Application of free and immobilized laccase for removal and detoxification of fluoroquinolones from aqueous solution

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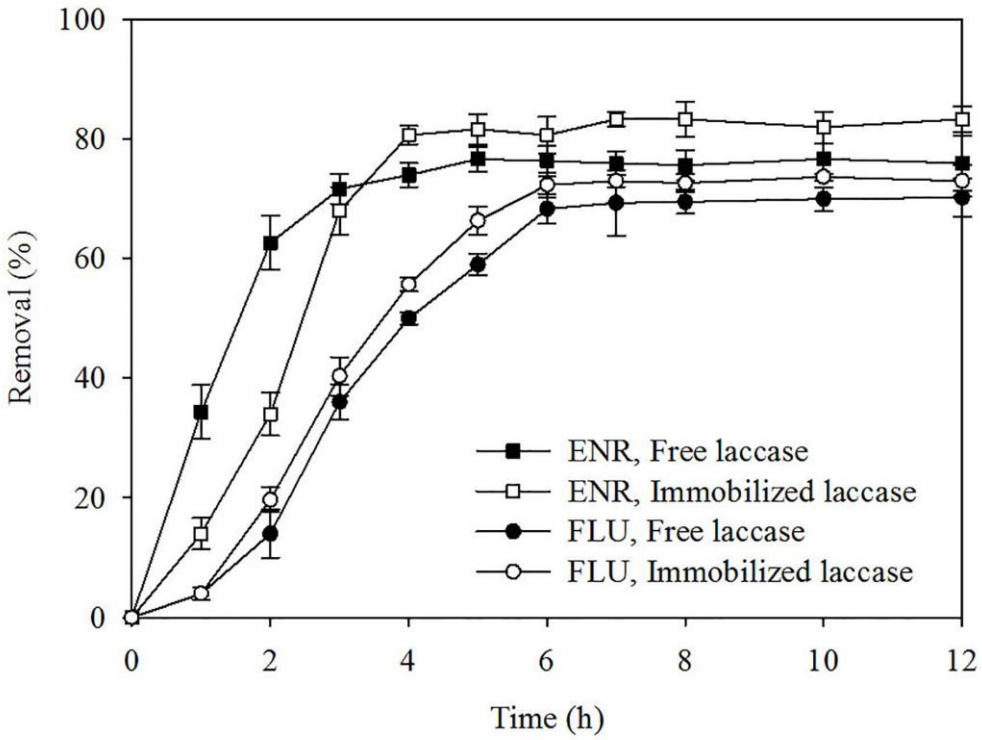
Graphical abstract



Abstract

Laccase from *Trametes versicolor* was immobilized by covalent bonds formation on CPC silica carriers. Elimination of two floroqinolone (FQ); enrofloxacine (ENR) and flumequine (FLU) using laccase in both free and immobilized form in the absence and presence of 1-hydroxybenzotriazole (HBT) and 4-Hydroxybenzoic acid (HBA) as mediators was investigated. Temperature, pH and storage stability of immobilized laccase was significantly improved compare to free laccase. In the absence of a laccase mediator, the initial concentrations of 50 mg L^{-1} of ENR and FLU decreased by 19 % and 28 %, respectively, after 6 h treatment using the immobilized laccase, while, the removal percentages were increased to 98 % and 96 %, respectively, when the immobilized laccase was used in presence of HBT. Whereas, the removal percentages of ENR and FLU were increased to 97 % and 88 %, respectively, when the immobilized laccase was used in presence of HBA. After twenty runs

of the enzymatic elimination (laccase-HBT system) of ENR and FLU, the immobilized laccase exhibited the relative removal of 17.63 % and 15.62 %, respectively. The results of microtoxicity test (growth inhabitation percentage of six bacterial strains) showed a significant decrease in toxicity of the laccase-treated ENR and FLU solution.

Keywords: Enrofloxacine, Flumequine, Immobilized enzyme, Laccase, Antibiotic removal.

1. Introduction

In recent years, environmental pollution by organic compounds has become a serious problem due to their adverse effects on many forms of life (Lopes and Furlong, 2001; Ashrafi *et al.*, 2016; Ashrafi *et al.*, 2016). A group of these compounds that has been widely used in the world is pharmaceuticals and personal care products (PPCPs). Among all the PPCPs, antibiotics occupy an important place due to their high consumption rates in both human and veterinary medicine (Nazari *et al.*, 2016; Kamani *et al.*, 2017; Panahi *et al.*, 2019). Fluoroquinolones (FQs) are among the most important classes of synthetic antibiotic used widely in veterinary and human medicine for therapeutic purposes and as a growth promoter (Fink *et al.*, 2012). Among the FQs, enrofloxacin and flumequine are two important antibacterial agents extensively applied in veterinary medicine (Rodrigues-Silva *et al.*, 2013). Due to low cost and broad spectrum activity of FLU against gram-negative bacteria and its efficacy for many microbial infections including respiratory, urinary, and digestive system (Williams *et al.*, 2007), it has been widely used in aquaculture to cure and prevent skin infections in fish (Ma *et al.*, 2012), and in animal husbandry as prophylactics to prevent diseases, or as chemotherapeutic agents to control diseases (Rodrigues-Silva *et al.*, 2013). ENR is largely used in poultry production (Ötker and Akmehmet-Balcıoğlu, 2005), cattle and swine farms (Sturini *et al.*, 2010), and treating respiratory and enteric bacterial infections. Due to the low bioavailability of FQs, it is mainly excreted as unchanged compounds in urine and feces, therefore, discharged into environment (Jia *et al.*, 2012). In addition, they cannot be completely removed by conventional wastewater treatment systems, therefore, residues of these compounds often appear in the environment (Wammer *et al.*, 2013). Subsequently, it can increase the risk for development of resistant bacteria and affect organisms (Ostadhadi-Dehkordi *et al.*, 2012; Rodrigues-Silva *et al.*, 2013), that may increase the risk of cancer development. The removal of ENR and FLU by processes such as Fenton and photo-Fenton

(Rodrigues-Silva *et al.*, 2013), photocatalyzed-doped TiO2 and UV (Nieto *et al.*, 2008), adsorption (Ötker and Akmehmet-Balcıoğlu, 2005; Yan *et al.*, 2013; Ashrafi *et al.*, 2016) and oxidation by chlorine dioxide (Wang *et al.*, 2010) techniques has been investigated in many studies. Recent studies have confirmed the ability of enzymes to eliminate the PPCPs (Ostadhadi-Dehkordi *et al.*, 2012; Suda *et al.*, 2012; Ashrafi *et al.*, 2015), and organic pollutants (Gholami-Borujeni *et al.*, 2011; Gholami-Borujeni *et al.*, 2011; Kalaiarasan *et al.*, 2014; Kamani *et al.*, 2018; Mehrabian *et al.*, 2018) as an interesting technique of degradation from the eco-friendly point of view, due to their higher efficiency and less toxicity of metabolites (Saratale *et al.*, 2011; Ashrafi *et al.*, 2013; Gholami-Borujeni *et al.*, 2016).

Laccases (benzendiol: oxygen oxidoreductase, EC 1.10.3.2) belongs to the group of multi copper-containing blue oxidases that is widespread in nature, produced mainly by fungi (especially white-rot basidiomycetes), brood type of plants, and bacteria (Balan *et al.*, 2012; Ostadhadi-Dehkordi *et al.*, 2012; Mogharabi and Faramarzi, 2014). Laccase catalyze the oxidation of a wide range of phenolic substrates coupled to the reduction of molecular oxygen to water molecule. However, applications of laccase may limit to oxidation of some substrates with high redox potentials, in the presence of redox mediators (usually a small molecule, that acting as an electron shuttle between the enzyme and the substrate) the spectrum of laccase substrates can be expanded to various non-phenolic substrates such as xenobiotics (Ostadhadi-Dehkordi *et al.*, 2012; Ashrafi *et al.*, 2013). Immobilization of laccases is necessary for stability and reusability, which enables the reusing of immobilized laccases and finally reduces the overall cost of enzymatic elimination (Fernández-Fernández *et al.*, 2013; Sadighi and Faramarzi, 2013). In that respect, enzymes have been immobilized on several supports by different mechanisms (Fernández-Fernández *et al.*, 2013), which among them, covalent binding supplies considerably more stable enzymes due to increasing the rigidity of enzyme structure and reducing protein unfolding (Fernández-Fernández *et al.*, 2013), and it was

preferred to other mechanisms (Jolivalt *et al.*, 2000). Many reports describe the improvement of thermal and other operational stabilities of immobilized laccase (Champagne and Ramsay, 2010; Dehghanifard *et al.*, 2013).

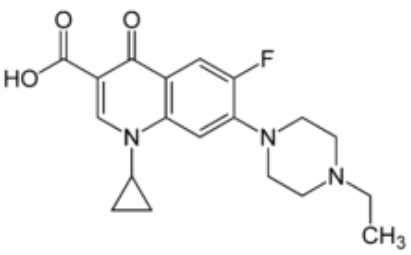
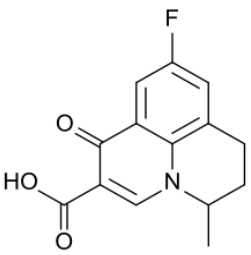
The aim of the present study was to investigate the application of free and immobilized laccase from *Trametes versicolor* as a biocatalyst onto silica-based for removal of ENR and FLU as a model, and evaluation of the effect of operational factors in the presence and absence of HBT, and HBA. The toxicity of laccase-treated samples was also evaluated using microtoxicity studies.

2. Materials and methods

2.1. Materials

The laccase (EC 1.10.3.2, activity > 10 U mg $^{-1}$) from *Trametes versicolor*, pre-silanized [3-aminopropyltriethoxysilane (APTES)] CPC silica beads, 1-hydroxybenzotriazole (HBT), 4-Hydroxybenzoic acid (HBA), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and a glutaraldehyde (50 %) solution were all purchased from Sigma-Aldrich (USA). Flumequine, enrofloxacin (Table 1), and all other chemicals were of the highest available grades.

Table 1 General characteristic of ENR and FLU.

Parameter	Characteristic	
Chemical name	Enrofloxacin	Flumequine
CAS number	93106−60−6	42835−25−6
Molecular formula	$C_{19}H_{22}FN_3O_3$	$C_{14}H_{12}FNO_3$
Molecular weight	359.4 g mol^{-1}	261.25 g mol^{-1}
Maximum wavelength	285 nm	248 nm
Chemical structure		

2.2. Preparation of immobilized laccase

Prior to the enzyme attachment, pre-silanized silica support was modified by suspending 10 mg of it into 1 mL of 0.5–4.5 % glutaraldehyde (vol/vol) solution (previously degassed under 2.0 bar vacuum pressure for 2 h) under stirring for 3 h in 0.1 M citrate buffer pH 4.5. The produced glutaraldehyde-activated CPC silica beads were washed for three times with citrate buffer (0.1 M, pH 4.5) and distilled water, and followed by drying in an oven at 40 °C for 24 h. The laccase immobilization on modified silica beads was carried out for 2 h at 4 °C under stirring, using 10 mg of silica, different concentrations of laccase stock-solution (0.25–2.5 U mL^{-1}) prepared in citrate buffer 0.1 M, pH 4.5. Then the support was separated and washed three times with 0.1 M of citrate buffer (pH 4.5) and 2 mol L^{-1} NaCL. The immobilized laccase were stored at 4 °C for the further use. All experiments were performed in duplicate.

2.3. Activity assay of free and immobilized laccase

The laccase activity was assayed using a UV/vis spectrophotometer (UVD 2950, Labomed, Culver City, USA) with ABTS as a laccase substrate (2 mM) in 0.1 M citrate buffer at pH 4.5 (Ashrafi *et al.*, 2013). For activity assay of laccase, 1 mL of free laccasse solution or 10 mg of immobilized laccase was added to 1 and 10 ml of the ABTS solution at 40 °C under shaking at 150 rpm, respectively (Rahmani *et al.*, 2015). The change in absorbance at 420 nm (ε_{420} = 36,000 M^{-1} cm^{-1}) was monitored after 10 min and the catalytic activity was calculated. One unit (U) of activity was defined as the amount of enzyme that oxidized 1 μmol of ABTS per minute (Tavares *et al.*, 2013).

2.4. Operational stability of free and immobilized laccase

The effect of temperature on stability of free and immobilized laccase were determined by incubating the samples in the range 4–80 °C, at pH 4.5 in citrate buffer 0.1 M for 6 h. The effect of pH on free and immobilized laccase stability was determined as the relative activity by incubating the samples under the variety of pH (ranging from 3.0–7.0 in citrate buffer 0.1 M) at 45 °C for 6 h. The storage stability experiments were conducted by incubating free and immobilized laccase in citrate buffer (0.1 M, pH 4.5) at 25 °C for 18 days and remained activity was measured.

2.5. Removal experiments of ENR and FLU

In order to study on the ability of the laccase in removal of ENR and FLU, elimination experiments were carried out in a batch reactor. ENR and FLU stock (500 mg L^{-1}) were prepared in citrate–sodium buffer (0.1 M) and appropriate dilutions of this stock were used for elimination experiments. Removal studies were performed by adding 6 U mL^{-1} of the free or immobilized laccase. The reaction solutions (final volume of 3 mL, pH 5 and 4.5 for ENR and FLU, respectively) containing 50 mg L^{-1} of each FQs were incubated at 45 °C and 150 rpm under dark for 6 h. The reaction tube (Eppendorf 15 mL) were removed every 1 h and the reaction was stopped immediately by adding 3 mL of HPLC grade methanol, and stored at − 20 °C for later analyze. Prior to determine the remained concentration of FQs the samples filtered through 0.45 μm membranes and then measured using high-performance liquid chromatography (HPLC). Control samples were maintained with heat-inactivated laccase (in the case of free laccase) and activated beads without laccase (for immobilized laccase). All the experiments had three replications, and the mean of them is reported for each experiment.

2.6. Effect of operational parameters on removal of ENR and FLU

2.6.1. Effect of temperature, pH, laccase activity and FQs initial concentration

The effect of temperature on enzymatic removal, was studied by incubating 50 mg L^{-1} of ENR and FLU solution in the presence of free or immobilized laccase (6 U mL^{-1}) at temperature range of 30 to 55 °C, pH 5 and 4.5, respectively. To study the pH effect, after adjusting the initial pH of the reaction solutions of each FQs (final concentration of 50 mg L^{-1}) using 0.1 M citrate–sodium buffer at values of 3.5, 4, 4.5, 5, and 5.5, the free or immobilized laccase (6 U mL^{-1}) was added to the reaction mixtures and incubated at 45 °C, 150 rpm for 6 h. In order to determine the effect of laccase activity on removal of FQs, 1–12 U mL^{-1} of the free or immobilized laccase was added to the reaction mixture (ENR and FLU with final concentration of 50 mg L^{-1} at pH 5 and 4.5, respectively) followed by incubation at 45 °C, 150 rpm for 6 h. For the evaluating of effects of each FQs initial concentration on removal percentage, the reaction mixtures (ENR and FLU with final concentration of 25, 50, 75, 100 and 150 mg L^{-1} at pH 5 and 4.5, respectively) were incubating after adding free or immobilized laccase (6 U mL^{-1}) at 45 °C, 150 rpm, for 6 h.

2.6.2. Effect of mediators on removal of FQs

The effects of HBT (final concentration of 0.5, 1, 1.5, 2, 2.5 and 3 mM) and HBA (final concentration of 1, 2, 3, 4, 5 and 6 mM) on removal of each FQs was studied by incubating of 50 mg L^{-1} of ENR (pH 5) and FLU (pH 4.5) solution in citrate–sodium buffer in presence of free or immobilized laccase (6 U mL^{-1}), at 45 °C, 150 rpm under dark. All The experiment had three replications.

2.7. HPLC and statistical analysis

The concentrations of FQs were measured using a high-performance liquid chromatography (HPLC). HPLC consists of a Knauer LPG pump, an EZ-chrom HPLC system manager program with a UV-visible diode array detector (k-2500). Separation was performed

using a column of MZ-analysentechnik ODS-3 C18 (4.6 mm × 250 mm) packed with 5-µm spherical particles. The samples were injected manually using an injection valve (SGE Australia). A methanol and water (18:82 vol/vol) mixture (pH 3, adjusted by acetic acid glacial), for ENR, and a water, methanol and acetonitrile (40:30:30 vol/vol) mixture for FLU, were used as mobile phase at 30 °C with a flow rate of 1.0 mL min^{-1}. The ENR and FLU retention times in HPLC analysis were 9.2 and 7.8 min, respectively. For the purpose of statistical analysis, all experiments performed in triplicate and results are expressed as the mean ± standard deviation (SD). The statistical significance between mean values was tested by the independent sample t-test and one-way analysis of variance (ANOVA) with Dunnett's T3 Post-Hoc test (SPSS 18.0, SPSS Inc). The significance level was set to 5 %.

2.8. Reusability of the immobilized laccase

In order to investigate the reusability of the immobilized laccase for removal of ENR and FLU, elimination experiments were carried out using 6 U mL^{-1} of immobilized laccase in presence of HBT (2mM) at 45 °C, pH of 5 and 4.5, respectively. The applied immobilized laccase was separated from substrate solution of each FQs (end of each cycle) by filtration and washed three times with citrate sodium buffer (0.1 M, pH 4.5). It was then immediately added again to other fresh substrate solutions of ENR or FLU, and after run time the residual of each FQs were analyzed and the relative removal of each FQs were calculated. All the reusability experiments were done in triplicate and mean of obtained results were reported.

2.9. Kinetic studies

First the velocity of enzymatic elimination of each FQs (Initial concentration 5–150 mg L^{-1}) in absence and presence of applied mediators assisted by free or immobilized laccases has been determined. Then, a Michaelis–Menten curve was drawn by plotting the obtained

velocity (V) against the each FQ concentrations (S). K_m (Michaelis constant) and V_{max} (maximal velocity) of free and immobilized laccases through each FQ were then calculated using the Lineweaver–Burk transformation of the Michaelis–Menten equation. All kinetic experiments were performed in three replications at 45 °C, in aqueous solutions buffered with citrate sodium at pH 5 and 4.5 for ENR and FLU, respectively.

2.10. Toxicity assay

A toxicity assay was conducted based on our previous work (Ashrafi *et al.*, 2013; Rahmani *et al.*, 2015), in order to evaluate the toxic effects of both the untreated and treated FQs solutions based on the inhibitory growth of six bacterial strains, (three gram-positive bacterial strains; *Staphylococcus epidemidis* ATCC 12228, *Staphylococcus aureus* ATCC 6538, and *Bacillus subtilis* ATCC 6633, and three gram-negative bacterial strains; *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, and *Klebsiella pneumonia* ATCC 10031). In brief, each tested bacterial strain was firstly cultivated in Mueller–Hinton broth to reach the OD600 of 0.2. Subsequently, the untreated FQs solution (different final concentrations for each FQs against each bacteria) and the samples obtained from enzymatic treatment of each FQs were separately added to the all set bacterial broth and incubated at 37 °C. Changes in the OD600 of each bacterial strain were then monitored and recorded every 1 h for 12 h. A negative control (cultivated bacterial strain in the absence of FQs) was also conducted for each experiment. The percentage of growth inhibition (GI %) was defined as $[(1 – D_{600S}/ OD_{600C}) \times 100]$, where OD_{600S} is the OD_{600} of sample and OD_{600C} is the OD_{600} of control (Ben Younes *et al.*, 2012; Rahmani *et al.*, 2015). All experiments were performed in triplicate.

3. Results and discussion

3.1. laccase immobilization

Glutaraldehyde is a reagent that used for immobilization of enzymes by many researchers (Champagne and Ramsay, 2010; Tavares *et al.*, 2013). Glutaraldehyde concentration influence the immobilization process (Tavares *et al.*, 2013), but there are no specific guidelines. In this work, in order to determine an optimum concentration of glutaraldehyde, pre-silanized CPC-silica beads were immersed in glutaraldehyde solution in the range from 0.5 to 4.5 % v/v. The results reveal that by increasing the concentration of glutaraldehyde up to 3 % the immobilized laccase activity increased (data not showed), but by further increasing a decrease in immobilized laccase activity was observed. This results was in agreement with Tavares et al. (Tavares *et al.*, 2013), who reported that when the glutaraldehyde concentration increased up to 5 %, the immobilized laccase (from *Aspergillus*) activity increased, while after further increasing of glutaraldehyde a decrease in activity was reported. Therefore, the concentration of 3% v/v of glutaraldehyde was selected and used in all experiments of immobilization procedure. In order to determine the optimum concentration of laccase for immobilization, it was evaluated by immersing 10 mg of silica support in different concentrations of laccase solution (0.25–2.5 U mL^{-1}) prepared in citrate buffer 0.1 M, pH 4.5. The results show that by increasing in the amount of initial laccase from 0.25 to 1.25 U mL^{-1}, the relative activity of immobilized laccase decreased slightly (from 95.3 to 92.3 %), but the amount of attached laccase per unit of support increased significantly (from 1.9 to 9.2 mg laccase per g of support). However, when the amount of initial laccase was above 1.25 U mL^{-1}, the amount of attached laccase per unit of support increased only slightly, while the relative activity of immobilized laccase decline sharply. So, the concentration of 1.25 U mL^{-1} for laccase solution was selected and used in all experiments of immobilization.

3.2. Operational stability of free and immobilized laccase

Enzyme stability is one of the most important characteristics, especially at industrial applications, which depends on its tolerance to deactivation over time under environmental conditions, generally temperature and pH (Sathishkumar *et al.*, 2012). Fig. 1(a–c) shows the normalized results of the storage, pH, and temperature stabilities of free and immobilized laccase. The profiles of thermal stability for free and immobilized laccase are shown in Fig. 1a. At temperature 4 °C after 6 h, the free and immobilized laccase exhibited the highest stability and there was no any losses in their activity. The activity of free laccase was decreased to 95.93, 91.05, and 87.7 % at temperature 20, 30 and 40 °C after 6 h, respectively. Whereas, there was no significant difference compared to immobilized laccase. However, at temperature 80 °C, only 8.13 % of relative activity of the free laccase was remained after 6 h, compared to immobilized laccase (57.37 %). This finding was in agreement with Rahmani, et al. (Rahmani *et al.*, 2015), who reported that the free and immobilized laccase on porous silica beads retains 25.6 % and 85.4 % of its relative activity at temperature 70 °C for 2 h. A description might be that stabilization of active conformation is increased by multipoint bond formation between the laccase molecule and the support, which improved thermal stability of the immobilized laccase (Zhu *et al.*, 2007). The similar result was reported by Arica et al. (Arıca *et al.*, 2009), who reported the free and immobilized laccase on non-porous poly (glycidyl methacrylate/ethylene glycol dimethacrylate [poly GMA/EGDMA-DAH]) beads retains 45 % and 7% of its relative activity after incubation at 65 °C for 2 h, respectively.

Fig. 1b shows the profile of free and immobilized laccase pH stability over the range 3–7 after 6 h. At pH 3, free and immobilized laccase lost more than 26 % and 45 % of its initial activity. However, at pH 7, free laccase loses more than 84 % of its initial activity, but, the immobilized laccase retained more than 70 %. The results indicate that the resistance of the immobilized laccase to broader range of pH was increased compared to free laccase. This results was in agreement with Mirzadeh et al. (Mirzadeh *et al.*, 2014), who reported that the

immobilized laccase of *Paraconiothyrium variabile* on CPC silica beads, exhibited a broader range of relative activity under extremes of pH conditions, compared to free laccase. Dodor et al. (Dodor *et al.*, 2004), reported that the free laccase from *Trametes versicolor* retained 3 % of its initial activity at pH 2, but, the immobilized laccase on kaolinite retained 40 % of its initial activity.

The pattern of decline in laccase activity during time as storage stability was shown in Fig. 1c. During the first 6 days storage there was not significant decrease (13 %) in the initial activity of immobilized laccase; by contrast, in the same time the free laccase retained only 46.34 % of its initial activity. After 16 days of storage at 25 °C, loss of activity of the immobilized laccase was about 63 % whereas the free laccase lost all its activity within same time. The similar result was reported by Arica et al. (Arıca *et al.*, 2009), who reported the free laccase at 4 °C lost all its activity within a 6-week, whereas the immobilized laccase on spacer-arm attached non-porous poly(GMA/EGDMA) beads lost only 48 % of its initial activity over the same period. The study of Annibale et al. (Annibale *et al.*, 1999), showed that laccase from *Lentinula edodes* immobilized on chitosan maintained 85 % of its initial activity during 2 months storage at 5 °C, whereas the loss of the free laccase was about 65 %.

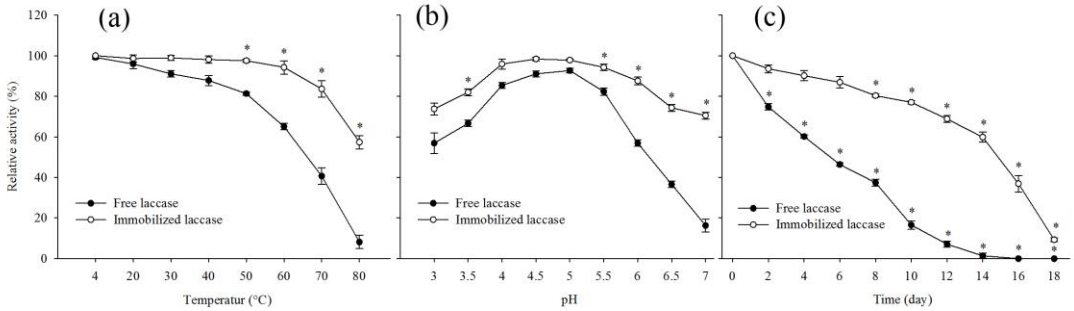


Fig. 1. (a) Temperature, (b) pH, and (c) storage stability of free and immobilized laccase. Data show the mean value ± SD (n = 3) and significancy (*) was checked after ANOVA analysis of obtained data (p-value < 0.05).

3.3. Removal of ENR and FLU using free and immobilized laccase

In order to demonstrate the time course of both FQs elimination by laccase, removal studies were performed by adding the free or immobilized laccase (6 U mL^{-1}) to the reaction solutions (50 mg L^{-1} of ENR and FLU at pH 5 and 4.5, respectively), and were incubated for 12 h. As shown in Fig. 2, both the free and immobilized laccase were efficiently able to remove ENR (74 % and 80.66 %) and FLU (68.33 % and 72.33 %) after 4 h and 6 h incubation, respectively. After that, there was no significant removal of both FQs. No removal was detected in negative controls. As shown in Fig. 2, the elimination of both studied FQs by immobilized laccase was a little more than free laccase after the time which the elimination curve reached to its highest levels. Our results on the immobilized laccase displayed a higher elimination than free laccase, was in agreement with previous studies by Rahmani et al. (Rahmani *et al.*, 2015), and Peralta-Zamora et al. (Peralta-Zamora *et al.*, 2003). This results show that the immobilization of laccase on CPC is a useful method for elimination of applied FQs.

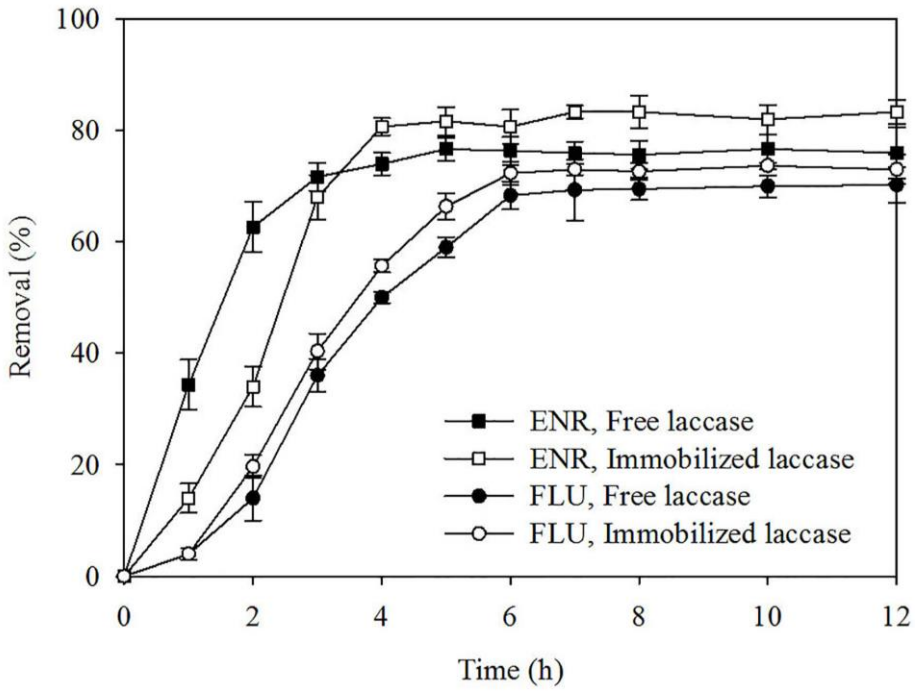


Fig. 2. Time course study of ENR and FLU removal using free and immobilized laccase. Data show the mean value ± SD (n = 3).

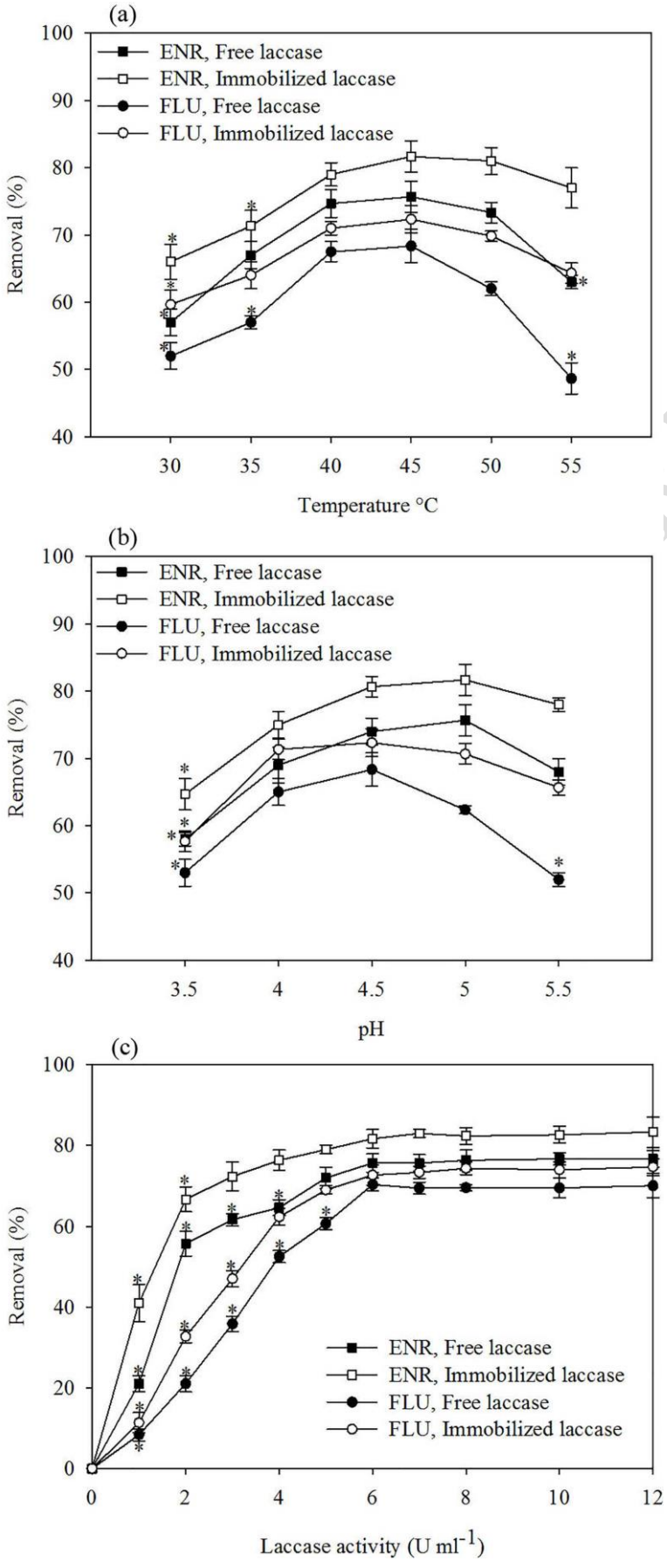
3.4. Effect of temperature, pH, and laccase concentration on FQs removal

As shown in Fig. 3a, the optimal range of temperature for both FQs removal was between 40 °C and 50 °C for both free and immobilized laccase. The highest removal of ENR (75.5 % and 81.5 % for free and immobilized laccase, respectively) and FLU (68 % and 72 % for free and immobilized laccase, respectively) occurred when the temperature was 45 °C. According to results the immobilized laccase showed higher removal of both applied FQs than that of the free laccase, and the optimal temperature was same degree for both free and immobilized laccase. These results were in agreement with the results of Wang et al. (Wang *et al.*, 2012), who reported that the immobilized laccase on magnetic mesoporous silica nanoparticles showed higher phenol degradation (69.2 %) than that of the free laccase (35.7 %) at the optimal temperature of 25 °C. In the case of free laccase, the removal percentage of both applied FQs decreased significantly at 55 °C, although, these decrease were not statistically significant in the presence of immobilized laccase. It may be illustrate by this fact that, due to thermal denaturation of the tertiary structure, the laccase loses activity (Kurniawati and Nicell, 2008), however, the immobilized laccase has relative stability against denaturing agents like temperature (Champagne and Ramsay, 2010; Fernández-Fernández *et al.*, 2013).

Effect of pH on applied FQs removal was studied at different pH values varying from 3.5–5.5 (Fig. 3b). The results show that the both free and immobilized laccase exhibit the maximal removal of ENR and FLU at pH 5.0 and pH 4.5, respectively. The difference between the optimal pH for ENR and FLU removal might depend on the substrate structure and oxidation mechanism (Weng *et al.*, 2013). As shown in Fig. 3b, the relative removal of free laccase at pH 3.5 decreased by 58 % (ENR) and 53 % (FLU), though, it decrease by 64.66 % (ENR) and 58 % (FLU) in the case of immobilized laccase. At pH 5.5, the decreases in the removal efficiency of both FQs by immobilized laccase was at a slower rate than that of

the free laccase. This results were in agreements with this fact that most of the fungal laccases like laccases from *Coriolus hirsutus*, *Trichoderma atroviride*, *Cerrena unicolor 059* and *Trametes versicolor* work optimally at mild acidic pH (4–6) (Sadhasivam *et al.*, 2008; Weng *et al.*, 2013; Rahmani *et al.*, 2015).

Fig. 3c shows the profiles of both free and immobilized laccase concentration role on the removal efficiency of ENR and FLU. As it can be realized from this figure, the removal of both ENR and FLU using free and immobilized form of laccase significantly increased when laccase activity was enhanced up to 6 U ml^{-1}, while after that (up to 12 U ml^{-1}), did not show a significant increase on both ENR and FLU removal. The recent study of Rahmani et al. (Rahmani *et al.*, 2015), showed that increasing of laccase concentration from 0.1 to 0.8 U ml^{-1} significantly increased the removal percentages of sulfonamides assisted by free or immobilized laccase, though, further increasing up to 1.2 U ml^{-1} did not have a significant effect on removal percentage. The same results were reported by Mogharabi et al. (Mogharabi *et al.*, 2012),who observed that decolorization of synthetic dyes significantly increased as enzyme quantity increased from 0.5 to 2.5 mg ml^{-1}. However, further enhancement of enzyme quantity up to 5 mg ml^{-1} did not have a significant effect on decolorization. Also, same result was observed in study of Asadgol et al. (Asadgol *et al.*, 2014), which showed that increasing of laccase quantity from 1 to 10 U ml^{-1} significantly enhanced both phenol and bisphenol A removal, however further increasing up to 20 U ml^{-1} did not have a significant effect on removal efficiency.


(a)
ENR, Free laccase
ENR, Immobilized laccase
FLU, Free laccase
FLU, Immobilized laccase
Removal (%)
Temperature °C
(b)
pH
(c)
Laccase activity (U ml-1)

373

Fig. 3. Effect of (a) temperature, (b) pH, (c) laccase activity on removal of ENR and FLU assisted by free and immobilized laccase. Data show the mean value ± SD (n = 3) and significancy (*) was checked after ANOVA analysis (Dunnett's T3 post-hoc test) of obtained data (p-value < 0.05).

3.5. Effect of HBT and HBA concentration on FQs removal

Results of removal study using free and immobilized laccase in absence and presence of HBT are showed in Fig. 4a. In the case of ENR, the removal efficiency by free and immobilized laccase without HBT was 75 % and 81 %, respectively, while removal percentages were significantly increased (90 % and 92 % for free and immobilized laccase) by increasing the HBT concentration up to 0.5 mM. By increasing the HBT concentration up to 2 mM, removal percentages were received to 95 % and 98 % for free and immobilized laccase, respectively. While, by further increasing of HBT concentration (3 mM) removal percentages were decreased by 87 % and 94 % for free and immobilized laccase, respectively (Fig. 4a). In the case of FLU, increasing of HBT concentration from 0 to 2 mM significantly enhanced removal percent from 68 % and 72 % to 90 % and 96 % assisted by free and immobilized laccase, respectively. However, at a higher concentration of HBT (3 mM), significantly decreased (72 % and 81 %, assisted by free and immobilized laccase, respectively) removal percentage (Fig. 4a). The removal percentages decreased in the high concentration of HBT, may be due to this fact that the nitroxide radical resulting from laccase oxidation of HBT could have toxic effect on laccase. Our results were in agreement with those of Mechichi et al. (Mechichi *et al.*, 2006), who observed no toxic effect of the HBT at concentrations between 0.125 and 2.5 mM on decolorization of Remazol Brilliant Blue R, however a concentration of 5 mM showed inhibition of decolorization and significantly decreased decolorization percentage.

In the case of HBA, removal experiments were studied at different concentration varying from 1–6 mM (Fig. 3b). According to results, removal of ENR and FLU (75 %, 81 %

400 for ENR and 68 %, 72 % for FLU using free and immobilized laccase without HBA,
401 respectively) were significantly increased (95 %, 96 % for ENR and 85 %, 88 % for FLU
402 using free and immobilized laccase, respectively) by increasing HBA concentration from 0 to
403 2 mM and 4 mM, respectively. While removal percentages for both ENR and FLU were not
404 significantly increased by further increasing the HBA concentration up to 6 mM (Fig. 3b). Of
405 the natural mediators (ethyl 4-hydroxybenzoate, methyl 4-hydroxybenzoate, reduced
406 glutathione, cysteine, methionine, 4-hydroxybenzaldehyde, oxidized glutathione and HBA)
407 tested by Maruyama (Maruyama *et al.*, 2007), HBA (5 mM) resulted in 80 % degradation
408 (more than other mediators testified) of imazalil assisted by laccase after 24 h of reaction.

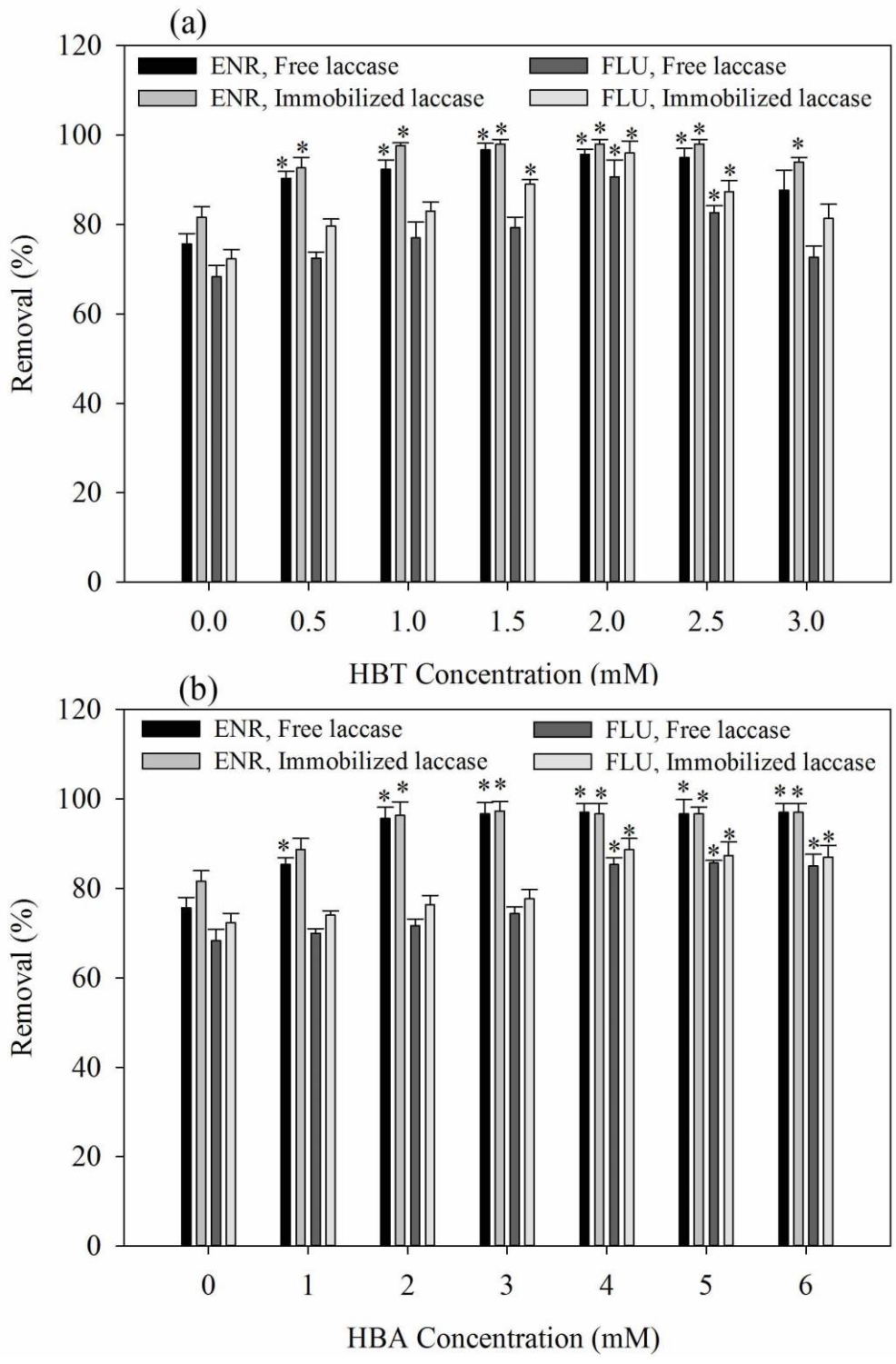


Fig. 4. Effect of (a) HBT and (b) HBA concentration on removal of ENR and FLU assisted by free and immobilized laccase. Data show the mean value ± SD (n = 3) and significancy (*) was checked after ANOVA analysis (Dunnett's T3 post-hoc test) of obtained data (p-value < 0.05).

3.6. Reusability of the immobilized laccase

Immobilization of enzyme facilitate their possible reuse for several reaction cycles and the overall cost of the process would be reduced (Champagne and Ramsay, 2010; Rahmani *et al.*, 2015). As shown in Fig. 5, the relative removal of both studied FQs was significantly decreased by increasing the cycle number of application. In the current study, the immobilized laccase exhibited the relative removal of 95.44 %, 77.67 %, 46.22 % and 17.63 % for ENR and 89.48 %, 65.27 %, 38.88 % and 15.62 % for FLU, after the 5th, 10th, 15th and 20th cycles number, respectively. The immobilized laccase on magnetic mesoporous silica nanoparticles retained 71.3 % of its initial degradation ability after 10 cycle of phenol degradation (Wang *et al.*, 2012). The same results was reported by Rahmani et al. (Rahmani *et al.*, 2015) who observed a 63.3 % and 82.6 % of initial removal activity of immobilized laccase remained after 10th cycles of sulfamethoxazole and sulfathiazole removal, respectively. However, a study by Sathishkumar et al. (Sathishkumar *et al.*, 2012), showed that no biocatalytic activity was retained after 8th cycles of diclofenac degradation. This declines in relative removal ability, indicated that laccase activity was decreased after each round, might be due to laccase inactivation by degradation products and other parameters (Sathishkumar *et al.*, 2012; Wang *et al.*, 2012; Fernández-Fernández *et al.*, 2013).

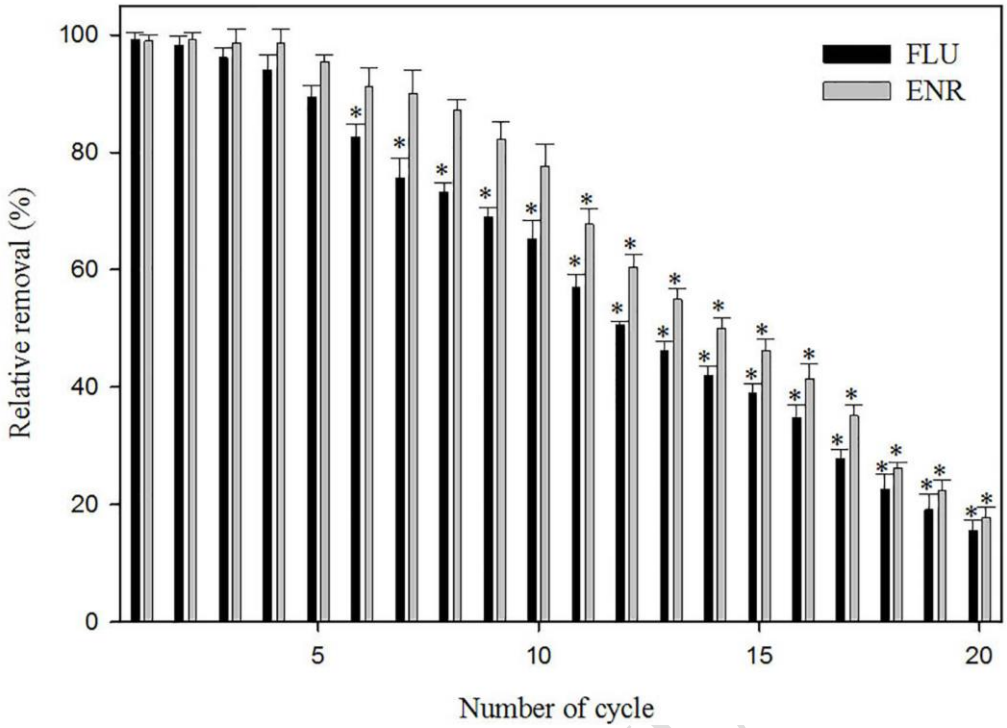


Fig. 5. Reusability potential of immobilized laccase cycles of ENR and FLU removal. Data show the mean value ± SD (n = 3) and significancy (*) was checked after ANOVA analysis of obtained data (p-value < 0.05).

3.7. Toxicity assays

In recent years, different toxicity assay techniques have been developed in order to examine the toxicity of pollutants and their products after degradation process (Ben Younes *et al.*, 2012; Ashrafi *et al.*, 2013). For this purpose, growth inhibition percentage (GI %) was measured (for three gram-positive and three gram-negative bacterial strains) for the evaluation of the toxicity of both applied FQs and their related treated solution. According to results (Table 3), the GI % of both applied FQs was significantly reduced for each treated ENR and FLU solution compared to untreated. The results showed that, the solution treated by laccase mediated with HBT has the lowest GI % compared to the others. A description might be that laccase mediated with HBT has the more removal efficiency of each FQs against the laccase without mediators and with HBA. The most GI % of ENR and FLU, was 78.2 % and 80 % for *E. coli* strain, which decreased to 34 % and 12.2 %, after treating by the laccase mediated with

HBT, respectively. This results were in agreement with Younes et al. who reported that the GI % of malachite green against *E. coli* was enhanced from 2 to 99 % after treatment by laccase (Ben Younes *et al.*, 2012). In our previous work, the obtained results of toxicity study using six standard bacterial strain showed that the GI % in presence of laccase-treated 13 synthetic dyes decreased significantly (Ashrafi *et al.*, 2013). The results of the Rahmani et al. (Rahmani *et al.*, 2015), study showed that the GI % of sulfathiazole and sulfamethoxazole was significantly reduced from 88 % and 81 % to 40.3 % and 28.6 %, following the addition of a laccase-HBT treated solution to cultivation media of *E. coli* strain. In the study of Pereira et al. (Pereira *et al.*, 2009), the GI % of Sudan Orange G reduced from 65.9 to 20 % for *S. cerevisiae* after treating by laccase.

Table 3. Growth inhibition percentage of untreated and treated FQs against six bacterial strains. Data show the mean value ± SD (n = 3).

Bacterial strains	FLU				ENR			
	Un-treated	Treated			Un-treated	Treated		
		Laccase	Laccase + HBA	Laccase + HBT		Laccase	Laccase + HBA	Laccase + HBT
S. epidemidis	79.5 ± 1.3	42.6 ± 2.1*	34.4 ± 0.5*	29.5 ± 3.1*	77.5 ± 0.8*	46.6 ± 2.3*	39.1 ± 0.4*	30.8 ± 0.7*
S. aureus	63.2 ± 0.9	32.2 ± 0.9*	19.3 ± 0.8*	14.8 ± 0.9*	72.0 ± 1.8*	49.3 ± 0.5*	38.3 ± 0.8*	28.5 ± 2.3*
B. subtilis	74.1 ± 1.8	35.4 ± 0.3*	20.9 ± 1.1*	19.3 ± 1.5*	71.7 ± 2.4*	44.4 ± 0.7*	34.1 ± 0.5*	22.1 ± 0.8*
E. coli	80.0 ± 0.5	32.2 ± 0.6*	20.2 ± 1.9*	12.2 ± 0.4*	78.2 ± 3.1*	58.1 ± 1.1*	43.6 ± 2.2*	34.0 ± 1.9*
P. aeruginosa	72.3 ± 0.8	46.5 ± 0.4*	41.1 ± 0.8*	37.6 ± 2.1*	75.4 ± 2.5*	61.2 ± 0.4*	46.5 ± 3.1*	38.5 ± 0.6*
K. pneumoniae	70.5 ± 0.3	46.4 ± 1.3*	24.7 ± 1.6*	14.6 ± 0.7*	74.2 ± 0.8*	51.6 ± 1.3*	37.9 ± 2.3*	31.5 ± 0.9*

* Signifcancy was determined using independent sample t-test (p-value < 0.05).

4. Conclusions

A CPC silica carriers was used for immobilization of laccase. Temperature, pH and storage stability of immobilized laccase increased significantly. Furthermore, the broader temperature and pH profiles than the free laccase have been exhibited by the immobilized laccase. The present study demonstrated that FQ antibiotics (ENR and FLU) are removed by treatment with the free and immobilized laccase. Additionally, it was confirmed that the removal percentages of ENR and FLU enhanced in the presence of the redox mediators HBT

and HBA. The immobilized laccase performed a good reusability. The removal efficacy of ENR and FLU was still as high as 46.22 % and 38.88 %, respectively, after 15th cycles umber. The results of microtoxicity test (growth inhabitation percentage, GI %, of three gram-positive and three gram-negative bacterial strains) showed a significant decrease in toxicity of the laccase-treated ENR and FLU solution.

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