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Removal of Some Textile Dyes from Aqueous Solution by using a Catalase-Peroxidase from *Aeribacillus pallidus* (P26)

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The textile dyeing wastewater is discharged to the environment in various occasions and causes severe environmental problems. Therefore, more effective treatment methods should be developed to eliminate dyes from the wastewater stream at the source. In this study, catalase-peroxidase enzyme was purified from *Aerobacillus pallidus* (P26) isolated from the Erzurum-Pasinler spring (Gen Bank NO: EU935591). A catalase-peroxidase (CP) using enzyme was purified by ammonium sulphate precipitation, CM-Sephadex ion chromatography and Sephacryl S 200 gel filtration chromatography changing. Optimum pH and temperature values were determined as 7 and 60 °C, respectively. In addition, for each substrate pairs, K_m and V_{max} values were calculated from Lineweaver-Burk graphs. The effect of some metal ions was also investigated on the activity of CP enzyme. Purified catalase-peroxidase from *A. pallidus* (P26) is then applied in the decolorization of Reactive Black 5, Fuchsine, Allura Red and Acid Red 37 in waste water. CP presents a potential as a viable alternative in the decolorization of textile wastewaters.

Key words: *Aeribacillus pallidus* (P26), Catalase-Peroxidase, Purification, Removal dye, thermal stability.

Thermophilic microorganisms are very important in the industry, because of their adaptation to high temperature, pH, salt concentration and high-pressure medium. Since the thermostable enzymes obtained from them are more stable than the mesophilic enzymes, they are widely used in food, leather, detergent, sugar, fruit juice, wine, and paper industry, in pharmacology, and as a hydrolyzing agent in beer, alcohol and starch production. In addition, they form an important field of study, because of their use in the production of acetic acid and vinegar, recycling of metals from the metal ores and waste materials, and for waste treatment[1].

The synthetic and semi-synthetic dyes which have a wide use in the various fields in industry are mostly used in the textile, paint, paper and printing industry. The discharge of colored waste water of these production plants directly to nature without any treatment have both a primary environmental impact of uncontrolled spread of the toxic-carcinogenic aromatic amines to the environment, and a secondary impact that cause the organisms in the water to be killed due to the blockage of sunlight on the water surface[2].

The peroxidases, the commercially important enzymes which are widely used in the industrial field and its purification and characterization is studied by many researchers by making use of different groups of organisms

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(POD; EC 1.11.1.7); catalyze the reaction between the hydrogen donor compounds and H_2O_2, which is a compound that receives these atoms, whereas the catalase enzyme (H_2O_2: H_2O_2oxidoreductase E.C.1.11.1.6), reduces or degrades one peroxide, as hydrogen peroxide and ROOH[2,3]. And the catalase-peroxidase (CP) enzyme; is an enzyme, containing a heme group, plays a role in the removal of H_2O_2, and typically contains 2 heme groups in the active region, and its reaction mechanism takes place over the tyrosine and histidine residues in the active regions[4]. While CP catalyzes the oxidation of organic and inorganic substrates by using hydrogen peroxide, it also additionally catalyzes the dehydrogenation of many compounds of phenolic and non-phenolic nature, such as phenols, hydroquinones and hydroquinone amines. 2-cresol, 2-toluidine, guaiacol, pyrogallol, leucho-malachite green, 4,4'-diaminodiphenylen amine, propionylpromazine, benzidine, o-tolidine, di-o-anisidine and some of the azo-dye derivatives are among these molecules[5].

Catalase-peroxidase was purified and characterized from various organisms such as *Bacillus* sp[6], *Bacillus stearothermophilus*[7], Alkalophilic *Bacillus* sp.[8], *Mycobacterium smegmatis*[9], *M. tuberculosis*[10, 11], *Anacystis nidulans*[12], *Synechococcus* PCC 7942[13], *Synechococcus* PCC 6301[12], *Synechocystis* PCC 6803[14], *Escherchia coli*[15], *Deinococcus radiophilus*[16] up to now. As a result of the literature review, however, a very small number of studies have been observed on the catalase-peroxidase enzyme, having thermo and pH stability. In this study, the termostable catalase-peroxidase enzyme was purified and characterized from *A. pallidus* (P26), and removal of some of the dyes, which are used in the textile industry and causing major environmental problems, from waste waters using pure peroxidase enzyme was investigated.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA), ABTS [2,2'-azino-bis(3-ethylbenzylthiazoline-6-sulfonic acid)], DEAE-sephadex, Sephacryl S-200, ethylene diamine tetra acetic acid (EDTA), dithioerythritol, ß-mercaptoethanol, tris(hydroxymethyl) aminomethane, H_2O_2, 4-methylcatechol, guaiacol, pyrogallol, hydroquinone, Reactive Black 5 (RB5), fuchsine ($C_{20}H_{19}N_3$ •HCl), allura red ($C_{18}H_{14}N_2Na_2O_8S_2$), Acid red 37 diammonium salt ($C_{18}H_{16}N_4O_8S_2$•$2H_3N$) and agents for SDS-PAGE were purchased from Sigma (USA). Sodium acetate (CH_3COONa), ammonium sulphate ($(NH_4)_2SO_4$), sodium chloride (NaCl), $BaCl_2$, $CuCl_2$, $MnCl_2$, $CoCl_2$, $ZnCl_2$, $FeCl_2$, $CaCl_2$, $NiCl_2$ $HgCl_2$, ethylenediaminetetraacetic acid (EDTA), sodium bicarbonate ($NaHCO_3$), sodium acetate (CH_3COONa), ascorbic acid ($C_6H_8O_6$) and sodium hydrogen phosphate monohydrate (Na_2HPO_4 x H_2O) were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Characterization of Thermophilic Bacteria

The isolation, purification and characterization methods of selected thermophilic bacteria used in this study was reported elsewhere[17].

Purification of Catalase-Peroxidase from *A. pallidus* (P26)

After filtering the crude enzyme extract it was centrifuged at 5000 rpm for 15 min and the enzyme homogenate was ammonium sulfate precipitated at 0-20% 20-40% 40-60% 60-80% and 80-100% intervals respectively. The obtained supernatant was saturated to 60-80%, using ammonium sulphate[18]. The precipitate was dissolved in 100 mM phosphate buffer (pH:7) and dialyzed against the same buffer.

Anion exchange chromatography

After the dialysis of the suspension obtained by ammonium sulfate precipitation, 100 mM phosphate buffer (pH:7.0) and previously equilibrated CM-sephadex A50 ion exchange column (2.5 x 30) were performed. The column was washed with the same buffer until the eluate protein detection fails. Then, the proteins attached to the column were eluted by applying NaCl gradient from 0 up to 1 M. The fractions were collected as 3 ml, with a 3 mL/min flow rate. Protein elution absorbance was measured at 280 nm spectrophotometrically. Activity was measured in the fractions using ABTS substrate. Active fractions were combined (pooled) and allowed to stand at 4 °C[2].

Gel filtration

The active fractions obtained from anion exchange column were combined, dialyzed,

desalted and concentrated by using an Amicon membrane concentrator with a 10 kDa cutoff. The obtained enzyme solution was applied to the Sephacryl S-200 column (120 cm × 1 cm), which was pre-equilibrated with 100 mM phosphate buffer (pH:7.0) containing 0.5 M NaCl, and the enzyme was obtained with the same buffer. All fractions were analyzed as described above. Active fractions were combined, concentrated and allowed to stand at 4 °C for later use[2].

Protein concentration

Protein concentration was determined spectrophotometrically (absorbance at 280 nm) in addition to the Bradford's method[19], using bovine serum albumin (BSA) as the standard.

Determination of peroxidase activity

This method, which is used to determine the POD enzyme activities in the study, is based on the formation of purpurogallin by pyrogallol catalyzed by peroxidase, in the presence of H_2O_2, at 430 nm wavelength and 55 ºC temperature. To summarize the POD activity measurement briefly: 2500 µl was taken from the 5 mM pyrogallol substrate solution, which was prepared in 0.1 M Na_2HPO_4 (pH:7.0) buffer, and 500 µl enzyme was added to determine the rate of increase in absorbance at 430 nm for 5 minutes of reaction.

To find the values of K_m and V_{max}, the peroxidase activity was determined by taking measurements at 420 nm, 340 nm and 412 nm respectively by using 5mM ABTS, Hydroquinone and 4-methyl catechol substrates. The blind prepared by adding the buffer, which contains the enzyme, instead of enzyme solution was used as the sample[20].

Catalase (CAT) Activity Determination

Catalase activity was found using the method developed by Havir and Mchale[21]. This method is based on to monitor the decrease in absorbance at 240 nm occurred while H_2O_2 transforms into O_2 and H_2O in the CAT activity measurement environment. The amount of enzyme that reduces absorbance by 1 µmol in 1 minute 25 °C was accepted as 1 enzyme unit and presented as the enzyme unit (EU/mL).

SDS polyacrylamide gel electrophoresis

A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed through the 10-30% discontinued electrophoresis method, which was defined by Laemmli[22]. 20 µg of protein was applied to each of the samples. Electrophoresis in a gelcastingapparatus (Bio-RAD) execution buffer (0.25 M Tris, 1.92 M Glycine, 1% SDS (pH:8.3) was applied at 4 °C.

Gel was dyed with 0.1% Coomassie Brilliant Blue R-250 in 50% methanol, 10% acetic acid and 40% distilled water for 1.5 h. Then the gel was washed with 50% methanol, 10% acetic acid and 40% distilled water until its surface becomes limpid. The electrophoretic pattern was photographed.

Molecular weight determination by gel filtration

Molecular weight of the catalase-peroxidase enzyme was performed by using column (3x70 cm) of Sephadex G100. The column was equilibrated until reaching 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; ß-lactoglobulin and lysozyme, 14 kDa) was applied to the column and was eluted from the column with the same buffer. Then, the pure catalase-peroxidase enzyme was applied to the column and was eluted under the same conditions. The flow-rate through the column was 20 mL/h. The elution volume was compared with standard proteins[23].

Studies on certain in vitro chemicals and metal ions

The impact of the metal ions of Fe^{3+}, 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 catalase-peroxidase enzyme was investigated. All metal ions were tested in three runs for each concentration. Catalase-peroxidase activity was measured in different concentrations of the inhibitor. The % activity values vs. concentration plots were obtained for each chemical or metal ion. I_{50} values were calculated for those that present inhibition[2].

Remediation of some textile dyes

In this study, it was investigated that whether the purified catalase-peroxidase enzyme can be used in the removal of some dyes used in industry or not. For this purpose, Reactive black 5, Fuchsine, Allura Red, Acid red 37 diammonium salt was used as 4 different dyes. The dye solutions were prepared at 50 mg/L concentration, and by taking 50 mL from these solutions 1 mL (5 µg protein), the purified catalase-peroxidase enzyme

was added. The reaction medium was set to pH:7.0 and 55 °C, which is the optimum pH and temperature for the enzyme, and samples were taken at 15th, 30th, 60th, 90th, 120th, 150th and 180th minutes from the medium, and the absorbance was measured spectrophotometrically for Reactive Black 5, Fuchsine, Allura Red, Acid Red 37 Diammonium Salt, at 597 nm, 547 nm, 513 nm and 504 nm, respectively. The results obtained are shown in the chart as Absorbency vs. mg dye plots[6].

Statistical analysis

All the tests were conducted in three runs in order to determine the laccase activities of the samples. Data were expressed in terms of mean ± standard errors. Statistical analyses were performed using SPSS version 10.0 software (SPSS Inc., Chicago, IL., USA), and the significant differences were determined with a 95% confidence interval ($p<0.05$) using Tukey's test.

RESULTS AND DISCUSSION

The bacterial strain isolated in this study was subjected to various biochemical and physiological tests. The results showed that the test strain was gram-positive, catalase, oxidase and amylase positive, forming endospore and mobile rod. The optimum pH and temperature for this strain was found as 7.5-8.5 and 55 ± 1 °C, respectively. The test strain was able to grow in a salt concentration range of 2%-5%. These met the criteria of thermophilic bacteria, which grew at temperatures above 50 °C[18].

16S rRNA Gene Sequence Analysis.

A total 1412 nucleotides of the16S rRNA from the test isolate (P26) were aligned and compared with sequences of related bacteria. On the basis of 16S rRNA gene sequence analysis, the isolate exhibited 99% resemblance to *Aeribacillus pallidus* (P26)[24]. The GenBank accession number for strain P26 is EU935591.

Purification and characterization of catalase-peroxidase enzyme from *A. pallidus* (P26)

The catalase-peroxidase enzyme was purified from the *A. pallidus* (P26) bacteria through three steps, as the extracellular ammonium sulfate precipitation, CM-sephadex and sephacryl S200 gel filtration chromatography. All purification steps of CP are shown in Table 1.

In the first step, ammonium sulfate saturation of CP enzyme in 00-90% interval was determined. The CP enzyme was precipitated in the 60-80% range, and 4.95 times purified with 46.2% efficiency, and applied to CM Sephadex ion exchange column. The enzyme gave a peak at the CM Sephadex ion exchange column in the second step, and purified 13.54 times with 39.1% efficiency.

Table 1. The purification process of catalase peroxidase (CP) from *A. pallidus* (P26).

Purification Steps	Volume (mL)	Ectivity (EU/mL)	Total Activity EU	Total Activity %	Protein Amount	Spesific Activity (mg/mL)	Purification Fold (EU/mg)
Extract	50	275	11000	100	3.81	72.2	-
$(NH_4)_2SO_4$ (%60-80)	20	254	5080	46.2	0.71	357.75	4.95
CM-Sephadex	20	215	4300	39.1	0.22	977.3	13.54
Sephacrty S200	20	196	3920	35.6	0.032	6125	84.8

Table 2. Vmax and Kmvalues of some substrates for catalase-peroxidase from *A. pallidus* (P26).

Substratlar	Vmax (EU/mL.dak)	Km (mM)
ABTS	5.95	3,53
Pirogalol	333.4	10
Hidrokinon	142.9	11.3
4-Metil katekol	20.83	6.2

Table 3. I_{50}values of some chemicals for catalase-peroxidase from *A. pallidus* (P26).

Chemicals	I_{50}
$HgCl_2$	1.67
$ZnCl_2$	1.06
$FeCl_2$	0.68
$BaCl_2$	0.544
EDTA	0.655

In the last step, the enzyme fraction obtained and concentrated from the ion exchange column was applied to the Sephacryl S 200 column. A single peak was obtained, and 6125 EU/mg protein specific activity was reached, with the 84.8 purification, and 35.6% efficiency. The elution profiles of the CP enzyme, which was purified from *A. pallidus* (P26) bacteria using anion-exchange chromatography and gel filtration chromatography, are shown in Fig. 1 and Fig. 2.

The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was revealed that the CP enzyme consists of 4 subunits of size 31 kDa (Fig. 3). The Sephadex G-100 gel filtration chromatography was used to find the molecular weight of the active form of the enzyme. First the standard proteins were passed through the gel filtration column, and log MA-K_{av} was plotted (Fig. 4). Then, the purified peroxidase enzyme solution was passed through the column. During these processes, absorbance of the eluates at 280 nm of the standard proteins and the activity of the fractions of the peroxidase enzyme was determined. It was found that the natural state of the enzyme is 120 kDa according to the standard plot (Fig. 4).

Calandrelli *et al*.[25] in their study, identified that the CP enzyme, which was purified from *Oceanobacillus oncorhynchi* subsp. *incaldaniensis* sub sp. nov., strain 20AG, was composed of four subunits of 72 kDa, as a result of the SDS-PAGE analysis, and its total molecular weight was 280 kDA according to data from the gel filtration analysis. Pongpom*et al*.[26] were observed that the molecular weight of catalase-peroxidase enzyme, purified from *Penicillium marneffei* fungus, was 82 kDa; and Levy *et al*.[27] were observed that the molecular weight of the CP enzyme, isolated from *Septoria tritici* was 61 kDa and has a tetramer structure.

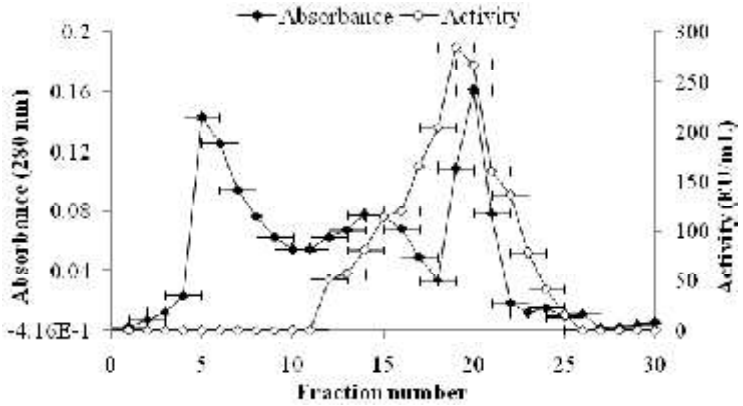

Fig. 1. Purification of catalase-peroxidase of *A. pallidus* (P26) by ion exchange chromatography using CM-Sephadex.

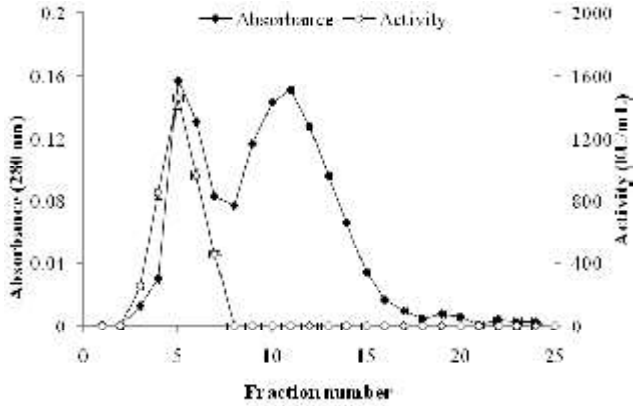

Fig. 2. Elution profile of protein and catalase-peroxidase of *A. pallidus* (P26) activity on Sephacryl S200.

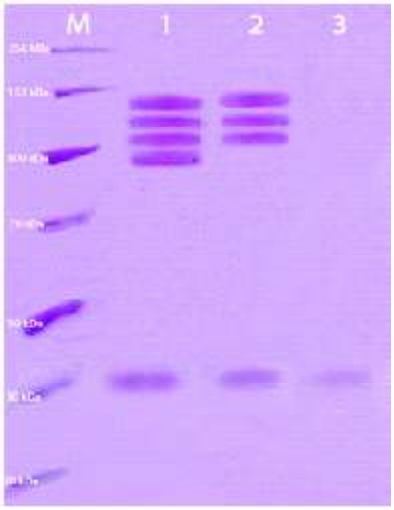

Fig. 3. SDS-PAGE catalase-peroxidase of *A. pallidus* (P26)

In one study, the catalase-peroxidase (CP) enzyme was purified from *Bacillus* sp. strain using ammonium sulfate, gel filtration chromatography techniques, and as a result, it was found that the molecular weight of CP was 165 kDa and composed of two sub-units[6]. Aptiz and Vanpee[28] was identified in their study that CP enzyme obtained from *B. sphaericus* organism has 13 kDa (dimer) and 26 kDa (tetramer) sub-structures. The results obtained appear to be in harmony with the literature data.

For the CP activity, optimum pH value was determined at different pH levels. As can be seen in Fig. 5, it was found as 7.0 by using 0.01 M phosphate buffer for the 4-methyl catechol, Pyrogallol and ABTS substrates.

In order to find the stable pH of the purified CP enzyme, the activity was measured for 10 days in 4 different buffers at pH 4.0-9.0 interval

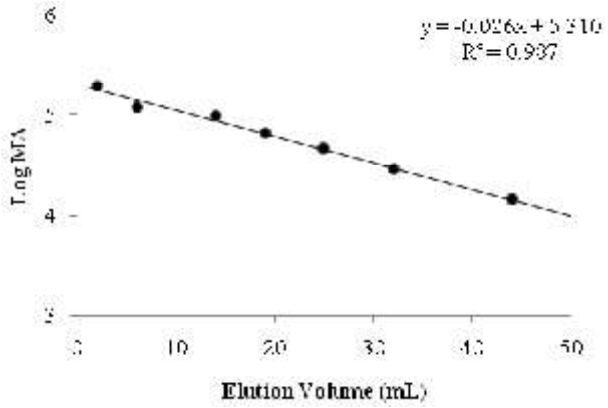

Fig. 4. Molecular weight determination of catalase-peroxidase of *A. pallidus* (P26) using gel filtration.

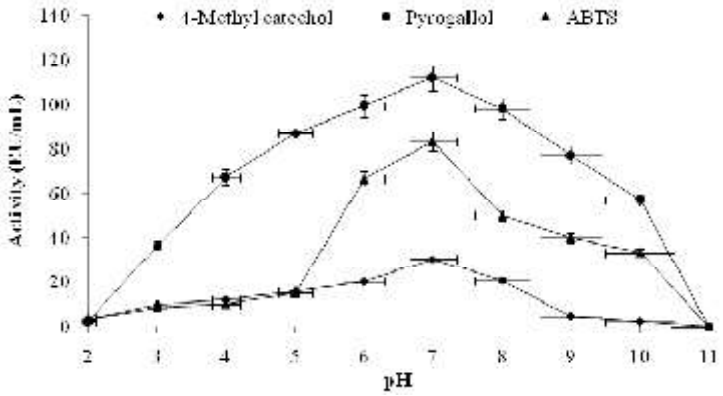

Fig. 5. Effect of pH on the activity of the purified catalase-peroxidase of *A. pallidus* (P26). Enzymes and substrate were dissolved either in 10 mM buffers of various pH. Other conditions were as given for the standard assay method.

of 1 unit difference. As can be seen in Fig. 6, it was identified that purified CP enzyme was more stable at pH 7.0.

The optimum pH of our CP was identical to that of CP from *Bacillus* SF and *Bacillus pumilus* (P9)[6], but was higher than that of the CPs produced from *Dichomitus squalens* (pH 5.5), *Irpexflavus* (pH 5.5) and *Polyporus sanguineus* (pH 5.5)[29]. However, it is less than the optimum pH of CP from *B. pumilus* (pH 8.0) and *Paenibacillus* sp (pH 9.0)[30].

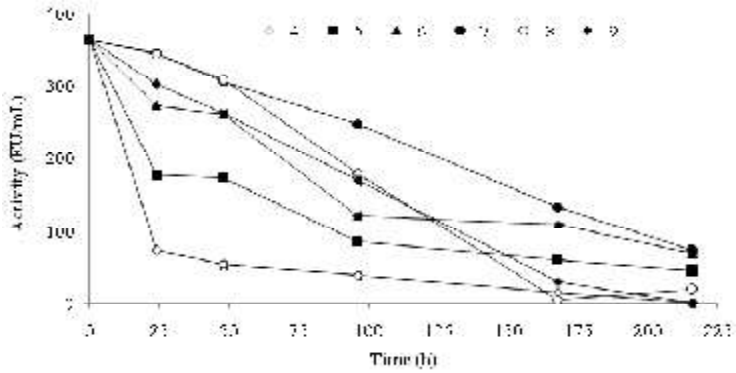

Fig. 6. Effect of pH on the activity and stability of the purified purifiedcatalase-peroxidase of *A. pallidus* (P26)

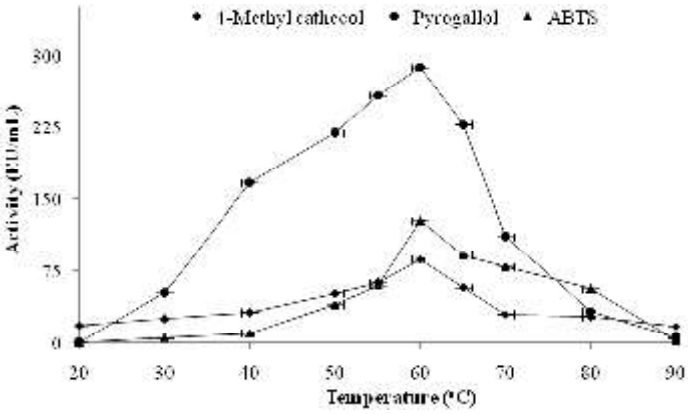

Fig. 7. Effect of temperatures on the activity of purified catalase-peroxidase of *A. pallidus* (P26). Activity was determined at different temperatures and at pH 4.0 over 10 min using the standard assay method

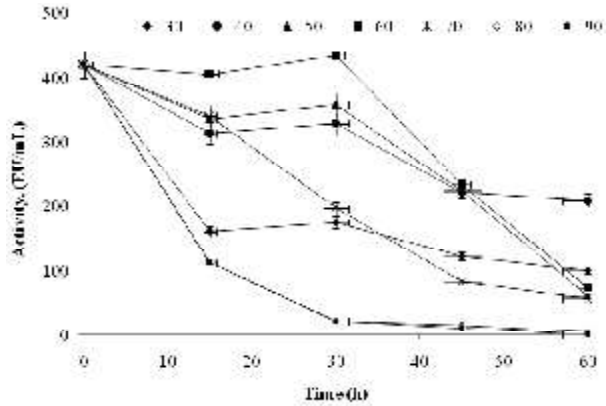

Fig. 8. Effect of temperature on the activity and stability of the purified purifiedcatalase-peroxidase of *A. pallidus* (P26)

Optimum Temperature

In order to find the optimum pH of the purified CP enzyme, 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 Fig. 5, it was found that the CP enzyme was highly active between 30 to 80 °C for 4-methyl catechol, Pyrogallol and ABTS substrates, and has the maximum activity at 60 °C. It was also found in the literature that the CP enzymes purified from some of the thermophilic bacteria had the maximum CP activity above 60 °C temperature similarly[31].

Stable Temperature

In order to determine the stable temperature values of the peroxidase enzyme

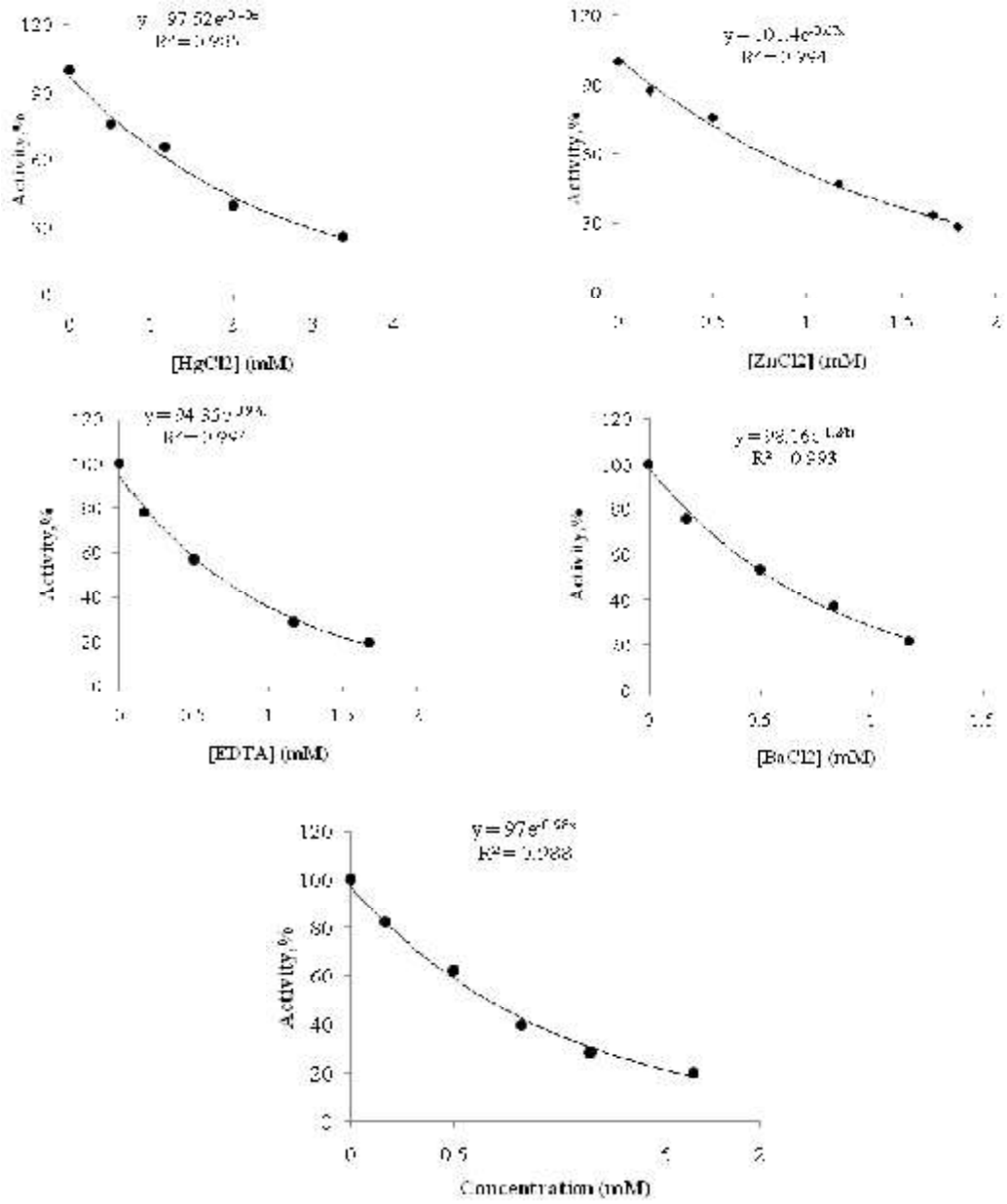

Fig. 9. The inhibition effects of $HgCl_2$, $ZnCl_2$ and $FeCl_2$ on activity of catalase-peroxidase from *A. pallidus* (P26).

derived and purified from *A. pallidus* (P26), measurements were made at regular intervals for 1 hour at 7 different temperature values in the range of 30 to 90 °C, at pH 7 (optimum pH) with the pyrogallol substrate. In the results, the percentage was calculated by comparing residual CP activity vs. unheated enzyme activity, and given in Fig. 8. It was determined that CP enzyme has the highest activity and stability at 60 °C. In addition, it was determined that 90% of the enzyme activity was maintained both at 40-50 °C and 60-70 °C within half an hour, and at the end of an hour still exhibited activity. Although *A. pallidus* CP maintains its thermal stability at high temperatures, purified catalase- peroxidase enzymes of *Rhodopseudomonas capsulata* and *Klebsiella pneumoniae* had merely active at temperatures as low as 30 °C[32,33]. The stability of the *A. pallidus* (P26) CP enzyme at high temperatures increases the availability of the enzyme in different industrial areas.

Results of V_{max} and K_m Values of Purified Peroxidase Enzyme from the *A. pallidus* (P26)

The studies on V_{max} and K_m values of the peroxidase enzyme were performed with 5 mM of ABTS, pyrogallol, hydroquinone and catechol substrates, in the presence of 22.5 mM H_2O_2. To achieve this, first the optimum activity measurements were made at five different substrate concentrations for H_2O_2, by fixing the concentrations of ABTS, pyrogallol, hydroquinone and 4-methyl catechol substrates and peroxidase enzyme, and the Lineweaver-Burk plots were obtained. By making use of the equations obtained from these plots, V_{max} and K_m values were calculated separately for each substrate, and the results are shown in Table 2. Enzyme had Km values of 3.53, 10, 11.3 and 6.2 mM for above mentioned substrates, respectively. On the other hand, the enzyme had V_{max} values of 5.95, 333.4, 142.9 and 20.83 EU/mL.min for each substrate respectively (Table 2).

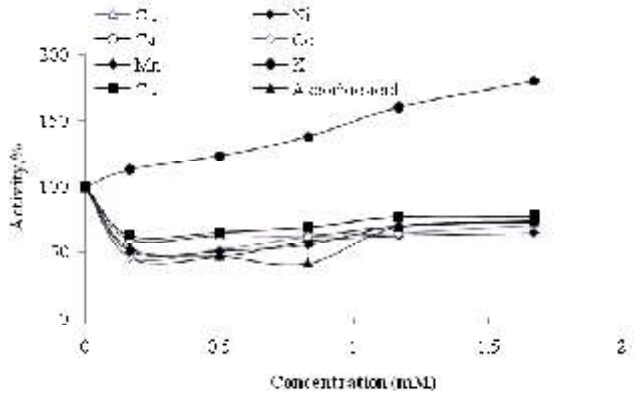

Fig. 10. The effects of some chemicals on activity of catalase-peroxidase from *A. pallidus* (P26).

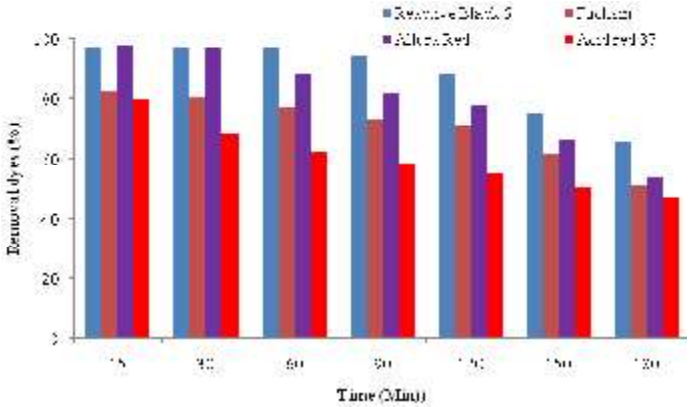

Fig. 11. The effect ofremoval oftextile dyesfrom waste water such as Reactive Black 5, Fuchsin, Allura Red and Acid Red 37 by using catalase-peroxidase from *A. pallidus* (P26).

Effect of Ca^{2+}, Mg^{2+}, Co^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, Citric Acid and Ascorbic Acid on the activities of peroxidase enzymes purified from *A. pallidus* (P26) extracellularly

The effect of Hg^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+}, Mn^{2+}, Fe^{3+}, K^{+}, Ba^{2+}, ascorbic acid and EDTA on the activity of the peroxidase enzyme produced and purified extracellularly from *A. pallidus* (P26) was investigated. The inhibition effects obtained in the study were shown in Fig. 9 and Fig. 10, as concentration vs. % activity plots. As shown in Fig. 9, it was found that Hg^{2+}, Zn^{2+}, Fe^{2+}, Ba^{2+} and EDTA inhibit the enzyme. I_{50} values were calculated for those that present inhibition and given in Table 3. It was determined that K^{+} activates the enzyme, and other metal ions inhibit at lower concentrations, but at higher concentrations they increased the enzyme activity (Fig. 10). The results suggest that the vast majority of metal ions do not inhibit the CP enzyme, and in fact activate, because of this feature it was found that CP is highly suitable for industrial applications.

Results for Removal of Certain Textile Dyes by Using Peroxidase Enzyme Purified from *A. pallidus* (P26) Extracellularly

The effect of peroxidase enzyme, which was derived and purified from *A. pallidus* (p26) extracellularly, on removal of certain textile dyes such as Reactive Black 5, Fuchsine, Allura Red and Acid Red 37 from waste water was investigated. At the end of 180 min. it was found that the enzyme removed the Reactive Black 5, Fuchsine, Allura Red and Acid Red 37 dye agents by 66%, 50.8, 53.6% and 47.3% respectively (Fig. 11) and the findings was observed to be in accord with the literature data[34].

CONCLUSION

The results suggest that the CP enzyme purified from *A. pallidus* (P26) is of very higher catalytic activity, durable against the metal ions, highly stable at different temperatures and pH levels, and cheap, and thanks to these features it can be used widely and effectively in the removal of the dyes that cause environmental pollution from waste water.

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