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IMMOBILIZATION OF CRUDE FUNGAL LACCASE FROM *GANODERMA SPP.* ON MODIFIED TITANIUM DIOXIDE NANOPARTICLES

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Abstract

In recent decades, enzyme immobilization on different supports occupies an important place in the modern biotechnology, given that it allows the design of green and sustainable production processes. Nanoparticles are very efficient supports for enzyme immobilization. The enzyme immobilized on nanoparticles is more stable than its soluble form and can be used in several operative cycles. In this study, the potential of modified titanium dioxide nanoparticles (Degussa P25, TiO_2) for crude fungal laccase (47.43 U/g) immobilization was investigated. The TiO_2 nanoparticles were modified with (3-glycidyloxypropyl) trimetoxylane under different conditions in order to obtain support with an optimal concentration of epoxy groups for immobilization. The obtained TiO_2 nanoparticles had different concentrations of epoxy groups on their surface, exactly 950 µmol/g (TiO_2M1) and 500 µmol/g (TiO_2M2), respectively. The immobilization was carried out at pH 5 and at room temperature for 4 h. The residual activity of immobilized laccase on TiO_2M2 was 33.40 %, while the residual activity of immobilized laccase on TiO_2M1 was 17.39 %. The immobilization efficiency was 26.75 % and 20.18 % for laccase immobilized on TiO_2M2 and immobilized laccase on TiO_2M1, respectively. The immobilization of crude fungal laccase on TiO_2M2 was further optimized by testing the influence of different contact time between laccase and TiO_2M2 (2 h ,3 h, 3,5 h and 4 h) as well as the effect of various pH values (pH 4, pH 5 and pH 6) on immobilization. The highest residual activity of 35.04 % and immobilization efficiency of 28.95 % were obtained for optimal contact time of 3.5 h between crude fungal laccase and TiO_2M2. The lowest residual activity (30.38 %) and immobilization efficiency (20.18 %) were obtained when immobilization was carried out at room temperature and at pH 5 for 2 h. The pH values had significant effect on immobilization. The optimal pH value was pH 5 with highest residual activity of 35.04 %, while the lowest residual activity of 14. 83 % was at pH 6. The residual activity of 21.21 % and immobilization efficency of 19.52 % were obtained when crude fungal laccase immobilized at pH 4. The immobilized laccase on TiO_2M2 was successfully used in 5 cycles of guaiacol oxidation. In the 2^{nd} cycle, the immobilized laccase had residual activity of about 60%, while after 3^{rd} cycle, immobilized laccase had residual activity of 40 %. The lowest residual activity of 14.83 %, immobilized laccase had after 5^{th} cycle.

Key words: *laccase, immobilization, white rot fungi, titanium dioxide nanoparticles*

Introduction

Laccases (E.C. 1.10.3.2., p-benzenediol: oxygen oxidoreductases) are cuproproteins. Laccases, like other polyphenoloxidases, have four copper atoms in their structure, which play an important role in the enzyme's catalytic activity, and are therefore they are also known as multi-copper oxidases, or blue oxidases (Brijwani, Rigdon, and Vadlani 2010). They can oxidize a wide range of aromatic and non-aromatic compounds, primarily phenols (ortho- and para-diphenols, phenolic acids), aromatic and aliphatic amines, benzenethiols, and inorganic compounds while simultaneously reducing oxygen to water (Cardullo, Muccilli, and Tringali 2022).

Laccases can be isolated from bacteria, fungi, lichens, plants, and some insects (Brijwani, Rigdon, and Vadlani 2010). Fungi, particularly white rot fungi with a highly developed ligninolytic system, are without a doubt the most important laccase producers. White rot fungi produce laccases that play an important role in lignin degradation, which in turn induces laccase production in this group of fungi (Gauthier, Cooper, and Yargeau 2008; Yang et al. 2017). Fungal laccases have been widely used in a variety of industrial and biotechnological processes in recent decades due to their low substrate specificity, environmental sustainability, and catalytic activity (Agrawal, Chaturvedi, and Verma 2018). They are used in the food, textile, wood, pharmaceutical, and chemical industries, as well as bioremediation, biodegradation, and biosensor production (Agrawal, Chaturvedi, and Verma 2018).

Although laccase has good catalytic oxidation ability, free laccase is extremely sensitive to environmental conditions, implying that the stability of free laccase is low under natural conditions (Z. Wang et al. 2021). This disadvantage restricts the use of free laccase in industrial processes that require high enzyme stability, longer duration, reusability, and and low cost biocatalyst (Zhou, Zhang, and Cai 2021). Laccase immobilization on various supports has been performed in recent decades to overcome these shortcomings (Li et al. 2018).

To date, various laccase immobilization methods and supports have been used with varying degrees of success. Because of their high surface-to-volume ratio, metal oxide nanoparticles are a promising immobilization support (Villalba-Rodríguez et al. 2022). They can also be made porous and in a variety of sizes and shapes. The small size of these supports, in general, improves the efficiency of the immobilized enzyme (Villalba-Rodríguez et al. 2022).

TiO_2 nanoparticles are one of the most ideal supports for laccase immobilization due to their high mechanical strength, physical and chemical stability, ability to establish interactions with amino and carboxyl enzyme groups, and biocompatibility (Hou et al. 2014). Furthermore, these nanoparticles are low in cost and toxicity, making them both economically and environmentally acceptable (Hou et al. 2014).

The aim of this study was for the first time to investigate the potential of TiO_2 nnoparticles modified in two ways with (3-glycidyloxypropyl)trimethoxysilane in immobilization of crude fungal laccase from white rot fungi *Ganoderma spp*. Additionally, the optimal conditions for laccase immobilization on TiO_2 nanoparticles with good performance, as well as the reusability of successfully immobilized laccase in guaiacol oxidation, were investigated.

Materials and methods

Microorganism

Fungus *Ganoderma spp.* was used for laccase production. *Ganoderma spp* was from the culture collection from the Department of Biochemical Engineering and Biotechnology of the Faculty of Technology and Metallurgy, Belgrade, Serbia.

Inoculum preparation

To create the inoculum, mycelium was grown on malt extract agar plates. Malt extract agar was sterilized before being poured into Petri dishes for inoculum preparation. It contained malt extract broth (20 g/L) and agar (15 g/L).

Laccase production

The cereal mix was used as a nutrient medium for fungal mycelium cultivation and laccase production. 50 g of waste substrate (dry matter content of 71.96%) were measured in 300 mL Erlenmeyer flasks. After autoclaving (121 ºC, 30 minutes), the sterilized substrate was used for fungal mycelium cultivation. Five (1x1 cm) squares of malt agar containing fungal mycelium were transferred to an Erlenmeyer flask with waste substrate and incubated at 30 ºC for 6 days. Laccase extraction was carried out in 0.1M Na-acetate buffer pH5.0. After that, the samples were shaken on an orbital shaker (170rpm, 25 ºC, 50 min). The samples were centrifuged at 6000 rpm after shaking. Laccase activity was determined in the resulting supernatant by spectrophotometric method at 470 nm, using method of Monssef and coworkers (Abd El Monssef, Hassan, and Ramadan 2016).

Laccase activity [U/mL] was calculated according to the equation:

$$E.A.[U/mL] = (A_{470} \times V_t)/(t \times V_e \times \varepsilon)$$ where is

E.A. laccase activity [U/mL], A_{470} absorbance at 470 nm, Vt total volume of reaction mixture (mL), t incubation time, ε extinction coefficient of guaiacol (0,6740 μM / cm), V_e laccase volume (mL).

Laccase activity [U/g] was calculated according to the equation:

$$E.A.[U/g] = E.A.[U/mL] \times V \div m_s,$$

E.A. laccase activity [U/g], E.A. laccase activity [U/mL], V total volume of buffer (mL), m_s mass of cereal mix (g).

Modification of TiO_2 nanoparticles

The porous titanium dioxide nanoparticles (TiO_2, Degussa P25) were modified in two ways using (3-glycidyloxypropyl)trimethoxysilane (GOPTMS) (Miljković, 2020).

***First type of modification*:** In a 50 mL flask, 300 mg TiO_2 nanoparticles, 1.2 mL GOPTMS, 36 mL of anhydrous toluene and 50 L of triethylamine were combined. The reaction mixture was incubated in an ultrasonic bath for 5 min and then modification was continued in presence of nitrogen on a magnetic stirrer (25 ºC, 600 rpm, 1h). The modified TiO_2/GOPTMS nanoparticles (TiO_2M1) were filtered, washed three times with 5 mL of toluene, and subjected to ultrasound for 5 minutes to remove unreacted GOPTMS. The prepared carrier was placed in a vacuum oven at 40 °C for 24 hours.

Second type of modification: In a 50 mL flask, 300 mg TiO_2 nanoparticles and 1.2 mL GOPTMS were combined. Then 36 mL of anhydrous toluene and 50 L of triethylamine were added, and nitrogen was continuously supplied throughout the modification reaction, which was performed on a magnetic stirrer (25 ºC, 600 rpm, 1h). Every 15 minutes, the balloon containing the reaction mixture was removed from the apparatus and placed in an ultrasonic bath for 5 minutes. The subsequent procedure was carried out in accordance with the previously described protocol. The obtained TiO_2M2 nanoparticles were used in further experiments.

Immobilization of laccase

On a roller shaker (25 ºC) in Na-acetate buffer pH 5, crude laccase from *Ganoderma spp*. (1mL) was immobilized for 4 hours on previously modified nanoparticles (20 mg). The supernatant was discarded after immobilization, the immobilizer was washed 3 times in Na-acetate buffer pH 5 (1 mL) and the activity of the immobilized laccase was determined by spectrophotometric method at 470 nm (Abd El Monssef, Hassan, and Ramadan 2016). Further optimization of laccase immobilization on modified TiO_2M2 nanoparticles was carried out based on the obtained activity of the immobilizer.

The contact time (2 h, 3 h, 3.5 h, 4 h) between the free laccase from *Ganoderma spp*. and the carrier (TiO_2M2) on a roller shaker was first optimized at the previously mentioned conditions, and then the influence of different pH (pH 4, pH 5, pH 6) on the immobilization of crude laccase on TiO_2M2 nanoparticles was investigated.

Activity of immobilized laccase was calculated according to the equation:

$$E.A._i\,[U/g] = (A_{470} \times V_t)/(t \times m_i \times \varepsilon),$$

where is E.A. immobilized laccase activity [U/g], A_{470} absorbance at 470 nm, V_t total volume of reaction mixture (mL), t incubation time, ε extinction coefficient of guaiacol (0,6740 µM / cm), m_i mass of immobilizer (g).

The residual activity was calculated according to the equation:

$$A(\%) = \frac{Activityofimmobilizedlaccase}{Activityoffreelaccase} \times 100$$

Immobilization efficiency was calculated to the equation:

$$E.I.[\%] = \frac{(C_i - C_s)}{C_i} \times 100,$$

where is E.I. immobilization efficiency [%], C_i protein concentration of free laccase [mg/mL], C_s protein concentration in supernatant [mg/mL].

Determination of immobilized laccase reusability

To determine the reusability of the *Ganoderma spp* immobilized laccase onto TiO_2M2 nanoparticles, it was used in the oxidation of guaiacol. The immobilizer was washed three times in 1 mL Na-acetate buffer (pH 5) after the first cycle of guaiacol oxidation and reused in the next cycle.

Results and discussion

***Ganoderma spp*. laccase production**

Crude laccase extract from *Ganoderma spp*. was produced by solid state fermentation (SSF) on agroindustrial lignocellulosic waste, cereal mix. Cereals are the most common and least expensive source of lignocellulosic agricultural waste used in the production of ligninolytic enzymes (F. Wang et al. 2019). Laccase-producing fungi, such as *Ganoderma* species, are able to degrade lignin in lignocellulosic biomass quickly and effectively because they release a powerful extracellular enzyme system that degrades lignin (F. Wang et al. 2019). Previous

researches have focused on the use of various types of lignocellulosic material, such as rice straw and husks, sunflower seed husks, and wheat bran, in the process of laccase production by *Ganoderma spp.* (Rodrigues et al. 2019).

After 6 days of fermentation, laccase produced by *Ganodema spp*. on cereal mix had activity of 47.43 U/g (31.62 U/mL). When using tamarind shell as a substrate for production, Manavalan et al. (2013) found low activity of *G. lucidum* laccase. After 15 days, the activity of laccase produced on the novel lignocellulosic biomass tamarind shell was only 0.182 U/mL in the case of *G. lucidum* (Manavalan et al. 2013). Furthermore, when grown on wheat bran, the activity of laccase from *G. lucidum* was 2,973 U/mL (Makalesi 2022). *G. lucidum* RCK 2011, on the other hand, produced laccase with an activity of 2098 ±130.50 U/g after 9 days SSF on wheat bran (Sharma et al. 2019).

The laccase activity obtained in this study can be increased by further optimizing production, extraction, and purification of enzyme, as well as determining the optimal laccase activity conditions.

Modified TiO_2 nanoparticles

Titanium dioxide (TiO_2, Degussa P25) nanoparticles are naturally amphoteric and have sizes ranging from 20 to 30 nm. They have a specific surface area of 50-60 m^2g^{-1} and a pore diameter of 17.5 nm (Almquist and Biswas 2002).

GOPTMS was used to modify TiO_2 nanoparticles in order to introduce epoxy groups and successfully immobilize crude laccase from *Ganoderma spp*. The nanoparticles were characterized before being used in the immobilization procedure, which involved determining the concentration of epoxy groups on their surface. The concentration of epoxy groups on particles modified according to protocol number one (TiO_2M1) was extremely high (950 µmol/g), whereas it was lower (500 µmol/g) on nanoparticles (TiO_2M2) modified according to protocol number two (Miljković, 2020).

The epoxy group concentration of 500 µmol/g is comparable to that of some commercial supports, such as Eupergit®, which has already been used for laccase and dextransucrase immobilization.

Imobilization of crude laccase on modified TiO_2 nanoparticles

Ganoderma spp. crude fungal laccase (47.43 U/g) was immobilized on both TiO_2M1 and TiO_2M2 nanoparticles. This is the first time that crude fungal laccase was immobilized onto TiO_2 nanoparticles modified with GOPTMS.

Immobilized laccase activity on TiO_2M1 nanoparticles was 8.25 U/g, while immobilized laccase on TiO_2M2 nanoparticles had activity of 15.84 U/g. According to the laccase activities obtained, laccase immobilized onto TiO_2M2 nanoparticles had a higher residual activity than laccase immobilized onto TiO_2M1 nanoparticles. Laccase immobilized onto TiO_2M1 and TiO_2M2 nanoparticles had residual activity of 17.39% and 33.40%, respectively (Fig. 1). Furthermore, laccase immobilized onto TiO_2M2 nanoparticles performed better than laccase immobilized onto TiO_2M1 nanoparticles. The immobilization efficiency of TiO_2M2 nanoparticles was 26.75%, while TiO_2M1 nanoparticles had an immobilization efficiency of 20.18% (Fig. 1).

The difference in residual activity and immobilization efficiency between laccase immobilized on TiO_2M1 and laccase immobilized on TiO_2M2 nanoparticles can be attributed to the different concentrations of epoxy groups on the nanoparticles' surfaces. A high concentration of reactive groups, such as in the case of TiO_2M1 nanoparticles, can affect the reduction of enzymatic activity due to enzyme binding to the functionalized support in several points (Banjanac et al. 2016; Grazu, López-Gallego, and Guisán 2012).

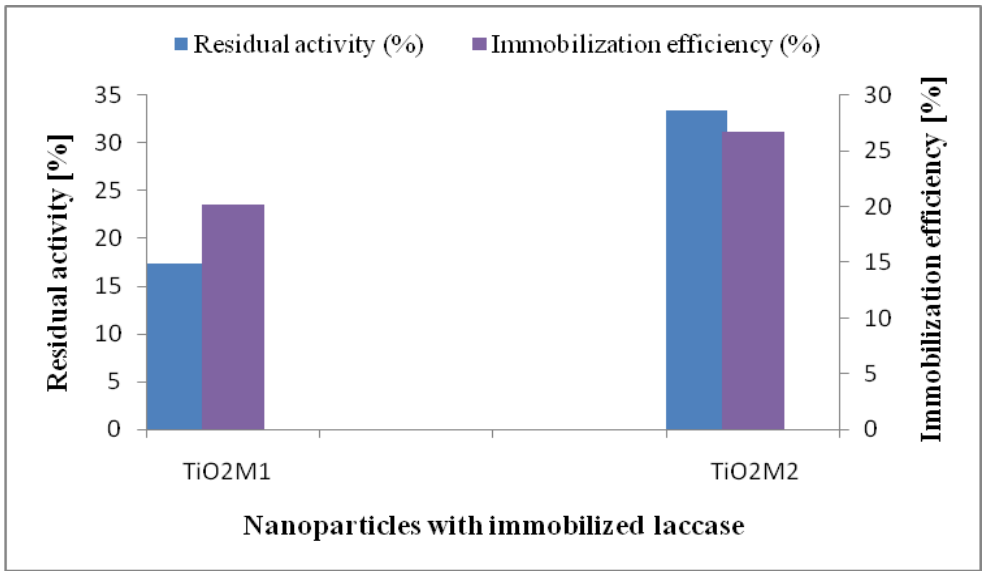

Figure 1. Resudual activity and immobilization efficiency for laccase immobilized onto TiO_2M1and TiO_2M2 nanoparticles

Optimization of immobilization of crude laccase onto TiO_2M2 nanoparticles

Immobilization of crude laccase from *Ganoderma spp*. on TiO_2M2 nanoparticles modified with (GOPTMS) was optimized by adjusting the contact time between the laccase and the nanoparticles, as well as the effect of different pH values on immobilization.

The effect of four contact times (2.0 h, 3.0 h, 3.5 h, 4 h) between the free laccase from *Ganoderma spp*. and the carrier,TiO_2M2 nanoparticles on immobilization was investigated. The shortest contact time of 2 h between *Ganoderma spp*. crude laccases and TiO_2M2 nanoparticles resulted in the lowest residual activity and immobilization efficiency. The residual activity in this case was 30.38 %, while the immobilization efficiency was 20.18 % (Fig. 2).

In the range of 2 h - 3.5 h, residual activity and immobilization efficiency values increased with increasing contact time (Fig. 2). The optimal contact time for obtaining the highest residual activity and immobilization efficiency was 3.5 hours. The maximum residual activity was 35.04 %, and the maximum immobilization efficiency was 28.95 % (Fig. 2). However, increasing the contact time between *Ganoderma spp*. crude laccases and TiO_2M2 nanoparticles for 4 h resulted in a decrease in residual activity and immobilization efficiency, which were 33.40 % and 26.75 %, respectively.

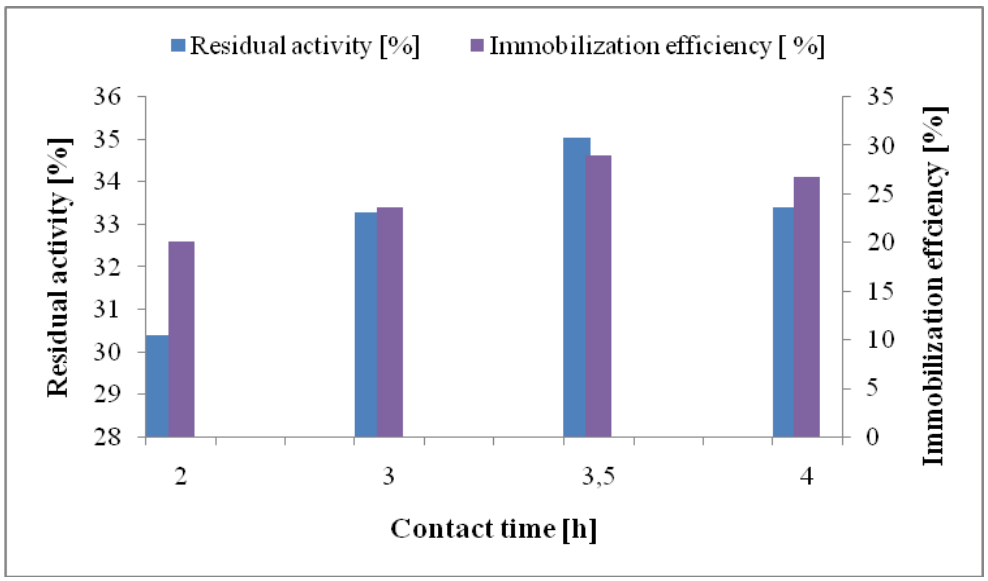

Figure 2. The effect of different contact times between crude laccase from Ganoderma spp. and TiO_2M2 nanoparticles on resudual activity and immobilization efficiency

The effect of three pH levels (4, 5, 6) on immobilization of crude fungal laccase from *Ganoderma spp.* was investigated. Both residual activity and immobilization efficiency were reduced by pH 4 and pH 6, with pH 6 having the largest impact where these two values were the lowest. The lowest residual activity and immobilization efficiency were 14.83 % and 16.56 %, respectively (Fig. 3). The residual activity and immobilization efficiency at pH 4 were marginally greater than those at pH 6, coming in at 21.21 % and 19.52 %, respectively (Fig. 3).

The highest values of residual activity and immobilization efficiency were achieved at pH 5, which was the optimal pH for the immobilization of crude laccase from *Ganoderma spp*. The highest immobilization efficiency was 28.95 %, whereas the highest residual activity of immobilized laccase on TiO_2M2 nanoparticles was 35.04 % (Fig. 3).

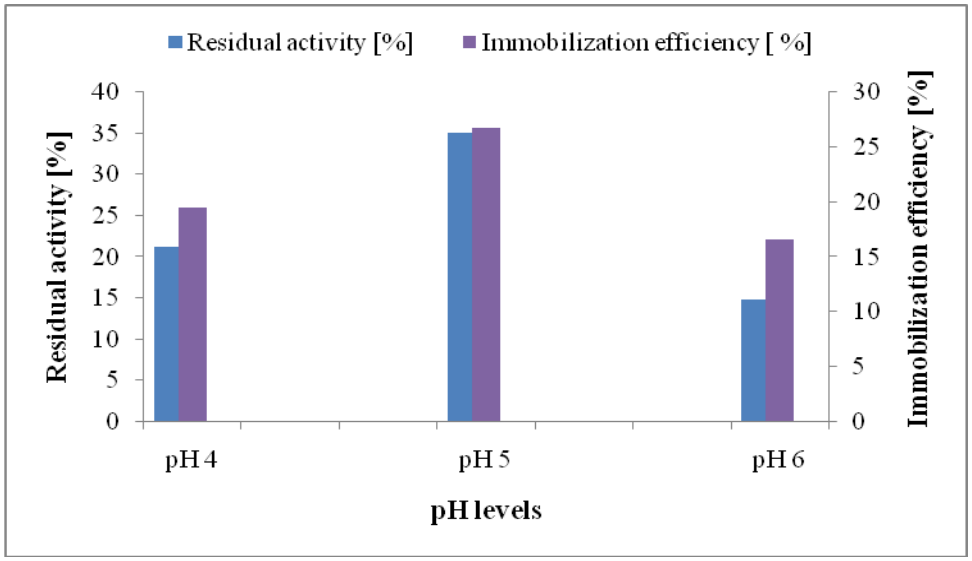

Figure 3. The effect of different pH levels on resudual activity and immobilization efficiency of immobilized Ganoderma spp. laccase

Contrary to our findings, the immobilization efficiency for *Trametes versicolor* laccase on the TiO_2/ZnO system was 99.4%, and the optimal pH for immobilization was 4 (Kołodziejczak-Radzimska et al., 2021). Under pH 7 and an 18-hour contact period, laccase immobilized on porous TiO2 nanostructures modified by (3-Aminopropyl) triethoxysilane (APTES) and glutaraldehyde (GLU) shown its highest level of activity (85.30±0.89 %)

(Isanapong et al., 2021). According to Wang and coworkers, laccase immobilized on the TiO2-montmorillonite complex exhibited that a pH 4.5 optimal pH for immobilization, with its residual activity of 74.72 % (Q. Wang et al. 2013). The immobilization efficiency for the crude fungal laccase from *Pleurotus ostreatus* that was immobilized on the APTES functionalized TiO_2 nanoparticle was above 80 % (Ji et al. 2017).

Reusability of laccase immobilized onto GOPTMS modified TiO_2 nanoparticles

Using a spectrophotometric method, the possibility of reusing immobilized laccase from *Ganoderma spp.* on GOPTMS modified TiO_2 nanoparticles was investigated during the oxidation of the phenolic substrate guaiacol.

Ganoderms spp. immobilized laccase was successfully used in 5 cycles of guaiacol oxidation (Fig. 4). The immobilized laccase retained more than half of its initial activity after the 2nd oxidation cycle, with a residual activity of 56.39 %. The immobilized laccase had a residual activity of 39.41 % after the 3rd cycle, but lost an additional 11.91 % during the 4th cycle. After 5th cycle, immobilized laccase had 14.83 % of its initial activity.

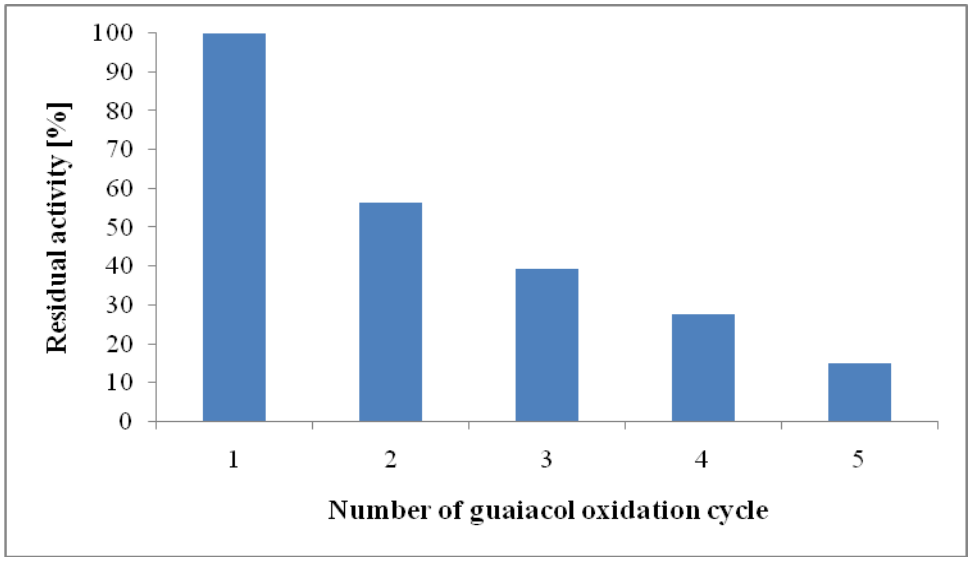

Figure 4. The reusability of laccase immobilized onto GOPTMS modified TiO_2 nanoparticles

Conclusions

TiO_2 nanoparticles are a potential support for enzyme immobilization due to their high surface-to-volume ratio, which increases the effectiveness of the immobilized enzyme. Furthermore, TiO_2 nanoparticles are non-toxic, inexpensive, and easily changed on the surface for use in enzyme immobilization. TiO_2 nanoparticles were chosen as a support for laccase immobilization in this study due to their biocompatibility and the possibility of using this immobilized enzyme in processes for eliminating hazardous contaminants from the environment such as dyes, pesticides, and pharmaceuticals.

The potential application of TiO_2 nanoparticles modified with GOPTMS in two ways in crude fungal laccase immobilization was investigated in this study. According to obtained results, both TiO_2 nanoparticles can be used in laccase immobiliztion.

The difference in immobilization efficiency and residual activity of *Ganoderma spp.* laccase immobilized on TiO_2M1 and TiO_2M2 nanoparticles is due to different epoxy group concentrations on nanoparticles' surface.

The optimal conditions for immobilization of *Ganoderma spp*. crude fungal laccase onto TiO_2M2 nanoparticles were 3.5 h contact time between laccase and nanoparticles and pH 5.

The maximum residual activity and imobilization efficiency obtained under optimal

immobilization conditions were 38.04 % and 28.95 %, respectively. In addition, the immobilized laccase was succesfully used in 5 cycles of guaiacol oxidation.

Acknowledgments

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