filtered and used for the examination. The reading was repeated three times. The laccase activity was fixed as 100% at the optimum pH or temperature.

## 3. Results and Discussion

### 3.1. Physico-chemical characterization

Figure 1 shows FTIR spectra of the immobilized laccase in comparison with the carrier (ox-MWCNTs). In the case of the ox-MWCNTs spectrum, the characteristic peaks at 2926, 2856, 1741, 1563, 1404, and 1381 $cm^{-1}$ were assigned to -$CH_2$ (str), -CH- (str), -C=O (str), -C-C- (str), -OH (bending) and -C-O (str) groups, respectively, which are attributed to the vibrations of the carbon skeleton after oxidation process [48]. On the other hand, in the case of the immobilized enzyme spectrum, not all the characteristic peaks belonging to the ox-MWCNTs appeared in the spectrum. However, the strong peak was appeared at 3432 $cm^{-1}$ which is assigning to -$NH_2$ groups of the protein enzyme. This may be due to the completely adsorption of the enzyme by the carrier through the physico-chemical interactions.

Figure 2 and Table 1 show TGA diagrams and weight loss (%) data at temperature range 30-1000ºC of the immobilized enzyme in comparison with the carrier (ox-MWCNTs). It can be noticed that Ox-MWCNTs exhibited high thermal stability (weight loss about 9.6% at > 455ºC).

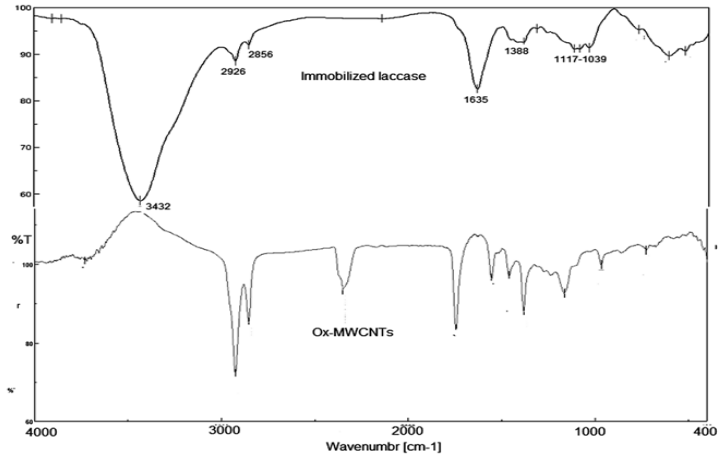


**Figure 1.** FTIR spectra of the immobilized laccase in comparison with the carrier (ox-MWCNTs).

**Table 1.** TGA data of the prepared materials

| Sample | Wight loss (%) at different temperatures (°C) | | | |
|---|---|---|---|---|
| | 40-120 | 120-281 | 281-350 | 350-780 |
| Ox-MWCNTs | 12 | ------ | ---- | 9.6 |
| Immobilized laccase | 15 | 13 | ------ | 72 |

**Table 2.** Particle size distribution and zeta potential analysis using DLS technique

| Sample | Particle size (nm) | Zeta potential (mv) |
|---|---|---|
| Ox-MWCNTs | 180±22.7 | -12.2±7.2 |
| Immobilized laccase | 396±17.9 | -5.6±3.4 |

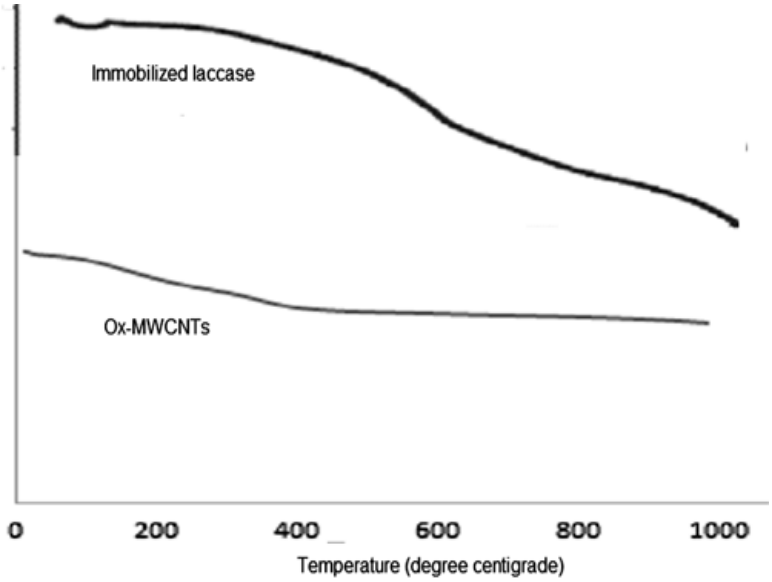


**Figure 2.** TGA diagrams of the immobilized laccase in comparison with the carrier (ox-MWCNTs).

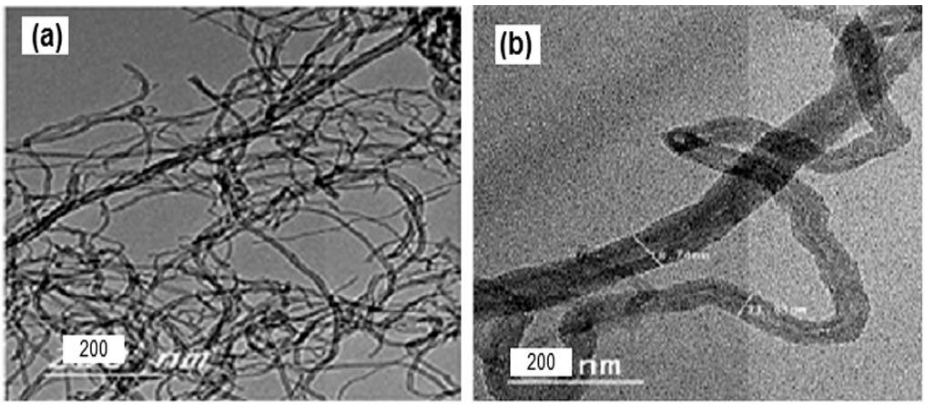


**Figure 3.** TEM images of (a) the carrier (ox-MWCNTs) and (b) the immobilized laccase at scale bar 200 nm.

After enzyme immobilization, it had three decomposition steps. The first starts at 40-120ºC, the second at 120-281ºC and the third one over 350ºC with weight loss about 15, 13 and 72%, respectively. This may be due to the loss of the unbound water and the presence of enzyme molecules. Moreover, these results proved that the successful laccase immobilization onto oxidized MWCNTs was taken place *via* covalent bonds.

Figure 3 shows TEM images of the immobilized enzyme in comparison with the carrier (ox-MWCNTs). It can be noticed that the diameters of the immobilized laccase were increased relative to that in case of the oxidized MWCNTs suggesting that adsorption of the laccase enzyme particles occurred in the matrices. This observation was confirmed by the particle size distribution analysis (Figure 4). Table 2 shows the particle size distribution analysis of the immobilized laccase onto MWCNTs in comparison with the carrier (ox-MWCNTs). The particle size of the carrier was increased from 180 to 396 nm after laccase immobilization. On the other hand, the ox-MWCNTs had high negative zeta potential value about -12 mv. While, after enzyme immobilization, it was about -5.6 mv. This may be due to the high tendency to flocculate and the presence of the attractive forces between the different charges (Table 2).

### 3.2. Biocatalytic activity evaluation

The silver nitrate solution with no laccase enzyme was used as the control, which showed no colour change during the incubation under the same conditions. While, after 1 h of incubation, the silver nitrate solution had slight colour intensity change in the case of laccase enzyme before and after immobilization, On the other hand, after 24 h of incubation, high colour changes could be obtained this may be due to the catalytic effect of the released enzyme from the carrier (ox-MWCNTs). The particle size of the $AgNO_3$ after 1 h of incubation with the immobilized laccase was around of 295±70.7 and 95±25.1 nm with PDI about 0.52.

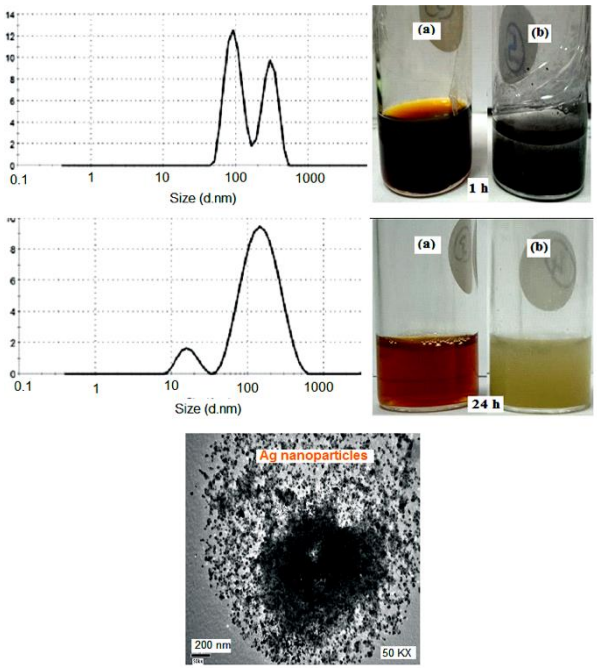


**Figure 4.** Particle size distribution analysis at room temperature using DLS technique and Photographs of the treated $AgNO_3$ with laccase (a) before and (b) after immobilization onto ox-MWCNTs after incubation for 1 and 24 h at room temperature in aqueous media.

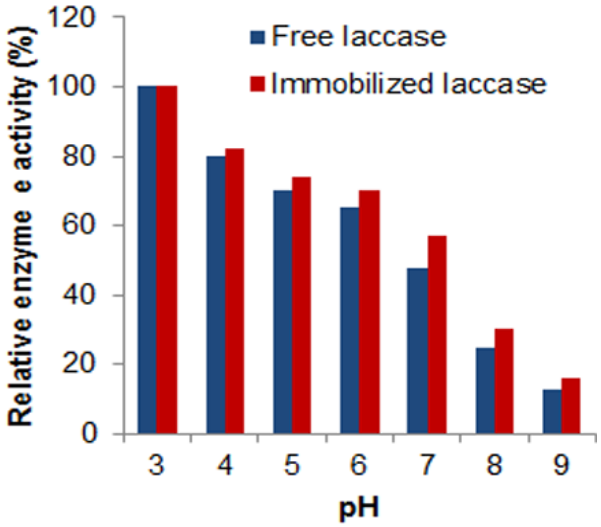


**Figure 5.** Effect of pH on stability of free and immobilized laccase onto ox-MWCNTs.

While, after 24 h of incubation, the size was around of 165±87.2 and 17±4.4 nm with PDI about 0.46. In other words, the first peaks (295 and 165 nm) may be assigned to the ox-MWCNTs dissociation particles, while, the other ones belong to the silver nanoparticles (95 and 17 nm).

## 3.3. Biochemical characterization

### 3.3.1. Effect of pH value on laccase activity

The optimum pH for laccases depends on the substrate and on its redox potential. Fungal laccases are usually stable at acidic pH, although pH stability varies considerably depending on the source of the enzyme [49]. In the present study, the influence of pH on the laccase activity was examined over a pH range from 3 to 8, as shown in Figure 5. It was observed that the optimum activity was recorded at pH 3 for both the free and immobilized laccase, with high activity level (100%). Besides, the free laccase had low activity in all pH range relative to the immobilized one, which proves that the immobilized laccase is relatively stable at different pH values. Moreover, at acidic pH range (4, 5 and 6), the immobilized enzyme retained 82, 74 and 70 % relative activity in comparison with that in the case of the free one (80, 70 and 65%, respectively).

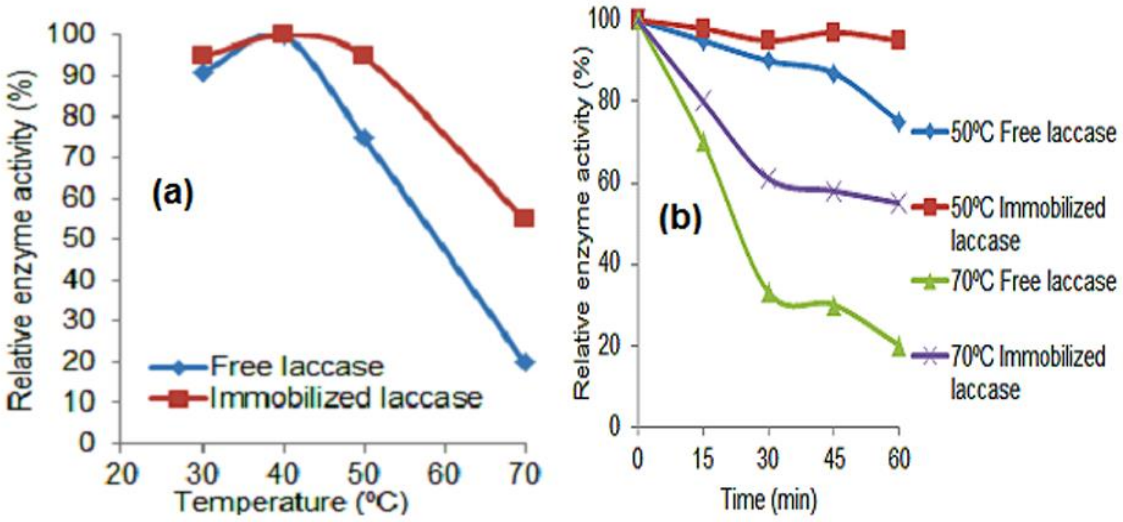


**Figure 6.** Effect of (a) temperature and (b) thermal stability at 50 and 70ºC on the activity of free and immobilized laccase.

Zofair [50] and Daronch *et al*. [51] reported recently that the maximum activity for both free and immobilized laccase was at pH 3. Patrick *et al.* [52] stated that the pH profile is the result of two opposing effects: the first effect is due to the redox potential difference between a reducing substrate (phenolic compound) and the Type 1 copper center of laccase, where the electron transfer rate is favored for phenolic substrates at a high pH; the second effect is generated by the binding of a hydroxyl anion to the type 2/type 3 copper centers of laccase, which inhibits the binding of oxygen, the terminal electron acceptor. Therefore, the activity at alkaline pH could be inhibited because of the increased amount of hydroxyl ions [53].

### 3.3.2. Effect of temperature and thermal stability on laccase activity

The effect of temperature on the laccase activity before and after immobilization was carried out, as illustrated in Figure 6a. It was noticed that when the temperature was increased, the activity of both enzymes increase till 40ºC, accompanying a slightly higher activity for the immobilized one relative to the free form. On the other hand, with a further increase in temperature up to 70ºC, the activity was decreased in the case of both enzymes. The optimal temperature was 40 ºC. Besides, the immobilized laccase onto ox-MWCNTs is more stable at a high temperature relative to the free one. At 70 ºC, the immobilized laccase retained 55% of its relative activity in comparison with that in the case of the free form (20%) under the same condition. These results are in agreement with the previous data [54] which reported that in spite of an increasing in the temperature enhances the conjugation between the laccase and the carrier, the enzyme activity can be stimulated. But, a further temperature increase result in the loss of enzyme activity because of the enzyme denaturation [55]. The improvement in the thermal stability of the immobilized laccase onto ox-MWCNTs may be caused by a strong enzyme-carrier interaction between the carboxylic acid groups at MWCNTs and the enzyme protein reaction, which enhances the stability at high temperature. Also, the molecular motions of enzyme could be protected, which may result in the improvement in the protein structure rigidity. Thermal stability is one of the most important parameters for enzyme applications in the industrial sectors. In this work, the thermal stability of the immobilized laccase onto ox-MWCNTs compared to the free form was investigated (Figure 6b). This was done by incubating the immobilized and free enzyme in a buffer (pH 3) at different temperatures (50 and 70ºC) for 1 h. After that the activities were determined as shown in Fig 6b. It was observed that when the temperature was increased up to 70 ºC, the free enzyme loses up to 80% of its activity, whereas the immobilized enzyme retains 45% of its relative activity under the same conditions. The inhibition in the free enzyme activity at a high temperature has also been previously reported [56]. Based on these results, laccase immobilization onto ox-MWCNTs improved the thermal stability of the immobilized enzyme relative to the free one.

## 4. Conclusion

Oxidized MWCNT can be utilized efficiently as a carrier for *Aspergillus sp.* laccase immobilization using simple mixing technique *via* ultrasonic treatment. FTIR, TGA and TEM confirmed the attachment of laccase to the ox-MWCNTs surface. Moreover, the prepared biohybrid material was successfully used for the treatment of silver nitrate aqueous solution to

produce the Ag nanoparticles. This biocatalytic activity exhibiting similar efficiency when using the free enzyme. Also, the immobilized enzyme results in an enhancement in the activity and thermal stability at a moderate temperature relative to that in the case of the free form. Overall, the results proved the immobilization reaction and the biocatalytic performance of laccase onto ox-MWCNTs as a bionanotechnological trend in all the industrial field of its utilization.

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## Conflict of interest

The authors have no conflicts of interest to declare that they are relevant to the content of this article.

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