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Purification and Characterization of Laccase from Thermophilic *Anoxybacillus Gonensis* P39 and its Application of Removal Textile Dyes

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Abstract

Textile dyes which are extensively used have important incomes commercially however they cause health and environmental problems. In this study, Anoxybacillus gonensis P39 (Gen Bank No:FJ808725) isolated from Erzurum-Ilica Spring and laccase from A. gonensis P39 was purified as 160 kDa molecular weight by using $(NH_4)_2SO_4$ precipitation, DEAE-cellulose and Sephacryl S200 coloumn with 4.16, 11.96 and 83.4 purification folds, orderly. Vmax and Km were calculated as 0.442 µmol/min.mg and 0.077 mM. Optimum pH and temperature were 5.0 and 60°C moreover, laccase was stable against high temperature and pH conditions. Although most of the metal ions were positively affected laccase activity, Mn^{2+}, Cu^{2+} and Fe^{2+} were strong inhibitors. Consequently, removal of some textile dyes was evaluated and the laccase reduced the Reactive Black5, Fuchsine, Allura Red and Acid Red 37 dyes by 22.1, 5.96, 1.34 and 1.15%, respectively so that A. gonensis P39 can be successfully used for bioremediation of waste water.

Keywords: *Purification; laccase; thermophilic bacteria; Anoxybacillus gonensis; textile dyes*

1. Introduction

The dyes, which are of major importance, are commonly used in different industrial fields such as cosmetics, food, paper and textile; however, they have negative effects on the environment. The dyes have potential to show photosynthetic activity due to decrease in luminous transmittance and toxic effects on living organisms [1]. The amount of commercial dyes used in the textile industry, particularly the synthetic–azo dyes, is rather high and the annual production of such dyes in the market is also as high as that. These synthetic–azo dyes are toxic, mutagenic and carcinogenic [2]. The textile dyes, when exposed to heat, light and different chemical oxidizing agents, on the other hand, are rather resistant to discoloration due to their chemical structure and as they are produced synthetically, it is rather challenging to enable the decoloration of most of these dyes. Because of the fact that, the disintegration products of these dyes are far more toxic, enzymatic methods are preferred instead of physico-chemical ones in the treatment of the wastes containing textile dyes [3]. Laccase is an enzyme which belongs to the oxidoreductase class. (EC 1.10.3.2; benzenediol: oxygen

oxidoreductase). As well as oxidizing the compounds associated with both phenolic and non-phenolic lignin, it can also de-oxidize the environmental pollutants resistant to biodegradation [4].Consequently, it is possible to use the laccase in many industrial areas, such as the removal of textile dyes, phenols and detoxification of wastes, since it does not have the substrate specificity [5, 6]. Laccases exist in 3 different classes as botanical, fungal and bacterial. It is found in numerous plants and white-rot fungi. However, the fungal strains to be used should be appropriate for inexpensive, fast and sensitive methods and screenings, and the laccase production should also be resistable and functional in the industrial field and it will be applied [7]. The bacterial laccase was identified, for the first time, in *Azospirillum lipoferum* [8]. The laccase, in the cell pigmentation in bacterium, serves in the oxidation of phenolic compounds [9, 10] and in the electron transport [11].The existence of laccase is determined in rather a small number of thermophilic bacteria, such as *Streptomyces lavendula* REN-711, *Pyrobaculum aerophilum* IM2, *Aquifex aeolicus* VF5, *Thermus thermophilus* and *Bacillus pumilus* [12-16]. In general, the laccase intracellularly exists in the bacterial cells. Thus, this leads to both less amount of enzyme generation and a decrease in the durability of the enzyme in industrial practices. Therefore, the aim of this study was that laccase producer bacterium, from isolations of Erzurum-Ilicahotsprings that have been already carryout out by our research group, will be used for research experiments. These researches will be purification and chracterization of laccase from bacteria located in Erzurum-Ilica hotspring and finally industrial applications of the laccase will be tested by removal of textile dyes-Reactive Black 5, Fuchsine, Allura Red and Acid Red 37.

2. Materials and methods

2.1. Isolation of thermophilic bacteria

Water samples were collected from Erzurum-Ilica hotspring and isolation and chracterization studies were carried out as decribed before [17].

2.2. 16S rRNA gene sequence analysis

16S rRNAgene sequencing analysis was performed as previous studies [18, 19].

2.3. Purification of laccase from A. gonensis **P39**

The test strain was identified and chracterized by 16S rRNA genotypic method and then it was cultivated for 24-48 hours at 55°C inNutrient Broth submerged medium. The crude enzyme extract had been filtered and centrifuged for 15 min at 5000 *x*gafter cultivation [20].

2.4. Ammonium sulphate precipitation of laccase

The crude laccase was used for ammonium sulfate precipitation at 0-20%, 20-40%, 40-60%, 60-80% and 80-100% intervals, respectively. Afterwards, to saturate the obtained supernatant at 60-80%, ammonium sulphate was used [21]. The precipitate was dissolved in 20 mM of Na-acetate buffer (pH: 5.0) and dialyzed against the same buffer.

2.5. Anion exchange chromatography

The suspension which was obtained through ammonium sulfate precipitation was dialyzed with the 20 mM of Na-acetate buffer (pH: 5.0) and previously equilibrated DEAE-cellulose ion exchange column (2.5 x 30) were studied. Until the eluate protein detection failed, the column was washed with the same buffer. The NaCl gradient from 0 up to 1 M was

applied to elute the proteins attached to the column. The flow rates of the collected fractions were determined to be 3 mL, with a 3 mL/min. The absorbance of protein elution was spectrophotometrically measured at 280 nm.

The enzyme activity was also pectrophotometrically measured at 734 nm by using ABTS substrate and the active fractions were combined and they were allowed to stand at 4 °C [22].

2.6. Gel filtration

By using an Amicon membrane concentrator with a 10 kDa cut off, the active fractions, which had been previously obtained from anion exchange column, were combined, dialyzed, desalted and concentrated. The already obtained enzyme solution was applied to Sephacryl S-200 column (120 cm × 1 cm), which was pre-equilibrated with 20 mM Na-acetate buffer (pH: 5.0) having 0.5 M NaCl and later the enzyme was obtained with the same buffer. All of fractions were analyzed as stated above. For later use, the active fractions were then combined, concentrated and they were allowed to stand at 4°C [23].

2.7. Protein concentration

The protein concentration was established spectrophotometrically according to the Bradford's method [24].

2.8. Determination of laccase activity

The laccase activity was determined by using ABTS substrate [25, 26]. The reaction mixture was prepared by adding 0.5 mL of the enzyme solution on top of the ABTS (3 mM) substrate, which was dissolved in 0.5 mL of 0.1 M acetate buffer (pH: 5.0), and then it was incubated at 55°C. The oxidation of ABTS was determined by monitoring the increase in absorbance at 420 nm. One unit of a laccase activity was described as the required amount of enzyme to oxidize 1 μmol of ABTS/min (ε_{420}:36,000 M^{-1} cm^{-1}) [6, 27]. The laccase activity was determined by taking measurements at 420 nm, 470 and 468 nm respectively, by making use of 3 mM ABTS, guaiacol and 2,6- dimethoxyphenol substrates in order to find out the Km and Vmax values. The blank, which was used as the sample, was prepared by adding the enzyme-containing buffer, instead of enzyme solution [26-31]. One unit of laccase was defined as the amount of enzyme that catalysed the oxidation of one micromol of substrate (guaiacol, ABTS and 2,6-dimethoxyphenol substrates) in a minute. The reactions were carried out in room temperature.

2.9. SDS polyacrylamide gel electrophoresis

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was realized through the 10-30% discontinued electrophoresis method, as defined by Laemmli [32], 20 μg of protein was applied to each of the samples. Electrophoresis in a gel casting apparatus (Bio- RAD) execution buffer (0.25 M Tris, 1.92 M Glycine, 1% SDS (pH: 8.3) was performed at 4°C. After the gel was dyed with 0.1% Coomassie Brilliant Blue R-250 in a solution containing 50% methanol, 10% acetic acid and 40% distilled water for 1.5 h, the gel was washed with 50% methanol, 10% acetic acid and 40% distilled water until its surface became limpid. The electrophoretic pattern was photographed.

2.10. Determination of molecular weight by gel filtration

The molecular weight of the laccase was measured by using column (3 x 70 cm) of Sephadex G100; the column was equilibrated until it reached zero absorbance, by using 0.05 M Na_2HPO_4, 1 mM dithioerythretol, pH: 7.0 buffer. Standard protein solution (bovine serum albumin, 66 kDa, egg ovalbumin, 45 kDa, pepsin, 34 kDa, trypsinogen, 24 kDa, s-lactoglobulin and lysozyme, 14 kDa) was applied to the column, and it was eluted from the column with the same buffer. After then, the pure laccase was applied to the column and it was eluted under the same conditions, the flow-rate through the column was determined as 20 mL/h. The elution volume was compared with standard proteins [33].

2.11. Determination of the effects of chemicals and metal ions on enzyme activity

The effects of such metal ions as Fe^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, Ba^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, EDTA and ascorbic acid in different inhibitory concentrations on laccase were investigated. All metal ions were tested in three runs for each concentration. Laccase activity was measured at different concentrations of the inhibitors.

2.12. Remediation of some textile dyes

In this study, whether the purified laccase could be used in the removal of some dyes used in industry or not was investigated. Therefore, Reactive black 5, Fuchsine, Allura Red, Acid red 37 were used in this study to attain the predetermined goals. The dye solutions were prepared at 50 mg/L concentration and the reaction preparation was prepared by taking 50 mL from these solutions and adding 1 mL (5 µg protein) purified laccase,. Each dyestuff was separately studied. The reaction medium was set to pH: 4.0 and 55°C, which were regarded as the optimum pH and temperature for the enzyme, the samples were taken at 15^{th}, 30^{th}, 60^{th}, 90^{th}, 120^{th}, 150^{th} and 180^{th} minutes from the medium. Afterwards, the absorbance was measured spectrophotometrically for Reactive Black 5, Fuchsine, Allura Red, and Acid Red 37 at 597 nm, 547 nm, 513 nm and 504 nm, respectively. The obtained results are shown in the chart as absorbance vs. mg dye plots. The colour intensity was measured spectrophotometrically in the reaction medium and the yield of transformation was calculated from the reduction in absorbance [34].

2.13. Statistical analysis

In order to determine the laccase activities of the samples, all of the tests were performed in three runs and the related data were expressed in terms of mean ± standard errors. Statistical analyses were performed by using SPSS version 20.0 software (SPSS Inc., Chicago, IL., USA), and significant differences were determined with a 95% confidence interval ($p<0.05$) according to Tukey's test.

3. Results and discussion

3.1. Results of 16S rRNA analysis

A total of 1431 nucleotides of 16S rRNA from the test strain were aligned and compared with sequences of related bacteria. The isolate exhibited 99% resemblance to *A. gonensis*, according to 16S rRNA gene sequence analysis [17].

3.2. Production, purification and characterization of laccase enzyme from A. gonensis P39.

In our previous edition, from the thermophilic *A. gonensis* P39 [17], the laccase enzyme was extracellularly purified and characterized. The laccase enzyme was purified extracellularly from the *A. gonensis* P39 in 3 steps, by using the ammonium sulfate precipitation, DEAE-cellulose and sephacryl S200 gel filtration chromatography (Table 1).

Table 1. Purification steps of laccase produced by *A. gonensis* P39

Purification Steps	Volume (mL)	Activity (EU/mL)	Total Activity (EU)	Yield (%)	Protein Amount (mg/mL)	Specific Activity (EU/mg protein)	Purification Fold
Extract	50	50.07 ± 0.13	2503.5	100	2.28 ± 1.11	21.96	-
$(NH_4)_2SO_4$ (%60-80)	25	40.24 ± 1.12	1006.0	40.2	0.44 ± 0.6	91.45	4.16
DEAE-Cellulose	25	36.78 ± 2.1	919.5	36.7	0.14 ± 3.1	262.7	11.96
Sephacryl S200	25	32.98 ± 0.17	824.5	32.9	0.018 ± 2.4	1832.2	83.4

The laccase was precipitated in the 60-80% range and the highest rate of precipitation was exhibited at 4.16 purification-folds with a 40.2% yield/efficiency. Then this precipitated was solved with a buffer and a sample was applied to DEAE-cellulose ion exchange column. The enzyme had a peak at DEAE cellulose ion exchange column in the second step, and further purified 11.96 times with a 36.7% yield. And in the final step, the obtained enzyme fractions from the ion exchange column were pooled and concentrated and this sample was applied to the Sephacryl S 200 column. A single peak was obtained, and 1832.2 EU/mg protein specific activity was reached with the 83.4 times purification and 32.9% yield (Table 1). The elution profiles of the laccase, which was purified from *A.gonensis* P39 bacteria using anion-exchange chromatography and gel filtration chromatography, are shown in Figure 1 and Figure 2. The Sephadex G-100 gel filtration chromatography analysis determined that the laccase enzyme consists of 160.694, 83.176 and 40.052 kDa of sub-units (Figure 3).

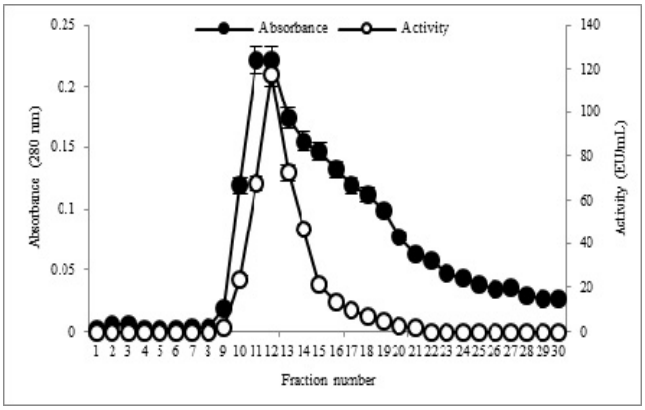

Figure 1. Elution profile of extracellular laccase produced by *A. gonensis* P39 from a DEAE-cellulose ion-exchange column chromatography in 20 mM (pH 5.0) Na-acetate buffer. (—o—) : OD at 280 nm; (—●): activity. The column was eluted at a flow rate of 80 mL/h with a linear gradient of NaCl from 0 to 1 M. The fraction volumes were 3 mL. The height of the columns was 20 cm

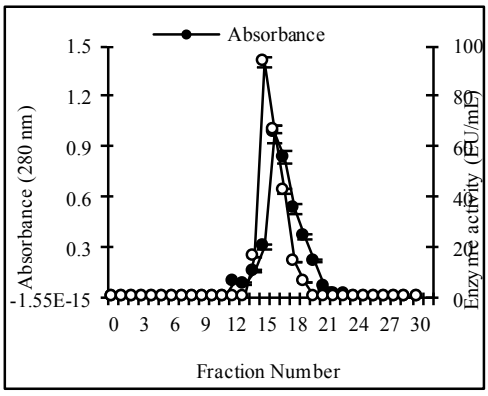

Figure 2. Elution profile of protein and laccase activity on Sephacryl S200

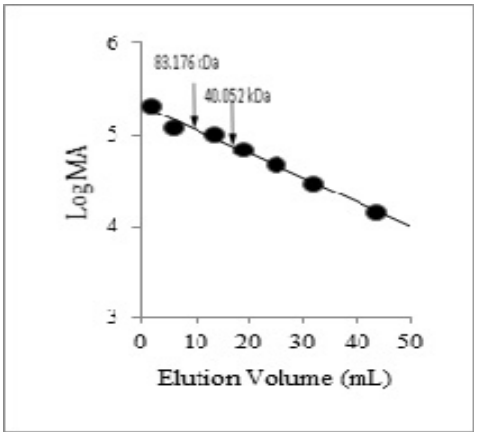

Figure 3. Standard *Kav*-Log MW graph of laccase from *A. gonensis* P39 using gel filtration

From this point, it was concluded that the enzyme was approximately 160 kDa with 4 sub-units that one sub-unit is 40 kDa and the other sub-units are the disintegration products of the enzyme. The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was also performed and a single band at a level of 40 kDa was observed (Figure 4).The molecular weight of the laccase from *Streptomyces griseus* (EpoA) and Sinorhizobium meliloti bacteria was found as 114 kDa and 90 kDa, and they were homotrimer [34-36]. In addition, the laccases obtained from the bacteria, *Thermus thermophilus* HB27, *Pseudomonas putida* GB1, and *P. maltophilia* have a single sub-unit, and their molecular weights were determined as 53, 50, 50 kDa respectively [37-39].

3.2. Determination of optimum pH and stability at different pH

In order to find the optimum pH of the laccase purified and derived from *A. gonensis* P39, seven different pH mediums were studied, ranging from pH 3 to 9. The buffers used were: acetate for pH 3 to 5, phosphate for pH 6 to 7, and Tris-HCl for pH 8 to 9. The activity measurements were made using ABTS substrate; the activity was calculated by using the obtained results. The enzyme activities in different pH were plotted and the results are shown in Figure 5. The optimum pH value was found as 5.0 for the ABTS substrate. The laccases from *Polyporus pinsitus, Myceliophthora thermophila* and *Streptomyces cyaneus* were variable and present in their activities in pH range of 4-6 [29, 30]. The laccase enzyme was incubated for 5 days at +4°C and the activity measurements were performed periodically, and the findings are shown in Figure 6. As can be seen in the plots, it was found out that the laccase maintains 55.6% of its activity after 5 days, and has a stable pH of 5.0 for ABTS.

3.3. Optimum temperatureand stability at different temperatures

In order to find the optimum temperature of the purified laccase, the activity was measured for each substrate in the temperature range of 20 to 90°C, by keeping it for 5 minutes at each temperature. As can be seen in Figure 7, it was found that the laccase was highly active between 30 to 80°C for ABTS substrate. The optimum temperature was determined as 60°C. Similarly, it was also found in the study conducted by Diamantidis et al. [40] that the laccase enzyme from *Azospirillum lipoferum* had the optimum temperature of 60°C. In order to determine purified enzyme's stable temperature, it was incubated for 60 minutes at 30-90°C. The activity measurements have been performed by standing the enzyme at the specified temperatures from 15 to 60 minutes and adding the substrate later.

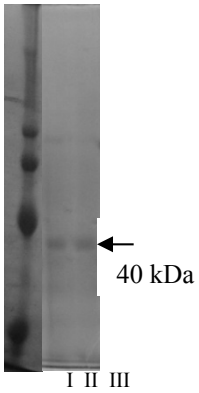

Figure 4. SDS-PAGE electrophoretic pattern of laccase [standart protein (β-Amylase, sweet potato, 200 kDa; alcohol dehydrogenase, yeast, 150 kDa bovine serum albumin, 97.4 kDa; rabbit muscle phosphorylase A, 66 kDa; egg ovalbumin, 45 kDa; pepsin, 29 kDa; carbonic anhydrase); cytochrome c, horse heart 12.4 kDa (I); II: and III: laccase (purified laccase from*A. gonensis* P39)]

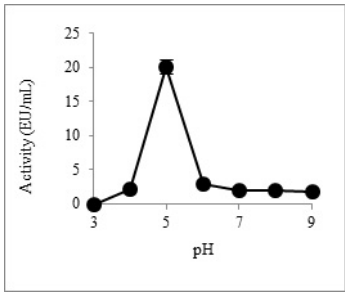

Figure 5. The effects of pH on the laccase activity

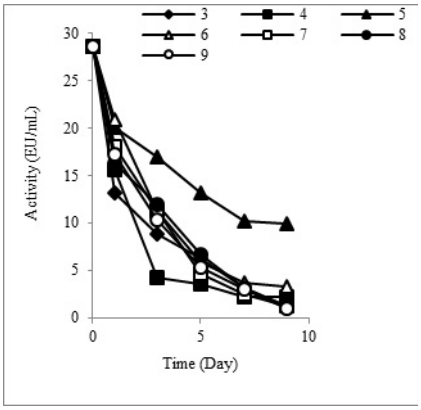

Figure 6. pH stability of extracellular laccase from *A. gonensis* P39 with ABTS substrate

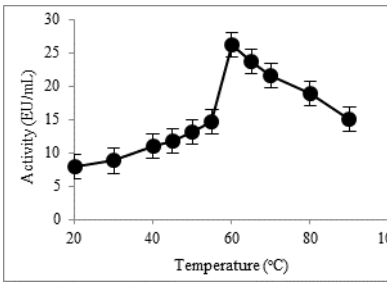

Figure 7. The effects of temperature on the laccase activity

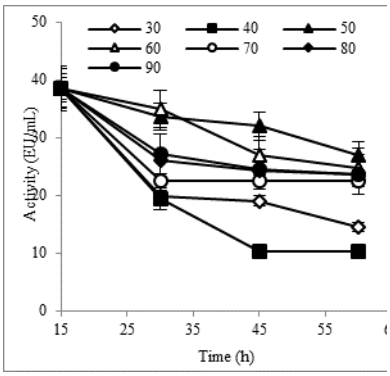

Figure 8. The stability of enzyme at different temperatures with ABTS substrate

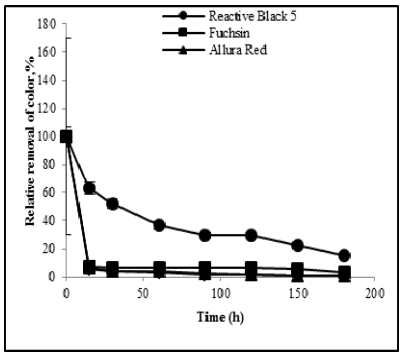

Figure 9. Time-dependent change of the azo dyes concentration

ABTS was used as a substrate. It was observed that purified enzyme from*A. gonensis* P39 maintains most of its activity after 1 hour at all temperatures (Figure 8). In general, bacterial laccases are stable at 70°C for half an hour and at 80°C for 10 minutes [4, 41]. However, the laccase that we have purified from *A. gonensis* P39 was more resistant to heat than the bacterial laccases purified previously. The stability of *A. gonensis* P39 laccase at high temperatures increased the usability of this enzyme in different industrial areas.

3.5. Determination of the Vmax and Km values of the laccase enzyme

The studies on Vmax and Km values of the peroxidase were performed with 5 mM of ABTS, Guaiacol (phenolic substrates) and 2.6 dimetoxyphenol (non-phenolic substrate). The kinetic parameters obtained by using different substrates were summarized in Table 2.

Table 2. V_{max} and K_m values for different substrate (ABTS, 2,6-dimetoxyphenol, Guaiacol)

Substrates	Vmax (µmol/min.mg)	Km (mM)
ABTS	0.067	0.084
2,6-dimetoxyphenol	0.075	0.105
Guaiacol	0.442	0.077

In order to calculate the Vmax and Km values for ABTS, 2,6-DMP and guaiacol substrates, Lineweaver Burk graphics were drawn. It was found that, Vmax was 0.442 µmol/minmg and Kmwas 0.077 mM of the pure laccase in the presence of guaiacol, and the highest affinity and the most effective oxidation of the enzyme was against guaiacol, in comparison with other substrates. Vmax values for the ABTS and 2,6-DMP substrates were 0.067 µmol/min.mg, 0.075 µmol/min.mg, and the Km values were 0.084 mM and 0.105 mM, respectively. Km and Vmax values were determined and the highest affinity was observed against guaiacol for purified enzyme. In a research, Km values were found as 10.23 and 2.66 mM for the laccase purified from *Fusarium solani* MAS2 [42] and *Trichoderma atroviride* [43] by using 2,6-DMP as a substrate.

3.6. Effects of Ca^{2+}, Mg^{2+}, Co^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, EDTA and ascorbic acid on the activity of the laccase

The effects of Ca^{2+}, Hg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Ba^{2+}, Ni^{2+}, Co^{2+}, K^{+}, EDTA, DTH and ascorbic acid on the activity of the laccase purified from *A. gonensis*P39 have been studied. The obtained values on inhibitory effects and concentration vs. activity values were shown in Table 3.

Table 3. The effects of some metal ions on enzyme activity

Chemical Compounds	Concentration (mM)	Laccase Activity (%)	Concentration (mM)	Laccase Activity (%)
None	-	100	-	100
DTH	1	25.5	5	14.9
Ascorbic acid	1	10.9	5	1.8
EDTA	1	21.99	5	7.9
Ca^{2+}	1	86.97	5	88.8
Hg^{2+}	1	112.7	5	127.5
Mn^{2+}	1	52.8	5	41.3
Zn^{2+}	1	112.6	5	153.9
Cu^{2+}	1	3.5	5	0
Fe^{2+}	1	62.4	5	10.9
Ba^{2+}	1	91.5	5	107.3
Ni^{2+}	1	142.5	5	379.2
Co^{2+}	1	61.6	5	181.2
K^{+}	1	65.9	5	83.6

As shown in Table 3, Hg^{2+}, Zn^{2+}, Ba^{2+}, Ni^{2+}, Co^{2+} and K^{+} metal ions have increased the activity of the laccase. However Mn^{2+}, Cu^{2+} and Fe^{2+} ions strongly inhibited the enzyme at both concentrations of 1 mM and 5 mM, EDTA has decreased the laccase relative activity to 21.9% at 1 mM of concentration, and completely inhibited at 5 mM of concentration. And neither ascorbic acid nor Ca^{2+} had any effect on the enzyme activity. The results suggested that the majority of metal ions did not inhibit but activated the laccase, and because of this feature, it was found that laccases were highly suitable for industrial applications.

3.7. Results for removal of certain textile dyes by using laccase enzyme purified from A. gonensis **P39**

The effect of laccase from *A. gonensis* P39, on removal of certain textile dyes such as Reactive Black 5, Fuchsine, Allura Red and Acid Red 37 from waste water, was investigated. Samples were taken at specific intervals from the reaction medium during 180 minutes of reaction, and spectrophotometric measurements were performed at wavelengths where the dyes exhibit a maximum absorbency (Figure 9).At the end of 150 minutes, it was observed that laccase reduced the Reactive Black 5, Fuchsine, Allura Red, Acid Red 37 dyes by 22.1, 5.96, 1.34 and 1.15% respectively (Figure9).The results suggested that purified laccase removed the Fuchsin, Allura Red, Acid Red 37 more efficiently than Reactive Black 5 dye. As shown in Figure 8, the chemical structures of these four dyes were different. Reactive

Black 5, Allura Red and Acid Red 37 were classified as azo dye. This purified enzyme effectivelly removed these dyes. In the previous studies, although some dyes were determined to inhibit the laccase activity [43], in our study, it was determined that the laccase purified from the bacterium, *A. gonensis* P39 was not inhibited, but, on the contrary, it almost removed / destained Reactive Black 5 at a rate of 80%, Allura Red and Acid Red at 37 and Fuchsin, on the other hand, at a rate of 95% from the environment. Previously, it was found out that a dye removal from the environment at an average rate of 70-75% was performed when the same coloring agents were tried to be removed by using the laccase that we purified from *Brevibacillus* sp. (Z1), which shows that the laccases obtained from different sources might have different degree of effects on the same coloring agents [5, 44, 45].

4. Conclusion

The crude extracellular laccase was produced by *A. gonensis* P39 and the laccase was purified using DEAE-celulose ion exchange chromatography and Sephacryl S200 gel filtration chromatography techniques. The results suggest that the laccase purified from *A. gonensis* P39 had a very high catalytic activity against the metal ions, at different temperatures and pH levels. Then, purified laccase was used to remove some purchased textile azo dyes for bioremediation purposes. In conclusion, the datas showed that the laccase obtained from the bacterium, *A. gonensis* P39 could be effectively used for the removal of coloring agents which have always been a major problem in terms of environmental pollution.

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