MAS Journal of Applied Sciences Uygulamalı Bilimler Dergisi ISSN: 2757-5675 masjaps.com

OPEN ACCESS

DOI: http://dx.doi.org/10.5281/zenodo.7956783

Araştırma Makalesi / Research Article

Examination of The Immobilization and Kinetics of The Laccase Enzyme on Various Clay Minerals

Fulya ÖZDEMİR[1*], Zeki YALÇINKAYA[2]

[1]Ufuk University Vocational School of Health Services, Department of Medical Services and Techniques, Ankara

[2]Van Yuzuncu Yil University, Faculty of Science, Department of Chemistry, Van

*Sorumlu Yazar (Corresponding author): fulyaozdemir83@gmail.com

Geliş Tarihi (Received): 28.01.2023 **Kabul Tarihi (Accepted)**: 01.03.2023

Abstract

In this study, the immobilization of the laccase enzyme, which has a wide application area in the industry, was examined. For this purpose, commercially obtained laccase enzyme was immobilized on various clays using the physical adsorption method. The effects of pH, temperature, substrate concentration, and storage time on the activity of free and immobilized laccase were examined. As a result of the studies, the optimum pH and temperature for free laccase were obtained as 5.5 and 40 °C respectively and the optimum pH and temperature for all immobilized enzymes (bentonite, diatomite, and Bardakçı) were 5.5-6.0 and 40 °C respectively. The effects of pH and temperature on the activity of the immobilized laccase showed that the properties of the immobilized enzyme were the same as those of the free enzyme. The resulting kinetic constant values turned out to be quite close to each other. In addition, it was shown that adsorption did not significantly affect the kinetic properties of the enzyme. Only 20%-30% of immobilized laccase activity disappeared in 2 months. K_M values for free enzyme and immobilized enzymes were found as 0.0700 mM, 0.0724 mM, 0.0831 mM, and 0.0935 mM and V_{max} values were 0.0695, 0.0216, 0.0236, and 0.0233 mM^{-1}, respectively. The K_M value of the immobilized enzyme is greater than that of the free enzyme and the V_{max} value is smaller. The increase in resistance of the immobilized enzyme to temperature change and storage time indicates that laccase immobilization on clay is beneficial for enzyme immobilization.

Keywords: Adsorption, enzyme immobilization, clay-minerals, laccase

1. Introduction

Clays are versatile materials used in ceramics and building materials, backing papers, fillers, drilling fluids, foundries, pharmaceuticals, and more in a variety of fields depending on their specific properties (Vaccari, 1999). There are two broad classes of clays (Reichle, 1986). Cationic clays, known as clay minerals are commonly found in nature. Anionic clays [or double-layered hydroxides (LDHS)] are rarely observed in nature but can be easily synthesized in the laboratory (Cavani et al., 1991). Bentonite, a naturally occurring mineral, is an inexpensive and readily available material for removing heavy metals and toxic compounds from wastewater. This property makes it a potential candidate for water recovery. Bentonite clay is used as an adsorbent to immobilize organic, inorganic, and toxic compounds because it can be changed without affecting the mineral structure of the clay. Studies have shown that bentonite natural pollutants can be effectively used to absorb aqueous solutions. However, the structure of natural bentonite is unstable and can be destroyed by toxic substances produced by the liquid during infiltration (Chen et al., 2020; Dinh et al., 2022). The diatomite is a sedimentary rock formed from the silica fossilized skeletons of the diatoms called hard cell walls and frustules (Ha et al., 2013). Diatomite recently, a reasonable price is frequently used to produce membranes of microfiltration in terms of abundance and high porosation (Yeom et al., 2016). Vasconcelos et al. prepared diatomic membranes by casting and lamination and found that the gas permeability of diatomaceous earth membranes increases in proportion to the increase in average pore size (Vasconcelos et al., 2000). Diatomite powder found in nature has a high adsorption affinity for elements in aqueous solutions such as Fr, Ni, Cu, Pb, Th, and Cd. Also, during its formation, the two-atom soil tends to merge with other mineral impurities surrounding the two-atom soil coating and reduce its original micro/nano-structured properties. Diatomaceous earth pollutants significantly affect the absorption of pollutants from a polluted environment (Ye et al., 2015). Bardakçı, moreover, is a cationic clay type found naturally around the Bardakçı village of Van province in Turkey. Since it is supplied from this region in the region, it is named with the name of the village. Enzymes are highly specific protein biocatalysts that can increase the reaction rate in the processes they catalyze more than chemically catalyzed reactions and allow the reaction conditions to be milder, managing all metabolic events in the living cell. Laccase (e.c. benzol-diol oxygen oxidoreductase) copper-containing molecular oxygen and an electron receiver use their catalytic cycle of an enzyme catalyzing the oxidation of various phenolic compounds and low-molecular-weight Quinones and is responsible for the production and use of energy. Laccase enzymes are obtained from 4 sources, including bacteria, insects, high-structure plants, and the most commonly used white-rot fungi. Among the fungi, which were in the hands of enzymes Lakkaz, *Coriolopsis gallica, Rhus vernicifera, Trametes hirsut to Pyricularia oryzae, Aspergillus sp, Corilous hirsutus, Trametes versicolor* in the lobby (Taşdelen, 2006; Yamak et al., 2009). Laccase, wood bio-bleaching, the food industry, and agricultural food wastes are impressive enzyme groups called blue-copper oxidases with effective results in the valuation of food waste and effective results in wastewater treatment. Although it is versatile and commonly used, there are a series of disadvantages such as sensitivity and high commercial costs of this enzyme. Different natural or synthetic supports can be used to provide enzyme immobilization (Basso and Serban, 2019; Bilal et al., 2019; Minussi et al., 2002; Nayak and Bhushan, 2019; Rahmani et al., 2020; Riva 2006). The use of free laccase in the industrial area, high production costs, and the difficulty of the enzyme separation, as well as problems such as pH, temperature, and inhibitors,

such as pH, temperature, and inhibitors, are confronted with problems such as environmental factors. All this causes stability to ensure the stability and the rapid loss of activity (Fathali et al., 2019; Li et al., 2018). In the solution to these problems, the immobilization method of laccase is used on water-insoluble supports. Immobilization is the process by which an enzyme bound to solid support transforms the catalytic form into a homogeneous heterogeneous (free enzyme) form (immobilized enzyme) (Deska et al. 2019; Ji et al., 2017; Rouhani et al., 2016; Shao et al., 2019; Tavares et al., 2015). As a result of the processes, the peptide structure of the bio-catalyzer is stabilized by interactions between the enzyme and its supporting material, and as a result, the enzyme increases the pH, temperature, operation, storage, and decisiveness of chemical reagents (Zdarta et al., 2018). In addition, it facilitates immobilization purification and produces desired products with higher yields (Yamak, 2007).

2. Material and Method

2.1. Chemical Substances Used

Laccase enzyme (was obtained from Sigma, St. Louis, MO, USA), syringaldazine (4-Hydroxy-3,5-dimethoxybenzaldehyde azine, $C_{18}H_{20}N_2O_6$, M_W:360.3 g/mol), DMP(2,6-dimethoxyphenol) (was obtained from Sigma, St. Louis, MO, USA), disodiumhydrogenphosphate ($Na_2HPO_4.2H_2O$), M_W: 178 g/mol, citrate ($C_6H_8O_7.2H_2O$), M_W: 232.124 g/mol, sodium hydroxide (NaOH), M_W: 40 g/mol, hydrochloric acid(HCl), M_W: 36.453 g/mol, clays:(bentonite, diatomite, Bardakçı).

2.2. Preparation of clay suspensions

Bentonite, diatomite, and Bardakci clays were dried in the oven after washing 3 times with deionized water. It was sieved after grinding and passing through a sieve with a pore size of 0.038 mm (400 mesh). Clay suspensions were prepared by taking 1.5 g of these sieved cellars and adding them to 50 ml of deionized water.

2.3. Determination of Activity

2.3.1. Measurement of free enzyme activity

For the measurement of laccase activity, we used syringaldazine as the substrate because the oxidation was measured with a UV spectrophotometer. Therefore, measurements of free laccase activity were made according to the method proposed by Leonowicz and Grzywnowicz (A Leonowicz et al., 1981). To measure the activity, the reaction was started by adding 1 mL of syringaldazine solution (0.5 mM) and 2 mL of laccase solution to 4 mL of citrate buffer (pH:5.3). The solution was stirred for 13 minutes in a water bath mixed at 20 °C. The absorption of the pink solution formed at the end of the reaction (15 minutes) was measured at 530 nm using a UV Visible Spectrophotometer. The reaction rate was calculated according to the following formula using the measured absorbance value and the slope of the syringaldazine standard curve (A_{530} / Δ_C).

$$Speed\ (V) = \frac{\Delta c}{\Delta t} = \frac{\Delta A_{530}}{\Delta t}\frac{\Delta c}{\Delta A_{530}}$$

Here, Δc indicates a change in syringaldazine concentration, Δt refers to a change in time and ΔA_{530} refers to a change in absorbance. The amount of enzyme that produces 1 μmol of product per minute at 20 °C is indicative of laccase activity (Gökgöz, 2006; Yamak, 2007).

2.3.2. Activity determination of immobilized enzyme (adsorbed)

Enzyme solution in 0.05 M sodium phosphate buffer (solution containing 1/5000 mg enzyme/ml sodium phosphate buffer) was used to adsorb the free enzyme into the clay. For this, 1 mL of enzyme solution and 4 mL of clay suspension

(bentonite, diatomite, and Bardakçı clay) were mixed at 30°C and gently shaken in the vortex for 60 minutes. An activity measurement as described above was performed by centrifuging the clay enzyme complex at 3000 rpm for 15 minutes and separating the supernatant. The underlying solid was washed 3 times with 0.05 M sodium phosphate buffer and centrifuged again. The substrate was mixed in vials with a 5 ml phosphate buffer to give a bound enzyme solution. Thus, pH, temperature, and storage time-bound solutions obtained K_M and V_{max} values of the enzyme to calculate and understand the impact of different substrate concentrations (Lineweaver-Burk equation) using activity measurements have been carried out as described above (Alkan, 1999).

2.4. Change of Substrate Concentration and Reaction Rate

To study how the change in substrate concentration affects the rate of enzymatic reaction 0.02; 0.05; 0.06; 0.1; 0.15; 0.2; 0.5; 1.00; and 1.5 mM syringaldazine solutions were prepared. The reaction rates were calculated by the method shown above. The change in reaction rate with substrate concentration is shown in Figure 2.2.

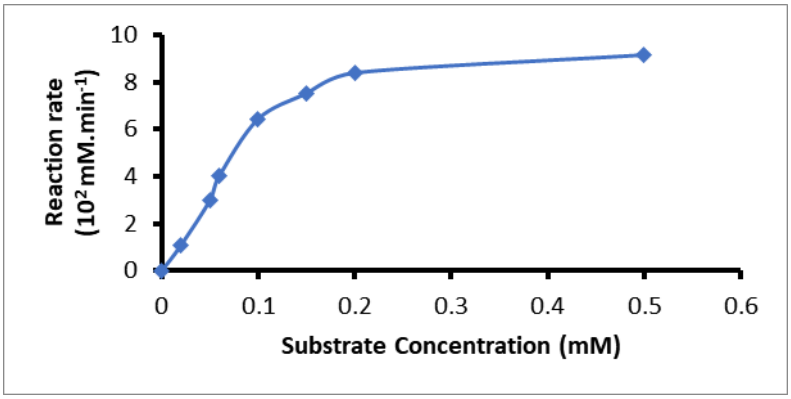

Figure 2.2. Change of reaction rate with substrate concentration (Michaelis-Menten graph).

2.5. pH Effect on Enzyme Activity

Enzymes are very sensitive to pH change. Generally, they are ineffective in very acidic or alkaline environments. Since pH is effective in the active site of the enzyme, it is one of the parameters that should be examined in enzyme characterization. Phosphate buffers ranging from pH 3-9 were prepared in experiments to see the effect of pH in free enzyme solutions. Activity at these pHs was determined by the method shown above. The temperature (20°C) and the concentration of syringaldazine (0.5 mM) were constant for 15 minutes, during which the reaction continued. To test the effect of pH on the activity of the binding enzyme, the binding enzyme was prepared with phosphate buffers at various pH (range of pH = 3-9), and activity at each pH activity determination was made according to the recipe described above. During the 15-minute reaction, the temperature of 20 °C and the concentration of syringaldazine $5x10^{-4}$ M remained constant.

2.6. Change of enzyme activity by temperature

To determine how the temperature affects the free and bound enzyme solutions, the activity determination of the samples at 20, 30, 40, 50, 60, and 70°C was performed as specified above. During the reaction, the pH of 5.3 and the concentration of syringaldazine, which was $5x10^{-4}$ M, remained constant.

2.7. Change of enzyme activity according to substrate concentration

To examine how the substrate concentration affects the activity of free

laccase and immobilized laccase, 5 (0.01, 0.02, 0.03, 0.04, and 0.05 mM) solutions of syringaldezine were prepared and the enzyme activity was determined as described above. pH (5.3), laccase concentration (0,01 mg/mL), and temperature (20 °C) were constant throughout the reaction.

2.8. Change of enzyme activity according to storage time

To investigate how storage time affects the activity of free laccase and immobilized laccase, the enzyme solution was prepared by the method shown above. From this solution at regular times (0, 5, 10, 18, 25, 33, 41, 50, and 60. days) by taking samples, the event was determined by the method described above. pH (5.3), syringaldazine concentration (0.05 mM), and temperature (20 °C) were kept constant during the reaction.

3. Results and Discussion

3.1. Effect of pH on enzyme activity and stability

To determine the optimum pH of the free enzyme, the absorbance changes and activity values of the solutions with pH values ranging from 3 to 8, as described above, are given in Table 3.1. The optimum pH of the free laccase enzyme was determined as 5.5. The variation of relative activity with pH is shown in Figure 3.1. In the literature, the optimum pH of laccase obtained from *Pycnoporus sanguineus* was found to be 3.0 when ABTS was used as a substrate and 5.0 when Syringaldazine was used (Bar, 2001; Yamak, 2007). It has been stated that the optimum pH varies between 5.0-6.3 when the laccase enzyme is obtained from different sources and different substrates are used (Al-Adhami et al., 2002; Lante et al., 2000). According to Simsek (2011), the optimum pH value of free laccase obtained from *Trametes versicolor* against ABTS substrate has been reported as 3.0. The optimum pH value of laccases against the same substrate may vary depending on the source from which they are obtained. In addition, the optimum pH of laccases obtained from the same source against different substrates can also be different. Laccase purified from *Trametes versicolor* has been described as having an optimum pH of 5.0 against a syringaldazine substrate (Yamak et al., 2009), and 3.3 against a substrate of 2,6-di meth oxyphenol (Andrzej Leonowicz et al., 1988).

Table 3.1. pH dependency of free laccase enzyme activity

pH	Absorbance (530nm)	Activity (Speed*1000)	Relative activity (%)
3.0	0.094	7.17	12
4.0	0.264	20.13	35
4.5	0.401	30.58	53
5.0	0.711	54.23	93
5.5	0.761	58.04	100
6.0	0.453	34.55	60
6.5	0.338	25.78	44
7.0	0.223	17.01	29
7.5	0.108	8.24	14
8.0	0.027	2.06	4

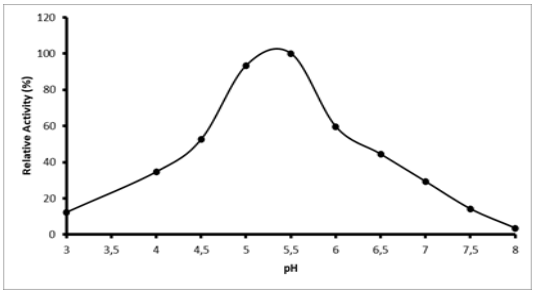

Figure 3.1. Graph showing the dependence of free laccase enzyme activity on pH

3.2. The Effect of pH on Immobilized Enzyme Activity

Activity values were obtained by using absorbance changes of solutions obtained at various pHs prepared as described above to investigate the effect of pH on the activity of laccase enzyme immobilized on clay minerals (bentonite, diatomite, and Bardakçı). Is given in tables 3.2-3.4. and the variation of relative activity with pH is shown in Figures 3.2-3.4. To compare how pH affects the activity of the free enzyme and immobilized enzymes, the relative activity and pH value of each condition are shown in the same graph (Figure 3.5). It was observed that the pH of immobilized enzymes also changed. The pH range of the immobilized enzyme is wider than the pH range of the free enzyme. This is because immobilization maintains enzyme activity over a wider pH range and shows that the immobilized enzyme is active even at a higher pH. While the optimum pH was 5.5 for laccases immobilized on free laccase, bentonite, and diatomite clays, it was 6.0 for laccase immobilized on Bardakci clay. However, the activity of the bound enzyme appears to be higher at alkaline pH than the free enzyme. This change in optimum pH may be ionic and polar interactions or secondary interactions such as hydrogen bonding and dipole-dipole interaction (Arica et al., 2000). In another study, Lante et al., When L immobilized on a polyethersulfone membrane was free, the pH of 6.3 was immobilized to 6.6 (Lante et al., 2000). While finding, by D'Annibale et al., found the optimum pH to be 4.0 when they immobilized laccase eupergite (D'Annibale et al. 2000). In another study by D'Annibale et al., Chitosan was cross-linked with glutaraldehyde and immobilized on it by laccase adsorption, it was observed that the optimum pH was 4.0 and did not change (D'Annibale et al., 1999). By immobilizing laccase purified from *Trametes versicolor* on poly(acrylamide-n-isopropyl acrylamide)/alginate and poly(acrylamide)/alginate hydrogels and using syringaldazine as substrate, the optimal pH of the enzymes was found to be pH 6 and pH 5.5, respectively (Yamak, 2007). According to Yamak (2007), the optimal pH of the laccase enzyme immobilized by adsorption on porous glass beads is 5.7.

Table 3.2. pH values for laccase immobilized in bentonite

pH	Absorbance (530nm)	Activity (Speed *10^3)	Relative Activity(%)
3,0	0,077	5,87	23
4,0	0,142	10,83	42
4,5	0,201	15,33	59
5,0	0,228	17,39	67
5,5	0,341	26,01	100
6,0	0,315	24,02	92
6,5	0,241	18,38	71
7,0	0,135	10,30	40
8,0	0,058	4,42	17

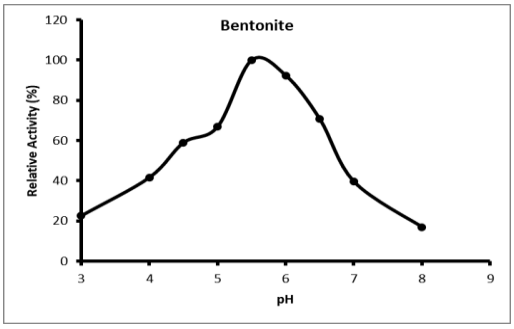

Figure 3.2. The effect of pH on the activity of the laccase enzyme immobilized in bentonite

Table 3.3. pH values for laccase immobilized in diatomite

pH	Absorbance (530nm)	Activity (Speed*1000)	Relative Activity (%)
3,0	0,021	1,60	6
4,0	0,064	4,88	17
4,5	0,115	8,77	30
5,0	0,267	20,36	70
5,5	0,379	28,91	100
6,0	0,361	27,53	95
6,5	0,218	16,63	58
7,0	0,133	10,14	35
7,5	0,105	8,01	28
8,0	0,026	1,98	7

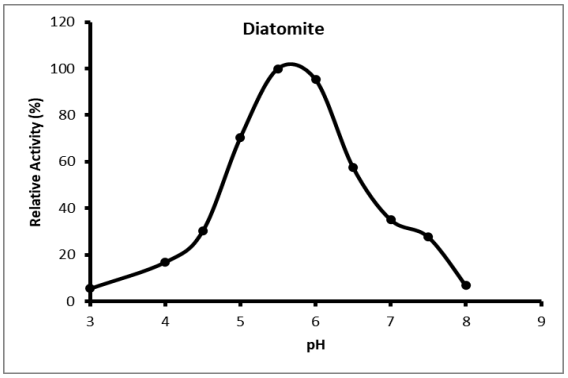

Figure 3.3. Effect of pH on the activity of laccase enzyme immobilized in diatomite

Table 3.4. pH values for laccase immobilized in Bardakci clay

pH	Absorbance (530nm)	Activity (Speed*1000)	Relative Activity (%)
3,0	0,062	4,73	16
4,0	0,117	8,92	30
4,5	0,165	12,58	43
5,0	0,259	19,75	67
5,5	0,348	26,54	90
6,0	0,386	29,44	100
6,5	0,339	25,86	88
7,0	0,232	17,69	60
7,5	0,113	8,62	29
8,0	0,031	2,36	8

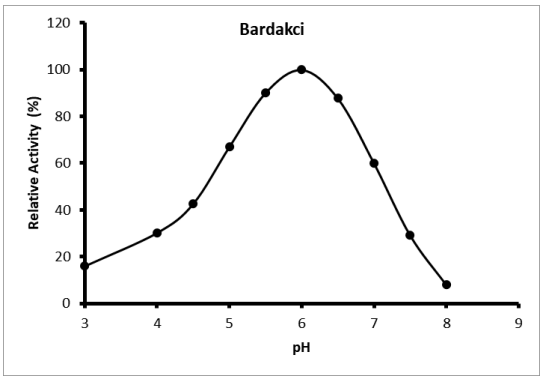

Figure 3.4. Effect of pH on the activity of laccase enzyme immobilized in Bardakçı clay

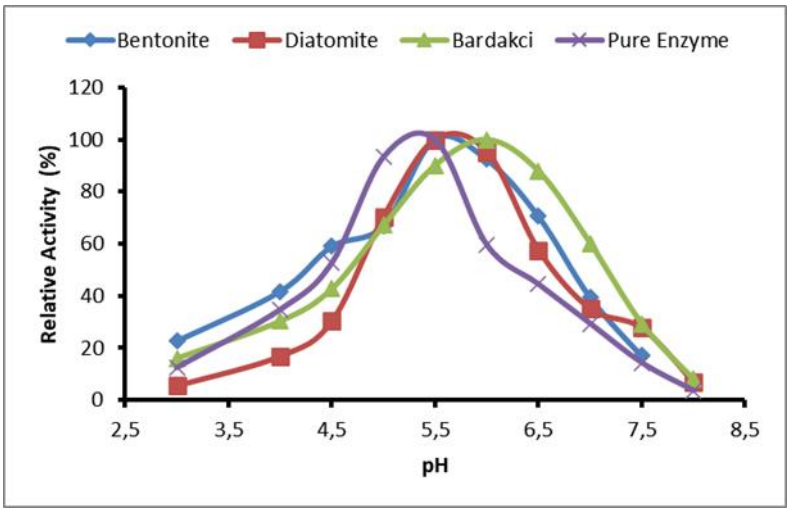

Figure 3.5. Change of % relative activity with pH for free and immobilized enzymes

3.3.The Effect of Temperature on Activity

To investigate how temperature affects the activity of laccase enzyme immobilized on free and clay minerals (bentonite, diatomite, and Bardakci), the relative activity values obtained by using the absorbance changes of the solutions prepared as described in section 2.3.2 are given in Table 3.5 and the relative activity change with temperature shown in Figures 3.6-3.8. The activity of the enzyme increases with increasing temperature (Figures 3.6-3.8). While free enzyme activity increased proportionally up to 40°C, maximum activity was observed at 40°C. While the immobilized enzyme activity increased proportionally up to 50°C, a value close to the maximum activity was obtained at 50°C. It was determined that the enzyme activity of the free enzyme decreased sharply after the temperature of 40°C and the relative activity of the enzyme decreased to 4% at 70°C. For the immobilized enzyme, it has been found that the enzyme activity drops sharply after the temperature of 50°C, and the relative activity of the enzyme decreases to 23% for bentonite and diatomite and 30% for Bardakci clay at 70°C (Table 3.5). The increase in the reaction rate is directly proportional to the temperature. But from a certain point, it starts to fall and stops completely. Enzymes are ineffective at high temperatures. Low temperatures reduce the enzyme's effectiveness. At 0 °C, the enzyme functions little or not at all; but it has not been observed that the cold impairs the structure of the enzyme. When the temperature is restored, the activity begins again (Alkan, 1999). Accordingly, it was observed that it was made more resistant to temperature by the enzyme immobilization process. According to Gokgoz (2006), this increase in optimum temperature is owing to the decrease in the conformational flexibility of the imprisoned laccase molecules, and therefore, the laccase molecule needs a higher temperature to perform its function and to come to a suitable conformation and rearrange it to attach to the substrate. The enzyme must have greater activation energy to show catalytic activity (Arica et al., 2000).

Table 3.6. Relative activity values of Pure Laccase and Immobilized Laccase (Bentonite, Diatomite, and Bardakçı) at various temperatures.

Temperature (°C)	%Relative Activity (Pure Laccase)	% Relative Activity (Bentonite)	% Relative Activity (Diatomite)	% Relative Activity (Bardakci)
20	85	93	96	96
30	94	98	97	97
40	100	100	100	100
50	54	98	91	91
60	17	46	46	46
70	4	30	23	23

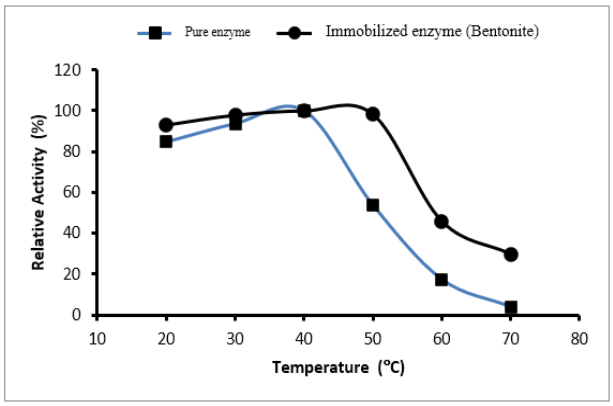

Figure 3.6. Effect of temperature on the activity of pure Laccase and immobilized Laccase (Bentonite)

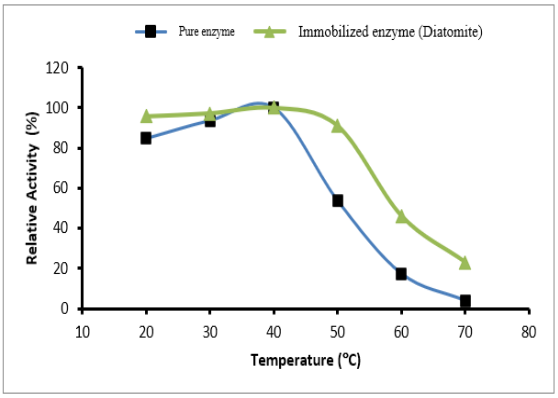

Figure 3.7. Effect of temperature on the activity of pure Laccase and immobilized Laccase (Diatomite)

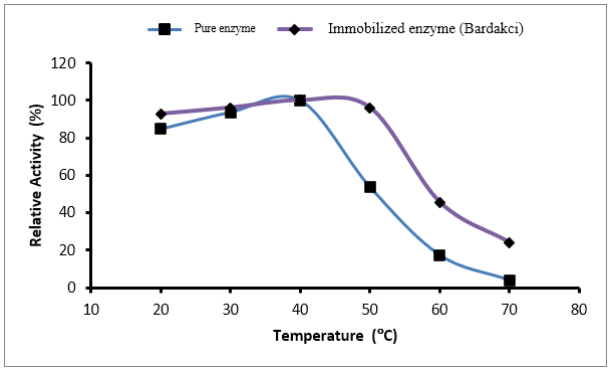

Figure 3.8. Effect of temperature on the activity of pure Laccase and immobilized Laccase (Bardakci)

Various findings have been found in the studies that the enzyme activity changes with temperature change. Al-Adhami et al., The optimum temperature of laccase immobilized by covalent bonding to free and cellulose was determined to be 60 °C (Al-Adhami et al. 2002), while D'Annibale et al. Its temperature was found to be 55°C (D'Annibale et al. 2000). Dodor et al. stated that the optimal temperatures of laccase bonded to kaolinite and free laccase by covalent bonding were 40 °C and 60°C, respectively (Dodor, et al., 2004). In the study of Lante et al., The optimum temperatures of laccase and free laccase immobilized on a polyethersulfone

membrane were found to be 35 and 40 ° C, respectively (Lante et al. 2000). In the study of D'Annibale et al., As a result of the immobilization of the laccase enzyme to chitosan, it was observed that the optimal temperature increased from 50°C to 60°C (D'Annibale et al. 2000). In the study of Rogalski et al., the laccase enzyme was immobilized on a porous glass surface and its optimum temperature was found to be 60°C (Rogalski et al. 1999). With the increase in temperature, first the tertiary structure of the enzyme molecule, and then the secondary structure (alpha-helical structure) deteriorates. The active center of the enzyme is also affected by these events and enzyme activity is lost (Mosbach et al.,1976). The optimum temperature of laccase immobilized on Eupergite by the covalent bonding method was found to be 50 °C (Hublik et al., 2000). The optimum temperature for laccase immobilized on kaolinite by covalent bonding was determined as 50 °C, and the optimum temperature of enzyme immobilized on kaolinite and nanoparticle was determined as 45 °C (Hu et al., 2007). The activity of the free enzyme is immobilized in Montmorillonite Analcime, the bound enzyme becomes active when the temperature reaches 60 °C and denatured at 70 °C. The free enzyme showed high activity at 30°C. Montmorillonite analcime showed its highest activity at 30°C and then began to denaturation (Uruç 2007).

3.4. Effect of Substrate Concentration on Activity

The enzyme's binding to its substrate and converting them into products is called enzyme kinetics. First, the substrate binds to the enzyme, forming the enzyme-substrate complex expressed by ES. This is referred to as the Michaelis complex. The chemical step of the enzyme reaction is then catalyzed and forms the product. In the example, an enzyme (E) binds to a substrate (S) to form an enzyme-substrate complex (ES) that is converted to a P product. Schematically this transformation can be represented as:

$$E + S \underset{k_r}{\overset{k_f}{\Leftrightarrow}} ES \overset{k_{cat}}{\rightarrow} E + P$$

where kf, kr, and kcat are rate constants. With some assumptions, the product formation rate (reaction rate v) is expressed in terms of the concentration of a substrate S:

$$v = \frac{V_{\max}[S]}{K_m + [S]}$$

This equation is the Michaelis – Menten equation. For the determination of V_{max} and K_M constants, parameters can be obtained by graphing the reaction rate by concentration and by performing nonlinear regression with the Michaelis-Menten equation (Leskovac 2003). But generally, graphical methods that provide linearization of this equation are used. Lineweaver – Burk plot is one of them. The Lineweaver-Burk plot is commonly used to display kinetic data. To obtain this equation, the Lineweaver-Burk equation is obtained by inverting the Michaelis-Menten equation. The linear form of the Michaelis-Menten equation is created.

$$\frac{1}{v} = \frac{K_m}{V_{\max}[S]} + \frac{1}{V_{\max}}$$

3.4.1.Determination of kinetic parameters for syringaldazine

To obtain kinetic data, solutions of different syringaldazine concentrations (0.01-0.05 mM) were prepared and the activity of the enzyme was calculated according to the method in section 2.3.1. During the reaction lasting 15 min, the pH was 5.3, the concentration of laccase enzyme was constant at 0.02 mg/mL, and the temperature at 20°C. Lineweaver-Burk plot was drawn for free enzyme by plotting the 1/S and 1/V values given in Table 3.6 (Figure 3.9.). As a result of the calculations, the KM of the non-immobilized enzyme is 0.0700 mM and the V_{max} is 0.0695 mM.min^{-1}. For the determination of kinetic data for the immobilized enzyme (bentonite, diatomite, and Bardakci clay), solutions of different syringaldazine concentrations (0.01-0.05 mM) were prepared and the activity of the enzyme was determined according to the method shown in section 2.3.1. During the reaction lasting 15 min, the pH was 5.3, the concentration of laccase enzyme was constant at 0.02 mg/mL, and the temperature at 20°C. Lineweaver-Burk graphs were drawn for the immobilized enzyme by plotting the 1/S and 1/V values given in Tables 3.7-3.9 (Figures 3.10-3.12.). As a result of the calculations, the K_M value of laccase immobilized in bentonite was 0.0724 mM and the V_{max} value was 2.16×10^{-5} M.min^{-1}; The K_M of laccase bound to diatomite is 0.0831 mM, V_{max} value is 0.0236 mM.min^{-1}, K_M of laccase bound to Bardakçı is 0.0935 mM and V_{max} is 0.0233 mM.min^{-1}. Simsek (2011), the K_M value of laccase (obtained from *Trametes Versicolor*) against ABTS substrate was found to be 5.69x10-2 mM at pH 5.0 and 25 °C. In the literature, K_M values of free laccase obtained from *Cerrena unicolor, Trametes hirsuta, and Pycnoporus sanguineus* against ABTS substrate in different experimental conditions are 0.183 mM (Bryjak et al. 2007) and $7.5x10^{-2}$ mM (75 μM), respectively (Almansa et al. 2004). The K_M value of the immobilized enzyme may increase or decrease. The decrease in the K_M of the immobilized enzyme tends to react more rapidly than the free enzyme; The increase indicates that the free enzyme needs more substrate to catch the reaction rate. The K_M constant is a measure of the enzyme's ability to bind to the substrate. K_M and V_{max} values for bound and unbound laccases are given in Table 3.10. In this study, it was observed that the K_M of the immobilized enzymes was higher than that of the free enzymes. The lower the enzyme's affinity for the substrate, the higher the K_M value. This plays a role in conformational changes and steric hindrance in the protein molecule, which reduces the possibility of immobilized enzyme-substrate complex formation (Georgieva et al. 2008). Gupta et al. Immobilized laccase to the gold surface with glutaraldehyde and found the K_M values for immobilized and free enzymes as 5.4 mM and 0,65 mM (Gupta et al., 2003). In the study conducted by D'Annibale et al., It was observed that the K_M constant of free laccase increased from 70 μM to 150 μM when eupergite was immobilized, and the V_{max} value decreased from 190 IUmg $^{-1}$ to 76 IU.mg-1 (D'Annibale et al. 2000). In the study of Rogalski et al., It was observed that when laccase was immobilized on the glass surface, the KM constant increased from 80 μM to 124 μM (Rogalski et al. 1999). In the study of Cabrita et al., It was stated that when laccase kaolinite is immobilized, the K_M constant decreases from 262 μM to 165 μM (Cabrita et al., 2005). In a study by De Quan et al., laccase 48 was immobilized on the platinum surface and the K_M constant was found to be 85 μM (Quan et al., 2004). In the study of Lante et al., laccase conjugated with chitosan and the K_M constant of 42 mM of the free enzyme was observed as 85 mM and 95 mM when conjugated (Lante et al. 2000).

Table 3.7. Effect of substrate concentration on free enzyme activity

Syringaldazine Concentration [S] (mM)	Absorbance (530nm)	Activity (V) (mM.min^{-})*10^{3}	1/[S] (1/mM)	1/V (min.mM^{-1}
0,01	0,117	8,92	100,0	112,06
0,02	0,183	13,96	50,0	71,65
0,03	0,254	19,37	33,3	51,62
0,04	0,364	27,76	25,0	36,02
0,05	0,431	32,87	20,0	30,42

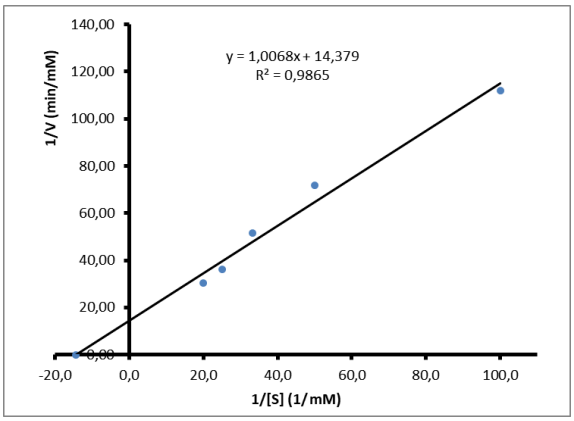

Figure 3.9. Lineweaver-Burk plot for Trametes Versicolor laccase

Table 3. 8. Effect of substrate concentration on laccase activity immobilized on bentonite clay

Syringaldazine Concentration [S] (mM)	Absorbance (530nm)	Activity (V) (mM.min^{-1}) *10^{3}	1/[S] (1/mM)	1/V (min.mM^{-1})
0,01	0,030	2,29	100,0	437,05
0,02	0,051	3,89	50,0	257,09
0,03	0,070	5,34	33,3	187,31
0,04	0,095	7,25	25,0	138,02
0,05	0,119	9,08	20,0	110,18

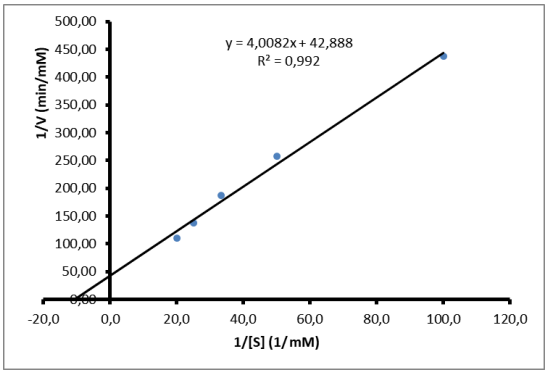

Figure 3.10. Lineweaver-Burk plot for bound laccase (bentonite)

Table 3. 9. Effect of substrate concentration on laccase activity immobilized on diatomite clay

Syringaldazine Concentration [S] (mM)	Absorbance (530nm)	Activity (V) (mM.min^{-1}*10^{3}	1/[S] (1/mM)	1/V (min.mM^{-1})
0,01	0,034	2,59	100,0	385,63
0,02	0,066	5,03	50,0	198,66
0,03	0,076	5,80	33,3	172,52
0,04	0,098	7,47	25,0	133,79
0,05	0,121	9,23	20,0	108,36

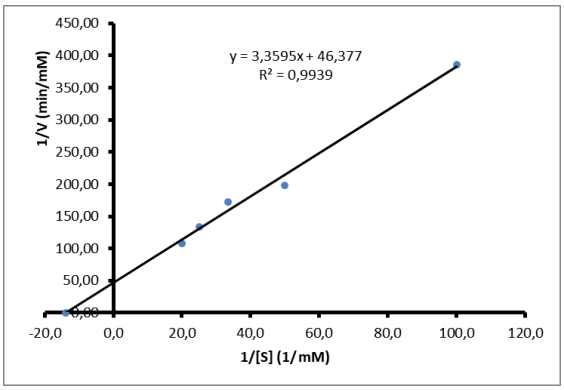

Figure 3. 11. Lineweaver-Burk plot for bound laccase (diatomite)

Table 3. 10. Effect of substrate concentration on laccase activity immobilized on Bardakçı clay

Syringaldazine Concentration [S] (mM)	Absorbance (530nm)	Activity (V) (mM.min^{1})*10^{3}	1/[S] (1/mM)	1/V (min.mM^{-1})
0,01	0,035	2,67	100,0	374,62
0,02	0,049	3,74	50,0	267,58
0,03	0,076	5,42	33,3	184,67
0,04	0,119	7,78	25,0	128,54
0,05	0,146	9,61	20,0	104,06

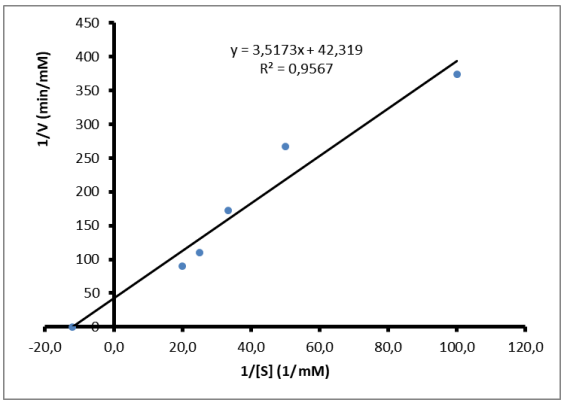

Figure 3.12. Lineweaver-Burk plot for bound laccase (Bardakçı)

Table 3. 11. K_M and V_{max} values for free enzyme and immobilized enzyme

Free and Immobilized laccase	K_m (mM)	V_{max} (mM.min^{-1})
Free laccase	0,0700	0,0695
Immobilized (Bentonit)	0,0724	0,0216
Immobilized (Diatomit)	0,0831	0,0236
Immobilized (Bardakci)	0,0935	0,0233

3.5.Shelf Life Stability of Free and Immobilized Enzymes

To examine the effect of storage time on the activity of the free enzyme, the activities of laccase solutions stored at +4°C for 60 days were determined as described in section 2.4.1. The change in the maximum activity of the free enzyme with storage time is shown in Table 3.11 and Figure 3.13. In a 60-day study with free enzyme, free enzyme preserved 28.6% of its initial activity. Dodor et al., D.E. found that the laccase obtained from Trametes Versicolor lost 90% of the activity of the free enzyme when stored at 4 °C for 4 months (Dodor et al., 2004). The stability of the immobilized enzyme in the storage process is one of the most important parameters after the immobilization of the enzyme. To examine the effect of the storage time on the laccase activity immobilized on clay minerals (bentonite, diatomite, and Bardakçı), the activities of the immobilized enzyme stored at +4 °C were determined at regular intervals for 60 days with the specified method. in section 2.4.1. The varying activity of laccase (bentonite) immobilized by shelf life is shown in Table 3.12 and Figure 3.14. The immobilized enzyme retained 70% of its initial activity at the end of the 60th day. The change in storage stability of immobilized laccase (diatomite) with storage time is shown in Table 3.13 and Figure 3.15. It was observed that the bound enzyme preserved 76.5 % of its initial activity on the 60th day. Table 3.14 and Figure 3.16 shows how the maximum activity of bound laccase (Bardakçı) changes with storage time. As a result of storing the immobilized laccase at +4 °C for 60 days, it was observed that 78.5% of its initial activity was preserved. When the free laccase is stored at +4 ° C, it was observed that the first activity of the 60th day has been maintained at 28.6% of the first activity, it was observed that the immobilized laccase has been stored under the same conditions, 70-80% of the initial activity on the 60th day was stored under the same conditions. That is, under the same storage conditions, it was observed that immobilized laccase loses its activity much more slowly than free laccase. This shows that immobilization makes the enzyme more stable. In a study by Gökgöz (2006), it was stated that laccase trapped in the PAAm-L gel retained 44% of its activity on the first day, while PAAm-KL1 and PAAm-KL2 retained approximately 68% of the gels with added carrageenan after 60 days (Gökgöz 2006). In the study of Al-Adhami et al., it was stated that the laccase enzyme immobilized to DEAE-Granocele retained 98% of its activity on the first day at the end of 4 months. In the study of Al-Adhami et al., it was stated that the laccase enzyme immobilized to DEAE-Granocele retained 98% of its activity on the first day at the end of 4 months (Al-Adhami et al. 2002). D'Annibale et al. In their study, it was stated that free laccase activity, which decreased to 17% in 6 months, decreased to 60% when immobilized to chitosan (D'Annibale et al. 2000). In the study of Quan et al., It was

determined that when laccase was covalently bonded to the platinum surface, it retained 80% of its activity in 2 months (Quan et al., 2004). In the study conducted by Yamak (2007), it was stated that the bound enzyme retained 91% of its activity on the 56th day when stored at 4°C. In the literature, it is reported that when laccase is immobilized by covalent binding to DEAE-Granocel 500, CM-Granocel, and acrylic carriers, 90% of its activity is preserved when the immobilized laccase is stored at 4°C for 4 months (Al-Adhami et al. 2002; Yamak 2007). When laccase is immobilized by the method of adsorption on chitosan, chitosan microspheres, and Fe^{3+} transition metal chelates dissolved in water with glutaraldehyde crosslinker, 10% of the activity of the enzyme immobilized on the chelate and water-soluble chitosan at 4°C after 3 months. It was reported that the enzyme immobilized on the microspheres lost 15% of its activity (Yang et al. 2006). It was determined that the source was Trametes Versicolor immobilized on laccase kaolinite at 4°C and after 90 days of storage, there was no loss of activity (Dodor et al., 2004). Laccase enzyme from Phlebia Radiata was immobilized on glass beads and after 180 days at 4°C, free laccase retained 3.7% of its first-day activity, and bound laccase retained 97.2% of its first-day activity (Rogalski et al. 1999).

Table 3.12. Effect of storage time on free enzyme activity

ccShelf life (day)	Absorbance change (530nm)	Activity (V) ($x10^3$mM. min^{-1})	Relative Activity (%)
0	0,462	35,24	100,0
5	0,452	34,47	97,8
10	0,441	33,63	95,5
18	0,416	31,73	90,0
25	0,363	27,69	78,6
33	0,331	25,24	71,6
41	0,258	19,68	55,8
50	0,205	15,64	44,4
60	0,132	10,07	28,6

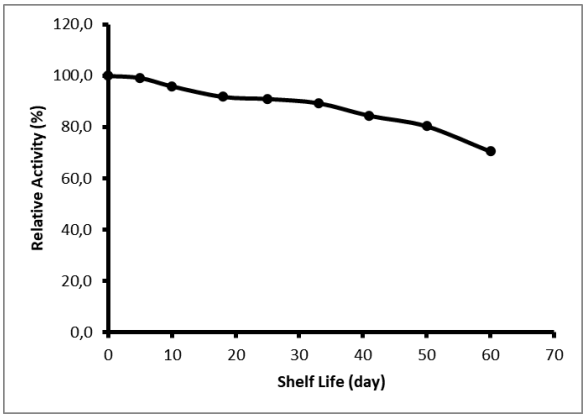

Figure 3. 13. Change of storage time of free enzyme with relative activity

Table 3.13. Effect of storage time on activity of the bound enzyme (Bentonite)

Shelf life (day)	Absorbance change (530nm)	Activity (V) ($x10^3$mM.min^{-1})	Relative Activity (%)
0	0,122	9,30	100,0
5	0,121	9,23	99,2
10	0,117	8,92	95,9
18	0,112	8,54	91,8
25	0,111	8,47	91,0
33	0,109	8,31	89,3
41	0,103	7,86	84,4
50	0,098	7,47	80,3
60	0,086	6,56	70,5

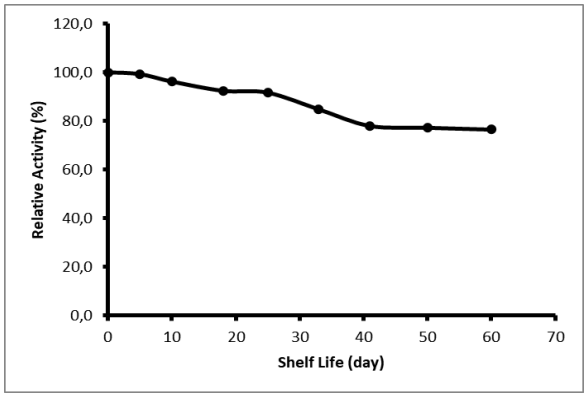

Figure 3.14. The relative activity change of the bound enzyme (Bentonite) with two months storage period

Table 3.14. Effect of storage time on activity of the bound enzyme (Diatomite)

Shelf life (day)	Absorbance change (530nm)	Activity (V) ($x10^3$mM.min^{-1})	Relative Activity (%)
0	0,132	10,07	100,0
5	0,131	9,99	99,2
10	0,127	9,69	96,2
18	0,122	9,30	92,4
25	0,121	9,23	91,7
33	0,112	8,54	84,8
41	0,103	7,86	78,0
50	0,102	7,78	77,3
60	0,101	7,70	76,5

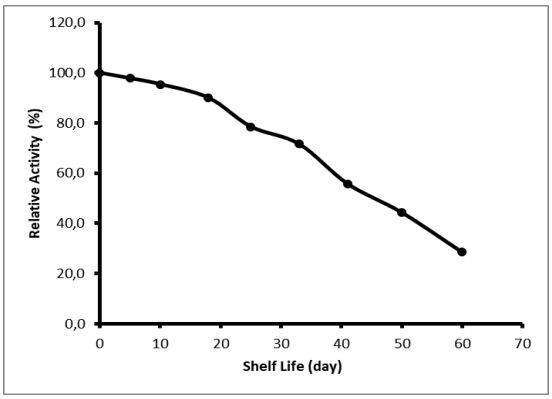

Figure 3.15. The relative activity change of the bound enzyme (diatomite) with a storage period of two months storage period

Table 3. 15. Effect of storage time on bound enzyme (Bardakçı) activity

Shelf life (day)	Absorbance change (530nm)	Activity (V) ($x10^3$mM.min^{-1})	Relative Activity (%)
0	0,121	9,23	100,0
5	0,120	9,15	99,2
10	0,117	8,92	96,7
18	0,112	8,54	92,6
25	0,110	8,39	90,9
33	0,102	7,78	84,3
41	0,101	7,70	83,5
50	0,097	7,40	80,2
60	0,095	7,25	78,5

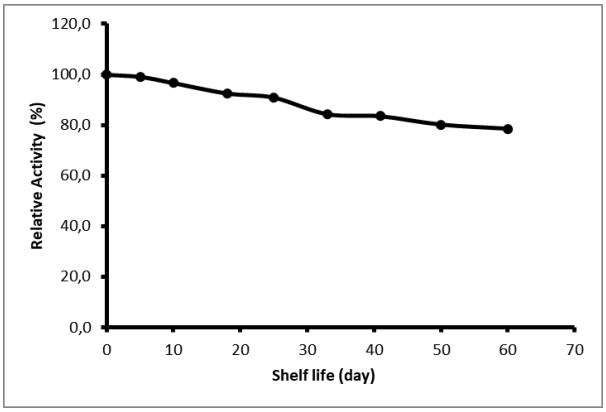

Figure 3.16. Relative activity change with a two-month storage period of the bound enzyme (Bardakçı) two months storage period

4. RESULTS

In our study, the laccase enzyme was bound on different clays by the adsorption method, and the properties of these enzymes were investigated. As a result of the experiments, the following conclusions have been reached: The optimum pH was determined to be 5.5 for laccase enzyme immobilized to the free enzyme, bentonite, and diatomite clay minerals, and 6.0 for laccase immobilized to Bardakçı clay. However, it was found that the activity of the immobilized enzyme was higher at alkaline pH than the free enzyme. It has been determined that immobilized laccases can be used with higher activity in a wider pH range than free laccase. The optimal temperature for the free and bound enzyme was determined to be 40°C. However, all bound enzymes showed an activity close to the maximum of up to 50°C. K_M of free laccase was determined as 0.0700 mM and V_{max} as 0.0695 mM.min^{-1}. K_M of laccase adsorbed in bentonite was 0.0724 mM and V_{max} was 0.0216 mM.$^{min-1}$; The K_M of the laccase adsorbed to diatomite was 0.0831 mM and the V_{max} was 0.0236 mM.$^{min-1}$, the K_M of the laccase adsorbed to Bardakçı clay was 0.0935 mM and the Vmax was 0.0233 mM.$^{min-1}$. It was determined that free laccase preserved 28.6% of its initial activity on day 60 when stored at 4 °C, and 70-80% of the initial activity of bound laccase was preserved on day 60 when stored under the same conditions. According to these results, immobilized enzymes can be used in various industrial areas due to their shelf-life stability, and higher activity than free enzymes in a wide temperature and pH range.

Declaration of Author Contributions

The authors declare that they have contributed equally to the article. All authors declare that they have seen/read and approved the final version of the article ready for publication.

Declaration of Conflicts of Interest

All authors declare that there is no conflict of interest related to this article.

Funding

This study was carried out with the project numbered 2010-FBE-YL055 and titled "Immobilization of Laccase Enzyme on Some Clay Minerals and Investigation of Kinetics". It was supported by Yüzüncü Yıl University Scientific Research Projects Presidency.

Acknowledgments

This study is the master's thesis of Fulya ÖZDEMİR. This study was conducted at Yüzüncü Yıl University, Department of Chemistry, Van, Turkey. In addition, this article is based on Ufuk University, Vocational School of Health Services, Ankara, Turkey, and Written at Ankara University, Department of Chemistry, Ankara, Turkey.

References

Al-Adhami, A., Abdulkareem, J.H., Bryjak, J., Greb-Markiewicz, B., Peczyńska-Czoch, W., 2002. Immobilization of wood-rotting fungi laccases on modified cellulose and acrylic carriers. *Process Biochemistry,* 37(12): 1387–94.

Alkan, S., 1999. Katalaz enziminin sepiolit, bentonit ve kaolin killeri üzerine adsorpsiyonu ve kinetiginin incelenmesi. Doktora Tezi, Yüzüncü Yıl Üniversitesi, Fen Bilimleri Enstitüsü, Van.

Almansa, E., Kandelbauer, A., Pereira, L. 2004. Influence of structure on dye degradation with laccase mediator systems. *Biocatalysis and Biotransformation,* 22(5–6): 315–24.

Arica, M.Y., Şenel, S., Alaeddinoğlu, N. 2000. Invertase ımmobilized on spacer-arm attached poly (Hydroxyethyl Methacrylate) membrane: preparation and properties. *Journal of Applied Polymer Science* 75(14): 1685–92.

Bar, M. 2001. Kinetics and physico-chemical properties of white-rot fungal laccases.

Basso, A., Simona S. 2019. Industrial applications of ımmobilized enzymes—a review. *Molecular Catalysis,* 479: 110607.

Bilal, M., Rasheed, T., Nabeel, F., 2019. Hazardous contaminants in the environment and their laccase-assisted degradation – a review. *Journal of Environmental Management,* 234: 253–64.

Bryjak, J., Kruczkiewicz, P., Rekuć, A., Peczyńska-Czoch, W., 2007. Laccase ımmobilization on copolymer of butyl acrylate and ethylene glycol dimethacrylate. *Biochemical engineering journal,* 35(3): 325–32.

Cabrita, J., Abrantes, L., Viana, A., 2005. N-Hydroxysuccinimide-Terminated Self-assembled monolayers on gold for biomolecules ımmobilisation. *Electrochimica Acta,* 50(10): 2117–24.

Cavani, F., Trifiro, F., Vaccari, A., 1991. Hydrotalcite-Type anionic clays: preparation, properties and applications. *Catalysis Today,* 11(2): 173–301.

Chen, Y., Liao, R., Yu, C., Yu, X., 2020. Sorption of Pb(II) on sodium polyacrylate modified bentonite. *Advanced Powder Technology,* 31(8): 3274–86.

D'Annibale, A., Stazi, S., Vinciguerra, V. 1999. Characterization of ımmobilized laccase from lentinula edodes and ıts use in olive-mill wastewater treatment. *Process Biochemistry,* 34(6–7): 697–706.

D'Annibale, A., Stazi, S., Vinciguerra, V. 2000. Oxirane-Immobilized lentinula edodes laccase: stability and phenolics removal efficiency in olive mill wastewater. *Journal of Biotechnology,* 77(2–3): 265–73.

Deska, M., Kończak, B., 2019. Immobilized fungal laccase as 'green catalyst' for the decolourization process – state of the art. *Process Biochemistry,* 84: 112–23.

Dinh, V., Nguyen, P., Tran, M. 2022. HTDMA-Modified Bentonite Clay for Effective Removal of Pb(II) from Aqueous Solution. *Chemosphere,* 286: 131766.

Dodor, D.E., Hwang, H., Ekunwe, S. 2004. Oxidation of anthracene and benzo [a] pyrene by ımmobilized laccase from trametes versicolor. *Enzyme and Microbial Technology*, 35(2–3): 210–17.

Fathali, Z., Rezaei, S., Faramarzi, M., Mehran, H. 2019. Catalytic phenol removal using entrapped cross-linked laccase aggregates. *International Journal of Biological Macromolecules*, 122: 359–66.

Georgieva, S., Godjevargova, T., Portaccio, M. 2008. Advantages in using non-ısothermal bioreactors in bioremediation of water polluted by phenol by means of ımmobilized laccase from rhus vernicifera. *Journal of Molecular Catalysis B: Enzymatic* 55(3–4): 177–84.

Gökgöz, M. 2006. Lakkazın poliakrilamit ve poliakrilamit-k-karragenan jellerine immobilizasyonu.Yüksek Lisans Tezi, Kırıkkale Üniversitesi, Fen Bilimleri Enstitüsü, Kırıkkale.

Gupta, G., Rajendran, V., Atanassov, P., 2003. Laccase biosensor on monolayer-modified gold electrode. *Electroanalysis: An International Journal Devoted to Fundamental and Practical Aspects of Electroanalysis* 15(20): 1577–83.

Ha, J., Oh, E., Song, I., 2013. The fabrication and characterization of sintered diatomite for potential microfiltration applications. *Ceramics International,* 39(7): 7641–48.

Hu, X., Zhao, X., Hwang, H., 2007. Comparative study of ımmobilized trametes versicolor laccase on nanoparticles and kaolinite. *Chemosphere*, 66(9): 1618–26.

Hublik, G., Schinner, F. 2000. Characterization and ımmobilization of the laccase from pleurotus ostreatus and ıts use for the continuous elimination of phenolic pollutants. *Enzyme and microbial technology*, 27(3–5): 330–36.

Ji, C.,Nguyen, L., Hou, J., 2017. Direct ımmobilization of laccase on titania nanoparticles from crude enzyme extracts of *P. ostreatus* culture for micro-pollutant degradation. *Separation and Purification Technology*, 178: 215–23.

Lante, A, Crapisi, A. Krastanov, A., Spettoli, P., 2000. Biodegradation of phenols by laccase ımmobilised in a membrane reactor. *Process Biochemistry*, 36(1–2): 51–58.

Leonowicz, A., Grzywnowicz, K., 1981. Quantitative estimation of laccase forms in some white-rot fungi using syringaldazine as a substrate. *Enzyme and microbial technology,* 3(1): 55–58.

Leonowicz, A., Sarkar, J., Bollag, J., 1988. Improvement in stability of an ımmobilized fungal laccase. *Applied Microbiology and Biotechnology,* 29(2): 129–35.

Leskovac, V. 2003. Kinetics of monosubstrate reactions. *Comprehensive Enzyme Kinetics*, 31–49.

Li, N., Tao, X., Liu, F., 2018. Influence of UHVDC in qinghai province on peak operation of northwest power grid. In 2018 2nd IEEE Conference on Energy Internet and Energy System Integration (EI2), IEEE, 1–9.

Minussi, R., Pastore, G., Durán, N., 2002. Potential applications of laccase in the food ındustry. *Trends in Food Science & Technology,* 13(6): 205–16.

Mosbach, K., Larsson, P., Lowe, C., 1976. [61] Immobilized coenzymes. In *Methods in Enzymology*, Elsevier, 859–87.

Nayak, A, Bhushan, B., 2019. An overview of the recent trends on the waste valorization techniques for food wastes. *Journal of Environmental Management,* 233: 352–70.

Quan, De., Kim, Y., Shin, W., 2004. Characterization of an amperometric laccase electrode covalently ımmobilized on platinum surface. *Journal of Electroanalytical Chemistry*, 561: 181–89.

Rahmani, H., Lakzian, A., Karimi, A., Halajnia, A., 2020. Efficient removal of 2,4-dinitrophenol from synthetic wastewater and contaminated soil samples using free and ımmobilized laccases. *Journal of Environmental Management*, 256: 109740.

Reichle, W.T., 1986. Synthesis of anionic clay minerals (Mixed Metal Hydroxides, Hydrotalcite). *Solid State Ionics*, 22(1): 135–41.

Riva, S., 2006. Laccases: blue enzymes for green chemistry. *Trends in Biotechnology*, 24(5): 219–26.

Rogalski, J., Dawidowicz, A., Jóźwik, E. Leonowicz, A. 1999. Immobilization of laccase from cerrena unicolor on controlled porosity glass. *Journal of Molecular Catalysis B: Enzymatic*, 6(1–2): 29–39.

Rouhani, S., Rostami, A., Salimi, A. 2016. Preparation and characterization of laccases ımmobilized on magnetic nanoparticles and their application as a recyclable nanobiocatalyst for the aerobic oxidation of alcohols in the presence of TEMPO. *RSC Advances*, 6(32): 26709–18.

Shao, B., Liu, Z., Zeng, G., 2019. Immobilization of laccase on hollow mesoporous carbon nanospheres: noteworthy ımmobilization, excellent stability and efficacious for antibiotic contaminants removal. *Journal of Hazardous Materials*, 362: 318–26.

Taşdelen, Ç. 2006, Proteaz enziminin fiziksel adsorpsiyon, kovalent ve iyonik bağlanma metotları ile immobilizasyonu. Yüksek Lisans Tezi, İstanbul Teknik Üniversitesi, Fen Bilimleri Enstitüsü, İstanbul.

Tavares, A., Silva, C., Drazic, G. 2015. Laccase ımmobilization over multi-walled carbon nanotubes: kinetic, thermodynamic and stability studies. *Journal of Colloid and Interface Science*, 454: 52–60.

Uruç, H., 2007. Katalaz enziminin montmorillonit analsim kili üzerine ımmobilizasyonu ve kinetiğinin incelenmesi. Yüksek Lisans Tezi Yüzüncü Yıl Üniversitesi Fen Bilimleri Enstitüsü, Van.

Vaccari, A. 1999. Clays and Catalysis: A promising future. *Applied Clay Science*, 14(4): 161–98.

Vasconcelos, P., Labrincha, J., Ferreira, J. 2000. Permeability of diatomite layers processed by different colloidal techniques. *Journal of the European Ceramic Society*, 20(2): 201–7.

Yamak, O., Kalkan, N., Aksoy, S. 2009. Semi-Interpenetrating polymer networks (semi-ıpns) for entrapment of laccase and their use in acid orange 52 decolorization. *Process Biochemistry*, 44(4): 440–45.

Yamak, Ö. 2007. Poli (Akrilamit)-Sodyum aljinat, poli (akrilamit-n-izopropilakrilamit)-sodyum aljinat, poli (akrilamit-n-izopropilakrilamit) hidrojellerine lakkaz immobilizasyonu. Yüksek Lisans Tezi, Gazi Üniveristesi Fen Bilimleri Enstitüsü, Ankara.

Yang, W., Wen, S., Jin, L. 2006. Immobilization and Characterization of Laccase from chinese rhus vernicifera on modified chitosan. *Process Biochemistry*, 41(6): 1378–82.

Ye, X., Kang, S., Wang, H. 2015. Modified natural diatomite and ıts enhanced ımmobilization of lead, copper and cadmium in simulated contaminated soils. *Journal of Hazardous Materials* 289: 210–18.

Yeom, H., Kim, S., Kim, Y., In-Hyuck Song. 2016. Processing of alumina-coated clay–diatomite composite membranes for oily wastewater treatment. *Ceramics International*, 42(4): 5024–35.

Zdarta, J., Meyer, A., Jesionowski, T.,Pinelo, M. 2018. Developments in support materials for ımmobilization of oxidoreductases: a comprehensive review. *Advances in Colloid and Interface Science,* 258: 1–20.

To Cite: Özdemir, F., Yalçınkaya, Z., 2023. Examination of The Immobilization and Kinetics of The Laccase Enzyme on Various Clay Minerals. *MAS Journal of Applied Sciences*, 8(2): 286-306. DOI: http://dx.doi.org/10.5281/zenodo.7956783.