Decolorization of textile dyes by partially purified *Pleurotus ostreatus* laccase
ازالة اصباغ النسيج بوساطة انزيم اللاكييز المنقى جزئيا من العزلة *Pleurotus ostreatus*

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Abstract

***Pleurotus ostreatus* produced 2.93 U/mg of laccase in solid state fermentation (SSF) using barley bran as substrate under optimum conditions. The optimum SSF conditions were: pH 6.5; temperature, 25Cº; inoculums size 3.5 mm and moisture content, 1:1.5 w/v. Laccase was partially purified 8.29 fold with specific activity 17.5 U/mg by ion exchange chromatography after curd enzyme concentrated by dialysis against the solid sucrose. Partially purified laccase had an optimum pH of 6.5 and was stable in the pH range from 6.5 to 7.5. The optimum temperature was 45 Cº and it displayed considerable stability within the range 15 to 45 Cº with 1h incubation as well as The ability of partial purified laccase to decolorize of textile dyes showed that the blue H3R dye was completely decolorized in all concentrations within first min while yellow FG and red 3B dyes were decolorized in different percentage.**

Key words: laccase, *P.ostreatus*, purification, decolorization, dyes

المستخلص

الفطر *Pleurotus ostreatus* ينتج 2.93 وحده\ملغم من انزيم اللاكيز في تخمرات الحاله الصلبه باستخدام نخالة الشعير ك مادة أساس تحت ظروف مثلى . الظروف المثلى باستخدام تخمرات الحاله الصلبه كانت: الداله الحامضية 6.5, درجة الحراره 25م , حجم اللقاح 3.5ملم و محتوى الرطوبة 1:1.5 وزن\حجم. انزيم اللاكيز تم تنقيته جزئيا 8.29 مرات بفعالية نوعية 17.5 وحده \ملغم باستخدام كروموتوغرافيا التبادل الايوني بعد تركيز الانزيم الخام باستخدام الفرز الغشائي باتجاه السكروز. الدالة الحامضية لانزيم اللاكيز المنقى جزئيا هو 6.5 وثباتيته تتراوح بين 6.5-7.5. درجة الحراره المثلى للانزيم 45م وأظهر ثباتيه كبيره ضمن الدرجات 15-45م بفترة حضن ساعه واحده. قابلية انزيم اللاكيز المنقى جزئيا على ازالة الصبغات المعقده أظهرت ان الصبغه الزرقاء H3R تمت ازالتها بصورة كامله في التراكيز خلال الدقيقة الاولى بينما الصبغة الصفراء FG والحمراء 3B تمت ازالتها بنسب مختلفة.

الكلمات المفتاحية: انزيم اللاكييز، الفطر*P.ostreatus* ، تنقية، ازالة الصبغات، الصبغه

Introduction

Laccases are (EC 1.10.3.2, diphenol oxidase) N-glycosylated multi copper oxidases belonging to the group of the blue copper proteins. They can catalyze the oxidation of many substances coupled to the reduction of molecular oxygen to water [1]. Laccases are widely found in fungi and higher plants [2] and also in a lower proportion in insects and bacteria. It was first described by Yoshida in 1883 when the enzyme extracted from the exudates of the Japanese lacquer tree *Rhus vernicifera*, from which the name laccase was derived [3]. Laccase production by white rot fungi was carried out using two types of fermentation submerged fermentation (SMF) and solid state fermentation. Solid-State Fermentation (SSF) is a fermentation process conducted in the absence of free flowing water, using either a natural support or an inert support as a solid material [4]. Fungal laccases have particular interest with regard to potential industrial applications, because of their capability to oxidize a wide range of toxic and environmentally problematic substrates. Oxidation reactions are comprehensively used in industrial processes, for instance in the textile, food, wood processing, pharmaceutical and chemical industries. Enzymatic oxidation is a potential substitute to chemical methods, since enzymes are very specific and efficient catalysts and are ecologically sustainable. One of promising sources for production of laccase has been white rot fungus, *Pleurotus ostreatus* and it has the ability to decolorize different textile dyes[5]. *Pleurotus ostreatus* is a commercially important edible mushroom, famous for its delicious taste and high quantities of protein, carbohydrates, minerals, and vitamins but low fat [6].The increasing use of synthetic dyes is alarming and their discharge as textile waste may cause substantial

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ecological damage. Biological decolorization of dye using microorganisms is an environmentally friendly and cost- competitive alternative to chemical methods. The current work was aimed to produce laccase in optimal conditions from *Pleurotus ostreatus* using barley bran as substrate, purification, characterization the enzyme and its role in textile dye decolorization.

Materials and methods

Fungal isolate

The isolate *Pleurotus ostreatus* was obtained from Department of Biotechnology, Collage of Science, Baghdad University. Fungal isolate was cultivated at 25°C on malt extract agar (MEA) and stored at 4°C.

Laccase production on solid substrate:

Solid state fermentation (SSF) medium consist of 7.5 gm barley bran has been used for producing the fungal enzyme .The substrate was humidified with a 1:1.5 (w/v) of mineral salt solution containing (0.2 gm KH_2PO_4, 0.1gm $MgSO_4.7H_2O$, 0.3gm NH_4CL_2 ,1.0 GM $CaCO_3$ in one liter distilled water, pH 5.6).The humidified medium was placed in 250 ml Erlenmeyer flasks and autoclaved at (121 °C , 20min).The sterilized medium was incubated with three mycelial plugs 5mm from 7 days culture of *P.ostreatus* (two flasks). Then flasks were incubated for 10 days at 25°C. Flask without inoculation was used as control [7].

Enzyme extraction

Laccase extracellular enzyme was extracted from barley bran culture using 60 ml (1: 12.5 w/v), of 0.1 M cooled citrate phosphate buffer, pH 5.6. The content of flasks were grind in mortar for 15 min in ice bath.The crud extracted were filtered through gauze, and then centrifuged [8].

Enzyme and protein assays

Laccase activity was followed spectrophotometrically at 525nm, through oxidation of syringaldazine to its quinone form ,using a molar absorptivity of 65000 for product [9].The reaction mixture contained 2ml citrate phosphate buffer (0.1 M, pH 5.6), 0.2 ml syringaldazine (0.5 mM in methanol solution) and 1ml of culture filtrate. The enzymatic activities were expressed as International Unit (U), defined as the amount of enzyme required to produce 1 µ mol product/min. Protein concentration was determined using standard curve of bovine serum albumin [10].

Purification of enzyme

Purification of laccase was carried out by ion exchange chromatography after curd enzyme concentrated by dialysis against the solid sucrose, and then loaded onto a DEAE –cellulose anion exchange column 3.5× 16 cm, equilibrated with 0.01 M citrate phosphate buffers pH 5.6 at flow rate 0.5 ml /min , with linearly increasing NaCl concentration gradient 0.2- 1M in the same buffer.The five fraction containing laccase activity of fractions were determined.

Laccase Characterization

Optimum temperature and pH were determined by performing enzymatic assays at different temperatures 5–85 Cº and pH levels 3–8.5, respectively. The pH level was adjusted using the following buffers: 0.1 M citrate phosphate buffer pH 3–5.5 , 0.1 M potassium phosphate buffer pH 6–7, and tris base buffer pH 7.5-8.5. The stability of the purified laccase at various temperatures was investigated by pre incubating the purified laccase at different temperatures between 15 and 85 Cº for 1 h, followed by determination of the residual activity. The effect of pH on the laccase stability was determined by incubating the purified enzyme at 25 Cº in different pH levels for 1 h and the residual activity was determined.

Decolorization of textile dyes

To test ability of fungal culture to textile dyes decolorization *P.ostreatus* cultured on malt extract agar contain three types of reactive dyes blue, yellow and red in different concentration 50,100,150,200,250 ppm, and incubated at 25Cº for 7 days. Dye decolorization noted by comparing with control (plates containing the medium and the dyes, without the fungus) [11]. Decolorization of textile dyes was investigated also by the partially purified laccase. The reaction mixture 1.6 ml contained 1 ml citrate phosphate buffer pH6.5, 0.5 ml partially purified laccase and 0.1 ml of three types of dyes for each dye five concentrations 50,100,150, 200, 250 ppm. The reaction was initiated when enzyme was added at optimum temperature 45Cº. Samples were withdrawn during 3min intervals and subsequently analyzed, by measuring the absorbance at 585 nm for blue H3R, 415nm for yellow and 535 nm for red H3B. The dye decolorization percentage was calculated as [12].

$$\textbf{Dye decolorization percentage } \% = \frac{\textbf{initial absorbance} - \textbf{final absorbance}}{\textbf{initial absorbance}} \times \textbf{100}$$

Results and discussion

Laccase production on solid substrate

Pleurotus ostreatus was cultivated in solid state fermentation medium containing barley bran as the main lignocelluloses' substrate humidified with a 66% (w/v) of mineral salt solution . The fungus produced 2.93 U/mg laccase in 10 days under optimum conditions of solid state fermentation. The optimum conditions were: p H 6.5; temperature, 25C°; inoculums size 3*5 mm and moisture content, 1:1.5 w/v. Gomez e*t al.*, (2005) found that barley bran is the best lignocelluloses' waste to produce laccase by solid state cultures of *Coriolopsis rigida* [13] .

Purification of laccase

The extracellular laccase from *P. ostreatus* was partially purified to 8.29 fold with a yield of 36.8 % (table 1), using two purification steps that included concentration by dialysis against the solid sucrose and DEAE - Cellulose column chromatography fig.(1).Ion exchange chromatography used in many studies of laccase purification from fungi. Studies used DEAE-Cellulose in laccase purification from *P. sapidus* and the purification fold was 3.14 and the yield 29.56 % [14]. This enzyme was also purified from *Hypsizygus ulmarius* by DEAE-Cellulose exchanger results in purification fold 5.11 and enzymatic yield 53.33 % [15].

Table (1): Purification steps of laccase from *P.ostreatus.*

	Volume ml	Protein mg/ml	Activity U/ml	Specific activity U/mg	Total activity U	Yield %	Purification fold
Crude enzyme	45	0.15	0.317	2.11	14.26	100	1
Concentrated with sucrose	15	0.20	0.488	2.44	7.32	51.33	1.15
Ion exchange DEAE- Cellulose	15	0.02	0.35	17.5	5.25	36.8	8.29

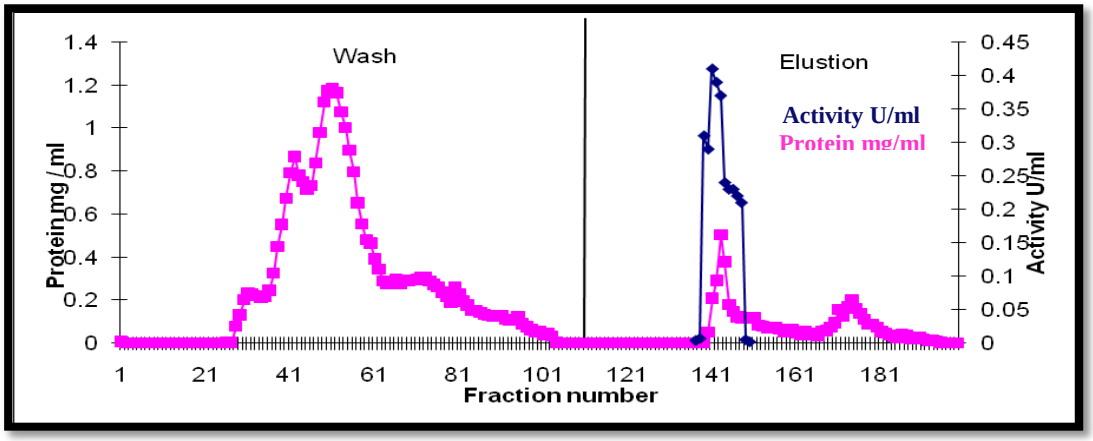

Fig (1): Ion exchange chromatography for laccase purification from *P.ostreatus* by using DEAE-Cellulose column (16×3.5 cm)equliliprate with citrate phosphate buffer (0.01 M, pH 5.6), eluted with citrate phosphate buffer with NaCl gradient (0-1) M in flow rate 30ml/ h. 3ml for each fraction.

laccase Characterization

Effect of pH on partially purified laccase

laccase activity was studied at varying pH pH 3- 8.5.The optimum pH of partially purified laccase produced by *P.ostreatus* was 6.5 for syringaldazin oxidation, while the activity decreased in pH below and above 6.5 figure (2). The partially purified laccase remained quite stable within the pH range of 6.5-7.5 after 1h incubation figure (3). The study of [16] they observed the optimum pH for *Pleurotus* sp. laccase activity was 4.5. The purified laccase produced by *S.commune* IBL-06 was stable within pH ranges 5-8 [17].

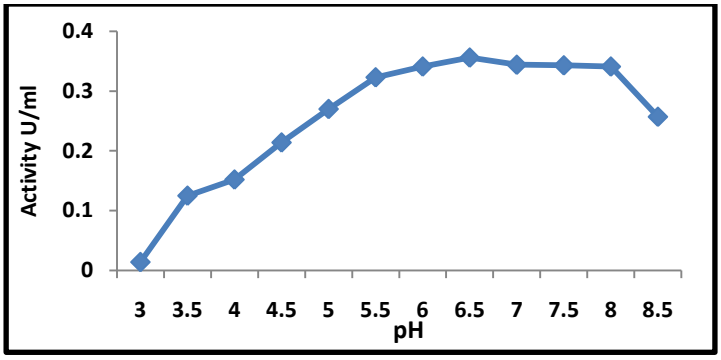

Fig (2): Optimum pH for partial purified laccase activity from *P.ostreatus*

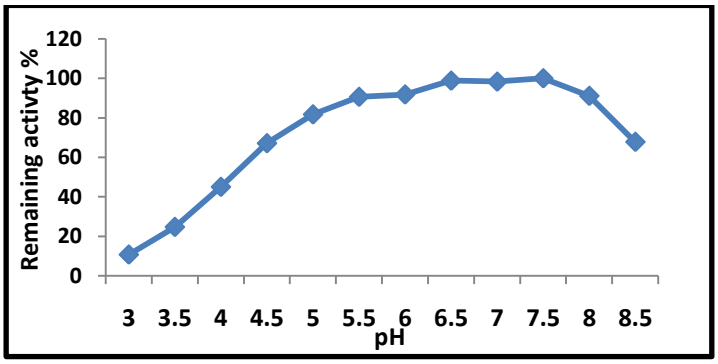

Fig (3): Optimum pH for partial purified laccase stability from *P.ostreatus*

Effect of temperature on partially purified laccase

The optimum temperature for partial purified laccase was 45Cº, laccase activity reached to 0.356 U/ml, then the activity was decreased with increasing temperature up to 45C˚ and minimum activity observed at 85 C˚ was 0.022 U/ml figure (4). Similar results was obtained by [17] reported that laccase from *Trametes versicolor* had 45C˚ as an optimum temperature . While Optimum temperature for laccase activity produced from *Pleurotus* sp. showed at 65C˚ [16]. The enzyme was stable in temperature between (15- 45) C˚, then the activity begun to decrease with increasing temperature at 85C˚ figure(5) , the enzyme retained only 4.41% of the initial activity. The thermal stability of the *Trametes versicolor* crude laccase was followed within the temperature interval 30-60 C˚ [18].

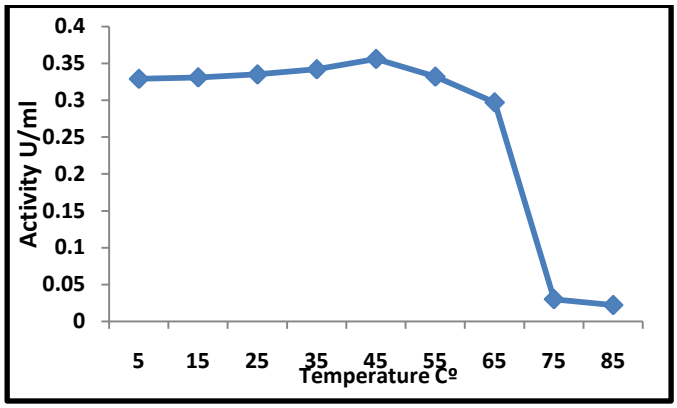

Fig (4): Optimum temperature for partial purified laccase activity from *P.ostreatus*

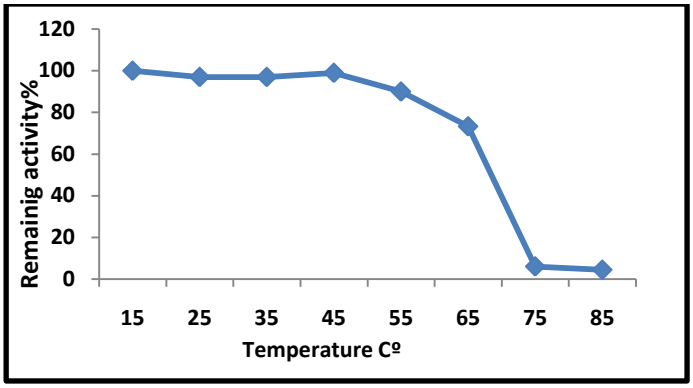

Fig (5): Optimum temperature for partial purified laccase stability from *P.ostreatus*

Decolorization of textile dyes:

The ability of *P.ostreatus* to decolorize textile dyes was tested first one on solid media. The fungus grew well and completely decolorized Blue dye H3R with concentrations 50,100,150,200 and approximately 98% with 250 ppm concentration figure (6A) contrasting to the yellow figure (6B), and Red were very slowly processed figure (6C) , dye decolorization was appeared only at 50 ppm concentration, this could be due to difference in chemical structures' of the dyes, as illustrated in figure (6).

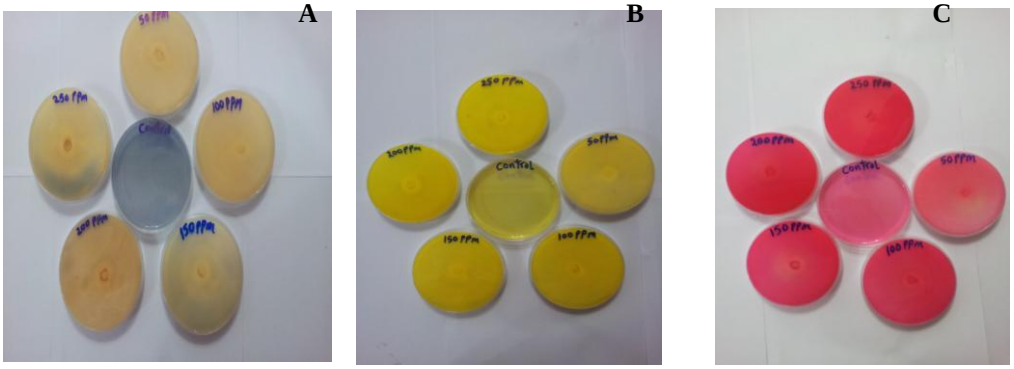

Fig (6): Decolorization of three dyes by *P.ostreatus* fungal isolate after incubation for 7days at 25C° using malt extract agar supplemented with each dye at different concentrations (50- 250 ppm)
(A) Blue H3R, (B) Yellow FG, (C) Red 3B

The white rot fungus *P. pseudobetulinus* showed good decolorizing results to blue H3R approximately 98% removed within 8 days but the yellow reached to 24% removal and red 3B reached 50% removal at 18 days [19]. Dye decolorization by partially purified laccase has been investigated using enzyme with activity 0.35 U/ml. The results showed that the blue H3R dye was completely decolorized in all concentrations within first min while yellow FG and red 3B dye was decolorized in different percentage which is probably due to the complexity of these two dyes structure [19] Table (2).

Table (2): Dyes decolorization by partial purified laccase

Concentration of dyes (ppm)	Decolorization of Yellow dye %			Decolorization of Red dye %		
Incubation time	1 min	2 min	3 min	1 min	2 min	3 min
50	1.33	1.92	2.27	51.13	54.50	56.25
100	0.99	1.26	1.62	46.92	47.15	48.95
150	0.52	0.88	0.90	30.91	30.70	30.65
200	0.39	0.37	0.37	19.5	19.4	19.3
250	0.23	0.22	0.21	8.2	8.2	8.2

The ability of partially purified laccase from *P.ostreatus* to decolorize reactive dyes (reactive blue 172 and reactive red 22). The reactive blue 172 was maximally decolorized ≈95% at 2h. treatment, followed by reactive red 22 ≈64% [20].

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