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Purification and Characterization of the Laccase Enzyme from the Basidiomycete Agaricus nevoi in Al-Alam in Salahaddin/ Iraq

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***Abstract*— Laccase (EC 1.10.3.2) is a type of enzyme known as a multicopper oxidase, which can oxidize a wide array of phenolic and non-phenolic substances, utilizing molecular oxygen to accept electrons. In our research, we assessed the capability of three macrofungal isolates—HM001, IN002, and SH003—to produce laccase. Initial tests showed that isolates HM001 and SH003 demonstrated the most significant activity, producing decolorization zones measuring 45 mm and 16 mm in diameter, respectively. Further quantitative tests revealed that isolate HM001 had the highest enzyme activity, reaching 50 U/mL by the twentieth day of incubation, making it the focal point for more detailed studies. Molecular analysis confirmed that this isolate was Agaricus nevoi, marking a novel discovery for Salahaddin Province, Iraq. We purified the laccase enzyme using a process that included ammonium sulfate precipitation at 70% saturation, followed by ion-exchange chromatography on DEAE-cellulose and size-exclusion chromatography using Sephadex G-150, enhancing the specific activity of the enzyme from 100 to 1000 U/mg. The purified laccase worked best at 37°C and a pH of 7–8, maintaining its stability within a temperature range of 32–37°C. We estimated the molecular weight of the enzyme to be 50 kDa. In conclusion, our findings indicate that Agaricus nevoi is a valuable new source of laccase, exhibiting robust enzymatic performance and stability under moderately warm and neutral pH conditions. This successful purification and characterization of the laccase underscore its potential for future use in biotechnology and industry, especially for the efficient oxidation of various phenolic substances.**

Keywords- Laccase enzyme, Purification, Fungal isolates, Ammonium sulfate

I. INTRODUCTION

Laccase (EC1.10.3.2) is a multi-copper oxidase (MCO) enzyme characterized through its ability to oxidize a wide variety of phenolic and non-phenolic compounds the usage of molecular oxygen as an electron acceptor, forming water, free radicals, and oxidized compounds [1-3]. The enzyme is isolated from fungi, especially from basidiomycetes, plants, some microorganism, and bugs. The homes of the laccase enzyme range depending on its organic source [4]. Given its ability to work on many different substrates and its efficiency, laccase has become a focal point in biotechnology and medical research. To fully explore its properties and potential uses, purifying laccase is essential. This usually starts with concentrating the protein using ammonium sulfate precipitation, followed by chromatography methods like ion-exchange and gel filtration to sort the proteins by charge and size. These steps help obtain a pure, stable enzyme ready for detailed study and practical applications [5-6]. The species Agaricus nevoi belongs to the phylum Basidiomycota, class Agaricomycetes, order Agaricales, and family Agaricaceae. It was first described by Wasser (1998) [7] as a new species of the genus Agaricus in Israel. Later, this species was also observed in Abu Ghraib, Baghdad, where it was morphologically characterized by a brownish cap measuring 2–5 cm in diameter and a stipe length of 2–5 cm. Moreover, its extracellular enzymatic activity has been investigated for several enzymes, including amylase, protease, xylanase, L-asparaginase, cellulase, and lipase [8]. Agaricus nevoi is considered a relatively rare species that typically inhabits Mediterranean-type environments, particularly pine forests under Pinus halepensis trees, where it grows as a saprophytic fungus, decomposing organic matter in the soil. It is classified as an edible species of moderate quality, meaning it is non-toxic and safe for consumption, though not highly valued commercially or gastronomically [9]. Based on the above, the current study aimed to extract the laccase enzyme from the basidiomycete fungus Agaricus nevoi, purify it, and determine its organic activity as an antimicrobial, antioxidant, and anticancer agent. This comes in light of the scarcity of similar clinical references in this fungus, as well as the need to identify the premier situations for its production.

II. MATERIALS AND METHODS

A. *SAMPLES*

Study Samples Were Collected From The Al-Alam/Salahaddin In Iraq, From The Soil And Trunks Of Dwelling And Dead Trees During The Period Extending From February To May. The Samples Were Transported To The Laboratory, Washed Thoroughly With Running Water, And Then Cultured On Potato Dextrose Agar Medium. They Were Then Incubated For Five-7 Days And Specified SH003, IN002, And HM001

1) *Initial Screening*

The ability of three fungal isolates to produce laccase enzyme was evaluated using potato dextrose agar (PDA) medium supplemented with 2.5% guaiacol as a qualitative indicator of laccase activity, following the method described by Pundir et al. (2016) [10]. The PDA plates were inoculated with the selected fungal isolates and incubated at 25°C for seven days. The formation of a reddish-brown

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coloration around the fungal colonies was considered a positive indication of laccase activity. The experiment was conducted in triplicate for each fungal isolate. The control treatment consisted of inoculating the same isolates on PDA medium without guaiacol to confirm that the color change resulted specifically from laccase enzymatic activity.

2) Secondary Screening for Laccase Production

For the quantitative evaluation of laccase production, a liquid potato dextrose medium was prepared and supplemented with the following components (g/L): K_2HPO_4, 0.4; glucose, 10.0; $ZnSO_4$, 0.001; peptone, 3.0; $FeSO_4$, 0.0005; $MgSO_4$, 0.5; $MnSO_4$, 0.05; KH_2PO_4, 0.6. The pH was adjusted to 6.0 prior to sterilization by autoclaving. After cooling, the medium was inoculated with six discs (6 mm in diameter) taken from 7-day-old fungal cultures of the selected isolates, each inoculated separately. The flasks were incubated in a shaking incubator at 27°C and 90 rpm for 26 days, following the method described by Chaudhary et al. (2016) [12]. All experiments were conducted in triplicate for each fungal isolate.

B. Estimation Protein Concentration

The protein concentration was determined according to the method of Bradford (1979) [11] using a bovine serum albumin (BSA) standard curve, as shown in Figure 1. Different concentrations of BSA (0–1 mg/mL) were prepared using distilled water, with two replicates for each concentration. Then, 2.5 mL of Coomassie Brilliant Blue G-250 reagent was added to each tube, mixed thoroughly, and left to stand for 2 minutes at room temperature. The absorbance was then Measured at 595 nm. The blank solution was prepared by mixing 0.45 mL of phosphate buffer solution with 2.5 mL of the dye reagent. The absorbance values obtained from the BSA standards were used to construct the standard calibration curve Fig.1. For the test samples, 0.05 mL of the enzyme extract was mixed with 0.45 mL of phosphate buffer and 2.5 mL of Coomassie reagent, and the mixture was left to stand for 2 minutes at room temperature before measuring absorbance at 595 nm.

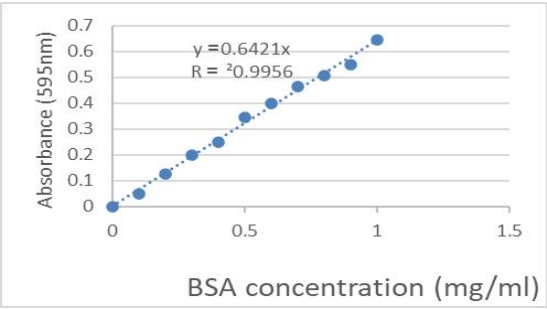

Fig.1: Standard curve for bovine serum albumin.

C. Assessment of Laccase Activity

Laccase activity was assessed by mixing 3 mL of sodium acetate buffer solution (prepared in section 9) with 1 ml of guaiacol substrate and 1 ml of fungal culture filtrate. This mixture was placed in centrifuge tubes and incubated at 6,000 rpm for 10 minutes at 4°C. One ml of the mixture was then taken and incubated at 37°C for 15 minutes, and the absorbance was measured at 450 nm. Enzyme activity is expressed in International Enzyme Units (IU), which is defined as the amount of enzyme required to convert 1 micromole of substrate to product in one minute under specified measurement conditions. It is sometimes referred to as V, which represents the rate of the enzyme reaction. Enzyme activity was calculated in units/ml using the following formula

$$E.A=\frac{A\times V}{t\times e\times v}$$

E.A represents the enzyme activity in units/mm³.

A represents the absorbance at 450 nm.

V represents the total volume of the reaction mixture in mm³.

t represents the incubation time of the reaction mixture in minutes.

e represents the extinction coefficient (Cm) (0.001M-1).

v represents the volume of the fungal growth filtrate in mm³ [13].

D. Enzyme Purification

According to Bradford (1976)[11]

The enzyme was purified through three main steps. In the first step, proteins were precipitated by the gradual addition of ammonium sulfate until the optimum saturation level was reached. The precipitate was collected by centrifugation at 6000 rpm for 20–30 minutes at 4°C. The supernatant was discarded, and the pellet was re-dissolved in phosphate buffer (0.5 M, pH 7). Dialysis was then performed using semi-permeable membrane bags (molecular weight cut-off 3500 Da) against the same buffer for 24 hours at 4°C, with buffer changes every 2–3 hours. Subsequently, the sample was subjected to ion-exchange chromatography on a DEAE-cellulose column equilibrated with phosphate buffer. Bound proteins were eluted gradually using increasing concentrations of NaCl (0.1–1 M) at a flow rate of 3 mL/min, and absorbance was monitored at 280 nm to detect the active fractions. The next step involved gel filtration chromatography using a Sephadex G-150 column (2 × 40 cm) equilibrated with the same phosphate buffer to separate the enzyme according to molecular size. The enzyme activity and protein concentration of each fraction were determined using the [11] method with bovine serum albumin (BSA) as the standard.

E. Molecular Identification

For molecular identification, the selected fungal isolate HM001, grown on culture medium, was sent to Macrogen Inc. (Seoul, South Korea) for ITS region amplification using the primers ITS1(5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4(5′-TCC TCC GCT TAT TGA TAT GC-3′), followed by sequencing using the Sanger method.

F. Enzyme Characterization:

According to Bradford (1976) [11].

1) Determination of Optimum Temperature for Enzyme Activity and Stability

The enzyme activity was measured under the standard assay conditions at various temperatures ranging from 30°C to 47°C. Thermal stability was assessed by pre-incubating the enzyme at these temperatures for 15 and 30 minutes, followed by rapid cooling, and then measuring the residual activity under standard conditions.

2) Determination of Optimum pH for Enzyme Activity and Stability

The enzyme activity was measured using guaiacol as the substrate within a pH range of 4–10, under the same assay conditions described previously , but without enzyme pre-incubation. For pH stability, equal volumes (1:1) of enzyme solution and buffer of the corresponding pH were mixed and incubated at 37°C for 15 minutes, followed by measuring the residual activity.

3) Determination of Molecular Weight

The molecular weight of the enzyme was estimated by gel filtration chromatography using a Sephadex G-150 column (2 × 40 cm) equilibrated with phosphate buffer. The elution volume (Ve) of the enzyme was determined and compared with a standard calibration curve generated from marker proteins of known molecular weights, which were eluted under identical chromatographic conditions.

G. Statistical Analysis

The experimental data were statistically analyzed using the Minitab software (version 17). Analysis of variance (ANOVA) was performed, and means were compared using Duncan's Multiple Range Test (DMRT) at a probability level of $P \geq 0.05$, according to the method described by Al-Rawi and Khalaf (2000).

III. Results and Discussion

A. Initial screening results

All isolates (SH003, IN002 and HM001) demonstrated the ability to produce laccase enzyme with different halo diameters. Isolate SH003 recorded a halo with a diameter of 45 mm, followed by isolate HM001, which achieved a halo with a diameter of 16 mm. Meanwhile, isolate IN002 achieved the smallest halo with a diameter of 8.5 mm after 10 days of incubation at 27 °C, as shown in Table 1 and Fig.2.

TABLE 1. Qualitative estimation of laccase enzyme production from three fungal isolates.

NUMBER OF DAYS AFTER CULTURING	ISOLATION CODE AND MEAN DIAMETER OF HALO			AVERAGE NUMBER OF DAYS
	HM001	IN 002	SH003	
3 DAYS	5.5 G	6.5 G	12.5 E	8.17 D
7 DAYS	9.0 F	7.0 FG	25.5 C	13.83 C
9 DAYS	14.0 DE	8.5 F	37.5 B	20 B
10 DAYS	16.0 D	8.5 F	45.0 A	23.17 A
AVERAGE PRODUCTION MEDIUM	11.13 B	7.63 C	30.13 A	

Values shown are averages of two replicates; similar letters mean no significant difference between them. Statistical analysis: Data were analyzed using one-way ANOVA, and differences were considered statistically significant at $P < 0.05$.

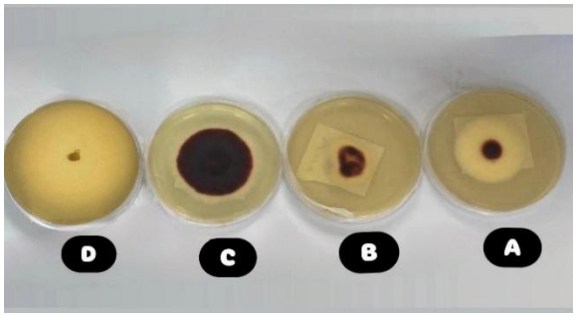

Fig.2: The diameter of the halos of three fungal isolates. A. IN002, B. HM001, C. SH003, and D. Control treatment.

The appearance of color is a clear indicator of laccase production, due to the presence of the basic substance " guaiacol " that stimulates its secretion. Guaiacol can be considered a reliable, specialized test for the activity of this enzyme. The brown color results from the oxidation of the substrate by alignolytic enzymes [14]. The appearance of light brown to reddish brown colors under and around the fungal colony is positive evidence of laccase activity and guaiacol oxidation [15]. In control dishes, no browning was observed, attributed to the absence of substrate and, consequently, to a lack of stimulation for enzyme production [16]. Studies indicate that the colored area surrounding the fungal colony, whether larger or adjacent to it, helps explain

the high yield and good activity of enzymes produced by the fungus [17].

The variation in the ability of pure fungal isolates to produce laccase is attributed to physiological differences among fungal species, as well as the diversity of their strategies for exploiting energy sources and nutrient medium components. Fungal genetics is also a major determinant of enzyme productivity, as it forms the basis for all its metabolic activities proceed.

The findings of the present study are consistent with several previous reports. For instance, Salem and Azeem-Abdel (2012) evaluated 60 fungal isolates belonging to 33 genera of both ascomycetous and basidiomycetous fungi. Their preliminary screening using guaiacol as a qualitative indicator revealed that all isolates were capable of producing laccase at varying levels. The Chaetomium globosum isolate exhibited the highest activity, forming a clearance halo of 40 mm around its 58 mm colony, whereas Mucor circinelloides showed the lowest activity, with a 4 mm halo around a 54 mm colony.

B. Secondary Screening Results

When conducting the secondary screening test (production medium), as shown in Table 2, it was found that the isolate HM001 produced the highest enzyme production on the twentieth day of incubation, with a value of 50 units/ml, while the fungal isolate SH003 achieved the highest enzyme production on the sixteenth day (36 units/ml). while the isolate IN002 achieved the highest enzyme productivity with a value of 40 units/ml on the

twentieth day of incubation. Since the quantitative test is the best and most reliable in many experiments, the isolate HM001 was chosen to isolate and purify the enzyme. This result was consistent with the results of the qualitative test, as it is noted from Figure (1) that both isolates HM001 and SH003 (regardless of the diameter of the two halos) colony size, which explains the high production of laccase enzyme in the isolate. The low growth may be attributed to reasons including that the medium may not be suitable for the growth of the fungal isolate due to its biological characteristics and preference for different growth conditions, as indicated by Mustafa [18]. He tested the species of the Agaricuc isolate on different agricultural media, It was found that the isolate grows very little or does not grow at all on the PDA agar medium due to the lack of suitable conditions for its growth in terms of the presence of a carbon source (sucrose-glucose), and because it prefers carbon sources in growth, we notice that it grows more in the secondary screening medium because the medium contains glucose. Therefore, the isolate was selected after quantitative testing, which demonstrated greater enzyme production in isolate HM001 compared to the other fungal isolates. The table also shows a decrease in enzyme production on day 26 for all isolates, which may be due to a lack of nutrients and the accumulation of toxic metabolites. It is also noted from Tables (1) and (2) that there is a difference between the isolates in their production of the laccase enzyme, which may be attributed to several reasons, including the difference in the source of isolation of fungal species, or it may be attributed to the presence of a difference in the genetic composition between the isolates, as each isolate possesses a group of genes responsible for producing the enzyme [19-20].

TABLE 2. Quantitative estimation of laccase enzyme production from three different fungal isolates and different incubation periods

Average production per day	IN002 Laccase activity (U/ml)	SH003 Laccase activity (U/ml)	HM001 Laccase activity (U/ml)	Incubation days for the fungus
24.67 d	20 g	18 g	36 e	2
25.67 d	20 g	19 g	38 de	4
26.33 d	20 g	19 g	40 cd	6
31.33 c	34 ef	20 g	40 cd	8
36.33 b	35 ef	34 ef	40 cd	12
36.67 b	36 e	32 f	42 bc	14
38.00 b	36 e	36 e	42 bc	16
38.67 b	38 de	36 e	42 bc	18
42.00 a	40 cd	36 e	50 a	20
38.67 b	38 de	34 ef	44 b	24
36.67 b	36 e	32 f	42 bc	26
	32.10 b	28.73 c	41.45 a	Average enzyme activity

The table shows that the enzyme activity increased to 150 units/ml and 0.6 mg/ml of protein using ammonium sulfate precipitation at 70% saturation with 2.5 purifications, resulting in an enzyme specific activity of 250 units/mg. The concentrated fraction from the previous step was then subjected to DEAE-cellulose ion exchange chromatography, which yielded an enzyme activity of 125 units/ml and 0.3 mg/ml of protein with 4.1 purifications

Values are shown as averages of two replicates. Similar letters indicate no significant differences

C. Laccase Enzyme Purification

The steps for purifying the laccase enzyme from the fungus Agaricus nevoi. Table 3 shows the enzyme activity, protein concentration, specific and total activity, as well as the number of times the enzyme was purified for each step.

TABLE 3. Steps for purifying the laccase enzyme from the crude enzyme extract of the fungus Agaricus nevoi

Purification steps	Size (mL)	Enzymatic activity	Protein concentration	Specific activity (U/mg)	Total activity (U)	purification folds	yield %
Crude enzyme	100	50	0.5	100	5000	1	100
Ammonium sulfate precipitation 70%	20	150	0.6	250	3000	2.5	60
DEAE-cellulose Ion exchange chromatography	24	125	0.3	416.6	2625	4.1	52.5
Sephadex- G150 gel filtration chromatography	21	100	0.1	1000	2100	10	42

Statistical analysis: Data were analyzed using one-way ANOVA, and differences were considered statistically significant at $P < 0.05$.

The results shown in Table 3 also indicate that the specific activity exceeded its value in the crude extract by a value of 416.6 units/mg, followed by gel filtration chromatography, as the enzyme and protein activity ratio was 100 units/mL and 0.1 mg/ml, respectively, The low protein content indicates that the enzyme was completely and correctly purified through 10 purifications and a significant increase in the specific activity of the enzyme from 100 units/mg to 1000 units/mg, indicating effective purification steps.The results of the current study are consistent with the results of several previous studies that confirmed the effectiveness of the adopted purification steps [21] purified the laccase enzyme from the fungus Aspergillus sp. using ion exchange chromatography on a DEAE Cellulose column, followed by a gel filtration process using a Sephadex G-100 column. This method yielded a specific activity of 465 units/mg, with a productivity of 6.21%. In another study, Baldrian [22] purified the laccase enzyme from the large mushroom Trametes versicolor using the same ion exchange and gel filtration techniques. This method achieved a specific activity of 840 units/mg with 2.89 purifications.

In addition to the effectiveness of the methods adopted in the current study, these methods are economical, as they mainly rely on traditional, low-cost steps used in most biological laboratories, such as ammonium sulfate precipitation, ion exchange chromatography, and gel filtration. These techniques are available in most biological laboratories and do not require advanced or expensive equipment. The salt precipitation method (ammonium sulfate) is a method used to concentrate and purify proteins by changing their solubility by increasing the ionic strength. At high salt concentrations, the protein precipitates from the solution. This process is known as salting out or

precipitation [23] while maintaining its solubility [24]. Ammonium sulfate also helps maintain the stability of the protein's natural structure during the precipitation process, improving the efficiency of the subsequent purification steps [24-25]. Ion exchange chromatography is also used in laccase purification because it relies on separating proteins according to their electrical charge at a specific pH. This method allows enzymes to be separated from impurities and other proteins with different charges, contributing to increased enzyme purity and improved activity. It is also considered an effective technique for recovering the enzyme with high purity without causing its degradation, as separation conditions such as pH and ion concentration can be adjusted to control the binding of proteins and separate them precisely [26]. Finally, gel filtration using a Sephadex G-150 column was suitable for separating the enzyme according to its molecular size, which was later determined to be about 50 kDa , as the separation range of this gel (5–300 kDa) accurately covers this size. This choice helped enhance purification efficiency and achieve near-homogeneity [27-1]. This method also preserves enzyme activity because it does not depend on changes in pH or organic solvents, making it suitable for the final steps in the purification of sensitive proteins [26].

Moreover, this method achieved high specific activity and good enzyme yield after purification, demonstrating its efficiency in enzyme isolation without significant loss of biological activity. The simplicity and reproducibility of the steps also make it suitable for large-scale industrial or research applications, enhancing its economic and practical feasibility in laccase production [28].

Fig.3 shows the absence of enzyme activity in the washing stage and its appearance only in the recovery stage, which indicates that the enzyme has a charge similar to the column charge, so it was trapped inside the column and only came out when the displacement solution was added, as 8 parts containing high enzyme activity appeared, shown in red in Fig.3 at ratios ranging from 240 - 170 - 150 - 140 - 100 - 70 - 70 - 60) U/mL.

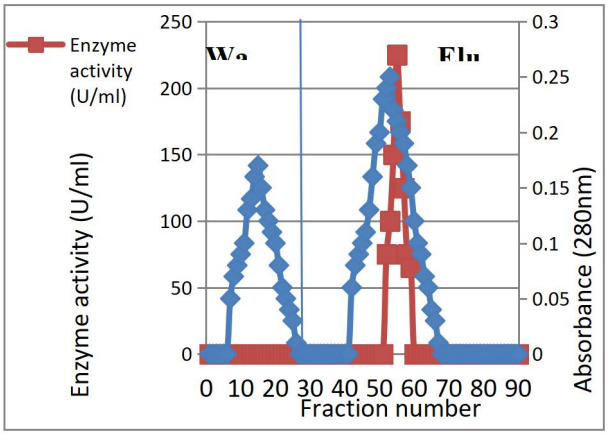

Fig.3: Ion filtration chromatography for purification of the laccase enzyme produced by the fungus Agaricus nevoi using a DEAE-Cellulose column (20 x 2 cm) at a flow rate of 30 ml/h.

In gel filtration chromatography, seven fractions with the highest enzyme activity were obtained, shown in red, at ratios ranging from (150-125-100-85-65-70-70) U/mL Fig.4.

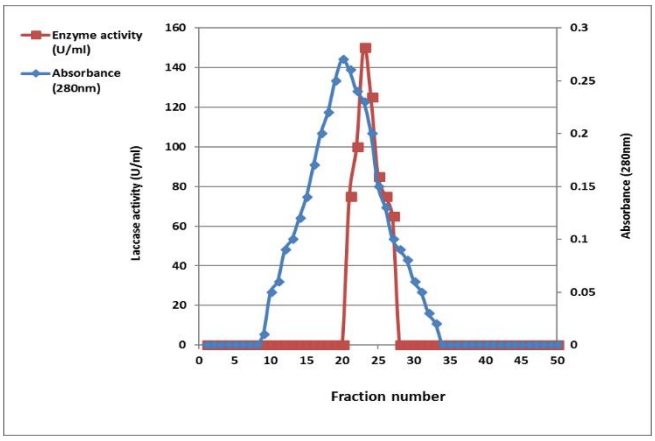

Fig.4: Gel filtration chromatography for the production of laccase enzyme from the fungus Agaricus nevoi.

D. Molecular Diagnosis

The BLAST analysis of the ITS region sequence amplified using primers ITS1(5′ -TCC GTA GGT GAA CCT GCG G-3′)and ITS4(5′ -TCC TCC GCT TAT TGA TAT GC-3′) (Figure 5) revealed a high sequence similarity with entries in the GenBank database. The sequence showed 99% identity with Agaricus nevoi (GenBank accession number KM657922.1), as summarized in Table 4 The obtained E-value was 0.0, indicating a very high degree of similarity and no significant differences between the sequences. These results confirm that the fungal isolate HM001 is genetically affiliated with Agaricus nevoi, representing the second local genetic documentation of this species in Iraq, The sample was registered in the global gene bank under deposit number 1.PX909784.

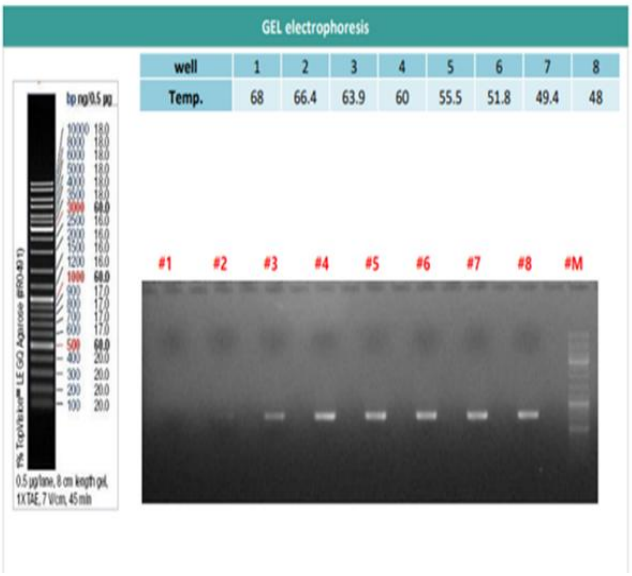

Fig 5: Gradient PCR Analysis .The gradient PCR of the fungal isolate HM001 using primers ITS1 and ITS4 showed clear amplification bands on agarose gel at an annealing temperature of 60°C, compared to other tested temperatures

Table 4. Genetic variations in the Ribosomal ITS1-5.8S-ITS4 gene of the Iraqi Agaricus nevoi isolate

C, G, T, and A indicate the nitrogenous bases cytosine, guanine, thymine, and adenine, respectively.

NO.	Location in Genebank	Nucleotide change	Type of substitution	Sequence ID	source	Identities
1	24 204 655 658 662	C/W G/R C/- T/G -/A	GAP GAP GAP TRANSTION GAP	KM657922.1	*Agaricus nevoi* isolate LAPAG257 18S ribosomal RNA gene, partial sequence	99%

W denotes weak nucleotides (A or T)
,R denotes purine nucleotides (A or G).
C/W and G/R represent ambiguous nucleotide substitutions according to IUPAC codes.
GAP (–) indicates a gap caused by insertion or deletion during sequence alignment.
–/A and C/– indicate nucleotide insertion or deletion.
Transitions represent substitutions between nucleotides of the same chemical class (purine purine or pyrimidine pyrimidine).

E. Results of Laccase Enzyme Characterization

1) Estimating the Optimal Temperature for Enzyme Activity and Stability:

Fig.6 illustrates the effect of temperature on enzyme activity. Activity increases with increasing temperature from 32°C to 37°C, which is the optimum temperature for enzyme activity. At 30°C, activity was 70 units/mL of protein, while it increased to 100 units/mL at 37°C. This positive effect of temperature, up to a certain point, is explained by the Arrhenius equation. Heat gives enzyme molecules kinetic energy, leading to continuous collisions with the substrate, increasing the reaction rate. As temperatures increase above 37°C, the reaction rate decreases due to the breakdown of hydrogen and ionic bonds. which enters into the enzyme's structure and maintains its active site. This explains the decreased activity of the enzyme (laccase) at temperatures above 37°C, with activity dropping to 75 units/ml at 47°C. High temperatures also lead to a change in the nature of the enzyme (denaturation) and a significant loss of activity [29]. These results indicate that the enzyme functions best under moderate thermal conditions.

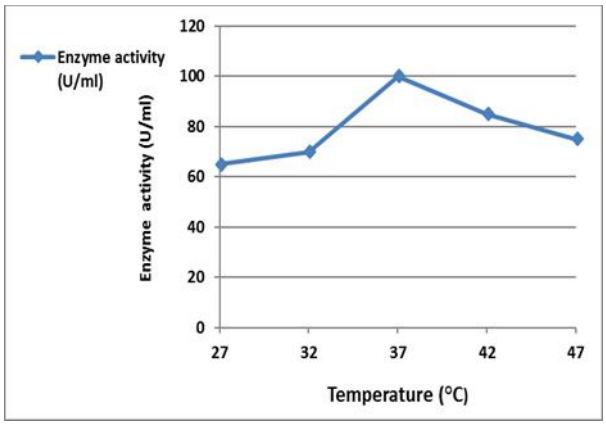

Fig.6: Effect of temperature on the production of laccase enzyme from Agaricus nevoi.

Regarding the thermal stability of the enzyme, it is noted from Fig.7 that the enzyme maintained its activity, which reached 100% within the temperature range (32-37°C), while the enzyme lost approximately 95, 80, and 55% of its activity at temperatures (42-47°C) and (52°C), respectively, as Figure (7) shows. The laccase enzyme has a limited range of thermal stability, as its thermal stability decreases at temperatures of 37°C to 52°C, reaching 55% of its activity. High temperatures above 45°C break down the various bonds in the enzyme, which are responsible for building the Enzyme's stability and may lead to enzyme degradation by destroying the three-dimensional structure of the protein and forming random polypeptide chains, or by causing the active site to change, which subsequently leads to enzyme inactivity [30].

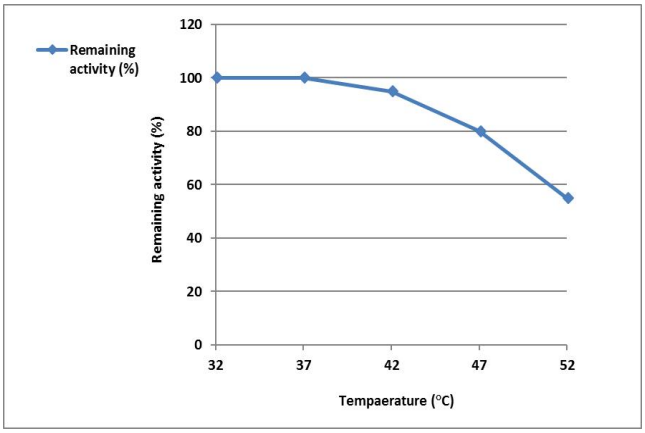

Fig. 7: The optimum temperature for enzyme stability.

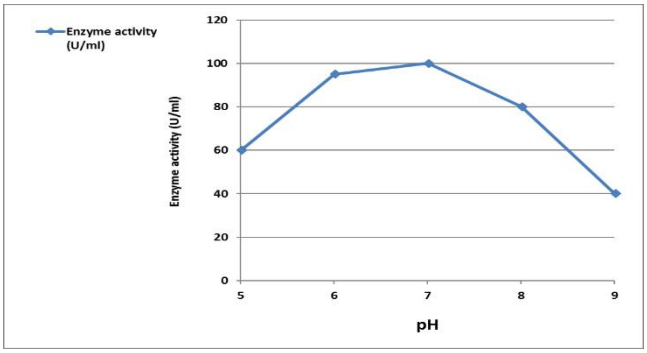

Fig.8: The relationship between pH and enzyme activity.

The effects of pH can be traced back to the ionization state of acidic or basic amino acids. Acidic amino acids contain carboxyl functional groups in their side chains, while basic amino acids contain amine functional groups in their side chains. Therefore, if the ionization states of amino acids in a protein change, the ionic bonds that help define the protein's three-dimensional shape can be altered. This, in turn, alters protein recognition or may render the enzyme inactive. Studies also show that changes in pH may not only affect the shape of the enzyme but may also cause a

change in the shape or properties of the substrate, so that the substrate is unable to bind to the active site or cannot undergo catalysis [42].

The results of the enzyme instability test at different pH levels (Figure 9) also showed that the purified laccase was stable at pH 8.0, as the enzyme achieved the highest activity of 100%, while its activity decreased by 50% at pH levels 5.0 and 9.0. The results of the study were consistent with the study by Contato and his group [34], which indicated that the purified laccase enzyme from the large fungus Pleurotus pulmonaria lost 60% of its activity after incubation at pH 5.0 for 24 hours. However, the enzyme maintained 100% of its activity after incubation at varying pH levels ranging from 4 to 10, after incubation at 37°C for only 15 minutes.

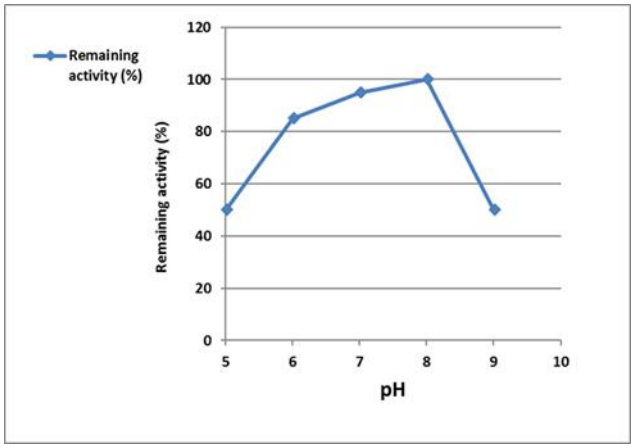

Fig.9: The optimal pH for stable enzyme activity.

The decrease in enzyme activity under highly acidic and highly basic conditions may be due to changes in the secondary and tertiary structures of the ionic state of the enzyme's active site and substrate (35). On the other hand, Moat and his group (36) discussed a group of factors that show the effect of pH on enzyme activity, the most prominent of which are: First, each enzyme has an optimal pH at which it exhibits the highest possible enzyme activity. However, the enzyme maintains part of its activity within a certain range of acidity or alkalinity around the optimal pH. Second, extreme changes in pH can lead to changes in the tertiary or quaternary structure of the enzyme, resulting in loss of stability or structural distortion (denaturation), especially in highly acidic or alkaline environments. Third, the effect of pH is not limited to the enzyme; it may also extend to the substrate itself, as it can cause changes in its structure or chemical reactivity, indirectly affecting the efficiency of the enzyme reaction. This effect is reflected in the shape of the activity versus pH curve.

3-Determining the Molecular Weight of the Enzyme

Gel filtration was used through a column filled with Sephadex gel to estimate the approximate molecular weight of the laccase enzyme, as shown in Fig.10. After passing the standard proteins and recording the elution volume (Ve) for each, the Ve/Vo ratio was calculated, and a curve was drawn linking the decimal logarithm of the molecular weight (log Mw) to Ve/Vo. which showed a clear linear relationship within the measurement range. The standard proteins showed a logical gradient in eluting from the column according to their molecular weights, with Alcohol Dehydrogenase (150 kDa) eluting while Lysozyme (14.3 kDa) lagged behind. A sample of laccase enzyme was passed through the column; its elution volume (Ve) was recorded, and the Ve/Vo ratio was calculated by applying. Ve/Vo on the calibration curve, the approximate molecular weight of the laccase enzyme was estimated to be about 50,000 kDa. The estimated molecular weight (50 kDa) indicates that the laccase enzyme is likely in its monomeric. Gel filtration is an effective and safe method for estimating the molecular weight of active enzymes without affecting their spatial structure or losing their biological activity [26]. It is important to note that the measured molecular weight reflects the natural structure of the enzyme in solution, not just its amino acid sequence, which is an advantage of this method over techniques such as SDS-PAGE [37].

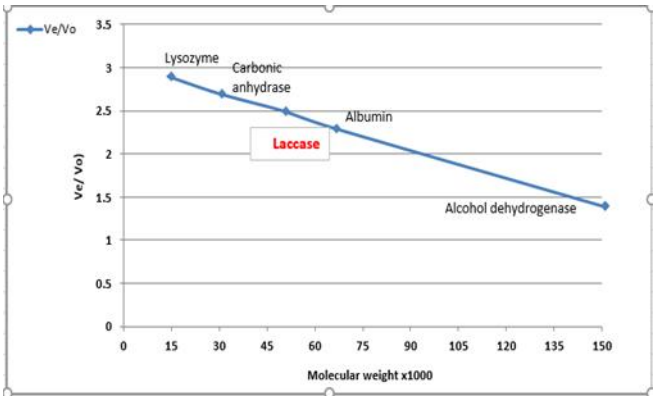

Fig.10: Molecular weight of laccase enzyme using Sephadex G-150 gel filtration chromatography.

The results indicate that proteins with higher molecular weights move through the gel filtration column at a higher speed and descends first, because they do not penetrate deeply into the charged particles in the column, unlike proteins with low molecular weights that enter into these particles, which leads to a delay in their descent. The molecular weight of the studied enzyme was estimated by calculating the ratio of the volume representing the descent of the high-molecular-weight Dextran Blue to the volume of the enzyme itself. The estimated molecular weight was approximately 50 kDa.

The results of the current study are consistent with several previous studies that showed that the molecular weight of laccase in most fungi and bacteria ranges approximately between 50 to 100 kDa [38-39].

One study showed that the molecular weight of the purified laccase enzyme extracted from the fungus Nectriella pironii was approximately 50 KDs [40]. This is close to the weight reported for the G. lucidum isolate, which ranged from 50 to 80 KDs (41). In another study, the molecular weight of the purified laccase enzyme from G. lucidum was estimated at 62.5 KDs, while it was 65 KDs in G. leucocontextum isolates [42-43,6]. A study on the Ganoderma lucidum isolate MDU 7 also showed Anoderma

lucidum MDU 7 showed that the molecular weight of the enzyme ranged between 40 and 66 KDs [44-45]. Therefore, the results of the current study indicate that the molecular weight of the laccase enzyme falls at the lower end of this range, enhancing the reliability and success of the method used

CONCLUSIONS

The purified laccase from Agaricus nevoi exhibited a relative molecular mass of approximately 50 kDa. The enzyme activity remained stable over a pH range of 7–8 and at temperatures between 32 and 37 °C, while it decreased rapidly above 37 °C. These findings indicate that the studied fungus is a promising source for the production of laccase with stable physiological properties, suitable for potential applications in medical, industrial, and environmental fields.

CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

REFERENCES

[1] Y. Yang, H. R. Hebron, and J. Hang, "High performance DNA purification using a novel ion-exchange matrix". *Journal of Biomolecular Techniques*, 19(4), 205–212. 2008.

[2] S. Sondhi and S. Saini, "Response surface based optimization of laccase production from Bacillus sp. MSK01 using fruit juice waste as an effective substrate," *PLOS ONE*, pp. 1–17, 2019, doi:10.1371/journal.pone.0229968.

[3] G. Janusz et al., "Laccase properties, physiological functions, and evolution," *International Journal of Molecular Sciences*, vol. 21, no. 3, p. 966, 2020, doi:10.3390/ijms21030966.

[4] P. Aza and S. Camarero, "Fungal laccases: Fundamentals, engineering and classification update," *Biomolecules*, vol. 13, no. 12, p. 1716, 2023, doi:10.3390/biom13121716.

[5] J. R. Whitaker, A. G. J. Voragen, and D. W. S. Wong, "Handbook of Food Enzymology," New York, USA: Marcel Dekker, 2003.

[6] D. L. Nelson and M. M. Cox, "Lehninger Principles of Biochemistry," *Recording for the Blind & Dyslexic New York*, 7th ed. New York, USA: W. H. Freeman, 2017.

[7] S. P. Wasser, "Species diversity in the tribe Agariceae Pat. (Higher Basidiomycetes) of Israel mycobiota," *Israel Journal of Plant Sciences*, vol. 46, no. 4, pp. 315–330, 1998.

[8] R. A. Chechan, E. M. Farhan, M. M. Muslat, and Z. M. Abdul-Qader, "Morphological characterization, molecular diagnosis, and enzymatic activity of some wild mushrooms in Baghdad Province, Iraq," Plant Archives , vol. 20, no 2, pp. 7437-7445, 2020.

[9] Cromushrooms, "Species profile: Agaricus nevoi Wasser," *European Mycological Database*, 2025.

[10] R. K. Pundir, V. K. Mishra, S. Rana, and M. Lakhani, "Screening of laccase producing fungi from soil samples—An in vitro study," *Electronic Journal of Biology*, vol. 12, no. 3, pp. 254–257, 2016.

[11] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein," *Analytical Biochemistry*, vol. 72, pp. 248–254, 1976.

[12] R. Chaudhary, S. Hooda, S. Beniwal, and A. Sindhu, "Partial purification and immobilization of laccase isolated from medicinal mushroom (Ganoderma lucidum)," *Indian Journal of Chemical Technology*, vol. 23, no. 4, pp. 313–317, 2016.

[13] T. Saito et al., "Purification and characterization of an extracellular laccase from a soil fungus," *Enzyme and Microbial Technology*, vol. 33, pp. 520–526, 2003.

[14] L.-L. Kiiskinen, M. Rättö, and K. Kruus, "Screening for novel laccase-producing microbes," *Journal of Applied Microbiology*, vol. 97, no. 3, pp. 640–646, 2004.

[15] A. B. Vantamuri and B. B. Kaliwal, "Isolation, screening and identification of laccase producing fungi," *International Journal of Current Microbiology and Applied Sciences*, vol. 4, no. 4, pp. 1014–1021, 2015.

[16] K. Amit et al., "Gel-based purification and biochemical study of laccase isozymes from Ganoderma sp.," *Frontiers in Microbiology*, vol. 8, p. 674, 2017, doi:10.3389/fmicb.2017.00674.

[17] Q. Wang, Y. Qian, Y. Ma, and C. Zhu, "A preliminary study on newly isolated high laccase-producing fungi," *Open Life Sciences*, vol. 13, pp. 463–469, 2018.

[18] S. M. Mustafa, "Production of pure isolates for cultivated mushroom (Agaricus bisporus) using tissue culture technology," 2018.

[19] H. Moallaei et al., "Enzymatic activity and molecular characterization of a secreted protease," *Iranian Journal of Public Health*, vol. 38, no. 1, pp. 25–33, 2009.

[20] C. U. Uma, "Production, purification and characterization of protease by Aspergillus flavus," 2011..

[21] P. Baldrian, "Increase of laccase activity during interspecific interactions of white-rot fungi," *FEMS Microbiology Ecology*, vol. 50, no. 3, pp. 245–253, 2004.

[22] A. A. Green and W. L. Hughes, "Protein fractionation on the basis of solubility," *Methods in Enzymology*, vol 1, pp. 67-90, 1955.

[23] P. T. Wingfield, "Protein precipitation using ammonium sulfate," *Current Protocols in Protein Science*, vol. 84, no. 1, 2016.

[24] F. B. Salisbury and C. W. Ross, "Plant Physiology, " Annals of Botany, vol. 71, pp. 91-94, 1993, USA: Ancestry Publishing.

[25] R. K. Scopes, "Protein Purification: Principles and Practice, 3rd ed," Springer, New York, USA, 1992.

[26] S. A.Hanan, "Al-Muthaffar,Biochemistry. Part One, Second Edition, " *University of Baghdad, Ministry of Higher Education and Scientific Research*. Page 492. 1990.

[27] M. Asgher, M. Irshad, and H. M. N. Iqbal, "Purification and characterization of lignin peroxidase," *BioResources*, vol. 7, no. 3, pp. 4012–4021, 2012.

[28] K. Miyanaga and H. Unno, "Reaction kinetics and stoichiometry," *in Comprehensive Biotechnology*, 2nd ed., vol. 2. Elsevier, 2011, pp. 33–46.

[29] V. W. Rodwell, "Structure and function of proteins and enzymes," *in Biochemistry*, 24th ed. USA: Prentice-Hall, 1996.

[30] K. M. Park and S. S. Park, "Purification and characterization of laccase from Fomitella fraxinea," *Journal*

of Microbiology and Biotechnology, vol. 18, no. 4, pp. 670–675, 2008.
[31] F. Xu et al., "Differences in redox potential and substrate specificity of fungal laccases," *Biochimica et Biophysica Acta*, vol. 1292, pp. 303–311, 1996.
[32] A. G. Contato et al., "Solid-state fermentation with orange waste for laccase production," *Acta Scientiarum Biological Sciences*, vol. 42, pp. 1–9, 2020.
[33] A. Lehmacher and H. Bisswanger, "Isolation of thermostable enzymes," *Microbiology*, vol. 136, no. 4, pp. 679–686, 1990.
[34] A. G. Moat, J. W. Foster, and M. P. Spector, "Microbial Physiology," *John Wiley & Sons*, New York, USA: Wiley, 2002.
[35] M. P. Deutscher, "Guide to Protein Purification,"*Gulf Professional Publishing*, vol. 182, 1990.
[36] P. Sharma, R. Goel, and N. Capalash, "Bacterial laccases," *World Journal of Microbiology and Biotechnology*, vol. 23, pp. 823–832, 2007.
[37] K. Brijwani, A. Rigdon, and P. V. Vadlani, "Fungal laccase: Production and applications," *Enzyme Research*, p. 149748, 2010.
[38] A. Góralczyk-Bińkowska et al., "Laccase activity of Nectriella pironii," *PLOS ONE*, vol. 15, no. 4, e0231453, 2020
[39] A. K. Sitarz et al., "Identification of a laccase from Ganoderma lucidum," *Enzyme and Microbial Technology*, vol. 53, no. 6–7, pp. 378–385, 2013.
[40] J. M. Chesworth, T. Stuchbury, and J. R. Scaife, "Amino acids and proteins," *in An Introduction to Agricultural Biochemistry. Springer*, 1998.
[41] A. M. Abdel-Azeem and F. M. Salem, "Biodiversity of laccase producing fungi in Egypt," *Mycosphere*, vol. 3, no. 5, pp. 900–920, 2012.
[42] M. M. Gyosheva et al., "Red list of fungi in Bulgaria," *Mycologia Balcanica*, vol. 3, no. 1, pp. 81–87, 2006
[43] H. Patel, A. Gupte, and S. Gupte, "Effect of culture conditions on laccase production," *BioResources*, vol. 4, no. 1, pp. 268–284, 2009.
[44] R. K. Sharma and D. S. Arora, "Production of lingocellulolytic enzymes," *Bioresource Technology*, vol. 101, no. 23, pp. 9248–9253, 2010.
[45] J. R. Whitaker, "Principles of Enzymology for the Food Sciences," *FOOD SCIENCE AND TECHNOLOGY*, vol. 61, New York, USA: Marcel Dekker, 1972