Iğdır Üniversitesi Fen Bilimleri Enstitüsü Dergisi, 14(1), 333-344, 2024
Journal of the Institute of Science and Technology, 14(1), 333-344, 2024

ISSN: 2146-0574, eISSN: 2536-4618

Kimya / *Chemistry* **DOI: 10.21597/jist.1354190**

Araştırma Makalesi / *Research Article*

Geliş tarihi / *Received*: 06.09.2023 **Kabul tarihi / *Accepted*: 12.10.2023**

Atıf İçin: Öztürk Kesebir, A., Şişecioğlu, M. Adıgüzel, A. Kılıç, D. ve Küfrevioğlu, Ö.İ. *(*2024). *Bacillus licheniformis* O12'den Yeni Bir Termostabil Lakkazın Tek Adım Afinite Kromatografisi Kullanılarak Saflaştırılması, Karakterizasyonu ve Renk Giderme Potansiyeli. *Iğdır Üniversitesi Fen Bilimleri Enstitüsü Dergisi*, 14(1), 333-344.

To Cite: Öztürk Kesebir, A., Şişecioğlu, M. Adıgüzel, A. Kılıç, D. ve Küfrevioğlu, Ö.İ. (2024). Purification and Characterization of a New Thermostable Laccase from *Bacillus licheniformis* O12 Using One-Step Affinity Chromatography and Its Potential for Decolorization. *Journal of the Institute of Science and Technology*, 14(1), 333-344.

Bacillus licheniformis O12'den Yeni Bir Termostabil Lakkazın Tek Adım Afinite Kromatografisi Kullanılarak Saflaştırılması, Karakterizasyonu ve Renk Giderme Potansiyeli

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Öne Çıkanlar:
- Lakkazın tek adımda saflaştırılmasında yeni bir afinite kolonu kullanıldı
- İzole edilen lakkaz çok yüksek sıcaklıklarda yüksek aktivite gösterdi
- İzole edilen lakkazın çok yüksek decolarizasyon yeteneğinin renk giderim olduğu gözlendi

Anahtar Kelimeler:
- Bacillus licheniformis
- Lakkaz
- Afinite Kromatografisi
- Karakterizasyon
- Renk giderimi
- Saflaştırma

ÖZET:

Lakkazlar çok çeşitli substratları oksitleyebilen bakır içeren enzimlerdir. Lakkazın bu özelliği sayesinde çevre kirliliğine neden olan bazı boyarmaddelerin renk giderimi yapılabilmektedir. *Bacillus licheniformis* gibi bazı bakteriler doğal olarak lakkaz enzimini üretir. Çalışmada lakkaz saflaştırması için yeni bir afinite kolonu test edilmiştir. Bu amaçla uygun ortamda yetiştirilen bakterilerin ürettiği hücre dışı lakkaz sefaroz 4B-L-tirozin-ρ-aminobenzoik asit afinite kromatografisi yöntemiyle izole edildi. Saflığı SDS-PAGE yöntemiyle kontrol edildi. *B. lichenisformis* O12'den afinite kolonu ile izole edilen lakkazın tekstil atık suyundaki bazı boyarmaddelerin renk giderici etkisi araştırıldı. Bu prosedürde herhangi bir mediyatör kullanılmadı. Sonuç olarak lakkaz %38,3 verimle 4,82 kat saflaştırıldı. Saflaştırılan enzimin moleküler ağırlığı SDS-PAGE yöntemiyle ~70 kDa olarak belirlendi. Enzim, pH 4.0'da ve 92°C sıcaklıkta optimum aktivite gösterdi. Enzimin, 60°C ve 92°C'de 12 saatlik inkübasyondan sonra bile %100 aktivitesini koruduğu görüldü. Kinetik parametreler ABTS, 2,6-DMP ve guaiacol gibi lakkaz substratları ile belirlendi. Ortama sadece izole edilen lakkaz eklenerek, herhangi bir redoks aracısı kullanılmadan %35 Reaktif siyah, %31 Asit siyahı 1, %28 Metilen mavisi ve %15 Asit kırmızısı 27 boyar maddelerinde renk giderimi elde edildi. *B. licheniformis* O12 lakkaz enziminin bu özellikleri, onu çeşitli biyoteknolojik ve endüstriyel uygulamalarda kullanım için potansiyel bir aday enzim haline getirmektedir.

Purification and Characterization of a New Thermostable Laccase from Bacillus licheniformis O12 Using One-Step Affinity Chromatography and Its Potential for Decolorization

Highlights:
- A new affinity column was used to purify the laccase in one step
- The isolated laccase showed high activity at very high temperatures
- It was observed that the isolated laccase had a very high decolarization ability

Keywords:
- Bacillus licheniformis,
- Laccase
- Affinity chromatography
- Characterization
- Decolorization
- Purification

ABSTRACT:

Laccases are copper-containing enzymes that can oxidize a wide variety of substrates. Thanks to this feature of laccase, some dyes that cause environmental pollution can be decolorized. Some bacteria, such as *Bacillus licheniformis*, naturally produce the enzyme laccase. A new affinity column was tested in this study. For this purpose, the extracellular laccase sepharose 4B-L-tyrosine-ρ-aminobenzoic acid produced by bacteria grown in suitable media was isolated by affinity chromatography method. Its purity was checked by SDS-PAGE method. The decolorization effect of some dyestable in textile wastewater of laccase isolated from *B. lichenisformis* O12 by affinity column was investigated. No mediator was used in this procedure. .As a result, laccase was purified 4.82-fold purification with a yield of 38.3% respectively, The molecular weight of the purified enzyme was determined as ~70 kDa by the SDS-PAGE method. The enzyme showed optimum activity at pH 4.0 and temperature 92°C. The enzyme was found to retain 100% activity even after 12 hours of incubation at 60°C and 92°C. The kinetic parameters were determined with laccase substrates such as ABTS, 2,6-DMP, and guaiacol. The purified laccase was decolorized with varied efficiencies such as 35% of Reactive black, 31% of Acid black 1, 28% of Methylene blue, and 15% of Acid red 27 without the use of any redox mediators. These properties of *B. licheniformis* O12 laccase enzyme make it a potential candidate enzyme for use in various biotechnological and industrial applications.

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INTRODUCTION

Laccases (EC 1.10.3.2) are copper-containing enzymes that catalyze the oxidation of a wide variety of compounds such as aromatic substances, organic, and inorganic substrates using molecular oxygen (Giardina et al., 2010; Jaiswal et al., 2015). They are usually found in plants, fungi, and bacteria (Dwivedi et al., 2010) Laccase was first purified from Rhus vernicifera, a Japanese plant, but most of the works have on fungi and bacteria (Yoshida 1883, Forootanfar et al., 2015). Bacterial laccases, unlike fungal laccases, which are very sensitive to high temperature and high pH, have some important properties such as activity at alkaline pH and high temperature, less dependence on metal ions, and less sensitivity to inhibitory substances (Santhanam et al., 2011, Singh et al., 2011). Laccases have recently attracted attention because of their use in pharmaceutical synthesis, organic synthesis, decolorization of textile dyes, biosensor development, ethanol production, food processing, xenobiotic degradation, biobleaching, and biopulping (Jaiswal et al., 2015, Dwivedi et al., 2010]. Most of the laccase enzymes isolated showed either low enzyme activity yield or sensitivity to extreme pH, temperature, metal ions conditions. This results in a loss of catalytic activity that limits major commercial and industrial processes. Therefore, isolating and characterizing novel bacterial laccases that are resistant to extreme conditions will help to increase their use for industrial applications. Heat-stable laccases can easily be used for the biotech bleaching of pulp and for the processing of colored industrial waste (Asgher et al., 2008). Some obstacles to the application of laccases in the biotechnology industry are neutral and alkaline pH and high metal ion content in wastewater (Murugesan et al., 2009; Xiao et al., 2012). Therefore, the use of alkaline pH-active laccases is another green technology alternative chemically as well as in many industrial applications such as the cosmetic industry, the textile industry, and waste water treatment (Dube et al., 2008, Saito et al., 2012). Laccase enzyme has been purified by many different methods such as ammonium sulfate precipitation and ion-exchange chromatography (Mehandia et al., 2020), gel filtration chromatography (Bozoglu et al., 2013; Rudakiya et al., 2020), affinity chromatography (Jaiswal et al., 2015; Sadeghian-Abadi et al. 2019), and three-phase partitioning (TPP) method (Kumar et al., 212). The high costs associated with the purification of laccase constitute one of the main problems limiting the industrial scale of this enzyme (Idris et al., 2017). Affinity chromatography, one of the most powerful separation techniques that provide efficient purification of biological molecules with high efficiency, purity, and activity, involves a small number of process steps and the processing time is short. It is suggested that the use of affinity chromatography in industrial applications can significantly facilitate the process and at the same time make it more cost-effective (Sadeghian-Abadi et al. 2019; Rezaei et al., 2017). We used sepharose 4B-L-tyrosine-ρ-aminobenzoic acid affinity chromatography to purify the laccase enzyme. This method was the first time tried for the laccase, our claim was to obtain the enzyme in one step. In our study, we showed that this method can be used for the laccase enzyme. In this study, the laccase from *Bacillus licheniformis* O12, which is known to be thermophilic, was purified with sepharose 4B-L-tyrosine-ρ-aminobenzoic acid affinity column and characterized. The potential of the purified laccase in the decolorization of dyes was also investigated.

MATERIALS AND METHODS

Chemicals And Materials

All chemicals were purchased from Sigma–Aldrich. The bacteria strain (*B. licheniformis* O12, Fig 1) used in the study was isolated and identified by Baltaci et al. 2017 (Baltaci et al., 2017)

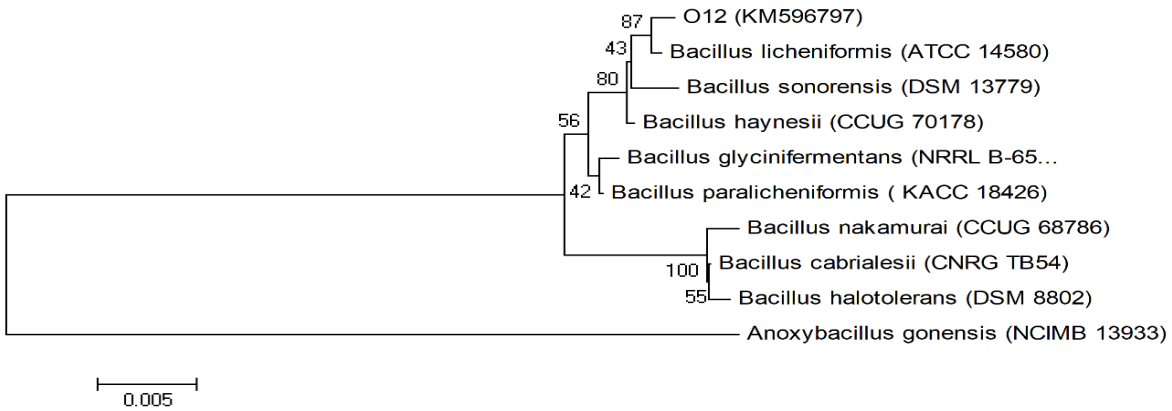

Figure 1. Phylogenetic tree constructed with neighbor joining method based on 16S rRNA gene sequence data of the *B. licheniformis* O12 strain. Anoxybacillus gonensis (NCIMB 13933) was used as out-group

Enzyme Activity Assay And Protein Determination

The laccase activity was determined by monitoring the oxidation of 2,2-azino-bis- (3-ethylbenzthiazoline-6-sulfonate) (ABTS) substrate. The activity mixture contained 2 mM Na-acetate buffer (pH 5.0), 1 mM ABTS and enzyme. This mixture was incubated at 55°C for 15 minutes and then absorbance at 420 nm was measured (ε_{420}= 36.000 1/M.cm). One unit of enzyme activity was defined as the amount of enzyme required to oxidase 1mM of ABTS per minute (Heinfling et al., 1998). Protein concentration was determined by the Bradford method and used bovine serum albumin as the standard (Bradford 1976).

Production and Purification Of Laccase Enzyme

Two different media were of used for laccase production and different concentrations of copper were added to these broth solutions. It was determined that the enzyme produced by the bacteria incubated for 4 days in the presence of 10 mM $CuSO_4$ in NB (Nutrient Broth) medium showed the best activation. Then, the broth solution was centrifuged at 13,000 xg for 30 minutes and the supernatant was removed. The supernatant was applied to the sepharose 4B-L-tyrosine-ρ-amino benzoic acid affinity column previously equilibrated and washed with 0.05 M Na_2HPO_4 pH 5.0 buffer. Elution was performed by preparing a 0.8 M ascorbic acid solution in tris-HCl buffer pH 8.5. Laccase activity and protein concentration were determined in eluates.

SDS- PAGE and Native PAGE Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was applied for the determination of molecular weight and control of purified laccase enzyme. SDS-PAGE was prepared according to the Laemmli method (Laemmli 1970). Protein bands were stained with Coomassie brilliant blue R250. Native PAGE analysis was done by using the same protocol used for SDS-PAGE without boiling the sample and without the use of denaturing agents (SDS, β-mercaptoethanol). For the confirmation of the laccase activity, the gel was incubated with activity buffer (2 mM Na-acetate buffer, pH 5.0) containing 5 mM ABTS in the dark until the green color has been observed (Afreen et al., 2017). The standard protein molecular weight markers (PageRegulerTM Prestained Protein Ladder) were used for analyzing protein samples.

Effect of pH on Activity and Stability of the Purified Laccase

The optimum pH for the laccase was determined in the pH range of 2.0–10.5 using different buffers. The pH stability of the laccase was studied by pre-incubating the enzyme in the pH 5.0 and 8.0 for 2 h. The residual laccase activity was calculated under standard assay conditions. The buffers (0.5

mM) used were glycine-HCl (pH 2.0-3.0), sodium acetate (pH 4.0–5.0), sodium phosphate (pH 6.0-8.0), and glycine-NaOH (pH 9.0–10.5).

Effect of Temperature on Activity and Stability of the Purified Laccase

The maximum temperature for the laccase enzyme was determined by performing the standard enzyme assay at different temperatures ranging from 30 to 92°C. The thermostability of the laccase was determined by pre-incubating the enzyme at 60 and 92°C for 12 hours and the residual activity was measured at various time intervals. The activity of the non-heated enzyme was considered as a control (100%).

Effect of Metal Ions, Various Chemical Reagents and Organic Solvents on Laccase Enzyme Activity

The effect of different metal ions (Al^{3+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+}, Cr^{2+}, Mn^{2+}, Zn^{2+}, Pb^{2+}), organic solvents (ethanol, methanol, 1-propanol, t-butanol, acetone, DMSO), surfactants (Tween-20, Tween-80, Triton X-100, SDS), oxidation agent (H_2O_2), and some chemicals (NaN_3, NaF, EDTA) on the purified laccase activity was investigated. For this purpose, 5 different concentrations of metal ions were added and activity was measured (1-5 mM). Organic solvents, inhibitors, surfactants, and H_2O_2, whose inhibition effect on laccase will be examined, were prepared at 1 mM concentration. It was added to the activity measurement medium at 5 different concentrations, and the activity change was measured under standard conditions.

Kinetic Assays

Kinetic properties of the laccase were investigated using different concentrations of ABTS, 2,6-dimethoxyphenol, and guaiacol. K_m and V_{max} values were determined from the Lineweaver-Burk plot. In addition, K_{cat} and K_{cat}/K_m were also calculated for each substrate.

Decolorization of Dyes by Using Laccase Enzyme from *B. licheniformis* O12

The ability of laccase to decolorization of azo dyes including Reactive black 5 (597 nm), Orange (622 nm), Acid red 27 (520 nm), Acid black 1 (620 nm), Congo red (350 nm), and Methylene blue (660 nm) was investigated. Decolorization experiments were carried out by incubating the enzyme with dyes for 15, 30, 60, 90, and 120 min absence of any mediator. Enzyme-free dye solutions were taken as a control. The percentage of decolorization was determined spectrophotometrically at respective of dyes maximal wavelengths (Lorenzo et al., 2006).

RESULTS AND DISCUSSION

Production and Purification of Laccase Enzyme

In order to obtain the laccase enzyme from *B. licheniformis* O12, we prepared two different media, adding different concentrations of $CuSO_4$ to these broths. Then we left it in a 55°C shaker, took some of the supernatant each day, centrifuged at 13,000 xg for 30 minutes and measured the activity. It was determined that bacteria growing on TSB medium containing 10 mM $CuSO_4$ for 4 days produced laccase enzyme at maximum yield (λ=0.943). The extracellular laccase obtained after centrifugation of the cultured medium was purified by sepharose 4B-L tyrosin-ρ-aminobenzoic acid affinity column. At the end of this process, the enzyme was purified to 4.82 fold with a yield of 38.3% (Table 1).

Table 1. Summary of the purification of the laccase from *B. licheniformis* O12

Purification Step	Volume (mL)	Total activity (U/mL)	Total protein (mg/mL)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	50	0.84	7678	0. 11	100	1
Affinity chromatography	7	0.32	607.32	0. 53	38.30	4.82

SDS-PAGE and native-PAGE methods were used to evaluate enzyme purity. The molecular weight of the purified enzyme was calculated by SDS-PAGE and found to be ~70 kDa enzyme. A single protein band was obtained for the purified enzyme (Fig 2). The purified enzyme was confirmed by native-PAGE analysis using ABTS as a substrate (Fig 2).

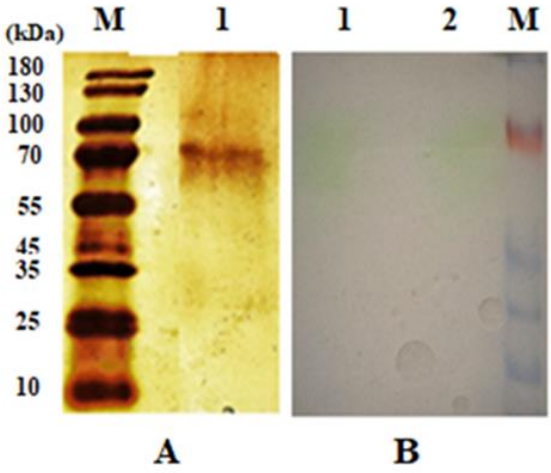

Figure 2. SDS-PAGE (A) and Native-PAGE (B) analysis of laccase from *B. licheniformis* O12.

M: Standard protein markers; A: Lane 1, The purified laccase from affinity chromatography; B: Lane 1-2, The activity staining band of purified laccase with ABTS

Effect of pH on Laccase Enzyme Activity and Stability

The pH profile of the *B. licheniformis* O12 laccase was determined in different buffers by varying the pH values. The laccase enzyme showed maximal activity at pH 4.0. It was observed that the *B. licheniformis* O12 laccase enzyme was active over a wide range of pH 2.0-10.5, the enzyme activity gradually decreased after pH 8.0 and still retained 79.61% of its activity at pH 10.5 (Fig 3). For investigation of pH stability, the laccase enzyme was incubated at acidic (pH 5.0) and alkaline (pH 8.0) pH values for 2 hours. At pH 5.0 and 8.0, the enzyme showed high stability (Fig 3).

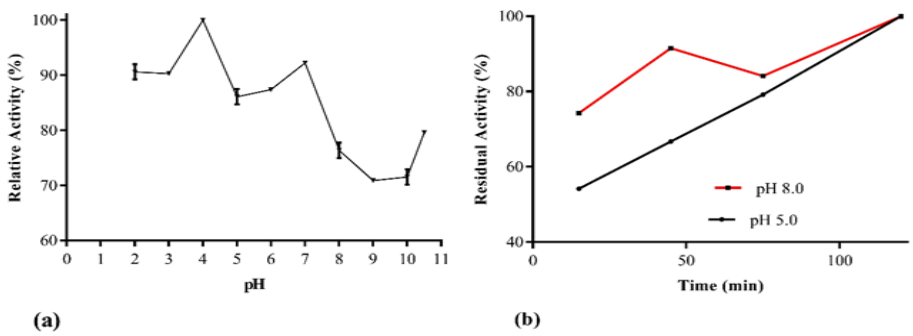

Figure 3. Effect of pH on activity (a) and stability (b) of laccase from *B. licheniformis* O12

Effect of Temperature on Laccase Enzyme Activity and Stability

The effect of temperature on *B. licheniformis* O12 was investigated at various temperatures between 30°C and 92°C. The optimum activity of the laccase enzyme was observed at 92°C; the enzyme was maintained more than 70% of its activity over a wide range of temperature (Fig 4).

The thermostability of the purified laccase was investigated by incubating the enzyme at 92°C (optimum temperature) and 60°C for 0 h to 12 h. As seen in Fig 4, the laccase activity increased with time at 92°C. The laccase enzyme retained approximately 100% of its initial activity at these temperatures even after 12h of incubation (60°C and 92°C).

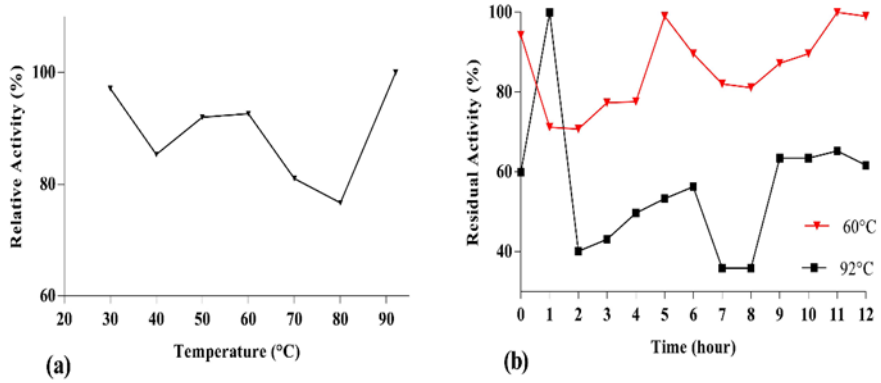

Figure 4. Effect of temperature on activity (a) and stability (b) of laccase from *B. licheniformis* O12

Effect of Metal Ions, Various Chemical Reagents and Organic Solvents on Laccase Activity

The effect of various effectors on laccase from *B. licheniformis* O12 was studied and is summarized in Table 2. It has been observed that the laccase enzyme is highly resistant to all the metal ions used. The highest inhibition was up to 40% with Pb^{2+} metal. Al^{3+}, Fe^{2+}, Hg^{2+}, and Cr^{2+} activated laccase enzyme in a concentration-dependent manner (1–5 mM). Where textile wastewater has high salt content, the effect of concentration-dependent metal ions can be significant (Kesebir et al., 2021). When organic solvents and surfactants are examined, it is seen that there is an inhibition of approximately 80%. As it can be observed from Table 2, the laccase enzyme was inhibited in the presence of non-ionic surfactants, (Tween-20, Tween-80, Triton X-100) whereas activated in the presence of anionic surfactant (SDS). In the earlier reports, it has been reported that the activity of laccase was enhanced by SDS (Mehandia et al., 2020; Frang et al., 2011). When H_2O_2 was examined, a 24% inhibition was observed. We also observed enzyme stability for more than 2 hours in the presence of various chemicals (Table 2). No inhibition was observed in NaF, NaN_3, and EDTA, which were considered laccase inhibitors. NaN_3 and NaF are generally effective inhibitors of laccases, EDTA does not show a strong inhibitory effect unless it is in a very high concentration (Sunil et al., 2011; Yang et al., 2014; (Jean and Lim 2017; Mehandia *et al.*, 2020). However, the laccase enzyme we obtained in our study was found to be highly resistant to these inhibitors. Similarly, Streptomyces viridochromogenes laccase has also been reported to be highly resistant in the presence of NaN_3 and NaF. Although the mechanism is unknown, it has been shown that the two-domain laccases are generally resistant to inhibitor NaN_3 (Trubitsina et al., 2015).

Table 2. Effects of metal ions, organic solvents, surfactants, and inhibitory agents on the laccase enzyme from *B. licheniformis* O12

(The measurement of organic solvents was monitored for 120 minutes.)

Effectors							
				Residual Activity (%)			
	Control	**1 mM**	**2 mM**	**3 mM**	**4 mM**	**5 mM**	
Al^{3+}	100	407.1±0.002	442.7±0.003	328.6±0.001	435.71	435.71	
Cd^{2+}	100	86.9±0.001	64.28	71.43±0.001	86.52±0.001	105.95	
Co^{2+}	100	78.81±0.057	76.43±0.056	129.76±0.001	102.38±0.001	104.76±0.001	
Cr^{2+}	100	123.81±0.001	166.66±0.002	247.62±0.001	328.57±0.006	369.05±0.001	
Cu^{2+}	100	107.14	107.14	80.95±0.001	114.29	107.14	
Fe^{2+}	100	125.95±0.001	157.14±0.002	185.71	311.91±0.027	226.19±0.002	
Hg^{2+}	100	107.14	145.24±0.001	169.29±0.001	157.14±0.002	183.34±0.001	
Mn^{2+}	100	57.14	65.48±0.001	74.29±0.001	89.29±0.001	83.1±0.001	
Pb^{2+}	100	59.5±0.001	71.43	76.2±0.001	92.90	83.6±0.001	
Zn^{2+}	100	87±0.001	87.14±0.001	86.7±0.001	83.34±0.001	97.14±0.001	
	Control	**15 min**	**45 min**	**90 min**	**120 min**	**135 min**	**165 min**
Ethanol	100	18.7±0.58	19.47±0.15	22.03±0.25	21.1±0.12		
Methanol	100	20.54±0.5	22.35±0.14	26.8±0.38	29.15±0.27		
1-propanol	100	19.4±0.36	22.16±0.14	26.3±0.3	30.3±0.61		
t-butanol	100	21.98±0.03	23.37±0.16	25.97±0.46	28.61±0.25		
Acetone	100	20.74±0.23	21.6±0.09	20.87±0.22	21.5±0.5		
H_2O_2	100	40.3±0.58	55.7±0.61	67.86±0.77	76.98±0.24		
Tween-20	100	17,377±0,14	18,72±0,027	15,1±0,66	21,41±0,53		
Tween-80	100	21.1±0.36	22.36±0.21	20.8±0.35	21.07±0.12		
Triton X-100	100	25.3±0.29	17.23±0.03	14.1±0.17	9.61±0.11		
SDS	100	124.7±0.6	133.7±0.31	161.9±1.8	166±1.1	178.96±0.34	185.5±0.8
	Control	**15 min**	**45 min**	**75 min**	**105 min**	**135 min**	**165 min**
EDTA	100	135.8±0.6	124.6±2.3	123.9±2.9	135±1.8	144.9±2.8	156.7±1.6
NaF	100	119.6±1.3	1343±2.6	144.8±1.37	144.16±0.7	159.2±2.31	166.6±1.1
NaN_3	100	119.4±0.26	138±2.8	140±1.8	143.8±1.6	184.4±1.8	195.5±2.04

Kinetic constants of laccase

The results of kinetic parameters for the oxidation of ABTS, 2,6-DMP, and guaiacol are shown in Table 3. The K_m and V_{max} values were found to be 0.0075, 0.0222, and 14300 mM, and 0.0103, 0.0033, and 0.0082 μM/min for ABTS, 2,6-DMP, and guaiacol, respectively. Laccase enzyme showed a higher affinity for ABTS substrate than 2,6-DMP and guaiacol. The lowest interest was in the guaiacol substrate.

Table 3. Kinetic properties of laccase from *B. licheniformis* O12 for various substrates

Substrates	**K_m (mM)**	**V_{max} (μM/ min. μL)**	**K_{cat} (1/min)**	**K_{cat}/K_m (1/mM min)**
ABTS	0.008	0.010	0.032	4.26
2,6-DMP	0.022	0.003	0.010	0.46
Guaiacol	14300	0.008	0.026	0.0000018

Decolorization of Dyes by Using the Purified Laccase

The decolorization of various azo dyes was investigated using laccase from *B. licheniformis* O12. The efficiency of decolorization was obtained in the order of Reactive black 5 (35.1%) > Acid black 1

(31.2%) > Methylene blue (28.32%) > Acid red 27 (15.45%) (Table 4 and Fig 5). Decolorization was not observed in Congo red and Orange dyes. The different decolorization rates can be seen depending on the specific catalytic properties of the enzymes and the structure of the dyes (Kumar et al., 2012).

Table 4. Decolorization of dyes with laccase from *B. licheniformis* O12

	Decolorization (%)			
Dyes	**15 min**	**60 min**	**90 min**	**120 min**
Acid black 1	5.25	6.5	11.46	31.2
Acid red 27	6	14.1	14.4	15.45
Reactive black 5	7.9	16.07	18.9	35.1
Methylene blue	12.16	22	26.3	28.32

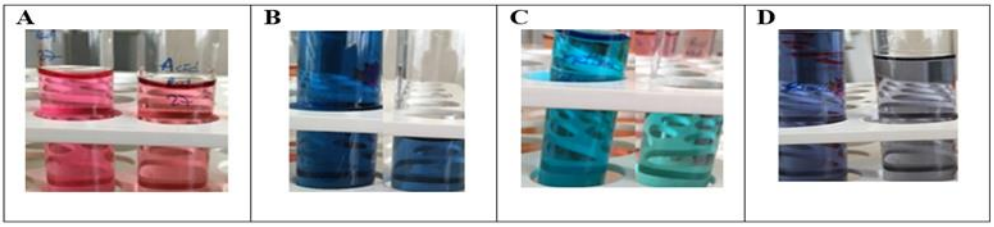

Figure 5. Decolorization of dyes using laccase from *B. licheniformis* O12. A) Acid red 27, B) Acid black 1, C) Methylene blue, D) Reactive black 5

In this study, we used sepharose 4B-L-tyrosine-ρ-amino benzoic acid affinity column for laccase enzyme for the first time and end of this process, the enzyme was purified to 4.82 fold with a yield of 38.3% (Table 1). In a study with fungi, laccase from Trichoderma harzianum was purified with 130.5 U/mg specific activity, 151.7-fold, and 0.39% yield using Concanavalin-A affinity chromatography (Sadhasivam et al., 2008). The laccase from papaya leaves was purified to 1377 fold by the same Concanavalin-A affinity chromatography with 62% yield and 41 U/mg specific activity (Jaiswal et al., 2015). In a similar study, a 10-fold purification and 40% yield were reported laccase from Chaetomium thermophilum using Concanavalin-A affinity chromatography (Chefetz et al., 1998). The laccase enzyme obtained from *Aquisalibacillus elongatus* was purified using a synthetic affinity column with 98.9 U/ mg specific activity, 99.8-fold, and 68.2% yield (Rezaei et al., 2017). In another study, the purification of laccase from *Brevibacillus* sp. Z1 was carried out using ammonium sulfate precipitation, ion exchange, and gel filtration chromatography with 93.3 fold and 30.2% yield (Bozoglu et al., 2013). The advantage of the method we use is that the enzyme can be obtained in one step.

B. licheniformis O12 laccase was similar to the molecular weight of laccases from *Brevibacillus* sp. Z1 (73.790 kDa) (Bozoglu et al., 2013) and *Alcaligenes faecalis* XF1 (71 kDa) (Mehandia et al., 2020).

The laccase enzyme from *B. licheniformis* O12 showed maximal activity at pH 4.0. This result was similar to *B. pumilus* W3 (pH 4.6) (Guan et al., 2014) and *B. licheniformis* CotA (pH 4.2) laccases (Koschorreck et al., 2008) Optimum pH value of laccase enzyme obtained by recombinant way using *B. licheniformis* O12 was determined as 5.0 (Kesebir et al., 2021) It was observed that the laccase enzyme obtained from *B. licheniformis* O12 showed stability in a weakly acidic environment similar to other

bacterial laccases (Baldıran 2006) and also maintained its activity at a basic pH value of 8.0. Most fungal laccases also show activity and stability under acidic conditions (pH 4.0-6.0) (Diamantidis et al., 2000; Baldıran 2006). The high relative activity and stability of *B. licheniformis* O12 laccase not only in acidic pH but also in neutral and alkaline pH will make it a potential candidate for different applications.

The optimum activity of the laccase enzyme was observed at 92°C; the enzyme was maintained more than 70% of its activity over a wide range of temperature (Fig 4). Optimum temperature value of laccase enzyme obtained by recombinant way using *B. licheniformis* O12 was also found as 92 °C (Kesebir et al., 2021). The optimum temperature of laccase was higher than that of the laccases from *Bacillus subtilis* X1 (60°C) (Guan et al., 2014) *Azospirillum lipoferum* (70°C) (Jeon and Park 2020), *Geobacillus* sp. JS12 (80°C), (Miyazaki 2005). and was similar to laccase from *Thermus thermophilus* HB27 (92°C) (Kim et al., 2015). Since *B. licheniformis* O12 is a thermophilic bacterium, we expected high activity of the laccase enzyme at high temperatures. However, a very high activity value was also observed at 30°C.

The stability of laccase from *B. licheniformis* O12 laccase was higher than previously reported other thermostable laccases such as *Thermus thermophilus* HJ6 (50 min at 85°C) (Fang et al., 2011) *Thermus thermophilus* HB27 (14 h at 80°C) (Kim et al., 2015), *B. licheniformis* DSM13 (1 h at 80°C) (Koschorreck et al., 2008) and *B. pumilus* W3 (10 h at 90°C) (Guan et al., 2014). These results show that the laccase enzyme we obtained from B. licheniformis O12 bacteria is quite thermostable. The high thermal stability of laccase makes it a particularly strong candidate for industrial and biotechnological applications where it can be exposed to high temperatures for extended periods of time.

The results of kinetic parameters for the oxidation of ABTS, 2,6-DMP, and guaiacol, ABTS is the most appropriate substrate for *B. licheniformis* O12 laccase with the lowest Km and maximum V_{max}. It was determined that the laccase enzyme obtained recombinantly from *B. licheniformis* O12 showed the highest affinity for guaiacole (Kesebir et al., 2021). Higher K_m values for ABTS have been reported for laccases from *T. harzianum* (Sadhasivam et al., 2008), Daedela quercina (0.038 mM) (Moon-Jeong et al., 2005), *Proteobacterium* JB (0.073 mM) (Xiao et al., 2003), *Trametes versicolor* (12.8 µM) (Shujing et al., 2013), *Trametes* sp. strain AH28-2 (25 µM) (Singh et al., 2011) and Mycena purpureofusca (0.296 mM) (Vantamiru et al., 2016). The catalytic efficiencies (K_{cat}/K_m) of the laccase from *B. licheniformis* O12 were found to be in the order: ABTS (4.26 1/mM.min) > 2,6-DMP (0.46 1/mM. min) > guaiacol (0.0000018 1/mM. min).

While working on the decolorization of the laccase enzyme, we did not add any mediators to the reaction medium. Different results can be obtained when the mediator is added. Decolarization of the laccase enzyme obtained by recombinant way using the *B. licheniformis* O12 was observed 51.2% for Acid black 1 and 36.2% for methylene blue (Kesebir et al., 2021). It has been reported that decolorization with CotA-laccase efficiently increased from 50% to 90% after 10 hours of incubation when methylsyringate was added as a mediator (Guan et al., 2014). Similarly, the important effect of laccase mediators in dye decolorization has been demonstrated in many studies (Sunil et al., 2011; Trubitsina et al., 2015; Bilal et al., 2017). The decolorization effect of the laccase enzyme increases in direct proportion to time. We observed decolorization within 2 hours in our study. When the time is extended, much more decolorization can be observed (Trubitsina et al., 2015) Decolorization of dyes is one of the most attractive biotechnological applications of bacterial laccases. Therefore, in subsequent studies, it may be aimed to increase the decolorization of dyes with laccase using a variety of mediators.

CONCLUSION

A new affinity method was used to purify the laccase enzyme from *B. licheniformis* O12. The resistance of laccase enzyme, which was purified in one step with sepharose 4B L-tyrosine p-aminobenzoic acid affinity column, to various chemicals and environmental effects that can be found in textile wastewater was investigated. It has been confirmed by studies that laccase is highly resistant to acidic and alkaline environments, high temperatures, metal ions and various chemicals. Afterwards, successful results were obtained in the decolarization study performed without adding a mediator.

ACKNOWLEDGEMENTS

This project This study was funded by TUBITAK project number 218Z032. Thank you for the institutional contributions of TUBITAK.

Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

All authors contributed to the study conception and design. Material preparation and analysis were performed by Arzu Öztürk Kesebir. The first draft of the manuscript was written by Arzu Öztürk Kesebir and Prof. Dr. Melda Sisecioglu and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript

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