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Purification and Characterization of Lignin Peroxidase Isozymes from *Humicola grisea* (Traaen) and Its Application in Bioremediation of Textile Dyes

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PEROXIDASES have numerous important applications in industries and biodegradation of organopollutants , so there is a need to explore more and more sources of enzymes with different characteristics. In the present study, lignin peroxidases (LiPs) producing by fungus *Humicola grisea* were extracted and purified using gel filtration and ion exchange chromatography. Protein and heme protein were eluted in different peaks about 6 of these peaks had LiP activity and this is an indication that LiP in *H. grisea* was isoenzymes. Four of these isoenzymes were characterized regarding their molecular mass, optimum temperature and optimum pH for activity, substrate concentration and effect of some metal ions. The four purified isoenzymes have maximum activities at different temperatures, where optimum temperature for H2 and H4 isoenzyme were at 60 ºC, and the optimum temperatures for H1 and H3 were at 40 ºC and 30 ºC, respectively. The maximum activities of the four isoenzymes were within the acidic range. The different LiP isoenzymes showed different Km and Vmax values representing the varied affinity of the four isoenzymes using veratryl alcohol as a substrate. Km values for H1, H2, H3 and H4 were 5.26, 5.0, 1.3 and 1.4, respectively. While Vmax values for the same isoenzymes were 1.25, 0.8, 0.87 and 1.1, respectively.

Keywords: *Humicola grisea*, Organopollutants, Textile dyes, Lignin peroxidases, Isoenzymes

Introduction

Synthetic textiles dyes belong to the most dangerous pollutants of the industrial effluents contaminate higher amounts of waste water. which lead to negative effects towards aquatic life and humans (Khaled et al., 2010 and Dhanjal et al., 2013).

These dyes may be acidic, reactive, basic, disperse, azo, diazo, anthraquinone-based and metal-complex dyes. Azo compounds represent a major environmental concern due to its potential toxic nature to the animals and humans consuming it (Senthil et al*.,* 2016)

Various strategies have been used to remove dyes from textile wastewater including chemical oxidation, physico-chemical techniques and electro-chemical methods (Pearce et al., 2003 and Pandey et al., 2007) . These methods are expensive and produce large amount of sludge (Poojary et al., 2012).

Microbial remediation techniques of textile effluents has received an increase attention because they are ecofriendly ,cheap and publically accepted (Gueu et al., 2007).

Especially, there is a growing interest for the enzyme degradation of dyes due to several advantages such as greater specificity, capability to operate over a broad concentration range of contaminants, better standardization, easier handling and storage (Rodarte-Morales et al., 2011).

In the biological removal of color from effluents, the employment of fungi and of their oxidative enzymes constitutes an alternative for treatment under aerobic conditions. The oxidative degradation of coloured compounds is significantly stimulated through the use of oxidative enzymes (Selene et al., 2007).

Fungal highly oxidative and non-specific ligninolytic enzymes are capable of mineralizing pollutant compounds and responsible for the de-colorization and degradation of many different dyes (Dos Santos et al., 2007).

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Lignin peroxidase (EC 1.11.1.14) belongs to the family of oxidoreductases. Lignin peroxidase (LiP) is an extracellular hemeprotein, dependent of H_2O_2, with an unusually high redox potential and low optimum pH. Due to their high redox potentials and their enlarged substrate range LiP have great potential for application in various industrial processes as detoxification of recalcitrant organopollutants, decolourization of textile effluents (Anuradha & Darah, 2006 and Sarvamangala, 2014).

Materials and Methods

Decolorization ability of different dyes

Five mycelial discs (1cm diameter) of *Humicola grisea* were inoculated into 100 ml Czapek Dox broth media, the cultures were kept for 3 days at 28 °C, the tested dyes (suncron blue and sunzol brilliant orange) were added aseptically to reach a concentration of 50 ppm to determine the extent of de-colorization, 2 ml from each flask content were removed immediately after addition of dye and after 2, 4, 6, 8 and 14 days following the addition of the dyes. The culture was centrifuged at 10,000 *g* for 10 min. The supernatant were measured spectrophotometrically at the maximum wavelength absorbance (ʎ max) for each dye (494 nm for orange and 592nm for blue dye). Experiments were carried out in triplicate and controls were performed without inoculation of culture. Percentage of de-colorization was calculated according to the following equation represented by Sidra et al.(2012)

$$\text{Decolorization (\%)} = [(\text{Absi-Absf}) / \text{Absi}] \times 100.$$

where:Absi refers to the initial absorbance and Absf refers to absorbance at appropriate day.

Peroxidases production

Broth Kirk & Tien media was used for production of enzymes. Five fungal discs (1cm in diameter) from periphery of actively growing culture for *Humicola grisea* were inoculated into 500 ml flasks containing 100 ml Kirk and Tien media, then incubated for 6 days at 28 ˚C. At the end of incubation period, mycelium and spores were separated by filtration through Whatman filter paper No.1, 95 % cold ethanol (1:1) was added to the crude extract overnight. Centrifugation at 4 ˚C for precipitation of protein was conducted at 13000 *g* for 15 min using centrifuge Sigma 330-K 12150-H), then air dried. The precipitate was dissolved in acetate buffer, pH 5.0, and kept at 4 ˚C for further investigations. Presence of LiPs and MnP were estimated according to Glenn et al. (1983) and Machado (2009), respectively.

Native PAGE

Peroxidases were analyzed using the native polyacrylamide gel electrophoresis (native PAGE) according to Laemmli (1970) using the vertical gel electrophoresis device (Appelex vertical electrophoresis unit)

The peroxidase isoenzymes bands stain reddish brown color.

Lignin peroxidase activity (Glenn et al., 1983 method)

Lignin peroxidase bioassay according to Tien & Kirk (1988) is based on monitoring the enzymes oxidation of veratryl alcohol (Sigma) to veratryl aldehyde. The reaction mixture contained 375 µl of 0.33 M sodium tartrate buffer, pH 3.0, 125 µl of 4 mM veratryl alcohol, 50 µl of 10 mM hydrogen peroxide, 450 µl distilled water and 250 µl enzyme (crude) for a final volume of 1250 µL. The reaction was measured at 310 nm in a UV spectrophotometer for 1 min at 30 °C using a quartz cuvette. Reaction was initiated by addition of hydrogen peroxide .The reference cuvette contain all components except the enzyme.

LiP activity was calculated using molar extinction coefficient value of 9300 M^{-1} cm^{-1} for veratraldehyde (Ticlo, 2008).

Purification of lignin peroxidases

The mycelia was filtrated through Whatman No.1, 2x acetone was added to the filtrate which was chilled for 4 h, then centrifuged at 14,000 rpm for 10 min to precipitate the protein. The crude protein was dissolved in 0.1M sodium acetate. Buffer, pH 5, then applied to the Sephadex G-100 column. The positive fractions for peroxidase were concentrated down to 1ml, then applied to 5 ml Q-Sepharose column (GE Healthcare, UK) using peristaltic pump (HBI, multistaltic pump, USA), with a flow rate of 1ml/min. 20 mM Tris HCl buffer (pH 7.0) were used as a mobile phase with increasing gradient of KCl from 100 mM to 1M. Protein and heme protein content were monitored in the fractions at 280 nm and 409 nm, respectively , and the ratio between them was calculated for each fraction. The activity of LiP was estimated only in fractions with high heme protein/protein ratio. Positive fractions were precipitated and then dissolved in 0.1M sodium acetate buffer, pH 5, and analyzed using SDS-PAGE according to Laemmli (1970).

Characterizations of purified LiP isoenzymes

Molecular mass determination

The molecular mass of the enzyme was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 12 % polyacrylamide according to the method of Laemmli (1970).

Optimal temperature for LiP isoenzymes activity

The optimum temperature for each isoenzymes was determine by varying the temperature of the reaction (30, 40, 50, 60 and 70 °C) of purified isoenzymes using veratryl alcohol as substrate.

Optimal pH for LiP isoenzymes activity

The response of purified isoenzymes to varying pH values (3, 4, 5, 6 and 7) at optimum temperature for individual isoenzymes was determined using the previously mentioned bioassay.

Effect of substrate concentration: determination of K_m and V_{max}

The effect of increasing substrate concentration on the activity of purified isoenzymes activity was assayed at different veratryl alcohol concentrations (1, 2, 3, 4 and 5 mM) at optimum pH and temperatures for each isoenzyme. The Michaelis-Menten constant (K_m) and maximum velocity of substrate hydrolysis (V_{max}) were measured.

Effect of some metal ions on LiP isoenzymes activity

The effect of various metal ions, in form of chloride, at 5 mM concentration, on the activity of purified LiP isoenzymes was measured spectrophotometrically using veratryl alcohol as substrate, at optimum pH and temperature for every isoenzyme individually. The metal tested were ferrous (Fe^{+2}), manganese (Mn^{+2}), calcium (Ca^{+2}), magnesium (Mg^{+2}), cupper (Cu^{+2}), sodium (Na^{+}), and potassium (K^{+}). The purified enzyme was pre-incubated for 30 min with the metal ion before assaying with veratryl alcohol. The activity was expressed as percentage relative activity considering the activity of purified LiP isoenzymes in the absence of metal ion as 100 %.

Results and Discussion

Purification of lignin peroxidases

Extraction and precipitation of crude protein

The crude protein obtained from the broth medium of *H. grisea* was concentrated by precipitation with cold ethanol described in the method section (Moubasher & Mostafa, 2015). The total precipitated protein was estimated to be 11.56 mg, heme protein represent about 68 % from the total protein precipitated. LiPs activity equal 2.8 U/ml. As a second step of purification, the precipitated protein was subjected to gel filtration on Sphedex G-100 column. Abedin et al. (2013) revealed that *Humicola grisea* exhibited maximum potential for high lignin degradation and showed higher lignin peroxidase activity.

Gel filtration

An elution pattern was obtained showing five well-separated peaks, these were designated P1, P2, P3, P4 and P5 (Fig. 1). The first peak was the major one with high protein concentration (as indicated by measurements of absorbance at 280 nm). Absorption peak at 409 nm (heme protein) corresponding to P1 was also detected. Results for LiP activity revealed that most of activity was associated with P1 (2.48 U/ml) while, in P3 LiP activity was (1.28U/ml) .Two small peaks P4 and P5 had less LiP activities. One protein peak without LiP activity (eluted in the region of P2) was observed. This peak did not appear to have an associated 409 nm absorption spectrum.

Ion exchange chromatography

Proteins from peak one was purified through another purification step, involving anion exchange chromatography on Q-Sepharose column, which resulted in several protein most of them were heme peaks (Fig. 2), these peaks were characterized by distinct LiPs activity. Peaks were designated from (P1-P8) whereas the four isoenzymes with highest activity were designed as H1, H2, H3 and H4.

LiPs and MnPs are actually mixtures of several isoenzymes, encoded by various genes and differentially expressed depending on the conditions. Farrell et al. (1989) separated ten hemeproteins from *P. chrysosporium* and designed H1-H10, by anion exchange HPLC, six of them were lignin peroxidases. Similarly, Stewart et al. (1992) identified that different related LiP genes in *P. chrysosporium* encoded different isoenzymes. Based on this fact they suggested that certain functional variability is needed; the main additional reason for the splitting in many isoenzymes is thought to be excessive glycosylation. Many researchers reported isoenzymes of multiple LiPs from white-rot fungi species (Tien & Kirk, 1988 and Wang et al., 2008).

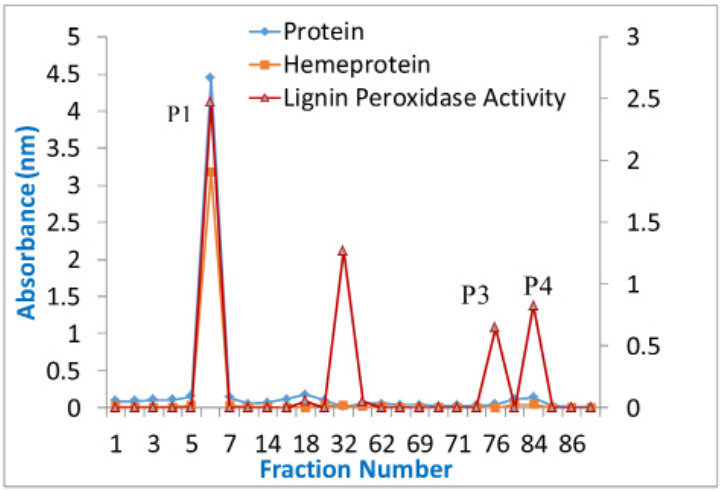

Fig. 1. Elution profiles of Humicola grisea protein after gel filtration, protein peaks designated as (P1-P4).

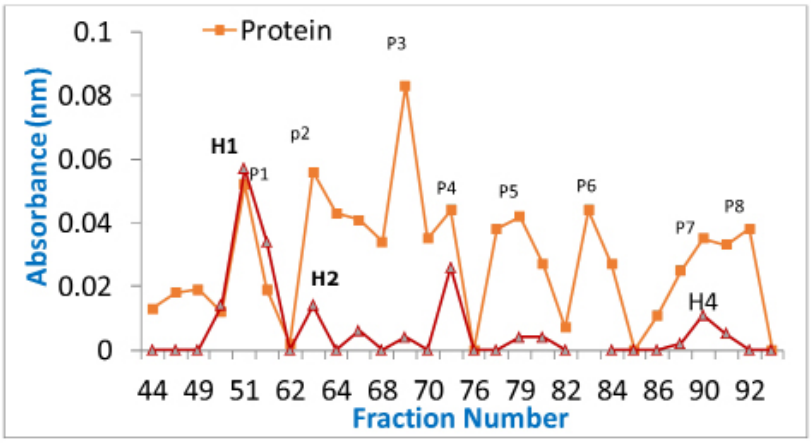

Fig. 2a. Elution profile Q-column chromatography of *H. grisea* Lignin peroxidases (protein and heme protein); The column (15 × 1.2 cm) was washed with 100 mM phosphate buffer, pH 7.0, at a flow rate of 1 ml/ min and then eluted with a linear gradient of 0 - 1 M NaCl. H1, H2, H3, H4 isoenzymes were selected for characterization.

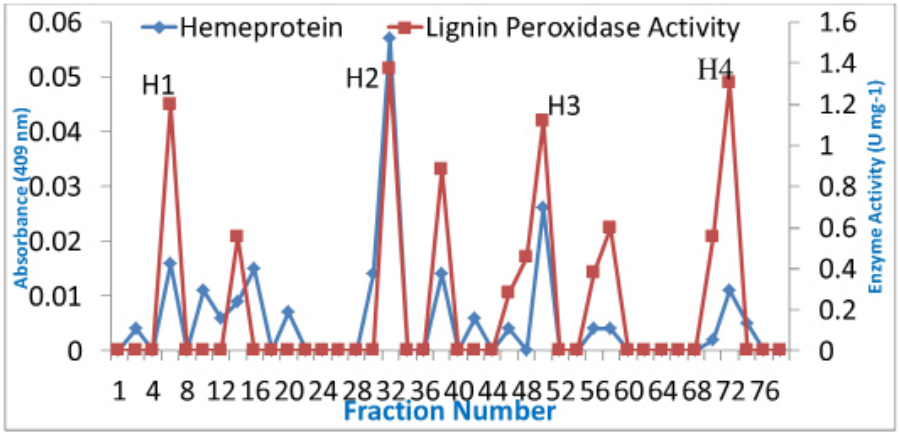

Fig. 2b. Elution profile Q-column chromatography of *H. grisea* Lignin peroxidases (protein and heme protein)): The column (15 × 1.2 cm) was washed with 100 mM phosphate buffer, pH 7.0, at a flow rate of 1 ml/ min and then eluted with a linear gradient of 0–1 M NaCl.

The isoenzymes with highest LiP activities were chosen for further characterization regarding their molecular mass, optimum temperature and pH, kinetic properties and the effect of some metal ions on its activity.

Characterization of the Isoenzymes

Estimation of molecular mass of purified LiPs isoenzymes

The LiPs isoenzymes H1, H2, H3 and H4, obtained after purification with Q –column that followed precipitation with cold ethanol and gel filtration, were run on 12% SDS-PAGE to estimate their molecular masses (Plate 1, 2 and 3). H1, H2 and H3 isoenzymes were single bands estimated to be about 70 KDa and H4 was estimated to be approximately 72 KDa. Relative to the standard molecular weight, it was expected to have the molecular masses of the three isoenzymes in the same range because they were eluted in the same peak (p1) during gel filtration. Molecular weight determinations are only approximate because glycoproteins do not bind SDS quantitatively and therefore migration in polyacrylamide gel is not linearly related to the log of molecular weight (Roberta et al., 1989).

The present results also gave an indication that after, two steps of purifications, one depending on the size and the other on charge seemed to be enough to get purified isoenzymes. This result is in accordance with LiPs isolated from *Phanerochaete sordida* YK-624, which was purified to homogeneity by anion-exchange and gel permeation chromatography (Wang et al., 2010).The molecular mass of LiP isoenzymes obtained in the current study was different from that of white-rot fungal LiP, according to Fakoussa & Hofrichter (1999); the subunit molecular mass range of WRF was 38- 47 kDa and that of peroxidases was 38- 50 kDa. These results agree to some extent with those reported by other researchers (Yadav et al., 2010). Roushdy et al. (2011) purified LiP from *Cunninghamella elegans,* which had a subunit molecular mass of 50 kDa. They are all heme containing glycoproteins (Tien & Kirk, 1984).

Effect of temperature and thermal stability on purified LiPs isoenzymes activity

The four LiP isoenzymes from *H. grisea* have different temperature profile for their activity (Fig. 3). All the isoenzymes except H1 have retaining their activity over a wide range of temperature (30--70) °C, they got maximum activities at different temperatures, H2 and H4 were (2.66 U/ml) and (4.62 U/ml) at 60°C, respectively, whereas H3 has maximum activity at (2.75 U/ml) at 30°C. H1 has maximum activity (4.93 U/ml) at 40 °C, and at 30 °C the activity was not much different (4.77 U/ml), but it is sensitive towards higher temperature, and completely deactivated at 70°C. As compared to previously reported *H. grisea* LiPs have wide range of thermal profile which enhances its potential for biotechnological applications. Relatively greater activity and high thermo-stability are attractive and desirable characteristics of an enzyme, for industrial applications (Iqbal et al., 2011).

The free LiPs from *Trametes versicolor* IBL-04 was optimally active at 60°C and further increase in temperature caused its deactivation (Asgher et al., 2012). The enzyme activity against temperature resistance depends on the source of the enzyme as well as on the assay conditions, especially pH and the nature of the substrate employed. Regarding thermal stability H3 is the most stable isoenzymes retaining 55% of its activity at 50 °C whereas other isoenzymes have narrow range of thermal stability; H1, H2 and H4 almost lost their activity at temperature more than 30 °C. The variability in the heat stability of peroxidase can be attributed largely to the particular enzyme structure. The enzyme stability can be attributed to non-covalent, electrostatic and hydrophobic interactions, as well as extra ion pairs, hydrogen bonds and the degree of glycosylation of individual isoenzymes (Adams, 1991)

Effect of pH on purified LiPs isoenzymes activity

The effect of pH on LiPs activity was studied using veratryl alcohol as substrate. The four isoenzymes have similar responses towards pH of the reaction mixture; the maximum activities were within the acidic range (Fig. 4). Data reveals highest LiPs activities for H2 and H4 (4.38 and 3.5 U/ml, respectively) was worked out at pH 3, whereas maximum activity of H1 was achieved 4.7 U/ml at pH 4. For all the three isoenzymes the activity decreased when the pH increase towards the neutral pH. These results are in agreement with that reported by Fakoussa & Hofrichter (1999) who stated that pH range for LiPs was between 2.0 and 5.0 withan optimum somewhere between 2.5 and 3.0. H3 had maximum activity at pH 6 that was different from the other isoenzymes. H3 was similar to LiPs of *Cunninghamella elegans,* since the optimum pH of *Cunninghamella elegans* LiPs was 6.0. Earlier studies reported optimum activities of various WRF LiPs to vary between pH 2–5. Alam et al. (2009) reported that the LiPs produced from *P. chrysosporium* showed more than 80% of the maximum activity at optimum pH 5, while according to Rodríguez-Couto et al. (2005) LiPs from *P. chrysosporium* was optimally active at pH 4.2.

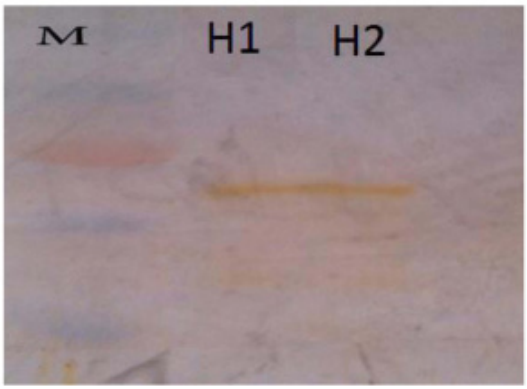

Plate 1. SDS-PAGE of purified LiPs from *H. grisea*. Lanes: H1 and H2, purified isoenzymes eluted from Q- column. Molecular mass standards markers (in kilodaltons) are shown on the left.

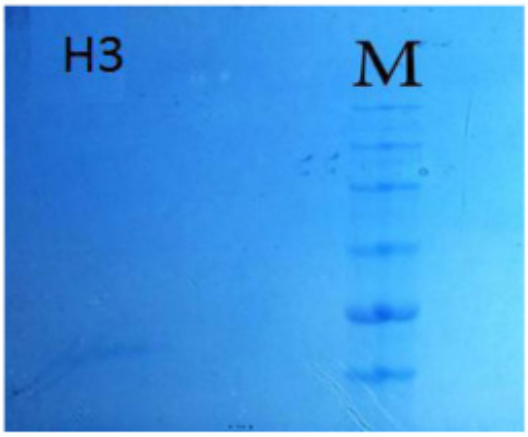

Plate 2 SDS-PAGE of purified lignin peroxidase from *H. grisea*. Lanes: H3, purified isoenzymes eluted from Q- column. Molecular mass standards markers (in kilodaltons) are shown on the right.

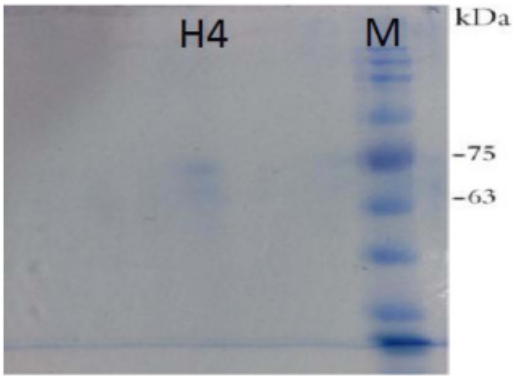

Plate 3. SDS-PAGE of purified lignin peroxidase from *H. grisea*. Lanes: H4, purified isoenzymes eluted from Q- column. Molecular mass standards markers (in kilodaltons) are shown on the right.

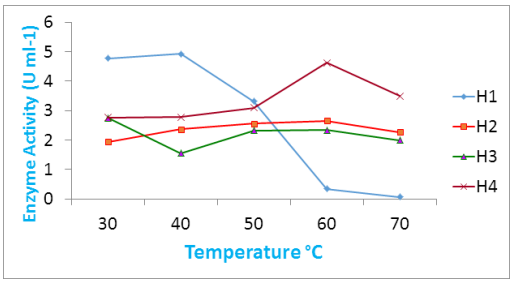

Fig. 3. Effect of temperature on LiP isoenzymes (H1, H2, H3, & H4) activity.

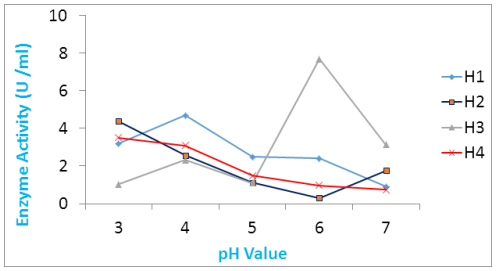

Fig. 4. Effect of different pH on LiP isoenzymes (H1, H2, H3, & H4) activity.

Effect of substrate concentration: determination of K_m and V_{max}

Effect of varying concentrations of veratryl alcohol (1, 2, 3 and 4 µM) on activity of the enzyme were calculated at optimum pH and temperature for each LiP isoenzyme. Reciprocal of varying veratryl alcohol concentrations (µM) were plotted against the respective initial specific activities (V). Michaelis-Menten kinetic constants (K_m and V_{max}) values were calculated by fitting the data into the Michaelis-Menten equation. The different LiP isoenzymes showed different Km and Vmax value representing the varied affinity of the four isoenzymes for veratryl alcohol as a substrate. The Km values for H1, H2, H3 and H4 were 5.56, 1.42, 1.25 and 1.4 µM, respectively; While the V_{max} values for the same isoenzymes were 0.53, 0.60, 0.45 and 0.58 U/mg, respectively. The values of Km were lesser than the values obtained with *Bjerkandera* sp. and P. *chresosporium* indicating the high affinity of *H. grisea* isoenzymes towards their substrate veratryl alcohol (Have & Teunissen, 2001).

Metal Ions

To further identify the nature of the isoenzymes under investigation, the effects of a number of metallic ions on LiP isoenzymes activity was studied by incubating the isoenzymes for 60 min with various metal ions (5 mM) and measuring the residual activity as percentage of initial activity. Among the different metal ions tested, Fe^{2+} at 5 mM concentration increased the activity of the four LiPs (Fig. 5), whereas, Mg^{2+} was showed peroxidase activity stimulation except for H3. The Mn^{2+} caused reduction in activity in all isoenzymes

except H2, which showed increase in activity up to 210% .In addition K^+ was inhibitor, giving residual activities of 26, 18, and 74%, for H1, H3 and H4, respectively, while positively affect H2 (128 %). The obtained results are in accordance with Gueu et al. (2007), they stated that different isoenzymes may response variably towards concentration of metal ions such as K^+ and Ca^{+2}. Metal ions, such as Fe^{2+}, can coordinate to oxidative site residues leading to activation of enzymes (Louie & Meade, 1999). Regarding the effect of Ca^{+2} and Cu^{+2} the results were in accordance to Fodil et al. (2012). The $Ca^{+2}$was required for the stabilization of the heme in the active site of peroxidases.

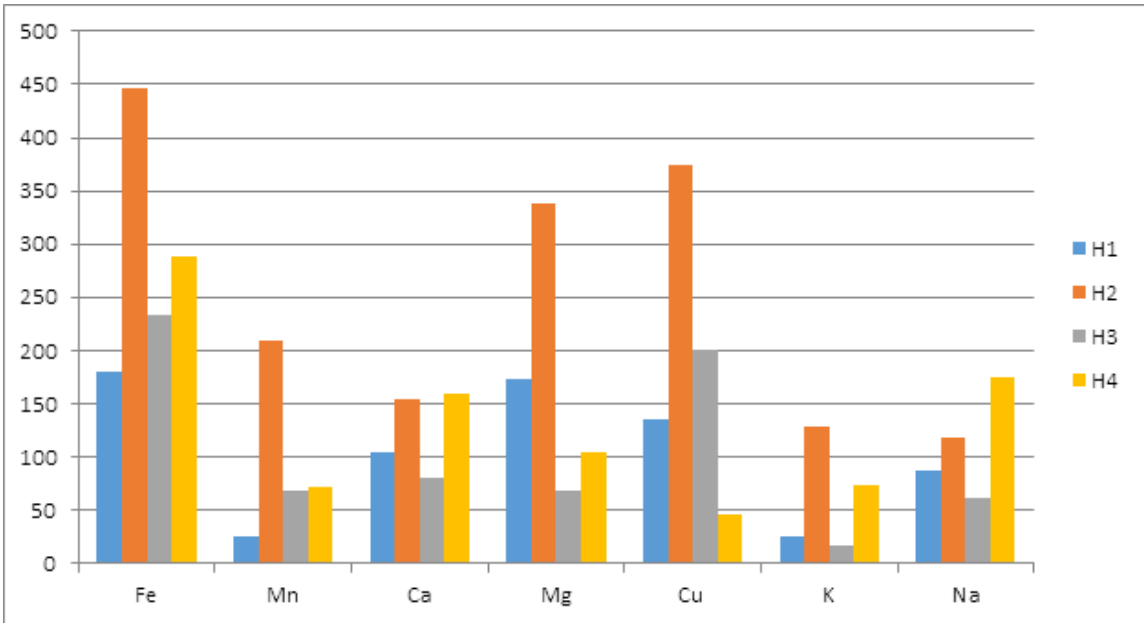

Fig.5.Effect of metal ions on activity of purified H. grisea LiPs isoenzymes H1, H2, H3 and H4, the chloride salt of each metal cation was used (5 mM). The activity was examined at pH and temperature optima for each isoenzyme.

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تنقية وتوصيف نظائر الانزيم اللجنين البيروكسيديز من *Humicola grisea* السنجابية وتطبيقه في المعالجة البيولوجية لأصباغ الغزل والنسيج

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تنقيةوتوصيف نظائر الانزيمات اللجنين البيروكسيديز من *Humicola grisea* وتطبيقه في المعالجة البيولوجية لأصباغ الغزل والنسيج لانزيم البيروكسيديز تطبيقات مهمه وعديده فى مجال التخلص من الملوثات العضويه و فى مجال الصناعه ولذلك فمن الضرورى البحث عن مصادر جديده للانزيم .وتهدف هذه الدراسه إلى انتاج انزيم اللجنين بيروكسيديز من فطر *Humicola grisea* حيث تم عزل و تنقيه الانزيم بطريقه الترشيح والتبادل الايونى.تم التوصل إلى ان انزيم البيروكسيديز من هذا الفطر له اربعه اشكال وتم تنقيتهم ودراسه خصائصهم وكانت كالتالى:درجه الحراره المثلى لنشاط اثنين منهم الثانى والرابع 60 درجه والاول والثالث 40 و30 درجه على التوالى .كما كانت ادرجه النشاط المثلى تحتاج إلى وسط حمضى.واظهرت قيم Km لـ H4, H3, H2, H1 5.26 و 5.0 و 1.3 و1.4 على التوالى.وكانت Vmax لهم 1.25 و 0.8 و 0.87 و 1.1 على التوالى.