Int. J. Bio-Inorg. Hybr. Nanomater., 7(1): 21-28, Spring 2018

The immobilization of laccase enzyme from Trametes versicolor on the surface of porous zinc oxide nanoparticles and studying features of the immobilized enzyme

Gh. Ghorbani[1], M. Ebrahimi[2*], N. Farhadyar[3]

[1] Department of Biotechnology, Faculty of Biological sciences, Varamin-Pishva Branch, Islamic Azad University, Varamin, Iran
[2] Department of Biochemistry and Biophysics, Faculty of Biological sciences, Varamin-Pishva Branch, Islamic Azad University, Varamin, Iran
[3] Department of Chemistry, Varamin-Pishva Branch, Islamic Azad University, Varamin, Iran

Received: 27 September 2017; Accepted: 29 November 2017

ABSTRACT: The laccase enzyme is the largest group of Oxidoreductase enzymes and is capable of oxidizing a wide range of organic substrates to water along with molecular oxygen resuscitation. ZnO nanoparticles are known for their specific properties such as chemical stability, high electrochemical coupling rates, and wide range of absorption of radiation as multifunctional compounds. In this study, ZnO porous nanoparticles were synthesized and then the laccase enzyme was stabilized from the source of vermicellum tramitis by surface absorption method on the surface of synthesized nanoparticles. In the following, kinetic parameters, temperature stability, reusability and sustainability of the stabilized enzyme were measured and compared with the free enzyme. Given the results obtained at all three temperatures (40, 50 and 60 °C), the stabilized enzyme shows more temperature stability than the free enzyme in desired time range. The kinetic parameters Vm and Km did not significantly change with respect to the free enzyme. Enzyme activity returned to zero after 10 cycles of use and recycling. While the free enzyme lost its activity after three weeks of maintenance in laboratory condition, the stabilized enzyme retained 30% of its initial activity. These results indicate that ZnO porous nanoparticles can be used as a suitable substrate for the stabilization of laccase enzyme by surface adsorption method, and improves the stability parameters of the enzyme without affecting the kinetic properties of the enzyme.

Keywords: *Immobilization; Laccase; Porous ZnO nanoparticles, Stability, Reusability; Tramitis versicolor*

INTRODUCTION

Laccases (benzendiol: oxygen oxidoreductase, EC 1.10.3.2) include the largest sub-group of copper-atomic aquatic oxidoreductases (MCO), which have the potential of recovering the copper ions to catalyze the oxidation of a wide range of aromatic substrates along with the resuscitation of molecular oxygen into water. Laccase was first identified in the Rhus vernicifera tree extract. Since then, Laccase has been found in different species of aschemic fungi, and thus fungal laccases have been identified as the most important MCOs group

(*) Corresponding Author - e-mail: ebrahimi@iauvaramin.ac.ir

(Gianfreda, *et al.*, 1999). Laccases are capable of catalyzing the direct oxidation of paradifenols, amino phenols, polyamines, aryldiamines and orthodiamines, as well as some organic ions. These enzymes coupled four electrons from resuscitation substrates to four electrons of oxygen bond breakdown (Giardina, *et al.*, 2010, Schlosser and Höfer, 2002). Laccase is one of the most important enzymes that have been studied in the field of phenolic compounds. Phenols such as 2,6-dimethoxyphenol, catechol, hydroquinone are suitable substrates for the Laccase enzyme in aqueous medium (Selinheimo, *et al.*, 2006). The enzyme has been widely used in various industrial and biotechnological fields, including the food industry (Couto and Herrera, 2006, Selinheimo, *et al.*, 2006), paper industry (Call and Mücke, 1997), fabric industry (Abadulla, *et al.*, 2000, Blánquez, *et al.*, 2004), nanobiotechnology (Ferry and Leech, 2005, Jarosz-Wilkołazka, *et al.*, 2004, Leite, *et al.*, 2003), and bioremediation (Gianfreda, *et al.*, 1999). The use of immobilization techniques is one of the most widely used and effective methods for reusing enzymes, facilitating the separation from process medium and improving the stability (Lee, *et al.*, 2011). The ability to easily separate the enzyme from the reaction mixture minimizes the protein contamination of products (Xu, 1996). Therefore, the reliability and efficiency of the desired reaction is improved by this technology. Also, the ability to reuse enzymes has economic benefits, which is a very important factor in discussing the costs associated with enzymes-catalyzed processes. In addition to easy separation and reusability, enzyme immobilization also has other benefits: increased storage stability, increased stability to unnatural heat, increased stability to organic solvents, and other severe and intolerable functional conditions (Kim, *et al.*, 2006). these special benefits of stabilized enzymes have led to their use in various industries (Sakurai, 1992). This has led to the development of various enzyme immobilization techniques. In these techniques, various supports have been used in conjunction with various methods for binding the enzyme to the support (Durán, *et al.*, 2002). One of the areas that have been considered in recent years is nano-sized compounds or nanoparticles. Zinc oxide nanostructures exhibit properties like semiconducting, piezoelectric, and piroelectric. These unique properties make zinc oxide particles one of the richest nanostructured materials. Under the special conditions, solid nanoparticles, nano-rings, nano-springs, nano-sheets, nanowires and nanoparticles of zinc oxide can be generated by steam-solid thermal sublimation method. Due to biocompatibility, these nanostructures can have new applications in biosensors and biotechnology and medical science (Kolodziejczak-Radzimska and Jesionowski, 2014). In the meantime, the use of physical binding methods is very important due to the much lower degradation effects on enzymes (Quiquampoix, *et al.*, 2002, Zimmerman and Ahn, 2010). In this study, porous ZnO nanoparticles were used as support for the stabilization of the laccase enzyme from the Tramitis versicolor, aims at improving thermal stability and reusability of this enzyme.

MATERIAL AND METHODS

Synthesis of porous zinc oxide nanoparticles

4 milliliters (2 M) of NaOH were mixed with 4 milliliters of (0.02 M) CTAB and then maintained at room temperature for 30 minutes. 6.3 grams of $ZnCl_2$ was dissolved in 50 milliliters of distilled water and the CTAB solution was added to it simultaneously with stirring. After an hour of stirring, the resultant precipitate was collected using a filter and washed with distilled water and ethanol. After collecting with filter, the precipitate was incubated at 100°C for 24 hours. The dried sample was then placed in a furnace at 600°C for one hour. Finally, the synthesized nanoparticle was evaluated using FTIR, XRD, SEM, and BET methods.

Enzyme immobilization on nanoparticle surface and laccase enzyme assay

In this study, the adsorption method was used to immobilization of the laccase enzyme on the surface of synthesized nanoparticles. In summary, 4 milligrams of the synthesized nanoparticle were mixed in 900 milliliters of McIlvane buffer (pH 6.5) and then homogenized using a sonication for 2 minutes. 100 µl of laccase enzyme solution (10 mg/ml) was added to the nanoparticle mixture and then was incubated for 4 h using a shaker incubator for 24 hours. Subsequently,

the mixture was centrifuged at 1200 rpm for 5 minutes. Then the supernatant was removed and the sediment was washed 3 times by adding 1 milliliter of McIlvane buffer. Finally, the remaining residue was mixed in 1 milliliter buffer and then homogenized for subsequent experiments. The optimized pH for immobilization of the enzyme was evaluated by performing the immobilization process using buffer pH 3, 4, 5, 6, and 7.

Measuring the Laccase activity

Laccase enzyme activity was measured using ABTS substrate and based on spectroscopic method. The measurement solution contains 50 µl of free enzyme (mg/ml) or immobilized enzyme (4 mg/ml), 950 µl of ABTS solution (0.4 mM) and 1 µl of McIlvane buffer (pH 6.5). Absorbance was measured over a period of 5 minutes from the onset of the reaction to a blank solution (enzyme-free solution) at 420 nm. Enzyme activity (U) was defined as the amount of enzyme required to oxidize 1 micromole ABTS per minute (Sheikhi, *et al.*, 2012, Tavares, *et al.*, 2012, Zhu, *et al.*, 2011).

Determination of kinetic parameters of free and immobilized laccase

To determine the kinetic parameters, the activity of laccase enzyme (free and immobilized) was measured at concentrations of 0.4, 0.2, 0.1, 0.05, 0.225 and 0.125 mM from ABTS substrate. By drawing the Line weaver-Burk chart and extrapolation of obtained line, the values of the parameters Vm and Km were determined.

Determination of the enzyme thermal stability

In order to determine the stability of the laccase enzyme, the enzyme solution (free and stabilized) was incubated at 30, 40 and 50°C, and 50 µl of enzymatic solution was removed at 5 minutes intervals and placed on ice for 2 minutes. The enzyme remaining activity was then measured in the desired solution.

Reusability and stability of the immobilized enzyme

The immobilized laccase enzyme was centrifuged after measuring at 1200 rpm. Then, like the enzyme immobilization, washing steps were performed and the enzymatic activity was measured again. These steps were repeated until the enzyme activity was reached to zero. Storage stability was performed by incubation of an enzyme (free and stabilized) solution at room temperature and measuring the activity of the enzyme at weekly intervals.

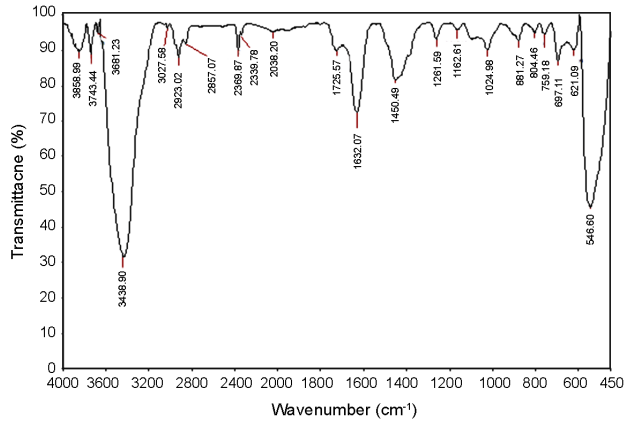
RESULTS AND DISCUSSION

FT-IR spectrum

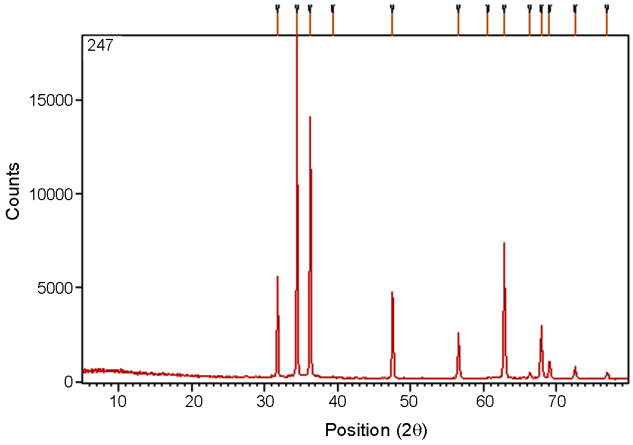
In the FT-IR spectrum of synthesized nanoparticles (Fig. 1-A), the wave number 3439 cm^{-1} corresponds to hydroxyl groups of water molecules coupled with the surface of nanoparticle by hydrogen bonding. The 415 cm^{-1} wave number refers to oxygen-zinc vibration bonds that indicate the bond is synthesized in the nanoparticle. The two spectra contained in 2923 and 2857 cm^{-1} are related to oxygen-to-carbon bonds, and peaks of 1453, 1271, and 1018 cm^{-1} are related to the same bond. The strong peaks at 436 and 546 cm are related to the Zn-O strain vibrations. The Strained Spectra in the graph (Fig. 1-B) shows a good crystallization of the synthesized nanoparticle, the crystalline phase of these nanoparticles is hexagonal and there is no severe impurity in the spectrum. From the spectrum obtained, the angle of the peaks (2Th) is 2.3 degrees and the size of the crystals is about 54.3. The image of SEM microscope (Fig. 1-C) shows a good distribution of zinc oxide particle size; these particles are spherical. The image along with the XRD spectrum shows that ZnO is crystallized.

The effect of pH on the immobilization of laccase

The pH variation plays an important role in changing the ionization state of ionized groups in proteins. These changes can have a significant effect on the interaction of protein with other molecules. For this reason, one of the factors involved in process of stabilizing proteins on various levels, including nanoparticles, is pH variation. In this regard, the effect of pH variation in the range of 3 to 7 on the stabilization of enzyme laccase on nanoparticles surface was investigated (Fig. 2). Based on the results, the highest enzyme activity obtained at pH= 6. However, within the pH range, the range of variation between the highest and the lowest recorded activity (respectively, pH 6 and 3) is approximately 40%. In this way, the opti-



(a)



(b)

(c)

Fig. 1 a) FT-IR spectrum of ZnO nanoparticles, b) XRD spectrum of ZnO nanoparticles, c) SEM images of ZnO nanoparticles.

mal pH for the immobilization process was considered as 6. In a study that reported on the stabilization of the laccase enzyme from *T. hirsute* and *M. albomyces* on the lignin surface by Saarinen, pH was introduced as a very influential factor in stabilization process.

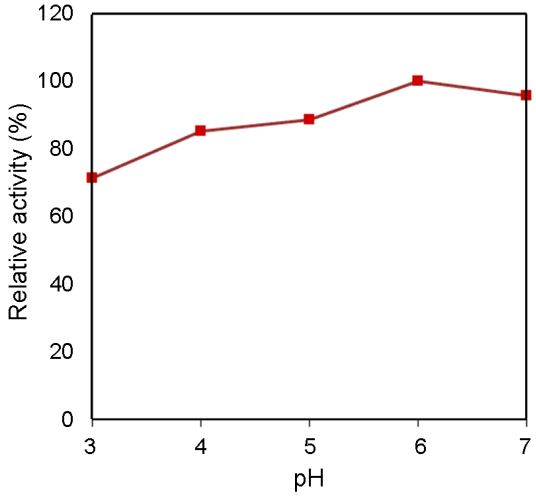


Fig. 2. Investigation of the effect of pH on the process of immobilization of the laccase enzyme on porous zinc oxide nanoparticles.

The pH suitable for stabilization in this study was 4.5 (Saarinen, *et al.*, 2008). In another study, pH 5 was reported as suitable conditions for the stabilization of the laccase enzyme from T. Versicolor on the surface of SiO2 nanowires (Patel, *et al.*, 2014). In the process of covalent stabilization, the laccase enzyme was activated from P. conchatus on the surface of poly-vinyl alcohol substrate, and the best pH was reported to be 4 (Yinghui, *et al.*, 2002). Therefore, the pH suitable for the stabilization process will be different depending on the used enzyme and type of substrate.

Comparison of kinetic parameters

The kinetic parameters, temperature stability and reusability are the most important features of a stabilized

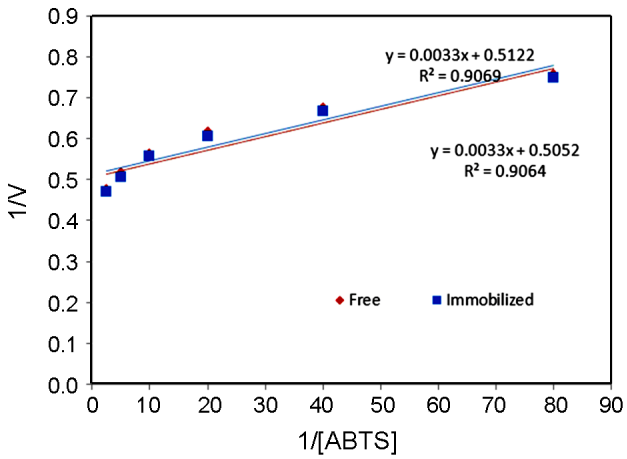


Fig. 3. Lineweaver-Burk diagram to determine the parameters of Km and Vm of free and immobilized lactate enzymes.

Table 1. Values of the parameters Vm and Km for free and stabilized laccase enzyme.

	Vm (U)	Km (mM)
Free Enzyme	1.961	0.006
Immobilized	2.000	0.007

enzyme. The rate of variation in kinetic parameters depends on the type of enzyme, type of substrate and the conditions of stabilization (Patel, *et al.*, 2014). The Lineweave-Burk chart is plotted in different concentration of ABTS substrate by measuring the enzyme activity (free and immobilized), and the line drawn from the points obtained with R2 is more than 90 (Fig. 3). Using the extrapolation of the obtained lines, the Vm value for free and immobilized enzymes was 1.9 and 2, respectively (Table 1). On the other hand, the Km value for free and stabilized enzymes was also determined to be 64×10^{-4} and 66×10^{-4} (mM), respectively. Thus, there is no significant difference between Km and Vm parameters in both free and stabilized enzymes.

One of the disadvantages of the immobilized process is the change in the parameters Vm and Km, so that the enzyme immobilization usually reduces Vm and increases Km. Under these conditions, the immobilized enzyme activity of free enzyme is reduced. In a study by Ahn, there was no change in kinetic parameters of Vm and Km in the laccase enzyme from *Trametes villosa* after stabilization on the aluminum hydroxide level. The change in enzyme contention and spatial suppression of the substrate to couple with the active site of the enzyme are among the most important factors in reducing activity and also increasing the Km enzyme. Failure to change these parameters could be a reason for the occupation of the enzyme active site after stabilization, and thus the efficiency of the method used to stabilize the laccase enzyme (Ahn, *et al.*, 2007). Given the fact that there is no significant change in the laccase enzyme immobilized on porous zinc oxide nanoparticles, the use of these nanoparticles and the method used in this study can be considered as an advantage.

Thermal stability of free and immobilized laccase

The residual activity of enzyme was used as an indicator for studying the temperature stability of enzyme laccase at the studied temperatures. The thermal stability of immobilized enzyme on the surface of nanoparticles was performed by measuring the remaining activity of enzyme (free and stabilized) at 40, 50 and

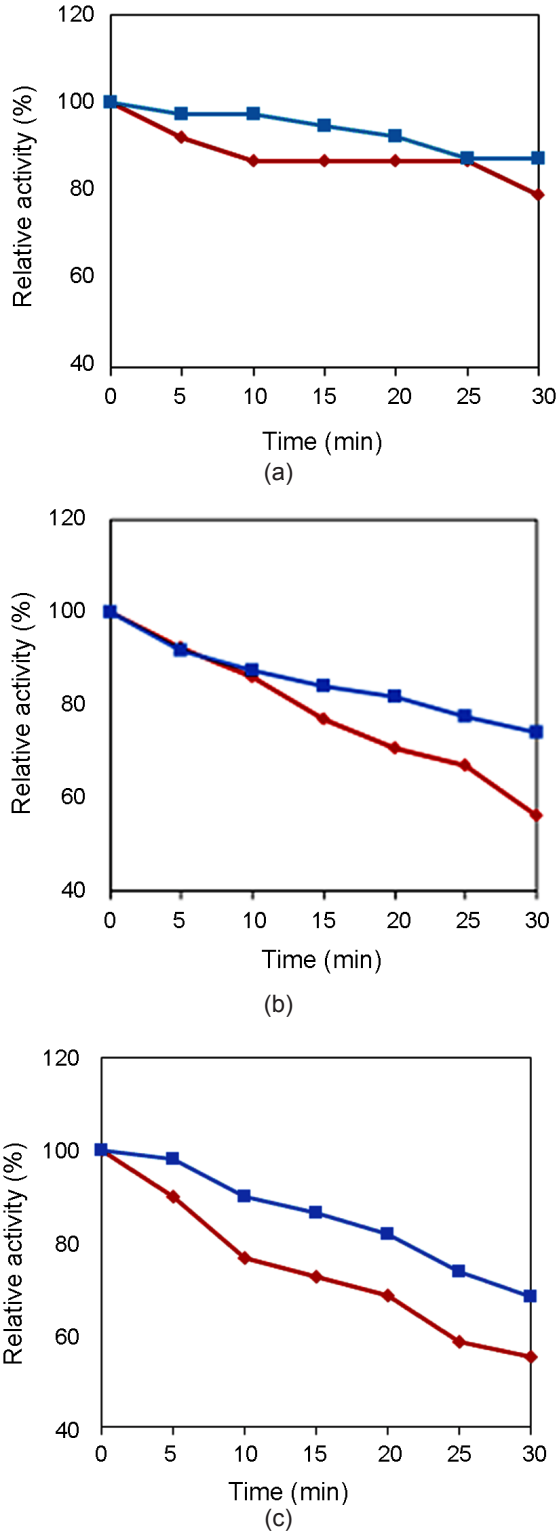


Fig. 4. Time vs. remaining activity chart to verify the temperature stability of free and immobilized lactate enzymes at 40 (a), 50 (b), and 60 (c) Celsius.

Table 2. Comparison of temperature stability parameters of free and immobilized laccase enzyme.

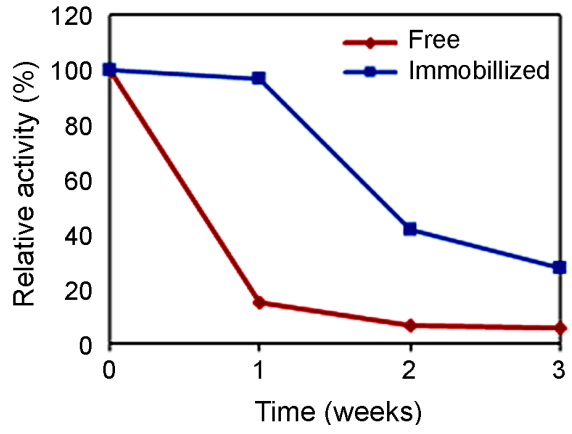
	30		40		50	
	Free	Immobilized	Free	Immobilized	Free	Immobilized
Kd	0.47	0.27	1.24	0.58	1.39	0.9
t 1/2	1.47	2.53	0.56	1.2	0.5	0.77

60°C. According to the results (Fig. 4), at 40°C after 30 minutes, the free enzyme activity remains about 78% of the initial activity, while the stabilized enzyme has maintained 87% of its initial activity. As the temperature increased to 50°C, the chart gradient, and namely the rate of deactivation of the enzyme in both free and immobilized conditions increased to 40°C, so that at the same time (30 minutes), the free enzyme activity decreased to 56% and the immobilized enzyme activity to 74%. At 60°C, the activity of the remaining free and immobilized enzyme decreased to 55% and 68%, respectively. The comparison between the temperatures indicated that with increasing temperature, the remaining activity of the enzyme in both free and immobilized states decreased. But in all three temperatures in free state, the enzyme activity decreased compared with the stabilized enzyme. Based on this, the immobilized laccase enzyme exhibited a significant thermal stability compared to the free enzyme at test temperatures. In other words, the use of nanoparticles will improve the temperature stability of the enzyme.

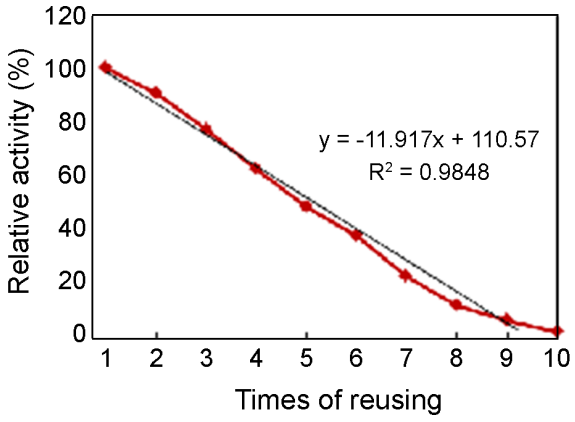
The rate of deactivation and the half-life of the enzyme are compared in Table 2. According to this table, the rate of enzyme deactivation in both free and immobilized conditions increases with increased temperature. However, at each temperature, the deactivation rate of immobilized enzyme is lower than that of the free enzyme. The immobilized enzyme thus has higher temperature stability and loose its activity less than free enzyme. In addition, the higher t1/2 is another reason for the stability of stabilized enzyme in comparison with the free enzyme. In the study on immobilization of laccase enzyme on SiO_2 nanoparticles, an 18-fold increase in half-life of the enzyme was reported after its stabilization (Yinghui, *et al.*, 2002).

Reusability and storage stability of immobilized laccase

Investigating the variation of immobilized enzyme activity after successive cycles of use, recycling and re-use of the enzyme indicates that enzyme activity decreases after each use (Fig. 5). The analysis of the obtained line shows that the reduction of enzyme activity is stabilized with a gradient of about 12%. In other words, after using the stabilized enzyme, its activity decreases by 12%, which will eventually be deactivated after 8 times. Investigating the storage stability of enzyme indicates that free enzyme activity is reduced by about 85% after a maintenance week at the laboratory temperature, until it actually reaches 5% of the initial activity in the third week. While in the immobilized enzyme in the first week, there is no



(a)



(b)

Fig. 5. Assessment of the stability of immobilized laccase enzyme (A) and re-usability (B).

significant change in enzyme activity. However, in the second and third weeks, 60% and 20% of the initial activity decreased, respectively. Although the activity of the enzyme has decreased in the third week, it is still significantly higher than the free enzyme.

CONCLUSIONS

According to the results, stabilization of the laccase enzyme using adsorption method on ZnO porous nanoparticles can be used as a suitable method for reusing and improving the temperature stability and maintenance of the enzyme without affecting the kinetic parameters.

REFERENCES

Abadulla, E., Tzanov, T., Costa, S., Robra, K. H., Cavaco-Paulo, A. & Gubitz, G. M. (2000). Decolorization and detoxification of textile dyes with a laccase from Trametes hirsuta. Appl. environ. microb., 66: 3357-3362.

Ahn, M. Y., Zimmerman, A.R, Martínez, C. E., Archibald, D. D., Bollag, J. M. & Dec, J. (2007). Characteristics of *Trametes villosa* laccase adsorbed on aluminum hydroxide. Enzyme and Microbial. Technol., 41: 141-148.

Blanquez, P., Casas, N., Font, X., Gabarrell, M., Sarra, M., Caminal, G. & Vicent, T. (2004). Mechanism of textile metal dye biotransformation by Trametes versicolor. Water Res., 38: 2166-2172.

Call, H. & Mucke, I. (1997). History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems. J. Biotechnol., 53: 163-202.

Couto, S. R. & Herrera, J. L. T. (2006). Industrial and biotechnological applications of laccases: a review. Biotechnol. Adv., 24: 500-513.

Duran, N., Rosa, M. A., D'Annibale, A. & Gianfreda, L. (2002). Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: A review. Enzyme Microb. Technol., 31: 907-931.

Ferry, Y. & Leech, D. (2005). Amperometric detection of catecholamine neurotransmitters using electrocatalytic substrate recycling at a laccase electrode. Electroanalysis, 17: 113-119.

Gianfreda, L., Xu, F. & Bollag, J.-M. (1999). Laccases: A useful group of oxidoreductive enzymes. Bioremediat J., 3: 1-26.

Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S. & Sannia. G. (2010). Laccases: a never-ending story. Cell. Mol. Life Sci., 67: 369-385.

Jarosz-Wilkolazka, A., Ruzgas, T. & Gorton, L. (2004). Use of laccase-modified electrode for amperometric detection of plant flavonoids. Enzyme Microb. Technol., 35: 238-241.

Kim, J., Grate, J. W. & Wang, P. (2006). Nanostructures for enzyme stabilization. Chem. Eng. Sci., 61: 1017-1026.

Kolodziejczak-Radzimska, A. & Jesionowski, T. (2014). Zinc Oxide-From Synthesis to Application: A Review. Materials (Basel), 7: 2833-2881.

Lee, S.Y., Lee, J., Chang, J.H. & Lee, J.H. (2011). Inorganic nanomaterial-based biocatalysts. BMB Rep, 44: 77-86.

Leite, O.D., Lupetti, K.O., Fatibello-Filho, O., Vieira, I.C. & de Barbosa, A.M. (2003). Synergic effect studies of the bi-enzymatic system laccase–peroxidase in a voltammetric biosensor for catecholamines. Talanta, 59: 889-896.

Patel, S. K. S., Kalia, V. C., Choi, J. H., Haw, J. R., Kim, I. W. & Lee, J. K. (2014). Immobilization of Laccase on SiO_2 Nanocarriers Improves Its Stability and Reusability. J. Microbiol. Biotechnol., 24: 639-647.

Quiquampoix, H., Servagent-Noinville, S., Baron, & M.-H. (2002). Enzyme adsorption on soil mineral surfaces and consequences for the catalytic activity. Enzymes in the environment. Marcel Dekker, New York, 285-306.

Saarinen, T., Orelma, H., Gronqvist, S., Andberg, M., Holappa, S. & Laine, J. (2009). Adsorption of different laccases on cellulose and lignin surfaces. Bioresource, 4 (1): 94-110.

Sakurai, T. (1992). Anaerobic reactions of Rhus vernicifera laccase and its type-2 Copper-depleted derivatives with hexacyanoferrate (II). Biochem. J., 284: 681.

Schlosser, D. & Hofer, C. (2002). Laccase-catalyzed

oxidation of Mn^{2+} in the presence of natural Mn^{3+} chelators as a novel source of extracellular H_2O_2 production and its impact on manganese peroxidase. Appl. Environ. Microbiol., 68: 3514-3521.

Selinheimo, E., Kruus, K., Buchert, J., Hopia, A., & Autio, K. (2006). Effects of laccase, xylanase and their combination on the rheological properties of wheat doughs. J. Cereal Sci., 43: 152-159.

Sheikhi, F., Ardakani, M. R., Enayatizamir, N., & Rodriguez-Couto, S. (2012). The Determination of Assay for Laccase of Bacillus subtilis WPI with Two Classes of Chemical Compounds as Substrates. Indian J. Microbiol, 52: 701-707.

Tavares, A. P. M., Pereira, J. A. N. & Xavier, A. M. R. B. (2012). Effect of ionic liquids activation on laccase from Trametes versicolor: Enzymatic stability and activity. Eng. Life Sci., 12: 648-655.

Xu, F. (1996). Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. Biochem., 35: 7608-7614.

Yinghui, D., Qiuling, W., Shiyu, F. (2002). Laccase stabilization by covalent binding immobilization on activated polyvinyl alcohol carrier. Lett. Appl. Microbiol., 35: 451-456.

Zhu, Y., Zhang, H., Cao, M., Wei, Z., Huang, F. & Gao, P. (2011). Production of a thermostable metal-tolerant laccase from Trametes versicolor and its application in dye decolorization. Biotechnol. Bioprocess Eng., 16: 1027-1035.

Zimmerma, A. R. & Ahn, M.-Y. (2010). Organo-mineral–enzyme interaction and soil enzyme activity. Soil Enzymology. Springer.

AUTHOR (S) BIOSKETCHES

Ghasem Ghorbani, M.Sc., Department of Chemistry, Varamin-Pishva Branch, Islamic Azad University, Varamin, Iran, *Email: ghasem71.ghorbani@gmail.com*

Mehdi Ebrahimi, Assistant Professor, Department of Biochemistry and Biophysics, Faculty of Biological sciences, Varamin-Pishva Branch, Islamic Azad University, Varamin, Iran, *Email: ebrahimi@iauvaramin.ac.ir*

Nazanin Farhadyar, Associate Professor, Department of Chemistry, Varamin-Pishva Branch, Islamic Azad University, Varamin, Iran