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Improvement of synthetic dyes decolorization by immobilized laccase from *Penicillium* sp. MN749552.1.

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ABSTRACT

The discharges of various dyes throughout industrial wastes are toxic to the environment. The transformation and decolorization of dyes by fungal laccases is an alternative method for dyes detoxification before their release into environment. Thus, laccase from *Penicillium* sp. MN749552.1 was purified to homogeneity (50 kDa) and immobilized onto chitin for application in dyes transformation. The immobilized enzyme exhibited appreciable immobilization efficiency. Laccase was applied for decolorization of numerous synthetic dyes linked to the chromophore groups of diazo, heterocyclic, anthraquinone and triarylmethane. At the corresponding absorbance maxima of each dye, there was a reduction in absorbance peak after being treated with free and immobilized laccase. Remazol brilliant blue R (RBBR) and malachite green (MG) displayed peak shift toward a shorter wavelength, compared to non-degraded dye. The enzymatic decolorization strategy of RBBR, MB and MG was improved in the presence of pyrogallol as mediator. Congo red (CR) dye was not effectively decolorized by the immobilized enzyme. The addition of laccase redox mediator improved the decolorization process. The most efficient decolorization process by immobilized laccase was at pH 5.0 and 30 °C. The chitin immobilized laccase exhibited good stability during 6 repeated cycles in MG decolorization. These results showed the efficient potentiality of immobilized laccase from *Penicillium* sp. MN749552.1 in the decolorization process of the four synthetic dyes from their aqueous solutions.

Keywords: Laccase, *Penicillium* sp., Decolorization, Immobilization, Redox mediator.

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INTRODUCTION

Laccases (EC1.10.3.2) are a part of polyphenol oxidases belonging to blue multi-copper oxidases. They have the capability to contribute in the oxidation of various substrates such as polyphenol, methoxy-substituted phenols, aromatic diamines, nonphenolic dyes and amino phenols [1-3]. Laccases include three copper atoms in their catalytic site arranged close to each other developing a tri-nuclear cluster and which are responsible for their characteristic blue color [4-6].

Laccases are distributed in higher plants, bacteria and fungi. The activities of laccases have been determined in various fungal species belonging to Basidiomycetes, Ascomycetes and Deuteromycetes [7, 8]. *Monocillium indicum* was described as the first fungal strain from Ascomycetes exhibited laccase activity [9].

Fungal laccases have been employed in different biotechnological applications due to it's higher redox capacity over the plant and bacterial laccases. They are implicated in baking industry, lignin degradation, bioremediation and beverage stabilization [8, 10-11]. The applications of immobilized enzymes in industry have a particular advantage over the traditional free enzymes because of tolerance to harsh condition, easy recovery and their stability. Adsorptions, encapsulation, entrapments, cross and covalent-linking are various techniques used in immobilization of the enzymes [12, 13].

Different synthetic dyes are applied in cosmetics industry, pharmaceutical and textile. The dyes are classified according to chromophore group into several groups like diazo, anthraquinone, heterocyclic, triphenylmethane, triarylmethane and acridine. The presence of dyes in industrial wastes affects the characteristics and quality of water [14, 15].

Various restrictions reduce applications of physical and chemical methods in decolorization of synthetic dyes. Therefore, the enzymatic detoxification and decolorization of dye effluent is one of the possible challenges in our world [16-18].

Reports have attested the ability of laccase in decolorization of various dyes [19, 20]. [21] stated that some phenolic and non-phenolic compounds can act as laccase mediators during decolorization process. [22] have applied various types of laccase mediators such as coumaric acids, acetosyringone, 1-hydroxybenzotriazole and Tempo in decolorization of the azo dye acid orange 51 by laccase from *Trametes trogii.*

The present investigation aimed to (i) purify and immobilize laccase from *Penicillium* sp. MN749552.1; (ii) evaluate the potentiality of free and immobilized enzyme in the decolorization of synthetic dyes which belong to various chromophore groups; (iii) determine the influence of mediator (Pyrogallol, PG) on decolorization process of various synthetic dyes; (iv) evaluate the influence of pH and temperature on the process for some synthetic dyes using immobilized laccase.

MATERIALS AND METHODS

Chemicals

Congo red (CR), remazol brilliant blue R (RBBR), methylene blue (MB) and malachite green (MG) were obtained from Sigma-Aldrich. These dyes were included as pollutant model for measuring degradation efficiency of laccase. The structure and characteristics of tested dyes are recorded in **Table 1**. All products were of analytical grade.

Experimental microorganism

Various fungi were isolated from the soil according to [23] and grown on modified Czapek's-Dox agar containing % (sucrose 3.0; $NaNO_3$ 0.2; K_2HPO_4 0.1; $FeSO_4.7H_2O$ 0.01; KCl 0.05 and $MgSO_4$ $7H_2O$ 0.05; pH 7.0) supplemented with guaiacol (0.01%) at 28 °C for 5 days and was sub-cultured periodically for extended periods of storage [24]. The appearance of deep brown color around each fungus was used as positive indication for laccase activity. The best laccase producing fungus was taken for genetic identification.

Genetic identification of the experimental fungus

Genomic DNA was extracted for PCR on 28S rRNA with the forward primer (5'-TCCGTAGGTGAACCTGCGG-3') and reverse primer (5'-GGTCCGTGTTTCAAGACGG-3'). The sequencing results were submitted to the GenBank database for sequence alignment using Blast tool (http://www.ncbi.nlm.nih.gov). Phylogenetic tree was conducted by CLUSTAL Omega software based on the Neighbor-Joining method [25].

Preparation of laccase extract

Penicillium sp. MN749552.1 was grown in 100 ml broth medium containing % (corn steep liquor 2.0; KCl 0.07; $NH_4H_2PO_4$ 1.2; $FeSO_4$ $7H_2O$ 0.1; $MgSO_4$ $7H_2O$ 0.5) supplemented with guaiacol (0.01%) for 5 days at 28 °C on rotary shaker (180 rpm). After the incubation time, the mycelia pellets were collected through filtration using Whatman filter paper No. 1 (Whatman, Piscataway, NJ, USA) and washed thrice using distilled water for removing the culture medium. The washed mycelium was homogenized in phosphate buffer (50 mM, pH 6) for 30 min and then centrifuged for 20 min at 10,000 g under 4 °C. The obtained clear supernatant was pooled and kept at -20 °C to represent the crude extract of laccase [4, 26].

Laccase assay and protein determination

Laccase activity was determined by the method of [27] using the guaiacol (1 mM as a substrate. The reaction mixture contained of 1.5 ml of 50 mM phosphate buffer (pH 5.0), 1 ml of enzyme extract and 0.5 ml of guaiacol. The reaction was incubated at 30 °C for 15 min. The absorbance of the color was read spectrophotometrically at 450 nm after 10 min. One unit (U) of laccase activity was defined as the amount of laccase which oxidizes one µmol of guaiacol in min under assay conditions [28]. Estimation of protein was carried out according to [29]. Bovine serum albumin (BSA) was used as standard.

Enzyme purification and molecular weight determination

The purification of laccase was carried out by the modified method of [30]**.** The crude enzyme extract was treated with $(NH_4)_2$ SO_4 (75%) with constant stirring at 4 °C for 24 h and centrifuged for 15 min at 12,000 g. The resulting precipitate was collected and then dissolved in 200 mM sodium acetate buffer (pH 5.0). The solution was dialyzed overnight at 4 °C versus the same buffer, using dialysis membrane (546-00051, Viskase Sales Corporation, Wako, Japan). The dialyzed enzyme was loaded on a column of DEAE-cellulose column which was pre-equilibrated by sodium acetate buffer (pH 5.0). The active fractions were collected, dialyzed and subjected to Sephadex G-200 column which was washed with the same buffer. The eluted fractions were assayed spectrophotometrically at 450 nm.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular mass was estimated using SDS-PAGE according to [31]**.**

Laccase immobilization

Laccase (5 gm/ml) was immobilization on chitin, agar-agar, calcium alginate and chitosan according to [21, 32]**.**

Decolorization of dyes by laccase

The efficiency of decolorization for the various dyes by free and immobilized laccase was monitored using a double beam spectrophotometer (UV/visible, Cary 100, Varian). The reaction medium consists of 150 mgL^{-1} in 100 mM Na phosphate buffer at pH 6.0 and free laccase (100 U) or 5 g immobilized laccase (60 U) in 50 ml. The mixture was incubated at 30 °C at 180 rpm for 24 h for all tested dyes including CR, RBBR, MB and MG. Control samples were carried out using boiled laccase. The percentage of dye decolorization was calculated by [33]:

$$\text{Dye decolorization \%} = \frac{Ao - At}{Ao} \times 100,$$

Where A_o is the initial optical denisty of the dye and A_t denotes the final optical density of the dye at time t.

Effect of redox mediator on the dyes decolorization by immobilized laccase

The influence of laccase redox mediator (Pyrogallol, PG, 2 mM) on decolorization of the various dyes was measured under standard assay conditions.

Decolorization of dyes by immobilized laccase at different pH and temperatures

To study the influence of pH on the decolorization process of the various dyes, the immobilized laccase (5 g) was incubated at pH range from 2.0 to 9.0 using the following buffers 100 mM sodium acetate (pH 2-6) and 100 mM Tris-HCl (pH 7-9) for 24 h at 30 °C in presence of 2 mM PG as mediator. Corresponding controls were prepared with boiled enzyme, while, blanks were prepared with all reaction mixture components without the investigated dyes to observe the changes of dyes color.

The effect of various temperatures (20-60 °C) on the decolorization process, 5 g immobilized laccase was added to the incubation medium in presence of 2 mM PG at (pH 5.0) under standard assay condition. In parallel, control samples with boiled enzyme and blanks without tested dyes were used under assay conditions.

Recycling of the immobilized beads

Since MG exhibited the most efficient decolorization percent, the reusability of the immobilized laccase was investigated using 150 mgL^{-1} dye in sodium phosphate buffer (100 mM, pH 5) throughout 6 cycles. The procedure was repeated according to [12].

Statistical Analysis

Assays were performed in three replicates and all values are the mean ± standard deviation (S.D.).

RESULTS AND DISCUSSION

Laccase purification

Penicillium sp. was identified using 28S rRNA sequence and the GenBank accession no. was MN749552.1 (Figure 1). Laccase purification from *Penicillium* sp. was done as mentioned in "Materials and Methods". The purity of the enzyme from Sephadex G-200 was about 34.1-fold with a specific activity of 231.9 Umg^{-1} protein using when guaiacol as a substrate (Table 2). The laccase elution profile exhibited a single major peak, indicating the homogeneity (Figure 2). The molecular mass of the purified protein was 50 KDa as determined by SDS-PAGE (Figure 3). Laccase from *Cerrena* sp. HYB07 exhibited 40% yield and 3.1-fold of purification [34]. Laccase from *Trichoderma harzianum* was purified to homogeneity with single protein band with 25-fold and specific activity 162.5 Umg^{-1} protein [21].

Laccase immobilization

The immobilized laccase onto chitin bead showed the highest immobilization efficiency (93.4%) compared to chitosan (82.5%), Ca-alginate (77.0%) and agar-agar (59.5%) beads (Table 3). The immobilization of laccase from various sources has been studied by various investigators [21, 35, 36].

Decolorization of different dyes by free and immobilized laccase

The decolorization of several synthetic dyes diazo [CR, λ_{max} 495 nm], anthraquinone [RBBR, λ_{max} 550 nm], heterocyclic [MB, λ_{max} 650 nm] and triarylmethane [MG, λ_{max} 620 nm] by free and immobilized laccase from *Penicillium* sp. MN749552.1 was evaluated. The UV-visible spectra of the different investigated dyes were

measured (Figure 4). CR and MB dyes showed a decrease in absorbance maxima without peak shifting, compared to control sample. However, the decolorized RBBR demonstrated a slight shift toward a shorter wavelength. In case of MG dye, the treated samples with free and immobilized laccase also exhibited a shift in the wavelength from 620 nm and 550 nm. This shift toward left side wavelength may be attributed to the modification in the dye structure.

The diazo dye CR was inefficiently decolorized by free and immobilized enzyme. Such difficulty of diazo dye decolorization was found by [21, 37]. This may be due to steric hindrances of aromatic rings as well as the possible reduction of accessibility of the functional groups to laccase. However, the decolorization of other examined synthetic dyes was improved by the immobilized enzyme. For this reason, the immobilized laccase was used to study the decolorization of the different dyes in presence of PG as redox mediator. The present results are agreeable with [37] who stated that the potentiality of enzymatic decolorization of the different dyes was improved by the immobilized laccase. The structure, simplicity, size and the redox potential of dyes are crucial factors to determine the ease of decolorization [38].

Effect of redox mediator (Pyrogallol) decolorization process by free and immobilized laccase

Decolorization of synthetic dyes from different chromophore groups by immobilized laccase in the presence or absence of 2 mM redox mediator (Pyrogallol, PG) was evaluated (Figure 5). These results illustrate that the enzymatic decolorization of both CR and RBBR dyes in presence of PG redox as mediator, exhibited considerable changes in the decolorization percentage during 24 h incubation. In the case of the heterocyclic dye, the immobilized laccase exhibited 20% decolorization without redox mediator. However, the presence of 2 mM PG as mediator improved the decolorization approximately by 3.7-fold after 24 h incubation. The triarylmethane dye presented the most effective decolorization percentage. In support, it was reported that the immobilized laccase displayed significant increase in the decolorization effectiveness in presence of 1-hydroxybenzotriazole (HBT) as redox mediator [21]. In addition, [39] observed the enhancement of dye decolorization through the participation of support material and enzyme catalysis.

Effect of different pH on decolorization process by the immobilized laccase-pyrogallol system

The decolorization of the tested dyes using the laccase-pyrogallol system was assayed as a function of pH exhibited its optimum at 5.0 (Figure 6). The higher decolorization percentage was observed for the tested dyes at pH 5.0 comparing to other pH values. Thus, the variation in pH has a negative impact on the decolorization process. These results are in harmony with the reports for *Trametes modesta* -laccase system which exhibited the most decolorization under pH 3–6 [40]. In addition**,** [15] reported an effective decolorization of dyes under acid conditions. Laccase was more stable under acid conditions [41].

Effect of different temperatures on decolorization process by the immobilized laccase in presence of pyrogallol system

The laccase-PG-dye decolorization systems were remarkably affected by the variation in the incubation temperature of reaction mixture (Figure 7). All the investigated dyes were decolorized effectively at various temperatures as compared to control. However, the efficiency of decolorization using immobilized laccase from *Penicillium* sp. MN749552.1 was remarkably increased at 30 °C. Textile dyes decolorization by laccase was elevated with rising the reaction temperature [40]. The most efficient decolorization of thirteen dyes belonging to different chromophore groups was at 35 °C [15].

Reusability of the immobilized laccase-pyrogallol system in the decolorization of malachite green

The decolorization of MG by the immobilized laccase in the presence of laccase mediator (PG) was assayed under the standard assay conditions. Generally, the dye decolorization declined more slowly during the elevation in the number of cycles as shown in Figure 8. Diffusion of enzyme from immobilization support and its inactivation may be the reasons of the reduction in decolorization activity. The high recycle stability of laccase has been found by [42, 43]**.**

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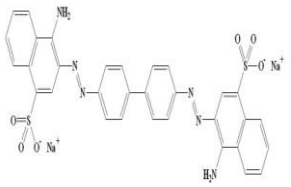
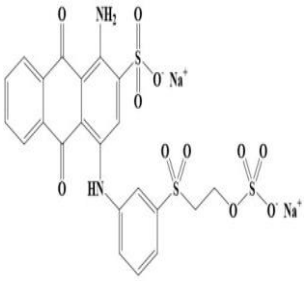
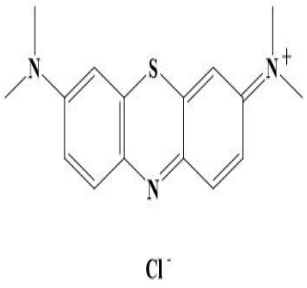
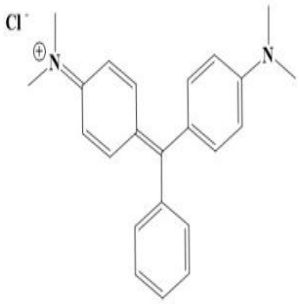
Table 1. Structures and characteristics of various synthetic dyes used in decolorization assays with laccase from *Penicillium* sp. MN749552.1 .

Dyes	Structure	Chemical formula	Classification	Molar mass	λ_{max}
Congo Red (CR)		$C_{32}H_{22}N_6Na_2O_6S_2$	Diazo	696.66	495 nm
Remazol Brilliant Blue R (RBBR)		$C_{22}H_{16}N_2Na_2O_{11}S_3$	Anthraquinone	626.54	550 nm
Methylene Blue (MB)		$C_{16}H_{18}ClN_3S.xH_2O$	Heterocyclic	319.85	650 nm
Malachite Green (MG)		$CH_{25}ClN_2$	Triarylmethane	364.91	620 nm

* λ_{max} is the absorbance maxima corresponding to each particular dye used in decolorization scanning assays with dye concentration of 150 mgL^{-1}.

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Table 2: Procedure of laccase purification from *Penicillium* sp. MN749552.1.

Purification stage	Total Activity (U)	Total protein (mg)	Specific activity (Umg^{-1})	Yield (%)	Purification fold
Crude extract	246 ± 1.9	36 ± 0.8	6.8 ± 0.1	100	1
$(NH_4)_2SO_4$ (75%)	220.8 ± 1.8	26.4 ± 0.4	8.3 ± 0.2	89.8 ± 1.1	1.2 ± 0.01
DEAE-cellulose	150.2 ± 1.6	3.4 ± 0.02	44.2 ± 0.5	61.1 ± 1.0	6.5 ± 0.2
Sephadex G-200	122.9 ± 2.3	0.53±0.03	231.9±1.8	50.0 ± 0.9	34.1 ± 0.9

Table 3: Immobilization of the purified laccase on different beads.

Beads	Added activity (Umg^{-1} protein)	Immobilized activity (Umg^{-1} protein)	Immobilization efficiency (%)
Chitin	60 ± 1.1	56.04 ± 0.9	93.4 ± 1.6.
Agar-agar	60 ±1.1	35.7 ± 0.6	59.5 ± 1.0
Calcium alginate	60 ±1.1	46.2 ± 0.8	77.0 ± 1.2
Chitosan	60 ± 1.1	49.5 ±0.7	82.5 ± 1.3

Figure (1): Phylogenetic tree of the *Penicillium* sp. MN749552.1. based on 28S rRNA.

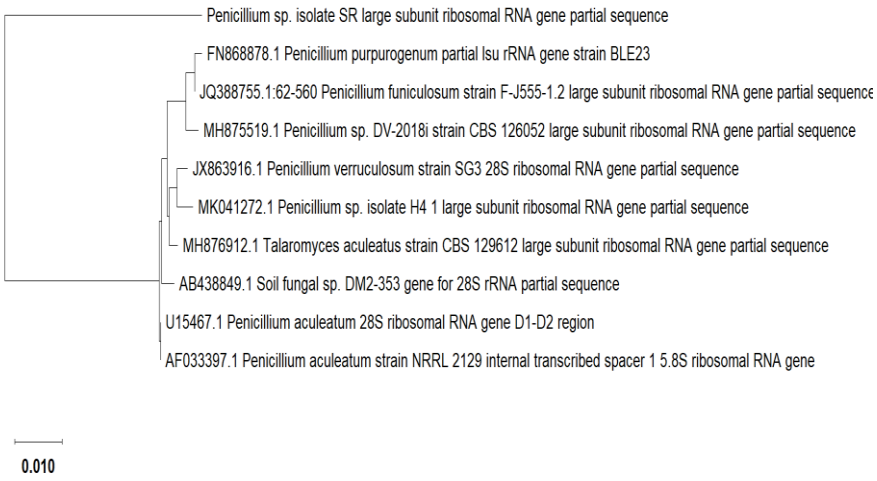

Figure (2): Elution profile of laccase from *Penicillium* sp. MN749552.1 eluted from Sephadex G-200.

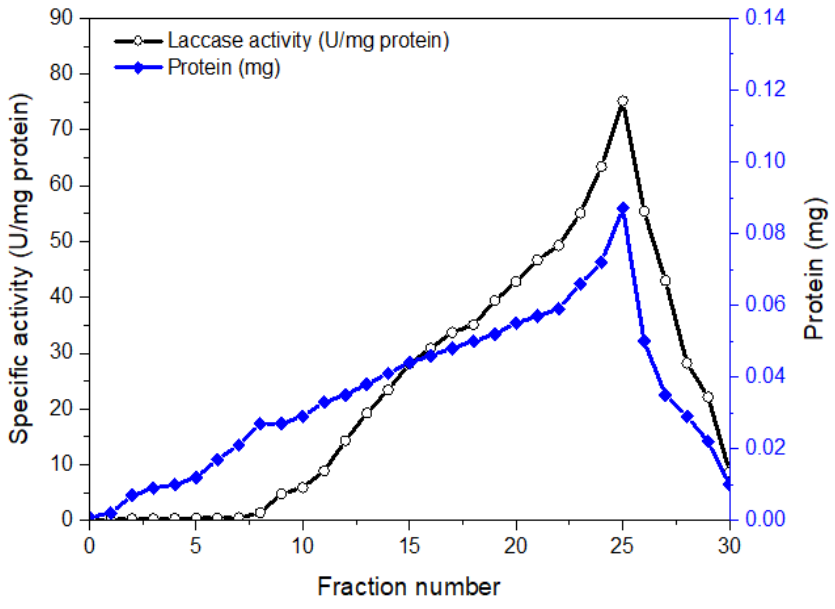

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Figure (3): SDS-PAGE of purified laccase produced by *Penicillium* sp. MN749552.1. Lane M: Markers and Lane PE: Purified enzyme.

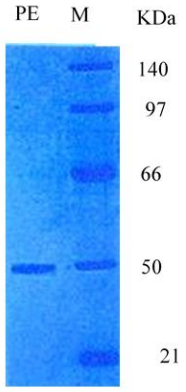

Figure (4): Variation in the UV-Visible absorption spectra of synthetic dyes: (A) congo red; (B) remazol brilliant blue R; (C) methylene blue; (D) malachite green. The concentration of dyes in the starting solution was 150 mgL^{-1} and was incubated at 30 °C and 24 h. Control dyes were monitored at 0 h.

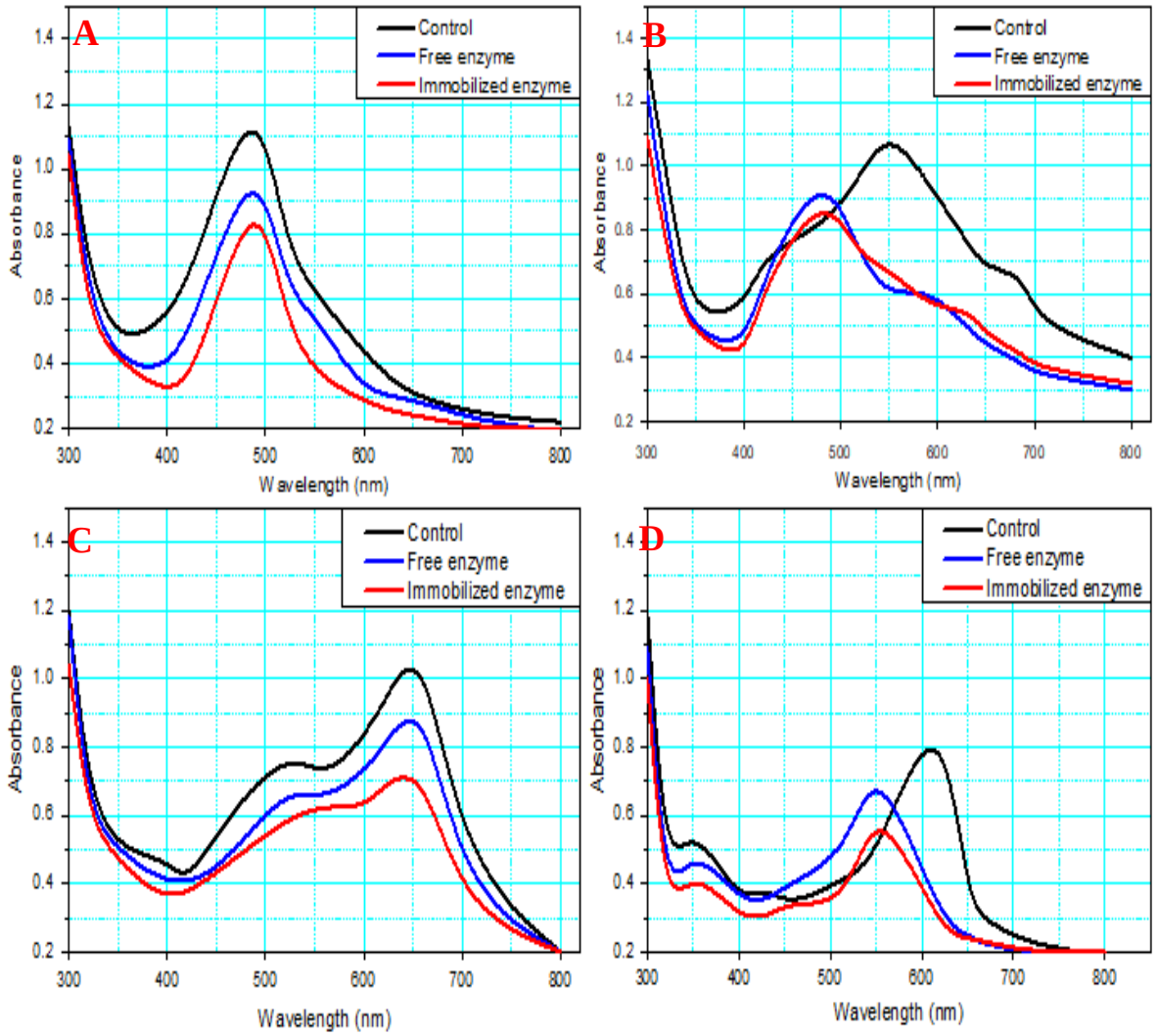

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Figure (5): Synthetic dyes decolorization with immobilized laccase (IL) in presence or absence of 2 mM PG-redox mediator. (A) CR; (B) RBBR; (C) MB; (D) MG were incubated at 30 °C for 24 h.

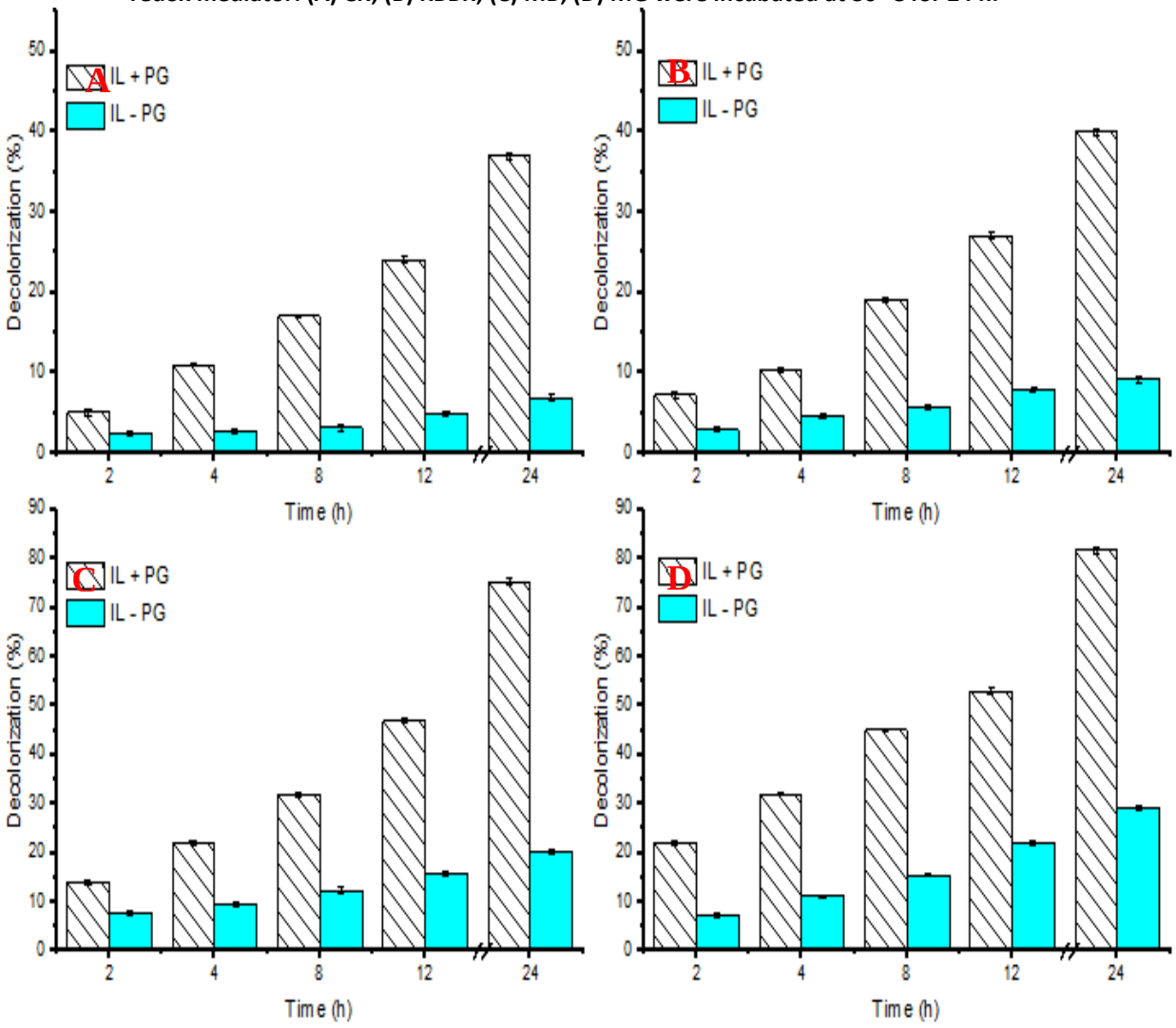

Figure (6): Synthetic dyes decolorization by immobilized laccase obtained from *Penicillium* sp. MN749552.1 at pH range of 2-9 in the presence of pyrogallol. The reaction mixture was incubated at 30 °C for 24 h.

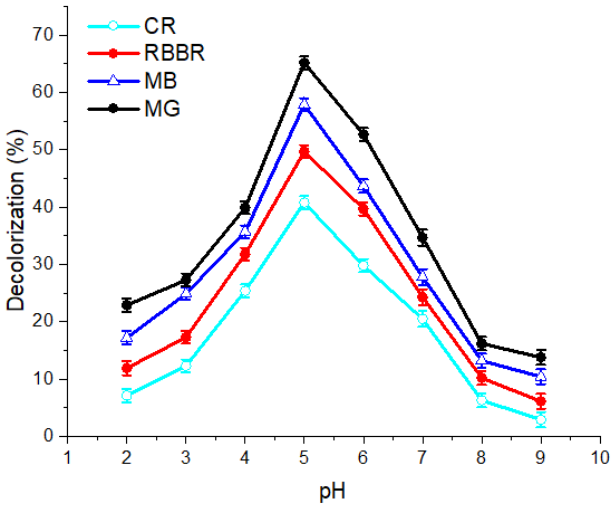

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Figure (7): Effect of temperature on different dyes decolorization by the *Penicillium* sp. MN749552.1 laccase–PG system. The reaction mixture was incubated at pH 5.0 for 24 h in the presence of 2 mM PG.

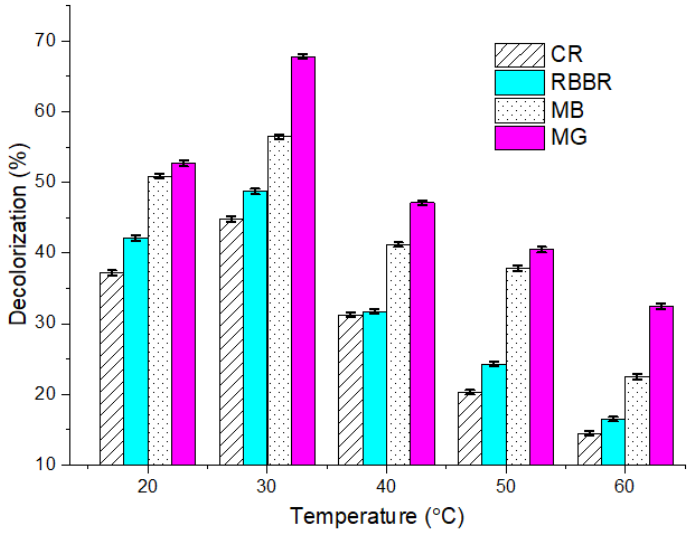
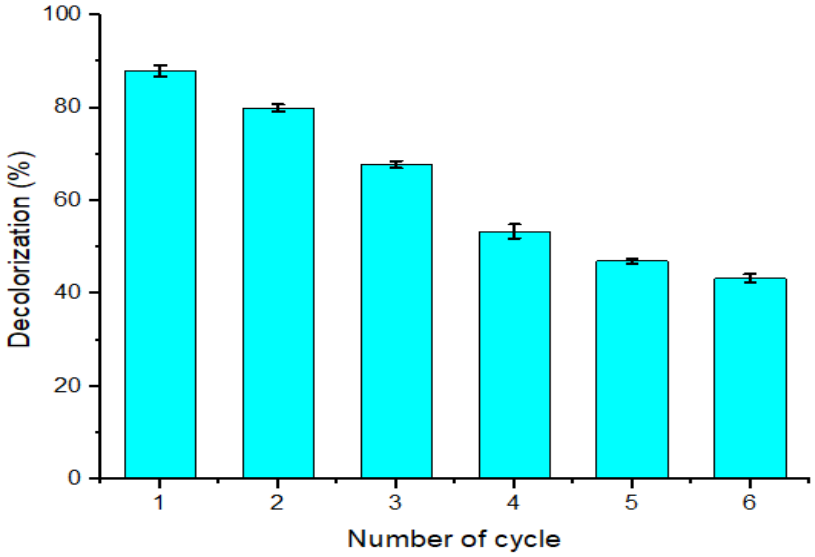


Figure (8): Reusability of the chitin immobilized laccase in the malachite green decolorization in the presence of 2 mM PG-redox mediator. Each cycle have 50 ml of the 150 mgL^{-1} fresh dye and incubated at pH 5.0, 180 rpm and 30 °C for 24h.



CONCLUSIONS

The purified laccase was successfully immobilized onto chitin beads and was further employed for degrading various synthetic dyes related to different groups of diazo, anthraquinone, heterocyclic and triarylmethane. Compared to the non-degraded dyes and free enzyme, immobilized laccase exhibited a remarkable reduction in absorbance peak of all tested dyes. When the degradation solution was supplemented with pyrogallol as redox mediator, the enzymatic decolorization of different synthetic dyes was enhanced particularly in case of Congo red dye. The decolorization efficiency has been affected by the variation of both the pH and temperature of the reaction mixture. The reusability experiments showed good stability of immobilized enzyme during six repeated cycles without a serious reduction in the decolorization percentage of malachite green dye. In conclusions, this study confirms the potential use of chitin immobilized laccase in the removal of different synthetic dyes from its aqueous solution.

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