CHEMICAL ENGINEERING TRANSACTIONS
VOL. 84, 2021
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.cetjournal.it
Guest Editors: Paolo Ciambelli, Luca Di Palma

ISBN 978-88-95608-82-2; **ISSN** 2283-9216
DOI: 10.3303/CET2184034

Highly-efficient Immobilized Laccase on Magnetic Support for Removal Aniline

Maria Sarno[a], Mariagrazia Iuliano[b*]

[a]Department of Physics "E.R. Caianiello, and Centre NANO_MATES University of Salerno Via Giovanni Paolo II ,132 - 84084 Fisciano (SA), Italy
[b]Department of Industrial Engineering University of Salerno Via Giovanni Paolo II ,132 - 84084 Fisciano (SA), Italy
maiuliano@unisa.it

Laccase has been immobilized on magnetic nanocellulose (MCNCs) and used for degradation of aniline from simulated wastewater. The MCNCs were prepared by self-assembly approach of magnetic nanoparticles covered by polyethyleneimine (Fe_3O_4@PEI) and CNCs by commercial microcrystalline cellulose. The laccase has been successfully immobilized on MCNCs by physical interaction, showing excellent performance. About 98 % of aniline were degraded after 6 h, with reusability for at least 5 cycles.

1. Introduction

Aniline is used as a raw material in many industries to produce pesticides and herbicides, pigments and dyes, pharmaceuticals and explosives, etc. (Yan et al., 2011).
These aromatic compounds are discharged directly in industrial wastewater and indirectly through the degradation of some production products. Indeed, large quantities of compounds accumulate in the environment, creating concerns because of their toxic nature to living forms. Moreover, aniline is identified as a potential carcinogen and found to convert hemoglobin into methemoglobin in the blood, thereby preventing oxygen uptake and damaging the spleen (Kamble et al., 2003)
Aromatic amine removal has been doing with several methods(Yan et al., 2011; Kamble et al., 2003), including physical processes, such as adsorption, pervaporation, reverse osmosis, and chemical (e.g., oxidation, photolysis, liquid membranes, and nanofiltration; and biological approaches). In particular, the biological treatment technologies, such as biodegradation or enzymatic catalysis, have low cost, high efficiency, mild reaction conditions, and no-production of secondary pollution. The enzyme catalysis processes can improve the wastewater treatment efficiency, save treatment time, and reduce infrastructure investment, belonging to the "environment-friendly" approach.
Laccase (EC 1.10.3.2) from white-rot fungi has excellent properties compared with other enzymes. It's a kind of copper-containing polyphenol oxidase which exists widely in plant, fungi, and bacteria. It can catalyze several recalcitrant pollutants, such as hydroxylated polychlorinated biphenyls, chlorophenols, polycyclic aromatic hydrocarbons. On the other hand, the free enzyme uses are limited because it has low stability to temperature and pH, and the difficulty in recovery from the reaction mixture. These disadvantages have encouraged the use of immobilizations to facilitate separation, recovery, and enhance activity.
In this contest, cellulose nanocrystals (CNCs), in recent years, were used as the carrier for enzyme immobilization thanks to their excellent properties such as low density, large specific surface, outstanding mechanical properties, etc.. (Zhou et al., 2013). On the other hand, though CNCs can be a good candidate for enzyme immobilization, the practical application is still limited due to the difficulty of recovery from the reaction system. Magnetic nanoparticles (MNPs) offer many advantages as supporting material for the immobilization of enzymes (Sarno and Iuliano, 2020) because of: (i) lower mass transfer resistance; (ii) high surface area for enzyme binding; and (iii) easy separation, just applying a magnetic field. Therefore, magnetic nanoparticles embedded in CNCs (MCNCs) can be a great alternative for the above problem. In this work, we have immobilized laccase on MCNCs for degradation of aniline from simulated wastewater. The MCNCs were

Paper Received: 12 July 2020; Revised: 1 November 2020; Accepted: 26 February 2021
Please cite this article as: Sarno M., Iuliano M., 2021, Highly-efficient Immobilized Laccase on Magnetic Support for Removal Aniline, Chemical Engineering Transactions, 84, 199-204 DOI:10.3303/CET2184034

prepared by self-assembly approach of magnetic nanoparticles covered by polyethyleneimine (Fe_3O_4@PEI) and CNCs by commercial cellulose microcrystalline. The laccase has been successfully immobilized on MCNCs by physical interaction, and it had to show excellent results during the degradation of aniline. This work emphasized the possibility of using an enzymatic approach for the removal of toxic pollutants and heavy-smelling from wastewater.

2. Materials & Methods

2.1 Materials

Microcrystalline cellulose (α-cellulose, 50 μm), polyethyleneimine (PEI, Mw ~ 10,000 g/mol), Laccase from Aspergillus sp., iron(III) chloride ($FeCl_3 \cdot 6H_2O$), sodium acetate anhydrous (NaAc), and all other chemicals were acquired from Aldrich Chemical Co. All chemicals were of analytical grade.

2.2 Synthesis of Fe_3O_4@PEI nanoparticles

The synthesis of Fe_3O_4@PEI nanoparticles was carried out using a solvothermal method (Sarno and Iuliano, 2019). Firstly, ~ 0.7 of $FeCl_3 \cdot 6H_2O$ was dissolved in 20 mL of ethylene glycol and ultrasonicated until to form a transparent solution, after 1.8 g of NaAc and 0.5 g PEI was added. The mixture was stirred vigorously for 20 min at 60 °C, transferred into an autoclave at 220 °C for 120 min.
After 120 min of synthesis, the nanoparticles were washed with ethanol several times in the presence of an external magnet and then dried at 60 °C for 24 h.

2.3 Preparation of MCNCs

The synthesis of MCNCs was carried out using a self-assembled method. In particular, CNCs are prepared from microcrystalline cellulose by acid hydrolysis. Briefly, 3 g of microcrystalline cellulose was mixed with 45 mL sulfuric acid (64 wt.%) and stirred vigorously at 40 °C for 90 min. After 90 min, the reaction was quenched with cold deionized water (450 mL). The sample was centrifuged at 5000 rpm for 15 min, and the supernatant was discharged. The cellulose was re-dispersed in deionized water and centrifuged again until reacing constant pH value. The suspension was sonicated for 10 min at an output power of 100 W while cooling in an ice bath to avoid overheating. Finally, the suspension was frozen overnight at −70 °C and freeze-dried. The MCNCs were prepared by self-assembly of CNCs with Fe_3O_4@PEI NPs. 200 mg of the CNCs was re-dispersed in 10 mL deionized water and sonicated at an output power of 100 W to be homogeneous. Then 10 mL of an aqueous dispersion containing 50 mg Fe_3O_4@PEI NPs were poured into the CNCs dispersion and mixed with intense agitation. Finally, the mixture was sonicated for 20 min at an output power of 100 W while cooling in an ice bath to avoid overheating. Subsequently, the resulting MCNCs were separated by a magnet and washed with deionized water, and freeze-dried for the use of an immobilized enzyme.

2.4 Immobilization of Laccase on MCNCs

5 mg of MCNCs was immersed in 11 mL of citrate buffer (pH 3.0). Then laccase solution (17 μL/mL) was added into the MCNCs dispersion, and the mixture was incubated in a shaker overnight at 4 °C. The immobilized laccase was separated by a magnet and washed to remove the unbound enzyme with distilled water. The laccase concentration was measured at 595 nm by using a Bradford Method (Bradford, 1976). The amount of adsorbed Laccase on MCNCs was calculated by measuring the initial and final concentration of the enzyme.

2.5 Aniline removal with Laccase on MCNCs

The reaction was carried out in triplicate at room temperature in a 50 mL batch reactor. Synthetic wastewater was comprised of a buffered solution containing aniline in concentration 1 mM in a total volume of 20 mL. Immobilized laccase was added to initiate the reaction, which was run for 8 h. The reaction was monitored at different time intervals, 2 mL aliquots of the reaction solution were sampled, the immobilized laccase was immediately recovered, and the reaction solution centrifuged, to remove possible sediment at 5000 rpm for 15 min with a Centrifuge 5430 R. The concentration of aniline in the supernatant was determined with UV-visible spectrophotometry

2.6 Anilines colorimetric assay

Aniline concentrations were measured by a colorimetric method (Wang et al., 2005). Briefly, 10 mM of TNBS, 100μL of 0.5 M phosphate buffer pH 7.4, 100μL of 20 mM sodium sulfite, 100 μL of different concentration of aniline, and 600 μL of H_2O were added. The blank was prepared in the same way as samples except that an

additional 100 µL of H_2O was added instead of aniline solutions. The solution absorbance was measured against the blank. The UV-VIS spectrophotometer was used to measure color at a wavelength of 430 nm.

2.7 Catalyst Characterization

MCNCs samples were monitored under SEM (Scanning electron microscopy) (TESCAN- VEGA LMH; 230 V) analysis. For thermogravimetric studies (TGA 2, METLER TOLEDO) was used under an airflow at 10 °C/min. FT-IR spectra were obtained by Nicolet iS50 FT-IR. X-ray diffraction measurements were also performed by a Bruker D2 X-ray diffractometer using CuKα radiation.

3. Result and discussion

3.1 Characterization of MCNCs and Immobilized Laccase

The surface of Fe_3O_4@PEI and MCNCs were morphologically studied by SEM. In Figure 1a-b the prepared CNCs were lamellar-shaped particles after acid hydrolysis of cellulose microcrystalline. As can be observed in Figure 1c-d, the nanoparticles of the Fe_3O_4@PEI show a spherical size distribution ranged from 100 to 230 nm, with an average size estimated to be ~209 nm. The prepared MCNCs present a sheet structure but with a rough surface due to Fe3O4@PEI NPs. The distribution of nanoparticles on the surface of CNCs was uniform, with small area showing aggregates, see Figure 1e-f. Figure 2 shown the digital photo of the CNCs before self-assembled with Fe_3O_4@PEI (Figure 2a) and of MCNCs (Figure 2b). MCNCs can be easily separated due to the magnetic nature, due to the presence of magnetic nanoparticles on the surface of CNCs, with an external magnetic field (Figure 2c).

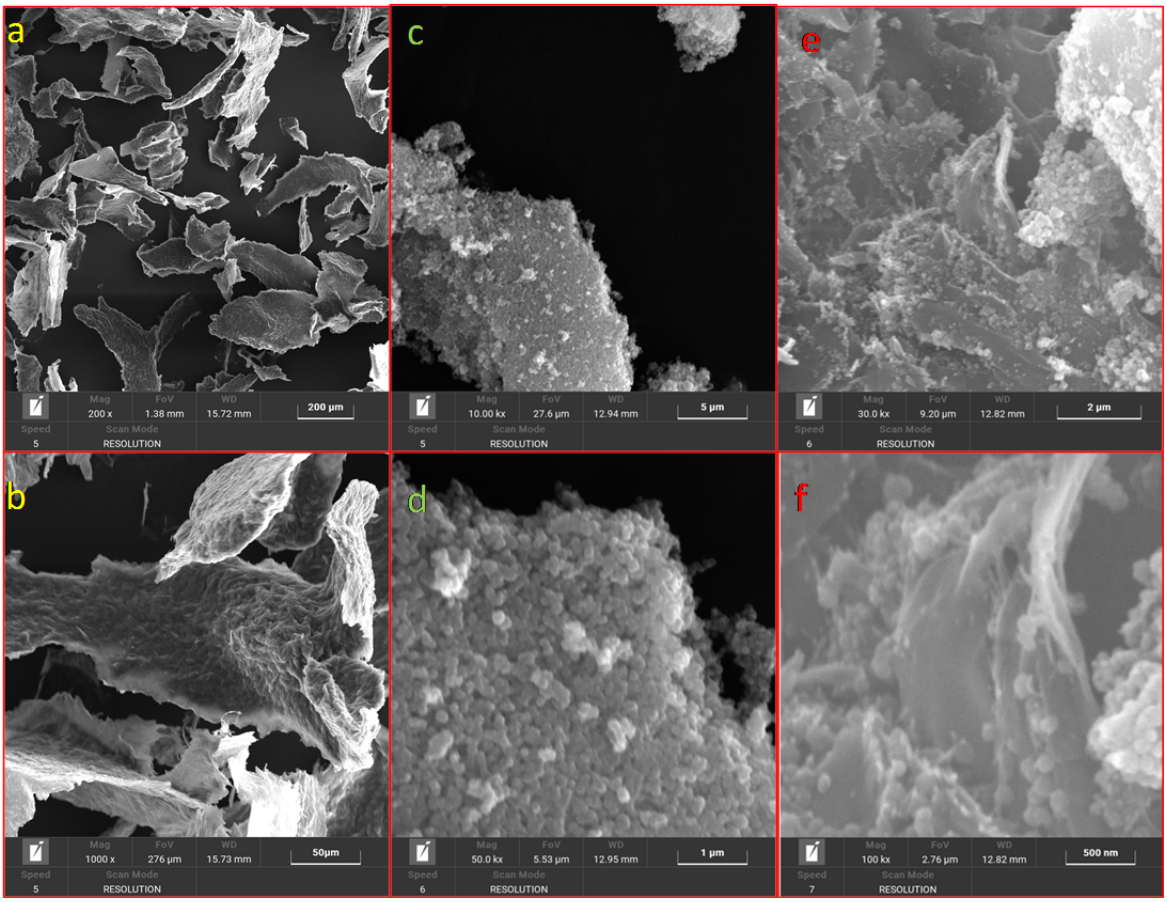

Figure 1. SEM analysis of Fe_3O_4@PEI (a,b) and MCNCs (c,d).

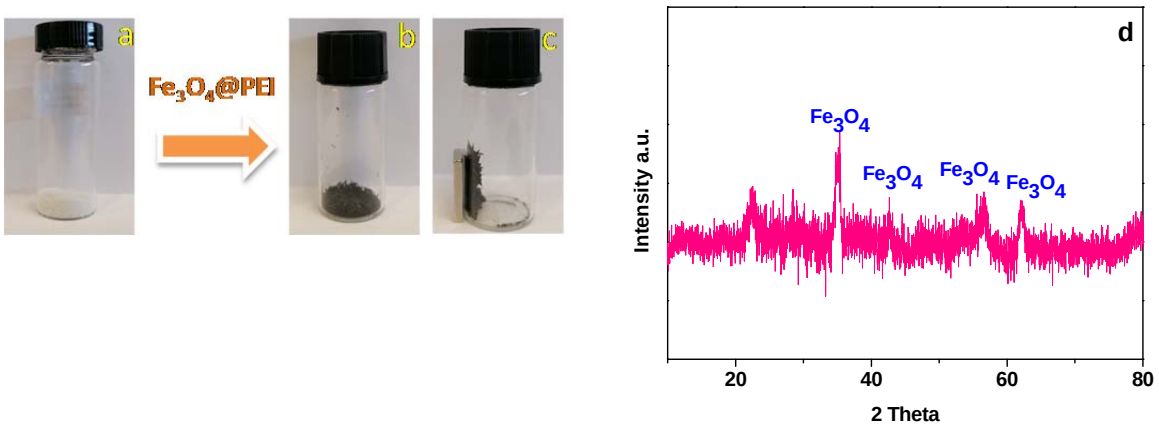

Figure 2. Digital photo of CNCs (a); MCNCs (b); and, MCNCs attracted by external magnet (c); XRD pattern of MCNCs (d).

A typical XRD pattern of MCNCs is shown in Figure 2d. In the pattern, five distinct characteristic diffraction peaks, belonging to Fe_3O_4 at 2 = 35.4°, 42.2°, 56.0°, and 62°, were seen. The peak at 22.7° was assigned to the crystallographic planes of (002) of the cellulose (Cao et al., 2016). These results indicated that the crystalline structure of cellulose was partially maintained during acid hydrolysis and the self-assembly process with magnetic nanoparticles.

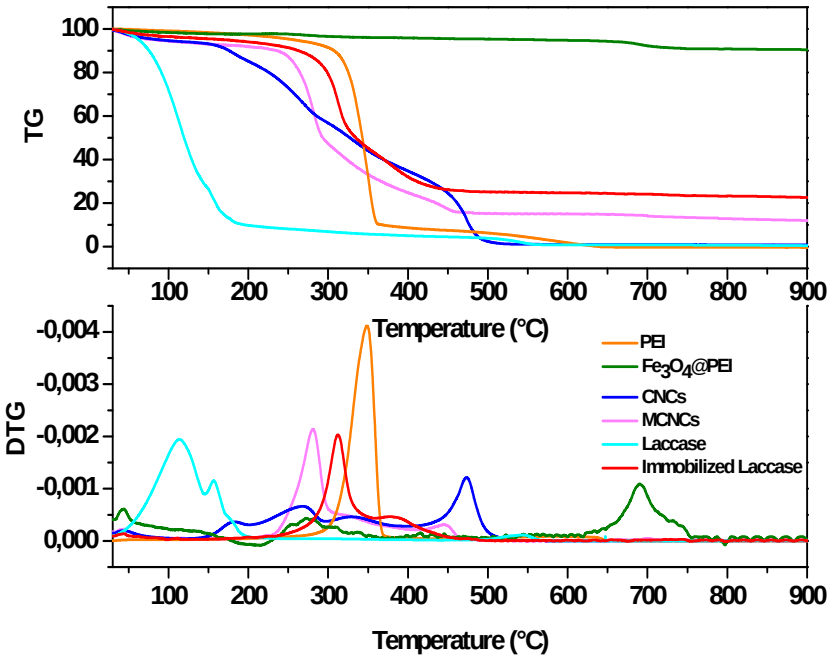

Figure 3. TG-DTG profiles of Fe_3O_4@PEI, PEI, CNCs, MCNCs , immobilized laccase, and Laccase (Sigma Aldrich (a);

TG-DTG profiles of PEI, Fe_3O_4@PEI, CNCs, MCNCs, immobilized laccase and laccase were shown in Figure 3. The Fe_3O_4@PEI TG curve shows small weight loss due to the decomposition of the PEI chains. The comparison between the TG-DTG profile of MCNCs and of CNCs, evidences the residual weight of 20 wt.% for MCNCs, probably due to magnetic nanoparticles' presence in the sample, while CNCs have shown complete decomposition at high temperatures. These results confirmed the complexation between the CNCs and Fe_3O_4@PEI NPs. Finally, the TG-DTG profiles of immobilized laccase demonstrated the presence of

laccase, proving the efficiency of the immobilization process. The subsequent FT-IR analysis further confirmed the conclusions. Figure 4 shows the FTIR spectra from 4000-500 cm^{-1} of Fe_3O_4@PEI, PEI, CNCs, MCNCs, immobilized laccase, and Laccase (Sigma Aldrich). The intense peak at 548 cm^{-1} in the spectrum of Fe_3O_4@PEI corresponds to the Fe-O vibration (Sarno and Iuliano, 2020). The light peaks visible at 2939 cm^{-1}, and 2821 cm^{-1} were due to the asymmetric and symmetric CH_2 stretching of the PEI chain. Moreover, the peaks around 1300 cm^{-1} and 1648 cm^{-1} are due to the -NH_2 scissoring vibration in PEI (Xu et al., 2009). The small peak at 1466 cm^{-1} can be attributed to the symmetric vibration of the carboxyl groups. The vibrational band at 1160 cm^{-1}, 1028 cm^{-1}, and the absorption peaks at about 672 cm^{-1} corresponds to C-O-C of CNCs. Furthermore, the signal at 553 cm^{-1} was a typical band of the β-glucosidic bond of sugar units. When the nanoparticles were added to the CNCs, in the spectra MCNCs, the peak at 553 cm^{-1} was hidden by the band of Fe-O vibration, which shifts at 559 cm^{-1}. In the spectra of MCNCs the bands at 1644 cm^{-1} shifted to 1640 cm^{-1} probability due to the hydrogen bond between CNCs and Fe_3O_4@PEI (Liu et al., 2012). After immobilization of the Laccase on MCNCs, the FT-IR spectrum in the region 1000-2000 cm^{-1} is dominated by the characteristic bands of the laccase (Karami et al., 2019).

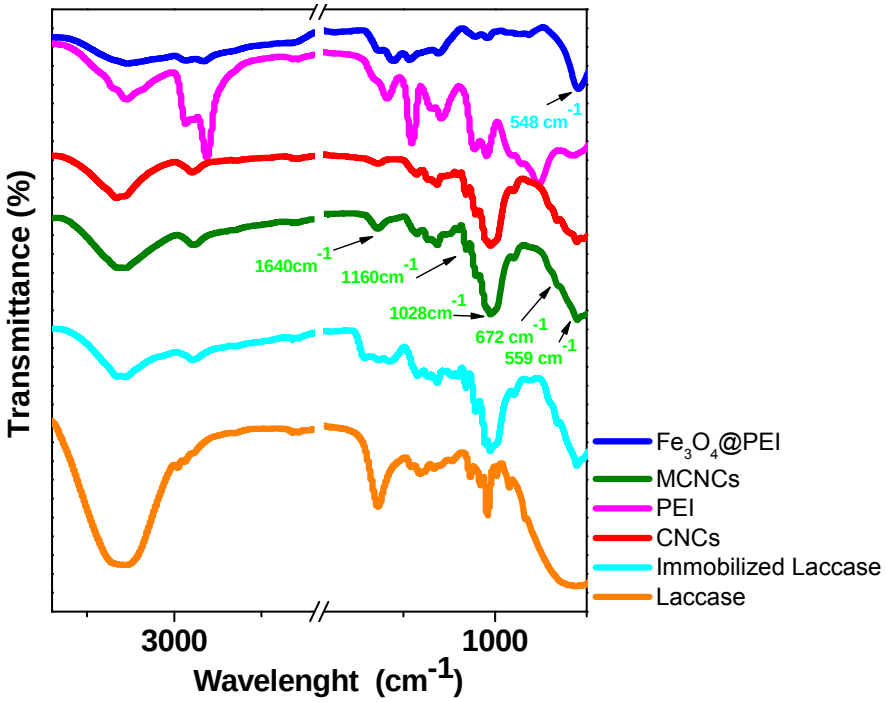

Figure 4. FT-IR spectra in the range of wavenumber 4000-500 cm^{-1} of Fe_3O_4@PEI, PEI, CNCs, MCNCs, immobilized laccase, and Laccase (Sigma Aldrich) (b).

3.2 Removal of aniline compounds by immobilized enzymes

The catalytic properties of immobilized Laccase on MCNCs were evaluated using aniline as reducing substrates.

Figure 5a shown enzymatic degradation. In the first 30 min, the degradation process of immobilized laccase was fast, achieving a degradation of up to 70%. The degradation of aniline in the presence of immobilized enzyme was as high as > 98% after 6 h. In particular, the enzymatic degradation by immobilized laccase could be attributed to the stability of immobilized laccases showing the rate of removal higher than that of free laccase during 8 h of reaction. The degradation of aniline by immobilized enzyme tended to be stable after 6 h maybe because of the decrease in pollutants compound and the increase in diffusion limiting effect due to produced polymers. These results show the excellent performance of MCNCs as support to improve enzyme activity and protect the enzyme towards the inactivation during the reaction.

The reusability of aniline degradation is shown in Figure 5b. The immobilized enzyme removal was 97% for the first two cycles. It decreases after three cycles probability due to the polymer's complexation, produced during enzyme degradation, with active sites of enzyme (Torres et al, 2003).

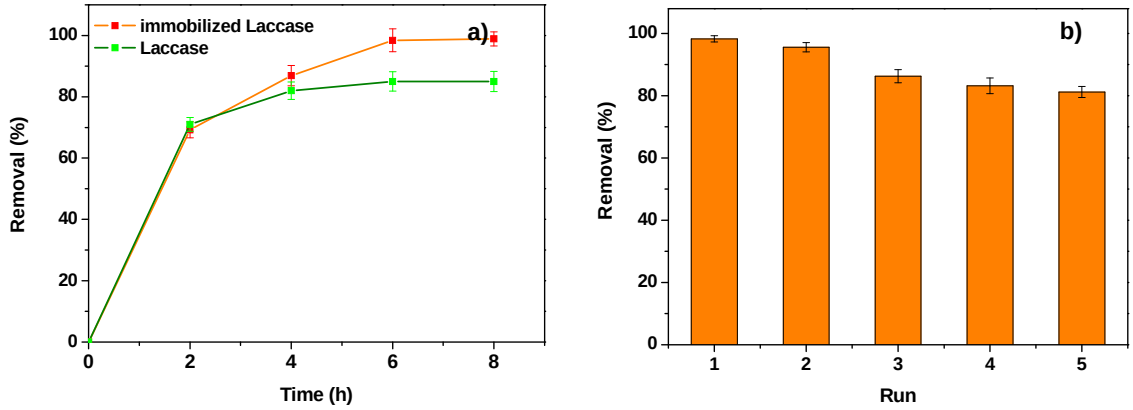

Figure 5: Effect of time on aniline degradation. Immobilization conditions: coupling temperature, 4 °C; coupling pH, 3; lipase concentration, 17 µg/ml; time, overnight. Degradation test conditions: reaction temperature, 25°C; catalyst concentration 0.25 mg/ml; reaction time 8 h (a). Effect of re-use of immobilized laccase. Degradation test conditions: reaction temperature, 25 °C; catalyst concentration 0.25 mg/ml; reaction time 8 h. Each point represents the mean of three experiments ± S.E.

4. Conclusions

Magnetic nanocellulose nanoparticles were used for the physical immobilization of laccase. The prepared MCNCs in the SEM image present a sheet structure with a rough surface due to Fe_3O_4@PEI NPs.The anchoring of laccase on MCNCs was confirmed by thermogravimetric analysis, FTIR spectra, and Bradford method. The immobilization capacity reached 85 % overnight. The immobilized laccase exhibited an excellent ability in the degradation of aniline in 6 h of reaction. Moreover, the immobilized laccase was easily collected by a magnet and retained its capacity in the next cycle. Hence, the immobilized laccase is a promising candidate for wastewater treatment.

References

Bradford M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Journal Analytical Biochemistry, 72, 248–254.

Cao S.L., Huang Y.M., Li X.H., Lou W.Y., Zong, M.H., 2016, Preparation and Characterization of Immobilized Lipase from Pseudomonas Cepacia onto Magnetic Cellulose Nanocrystals, Scientific Reports 6, 20420.

Kamble S.P., Sawant S.B., Schouten J.C., Pangarkar V.G., 2003, Photocatalytic and photochemical degradation of aniline using concentrated solar radiation. Journal of Chemical Technology and Biotechnology, 78, 865-872.

Karami C., Taher M.A., 2019, A catechol biosensor based on immobilizing laccase to Fe3O4@Au core-shell nanoparticles, International Journal of Biological Macromolecules, 129, 84-90.

Liu Z., Wang H., Li B., Liu C., Jiang Y., Yu G., Mu X., 2012, Biocompatible magnetic cellulose–chitosan hybrid gel microspheres reconstituted from ionic liquids for enzyme immobilization, Journal of Materials Chemistry A, 22, 15085-15091.

Torres E., Bustos-Jaimes I., Le Borgne S., 2003, Potential use of oxidative enzymes for the detoxification of organic pollutants, Applied Catalysis B: Environmental, 46, 1-15.

Sarno M., Iuliano M., 2020, G_Fe_3O_4/Ag supporting Candida rugosa lipase for the "green" synthesis of pomegranate seed oil derived liquid wax esters, Applied Surface Science, 510,145481.

Sarno M., Iuliano M., 2019, Immobilization of horseradish peroxidase on Fe_3O_4/Au_Go nanoparticles to remove 4-chlorophenols from wastewater, Chemical Engineering Transactions, 73, 217-222.

Yan H.X., Yang J., Chen C., Yin C., Xiao H., 2011, Chen Synergistic removal of aniline by carbon nanotubes and the enzymes of Delftia sp. XYJ6, Journal of Environmental Sciences-China23, 1165-1170

Wang L., Taylor K.E., Bewtra J.K., Biswas N., 2005, A simple colorimetric method for analysis of aqueous phenylenediamines and aniline. Journal of Environmental Engineering and Science, 4, 423-427.

Xu Z., Shen C., Hou Y., Gao H., Sun S., 2009, Oleylamine as Both Reducing Agent and Stabilizer in a Facile Synthesis of Magnetite Nanoparticles, Chemistry of Materials, 21, 1778-1780.

Zhou C., Shi Q., Guo W., Terrell L., Qureshi A.T., Hayes D.J., Wu Q., 2013, Electrospun Bio-Nanocomposite Scaffolds for Bone Tissue Engineering by Cellulose Nanocrystals Reinforcing Maleic Anhydride Grafted PLA, ACS Applied Materials & Interfaces, 5, 3847-3854.