EVALUATION THE PROPERTIES OF PURIFIED LACCASE EXTRACTED FROM SOME LOCAL PLANTS UNDER THE OPTIMUM CONDITIONS

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ABSTRACT

This study set out to screen 36 common plants which have the greatest level of laccase enzyme activity. It revealed that the enzymatic activity of fenugreek seeds was the highest in comparison with other plants. The optimal enzyme-specific activity was 5340.38 units per milligram protein which were obtained by extracting the enzyme with a sodium phosphate buffer at a concentration of 0.02 M and pH 8.0, at a ratio of 1:40 (weight to volume), and extracting time of 210 minutes. The enzyme yield was 27.6% after extraction and purification by gel filtration using Sephacryl S-3 after 1.01 purification fold. The optimum circumstances for enzymatic activity and stability were found by using 0.1 M sodium acetate as a buffer at pH 5. Also, the maximal activity and stability of purified laccase was obtained at 20 ^{o}C for 15 min by using o-tolidine as a substrate. This research sheds light on how to isolate and characterize the laccase enzyme, an important biochemical with numerous biotechnological and technological uses through fenugreek seeds as a source of the laccase.

Key words: laccase enzyme, plants, fenugreek seed, extraction.

مجلة العلوم الزراعية العراقية -2023 :54(4):1101-1112 تليع وآخرون

تقييم خواص اللاكيز المنقى والمستخلص من بعض النباتات المحلية تحت الظروف المثلى

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المستخلص

تهدف الدراسة الحاليه إلى غربله 36 نباتًا شائعًا لغرض اختيار النبات الامثل كمصدر لإنزيم اللاكيز. حيث كشفت الدراسة أن بذور نبات الحلبة كانت في انتاج الانزيم الأعلى مقارنة بباقي النباتات حيث امتلكت اعلى فعاليه نوعيه (5340.38 وحدة لكل ملغرام بروتين) عند الاستخلاص باستخدام محلول فوسفات الصوديوم بتركيز 0.02 مولاري ورقم هيدروجيني 8 ، بنسبة 40:1 (وزن إلى حجم) ، ووقت الاستخلاص 210 دقيقة.. بلغ مردود الإنزيم 27.6٪ عند تنقيته بطريقه كروماتوكرافيه الترشيح الهلامي باستخدام مادة سيفاكريل س–300 بعدد مرات تنقيه 1.01 ، كما واظهر الانزيم المنقى اعلى فعاليه وثبات انزيمي عند رقم هيدروجيني5 باستخدام(0.1 مولاري) بفر خلات الصوديوم كما اظهر الانزيم اعلى فعاليه وثبات حراري عندر درجه 20 م ولمدة 15 دقيقه باستخدام التوليدين كافضل مادة اساس.سلط البحث الحالي الضوء على كيفية عزل وتوصيف إنزيم اللاكيز، وهو مادة كيميائية حيوية مهمة لها العديد من الاستخدامات التكنولوجية الحيوية والتي يمكن استخلاصها من بذور نبات الحلبه.

الكلمات المفتاحية: إنزيم اللاكيز، النباتات، بذور الحلبة، الاستخلاص

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INTODUCTION

The enzyme laccase (EC 1.10.3.2) is a blue copper protein that exists outside cells, it is classified as a multi-copper oxidase. Through a radical-mediated reaction mechanism, laccase uses oxygen at the molecular level for facilitating the oxidation of several aromatic and non-aromatic compounds. One-electron oxidation is performed by ligninolytic enzymes, leading to the production of cation radicals that degrade contaminants (12). To produce more hydrophilic derivatives, these radicals can break C-C bonds or introduce hydroxyl groups to start chemical processes. Co-metabolism of these products with the strains mentioned by Mester and Tien (27) when an appropriate carbon source or carbon dioxide is present. The Japanese lacquer tree *Rhus vernicifera* was the original source of laccase, which was initially reported in 1883 by Yoshida (37). Fungi, higher plants, and bacteria have laccases. Laccases are also present in insects, although at a far lower frequency. Some plant species, such as the sycamore, the mango, the mung bean, the peach, the pine, and the prune, have been found to have laccases (23). According to Youn *et al.* (41), laccase could form reactions with the phenolic hydroxyl groups that are present in lignin, and it also has a wide substrate selectivity for aromatic compounds that contain hydroxyl and amine groups. Industrial applications of laccases include decolorizing wine, bleaching paper, creating chemicals from lignin, and cleaning up polluted environments (39). Rittstieg et al. (32) found that a purifying of laccase is an important step because compounds from the host fungus, which may act as natural mediators, or the presence of similar enzymes with very different reaction kinetics can all make it hard to find accurate kinetic parameters. The goal of this research was to determine, with as much precision as possible, under what circumstances laccase could be extracted from different plant species, purified and characterized most effectively utilizing locally accessible chromatographic techniques.

MATERIALS AND METHODS

Plants collection: Thirty-six plants were effortlessly obtainable from the Iraqi markets involving Turnip (*Brassica rapa*), Fenugreek seeds (*Triganeila foenum graecum*), clove (*syzgium aromticum*), Spinach (*spinacia oleracea*), Red cabbage (*Brassica oleracea var*), green cabbage (*Brassica oleracea var*), mallow (*malva. Sylvestris*), Albizia(*Albiziq lbbeck*), Broccoli lentil(*Brassica oleracea*), Thyme(*Thymus vulgaris*), Myrtus communis, pear(*pyrus communist*), Carps (*cyprinus curpio*), Dill(*Anethum graveolens*), cotton(*Gossypium barbadense*), cucumber(*cucumis sativas*), Black seed (*Nigella sativa*), chard(*Beta vulgaris subsp.cicla*), cauliflower(*brassica oleracea var. botrytis*), garlic(*Allium sativum*), peas(*pisum sativ um*), yellow apple(*Malus domestica*), coriander(*coriaudrum sativum*), sesame(*sesamum ibdicum l*), Dodonaea, Green pepper(*Capsicum annuum*), chives(*Allium schoenoprasum*), celery(*Apium dulce*), Radishes(*Raphanum sativus*), Green apple(*Malus viridis*), onion (*Allium cepa*), red apple (*pyrus Malus*), potato(*colanum tubersum*) and Tomato(*salamum lvcopersicum*). The plant materials were subjected to availability and laccase extraction capacity tests as part of the screening process. To prepare plant specimens, they were cut into small pieces and then dried at room temperature.

Chemicals

Three buffer solutions, namely sodium acetate (99% $C_2H_3NaO_2$ BDH, England,), phosphate-buffered saline ($C_{l2}H_3K_2Na_3O_8P_2$, BDH, England), tris-base ($C_4H_{11}NO_3$, Hi Media, India), and o-tolidine, $HgCl_2$, $ZnSO_4$, KCl, $FeSO_4$, EDTA, $CuSO_4$, $CaCO_3$, and cysteine, were bought from Hi-media.

The process of extracting and recovering the laccase enzyme: One gram of thirty-six plant was mixed with 40 ml of 0.02 M sodium phosphate buffer solution at pH 7 to extract laccase from the tested plant. The extract solutions were centrifuged at 10000 rpm for 15 minutes. Enzyme activity, protein content, and specific activity were by testing the consequent clear liquid, also called the crude extract (16).

The Laccase activity measurement

To measure the laccase activity; o-tolidine was used as a substrate according to the method described by Al-Assadi (1). Firstly, o-tolidine oxidation detection was measured using a

spectrophotometer to screen the clear solution absorbance at 366 nm where: e 366 = 27,600 in units of M^{-1} cm^{-1}. Secondly, the laccase protein concentration was determined using the Bradford (8) technique. Then, enzyme activity was calculated by a formula described by Kalral et al., (19) as follows:

$$\textbf{Laccase activity (U/ml)} = 10^6 \times A \times V / t \times e \times v \quad \textbf{(1)}$$

Where A is the 366 nm absorbance and V is the total volume (ml) of the combination. v = milliliters of enzyme, t = minutes of incubation. e: the substrate extinction coefficient (e 366 = 27,600 in M-1 cm-1) and is the cuvette's diameter (in centimeters). The quantity of enzyme needed to oxidize 1 µmol of substrate per minute was defined as one unit of enzyme activity. (24**)**.

Optimum conditions for laccase extraction:

Type of extraction buffer: Three types of buffers were examined to select the best one to be used for further experiments, the experiments were done by mixing the best plant samples obtained from the previous experiments. The experiments were conducted by mixing the plant sample for 15 minutes at room temperature, with 0.02 M sodium acetate (pH 4, 5, and 6), 0.02 M phosphate-buffered saline (pH 7, 7.6, and 8), and 0.02 M tris-base (pH 9, and 10) .The best buffer in terms of enzyme activity, protein content, and specific activity was measured using the same procedures (19,8,17).

Concentration of extraction buffer

The type best buffer was derived from a previous experiment of 0.01, 0.02, 0.05, 0.1, 0.15, 0.2, 0.25, and 0.3 M was experimented to find the optimum concentration. The best concentration buffer in terms of enzyme activity, protein content, and specific activity was measured using the same procedures (19,8,17).

Extraction time

The purpose of this experiment was to identify the optimal extraction time ranging from (5-240) min for laccase by employing best concentration buffer and pH. The activity of the extracted enzyme was tested, protein content and specific activity were measured. Optimum extraction time was stated in the next experiments.

Extraction ratio: one g of fenugreek seeds was mixed with best concentration buffer and extraction time obtained from previous experiments using the extraction ratio (w:v) of 1:40, 1:45, 1:50, 1:55, 1:60, 1:65, and 1:70 to find the optimal buffer ratio for fenugreek seed enzyme extraction(4). Followed by centrifuging at 10000 rpm for 15 minutes. The measurements of enzymatic activity, amount of protein, and specific activity were conducted (19,8,17).

Purification of enzyme with gel filtration chromatography

Sephacryl S-300 preparation : A suspension of approximately 20 grams of Sephacryl S-300 was prepared in 500 millilitres of distilled water at a temperature of 90 ^oC. The suspension was subjected to gentle agitation for a duration of 3 hours to facilitate the swelling of the gel beads. Following this, the mixture was stored overnight in a refrigerator at a temperature of 4 ^oC, in accordance with the instructions provided by the manufacturer (Pharmacia-Sweden). The gel underwent degassing through the utilisation of a vacuum pump. Subsequently, it was carefully placed within a glass column measuring 31×1.5 cm. The gel was then subjected to equilibration using the identical sodium phosphate buffer that had been employed during the washing and resuspension procedures.

Enzyme fractionation using a Sephacryl S-300 column: The enzyme was passed through a Sephacryl S-300 column, and the elution phase was performed with 0.1M sodium phosphate buffer solution, pH 7.6, at a flow rate of 20 ml/h and a fraction volume of 3 ml. At a wavelength of 280 nm, the protein concentrations in each fraction were calculated, after which the enzyme activity of these fractions was measured and the effective fractions were collected, the volume was measured, the activity and protein concentration were estimated, and then the volume was concentrated and distributed among tubes, frozen, and stored for future experiments (4).

Characterisation of laccase enzyme

Evaluation of the effects of pH and temperature on laccase activity and stability: The effects of reaction pH and temperature were investigated by measuring

the activities of laccase at a range of pH and temperatures. The stabilities of the laccase were determined as follows (18). Then enzyme activity and the residual activity (%) were plotted against pH and the temperatures (34, 5).

Specificity of laccases

Different substrates, such as toluidine, tannic acid, guaiacol, and catechol, were used to examine their effects on laccase activity. These substrate solutions were prepared by adding 10 mM of each substrate to 15 mL of 0.1 M sodium acetate buffer at pH 5. The best substrates for laccase activity were identified by analyzing the relationship between enzyme activities and substrates.

Influence of certain chemicals on laccase activity: On partially pure laccase activity, the effect of several chemical substances, such as $HgCl_2$, $ZnSO_4$, KCl, $FeSO_4$, EDTA, $CuSO_4$, $CaCO_3$, and cysteine, was investigated. Each compound solution was dissolved in 0.1 M of sodium phosphate buffer at a pH of 5 to produce a solution that was either 5 or 10 mM in concentration. The enzymatic solution was incubated with the metal ion solution at 1:1 (v/v) ratio for 15 minutes at 20 degrees Celsius. After the incubation period, the enzymatic activity was measured and compared to the control. Following that, the percentage of remaining activity was measured (1).

RESULTS AND DISCUSSION

Determination of the best plant material: This study investigated the influence of plant type on laccase enzyme extraction, and the findings showed that there were substantial differences in the enzymatic activity of the 36 plants that were tested. Turnip plant had the highest laccase specific activity, but because it is a seasonal plant, it is only available during the winter months. Fenugreek seeds had the second highest laccase specific activity, measuring 1141.47 U/mg in a 0.02 M sodium phosphate buffer at pH 7. Tomatoes, on the other hand, had the least amount of specific activity (Fig. 1). Al-Heety (2) suggests that variations in enzymatic activity among plant sources may have origins in both genetics and environmental conditions like temperature, pH, and soil composition. Aziz et al. (5) found that *Malva parviflora* had a maximum enzyme-specific activity of 420 units/mg proteins

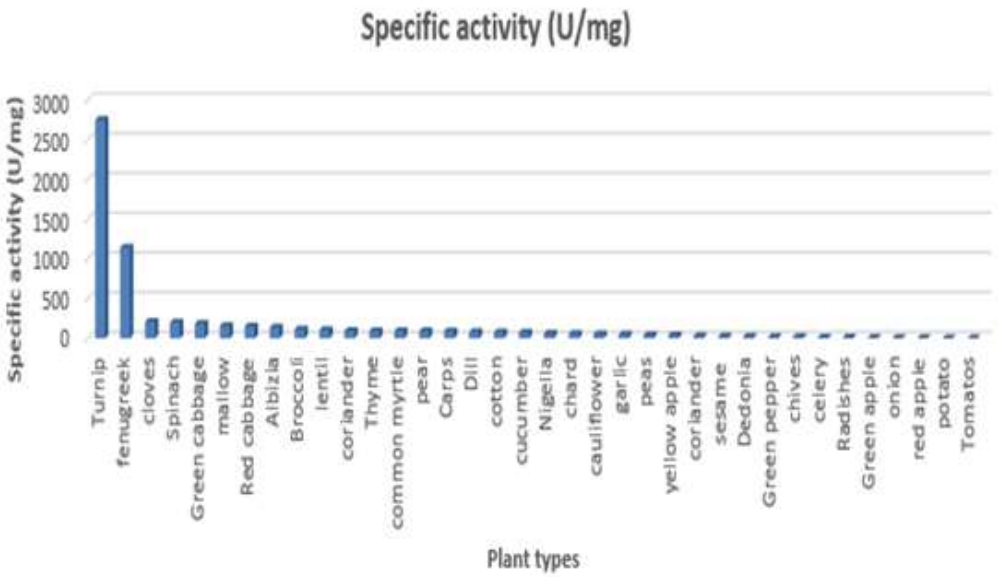

Figure 1. Laccase enzymes extracted from different types of plants using 0.02 M sodium phosphate buffer pH 7 at 25 °C for 15 min,with ratio1:40(w:v)

Type of extraction buffer

The specific activity of laccase was estimated after extraction using different buffers, and the findings are presented in Figure 2. Accordingly, the sodium phosphate buffer with a concentration of 0.02 M and a pH of 8.0 was the ideal extraction buffer since it had a specific activity of 1293.31 U per mg of protein. The specific activity of other buffers with various pH levels was quite low. According to Gawlik-Dziki and colleagues (15), most plant species exhibit the highest levels of enzyme activity at or very close to pH 7. The presence of H+ and/or OH- ions in

an enzyme creates competition with hydrogen and ionic bonds, enzymatic denaturation occurs as a result Tortora et al., (38). A buffer solution that can preserve protein stability throughout the entirety of the procedure needs to be chosen. This is necessary to keep the extraction process on schedule and effective, as well as to avoid the necessity of switching buffer solutions in the middle of the process (20). According to Al-Sa'ady and Hilal (3), the optimal buffer for the laccase extraction from *M. parviflora* was a solution of 0.2 M sodium acetate at a pH of 6. This buffer offers a specific activity level of 1.8 U/mg and was found to be the perfect choice. According to Niladevi and Prema (29), the value pH for *Streptomycetes* sp laccase extraction ranges between 7 and 7.5.

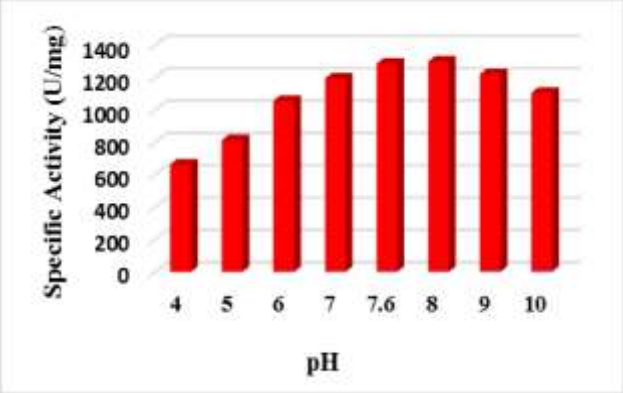

Figure 2. Effect of the type of buffers on laccase extraction from fenugreek seeds at 25°C for 15 min

The concentration of extraction buffer

Effect of the concentration of extraction buffer on the activity of laccase extracted from fenugreek seeds was studied. Result illustrated in figure (3), a solution of 0.02 M sodium phosphate buffer at a pH of 8 is the optimal choice for the process of extracting laccase from fenugreek seeds. This concentration produced the maximum specific activity, which was measured at 1295.29 U/mg protein. Indicating that the concentration of buffer can have a substantial effect on the enzymatic activity, the low concentration of 0.01 M resulted in a low specific activity of 322.96 U/mg protein. The high concentration of buffer has a negative impact on the activity of laccase. This is likely due to the presence of numerous ionic groups that make the job of enzymatic activation more difficult. According to Al-Heety (2), it is essential to pick the right buffer concentration to optimize the extraction process and preserve the enzymatic activity.

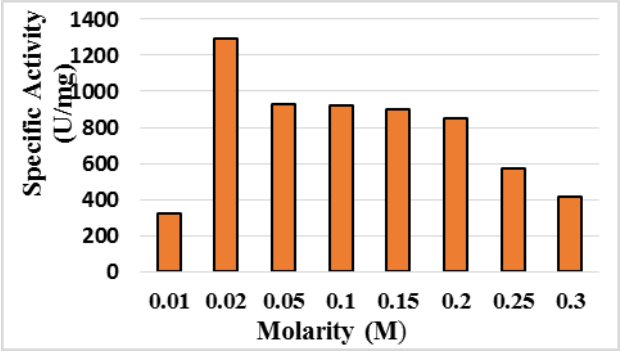

Figure 3. Influence of buffer concentration on extract laccase from fenugreek seeds at 25°C for 15 min

Extraction time

The purpose of this experiment was to identify the optimal extraction time for laccase by employing a sodium phosphate buffer with a pH of 8.0. The specific activity of the extracted enzyme was tested, and the results are depicted in Figure 4, which displays the outcomes of 10 distinct extraction times ranging from 5 to 240 minutes each. After 210 minutes of extraction, the findings showed that a specific activity of 5,340.38 U/mg protein was obtained, which was the maximum possible value. It is important to note that the optimal extraction time may differ depending on the source of the enzyme and the materials that are present in that source. These materials might interfere with the enzyme, and contaminants can impair the stability of the protein extract regarding its ability to resist decomposition. In order to maximize the effectiveness of the enzyme extraction process and achieve the maximum possible level of specific activity, it is necessary to identify the optimal extraction duration for each unique source of enzyme. This finding is consistent with previous studies that highlight the importance of optimising extraction conditions to obtain high-quality enzyme extracts (30).

Extraction ratio

Different buffer ratios were using to determine the optimum for maximum laccase activity under optimum conditions examined previously. The extract ratio of 1:40 ratio had the highest specific activity, measuring 5128.09 U/mg protein; specific activities were also detected at 3781.45, 1768.59, 1084.11, 914.05, 902.88, and 876.76 U/mg protein with

the other ratios (Fig. 5). The extraction ratio varies depending on the enzyme's composition and concentration. As the concentration of the extraction solution rises, the rate at which the enzyme-reactant complex is formed slows down, reducing the specific activity of the enzyme (21). The proportion of the herb used in the extract, known as the native extract ratio, can vary. As a result, the equivalent dry weight of the plant can vary depending on how much of the herb is used in each preparation. According to Predescu et al. (31), a low native extract ratio is associated with a high yield of extractable material from the herbal source.

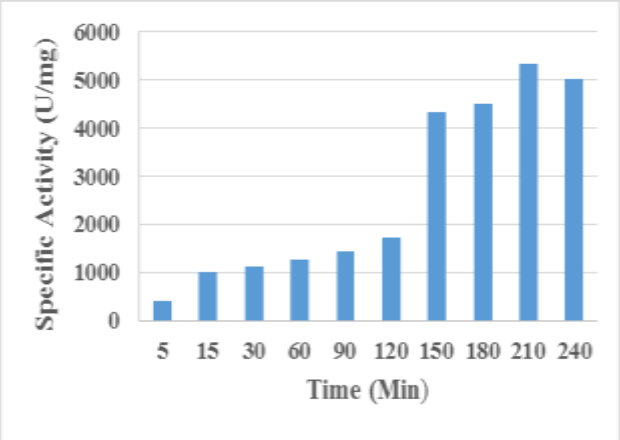

Figure 4. Laccase extraction from fenugreek seeds affected by extraction time at 25°C using 0.02 M of sodium phosphate buffer

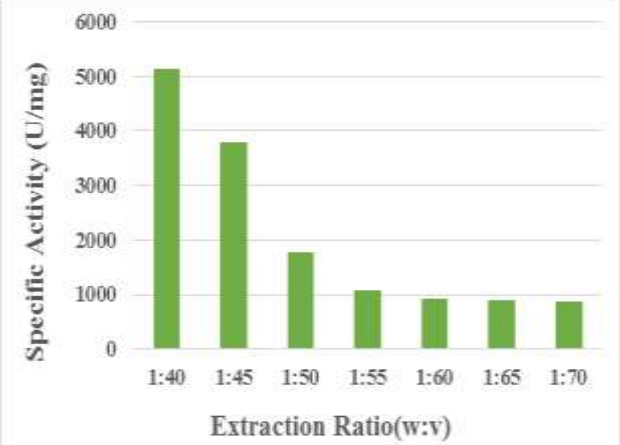

Figure 5. Laccase extraction from Fenugreek seeds affected by extraction ratio at 25°C using 0.02M of sodium-phosphate buffer pH 8,time 210min

Purification of laccase

Fenugreek seeds were homogenized with sodium phosphate buffer (0.02 M, pH 8.0) at a ratio of 1:40 (w:v) for 210 min, and the laccase enzyme was subsequently extracted by cooling centrifugation at 10,000 rpm. The crude laccase was subjected to purification through gel filtration using Sephacryl S-300 in order to remove unwanted proteins and other components.

Gel filtration chromatography

To begin, a 0.1 M sodium phosphate buffer at pH 7.6-equilibrated Sephacryl S -300 gel column (31 cm × 1.5 cm) was used to filter crude enzyme extract. The analysis highlights a pair of protein crests in proportions eluted from this filtration, with a further additional high point indicating the presence of laccase (Fig. 6), so the appropriate fraction of the filtrate can be collected. Table 1 demonstrates that there was only one protein peak and one laccase activity peak in the fractions eluted from the column. Active laccase was present in all of fractions 12–31, with a maximal specific activity of 5228.9 U/mg protein with 1.01-fold of purification and a yield of 27.6% overall. Purification using Sephadex G-100, the crude laccase recovered from *Trametes polyzona* WRF03 (TpL) revealed a single peak at 280 nm in UV spectra, confirming the presence of a single, distinctive feature. With a purity level 13 times higher than 2%, post-gel filtration laccase had a protein-specific activity of 21525 U/mg when measured with ABTS (13). Laccase from *M. parviflora* was purified using Sephadex G-150 to obtain a protein with a specific activity of 47830 U/mg and a purity of 94.4% (5).

Table 1. Purification steps of laccase from fenugreek Seeds

Sample	Volume (ml)	Enzyme Activity (U/ml)	Protein Conc. (mg/ml)	Specific Activity (U/mg)	Total Activity (U)	Purification Fold	Yield (%)
Crude Extract	40	33973.6	66	5147.5	13589440	1	100
Gel Filtration Chromatography using (Sephadex G-300)	57	64838.5	12.4	5228.9	3695795.03	1.01	27.6

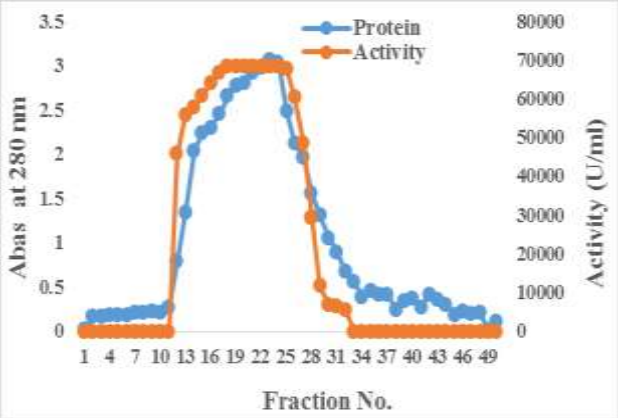

Figure 6. The purification of laccase from fenugreek seeds was conducted using gel filtration chromatography employing a Sephacryl S-300 column with dimensions of 31 cm × 1.5 cm. The column was equilibrated and eluted using a sodium phosphate buffer (0.1 M, pH 7.6) at a flow rate of 20 mL/h, with each fraction collected in 3 mL

Characterization of partial purified laccase

The effects of different parameters on the purified laccase are determined

pH's influence on laccase activity

The effect of the pH of three different buffers on the activity of partially purified laccase were studied. A o-tolidine was used as a substrate with varying pHs, including 0.2 M sodium acetate buffer (pH 4, 5, and 6), 0.2 M phosphate buffer (pH 7 and 8), and 0.2 M of tris-base buffer (pH 9 and 10). The optimum pH for laccase activity was discovered by estimating the activity and then plotting the relationship between enzymatic activities and pH values. According to the findings, an enzyme's activity is at its highest when the pH is 5, this results in enzyme activity reached 68322.98 U/mL. The decline in activity was also seen to be noticeably more pronounced in the alkaline medium as compared to the acidic medium. The pH may affect the organization of both the enzyme and the substrate, The ionization of groups within the active site of an enzyme, as well as the ionization of groups within the substrate (11). This is one of the many ways that pH can affect enzymatic activity. The observed phenomenon of increased substrate oxidation at higher pH levels may be attributed to the significant difference in redox potential between the phenolic substrate and the T1 copper. However, when hydroxide anions (OH^-) bind to the T2/T3 coppers, laccase activity is inhibited because this stops electrons from readily passing across the T1 and T2/T3 centers. Consequently, laccase activity is reduced (40).

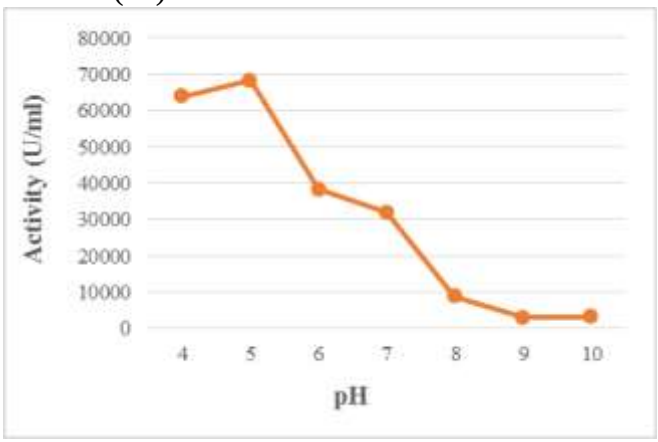

Figure 7. The influence of varying pH values (ranging from 4 to 10) on the activity of purified laccase derived from fenugreek seeds when o-tolidine is used as the substrate

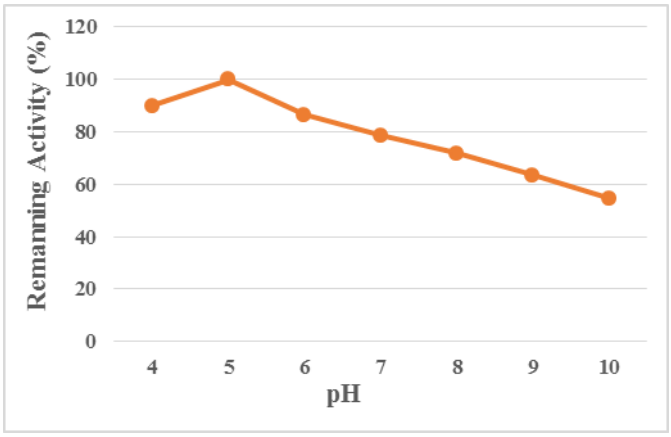

Figure 8. Illustrates the impact of varying pH values (ranging from 4 to 10) on the stability of laccase extracted from fenugreek seeds, with o-tolidine used as the substrate

Effect of pH on the stability of partially purified laccase: The pH of enzyme stability was studied because it is an important criterion to determine the optimum conditions for purification and storage of the enzyme. At varying pH levels (ranging from 4 to 10), a mixture of partially purified enzymes and buffers was combined in equal amounts (1:1 ratio). Thereafter 15 minutes in a water bath maintained at 37^{o}C. The tubes were placed straight into an ice bath for further examination. After measuring the enzymatic activity, the remaining enzymatic activity was calculated for each pH value and plotted to

determine the optimum pH for laccase stability. This was done to determine the best pH. According to the findings presented in Figure (8), the most favourable conditions for the stability of laccase are reached at pH levels ranging from 4 to 7. While enzyme activity stayed at 100% when the pH was 5, it remained at around 89.81% when the pH was 4. In addition, it preserved around 86.56 percent and 78.72 percent of its initial activity when the pH was 6 and 7, respectively. A decline in activity was observed on both sides of the optimal pH values, with residual activities above 71.83% at pH 8, and 63.44% and 54.50% at pH 9 and 10, respectively. It was shown that the enzymatic activity decreased as the pH level became more alkaline. It is possible that the influence of pH stability on the structure of the enzyme is responsible for the general drop-in activity that is observed at pH levels that are outside of the optimum range. The secondary and tertiary structures of the enzyme molecule are altered because of this, which may result in the enzyme becoming denatured or in alterations to the ionic state of the active site. According to Segel (35), enzymes lose their activity when exposed to buffer solutions with pH values that fall outside of the optimum range.

Effect of temperature on laccase activity

Temperature is an important factor which affects enzyme activity. The favourable temperature for laccase activity may differ with different laccase sources. The activity of partially purified laccase was determined at temperatures of 20, 37, 45, 50, 55, and 60 ^{o}C. The optimal temperature for laccase activity was then determined by analyzing the correlation between enzyme activities and temperature. The optimal temperature for laccase activity was determined by subjecting the purified enzyme and substrate combination to various temperatures ranging from 20 °C to 60 °C, with an incubation period of 15 minutes. The enzyme's activity was found to be significantly higher at 20 °C (as shown in Figure 9). At 20 °C, laccase activity was at its highest (65607.14 U/mL), but it dropped to its lowest (17698.94 U/mL) at 60 °C. Due to the fact that within a given temperature range, the rate of enzymatic interaction accelerates as a result of enhanced energy kinetics and collisions between enzyme molecules and substrate, laccase activity was also seen to drop below 40 °C. Muro et al. (28) noticed that enzymes lose their three-dimensional structure and enzymatic function when exposed to temperatures above certain thresholds, a process known as denaturation.These findings corroborated Kim et al. (22) works, who employed o-tolidine as a substrate to demonstrate that the optimal temperature for the activity of enzyme isolated from *Phlebia termellen* is 20 °C. *Cladophora* sp. laccases remained active at 35 °C (2).

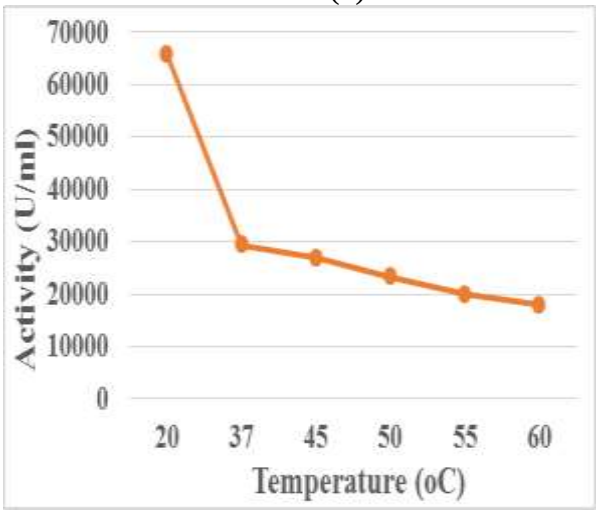

Figure 9. Effect of different ranges of temperatures (20 °C–60 °C) on fenugreek seed laccase activity using o-tolidine as a substrate at pH 5

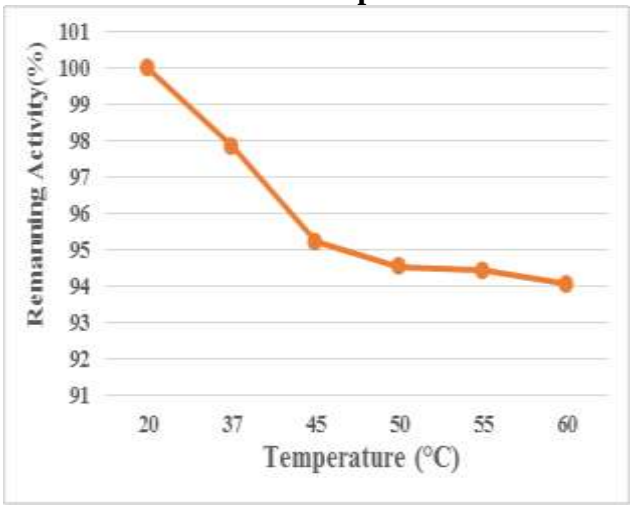

Figure10. Impact of varying temperature ranges (20 °C–60 °C) on the stability of laccase derived from fenugreek seeds, with o-tolidine utilized as the substrate and pH 5

Effect of temperature on laccase stability

The partially purified laccase enzyme was subjected to a series of temperature treatments (20, 37, 45, 50, 55, and 60°C) for a duration of

15 minutes. Subsequently, the enzyme was promptly transferred to an ice bath. Following that, the enzymatic activity was evaluated at the temperature that maximizes enzyme activity. The remaining activity percentage was estimated. Figure (10) shows that incubation of laccase at temperatures higher than the optimal for stability (20 °C) resulted in a slight decrease in the enzyme's remaining activity, with maximum remaining activity being achieved at 20 °C. The enzyme was still 94% as active at 60 °C as it was at room temperature. At lower temperatures, most enzymes are more stable, which is why they need to kept in the fridge or freezer. The fact that laccase activity decreases at 60 °C demonstrates its sensitivity to high temperatures, which may alter the protein's three-dimensional structure by damaging R-groups of amino acids. Denaturing proteins in this way causes them to stop working as enzymes (9).

Effect of substrates on Laccase specificity

In order to investigate the specificity of purified laccase extracted from fenugreek seeds toward different substrates. Different substrates were added to the purified enzyme and the reaction mixture was incubated, then the activity determined. As can be seen in Fig. (11), when o-tolidine was used as a substrate, the enzymatic activity was significantly higher than when other substrates were used. Activity of laccase was as high as 7686.34 U/mL but dropped when other substrates were used. Furthermore, laccase activity reached 34161.49, 11956.52, and 3416.15 U/mL when using tannic acid, guaiacol, and catechol, respectively; the findings of this study indicate that the laccase obtained from fenugreek seeds exhibits enhanced oxidation capabilities when o-tolidine is employed as the preferred substrate, surpassing its performance with other phenolic compounds. Orthotolidine was shown to be the phenolic molecule most amenable to oxidation by the laccase enzyme isolated from *Cladophora* sp. (2).

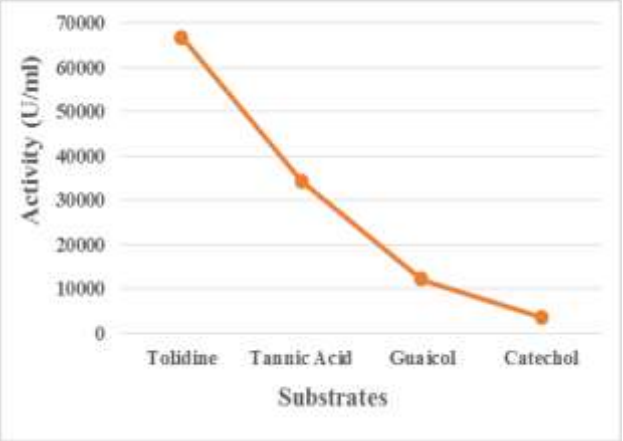

Figure 11. Illustrates the impact of various substrates on fenugreek seeds purified laccase activity at a temperature of 20 °C and pH 5

Influence of certain chemicals on laccase activity: Heavy metals normally found in the environment, might affect the activity or stability of enzymes. The effect of some heavy metals and chemicals agent on the activity of the purified laccase was examined. According to the findings of Fig. 12, the concentration of KCl that produced the greatest increase in laccase activity was between 5 and 10 mM. According Cliff et al. (10), metals can be assimilated as a component of enzyme cofactors, which boost the activity of the enzymatic reaction, and they can also be adsorbed to the surfaces of cells and precipitated as a result of the metabolic process. In addition, the enzyme was activated by $CuSO_4$ and $FeSO_4$ at concentrations of 5 and 10 mM, respectively. Telke et al., (36) elucidated that $CuSO_4$ has a role in enhancing the rate of o-tolidine oxidation. Additionally, it was stated that the stimulation of laccase activity by CuSO4 could potentially be attributed to the binding of copper ions to the type-2 copper binding sites. Due to its natural occurrence as a biogenic metal in the environment, the concentration of copper can significantly influence the activity of enzymes, affecting their ability to perform their functions and maintain their stability. The effect that metal ions have on the activity of laccase might change depending on where the enzyme came from, often known as its source. At doses of 5 and 10 mM, it was noted that the presence of $HgCl_2$ inhibited enzymatic activity to the point where it reached levels that were lower than the activity that was being

controlled. These outcomes are consistent with the majority of studies that investigated the influence of these compounds on laccase activity. According to the study conducted by Farnet et al (14) the inhibition of laccase activity was attributed to the effect of metal ions, particularly chloride ions. The enzyme was inhibited when $HgCl_2$ was present, which demonstrated the presence of sulfhydryl (SH) groups inside the active region of the enzyme, which were oxidized when $HgCl_2$ was present. According to Maher and Cordes (26), the presence of $HgCl_2$ in the solution that is being used for the processing of the substrate might lead to the development of a complex with the enzyme. This complex would prevent the enzyme from attaching to the substrate, which would slow down the process of product creation. During incubation with a $ZnSO_4$ solution, there was an increase in the enzyme's enzymatic activity at both 5 and 10 mM, with the respective increases being 150.34% and 106.50%. The increase in laccase activity was seen with $CaCO_3$ at concentrations of 5 and 10 millimolar (109.80% and 115.86%, respectively). These findings are consistent with those that were published by Bao et al (6) and Ma et al. (25), who found that the enzymatic activity was significantly improved by 1 and 10 mM $CaCO_3$ (12% and 35%, respectively). Ca^{+2} has the capacity to promote laccase activity at concentrations as low as 1 mM, even when the concentration is not very high. Ca^{+2} ions are necessary for the catalytic activity of laccase due to this reason; however, they are not necessary for the structural stability of laccase. EDTA and cysteine, when present at doses of 5 and 10 mM, appear to have the opposite effect on the activity of laccase, which have been previously reported as an efficient laccase inhibitor (1, 2). This stands in opposition to the other attributes of laccase. The resistance to EDTA as a chelating agent can be attributed to its limited ability to access the structural copper atoms located in the active site, which are necessary for the catalytic activity of the enzyme (33). As for cysteine, which may suggest no strong reduction of the disulphide bridges between domains 1 and 2, which stabilizes the structure of laccase(7).

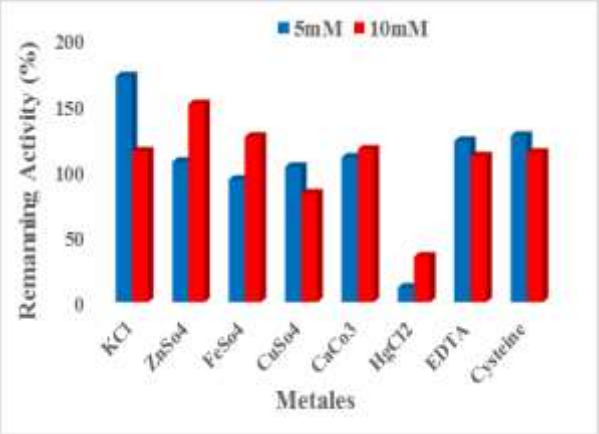

Figure 12. The impact of metal ions and other inactivating agents on the purified laccase enzyme derived from fenugreek seeds

REFERENCES

1. Al-Assadi, S. I. H. 2017. Biodegradation of Industrial Dyes in a Bioreactor by Free and Immobilized Laccase from Local Isolate of *Pseudomonas aeruginosa SR3*. Ph. D. Dissertation, Biotechnology, College of Sciences, Baghdad University. Iraq

2. Al-Heety, B. F. 2018. Biochemical Study of Laccase Enzyme Extracted from Some Local Algae. M. Sc. Thesis, Biotechnology, College Education for Pure Science, Anbar University. Iraq

3. AL-Sa'ady, A., J. and M., H. Hilal. 2020. Determination of the optimum conditions for extracting polyphenol oxidase and laccase enzymes from *Malva Parviflora* and their role in the decolorization of some dyes. Iraqi Journal of Science. 61(2): 306-313.

4. Aziz, G. M., S. I., Hussein, S. D., Abbass, A. L., Ibrahim, and D. K. Abbas. 2021. Degradation of reactive dyes using immobilized peroxodase purified from *Nigella Sative*. Iraqi Journal of Agricultural Sciences, 52(6):1365–1374.
doi: 10.36103/ijas.v52i6.1476

5. Aziz, G.M., S. I., Hussein, M. J., M-Ridha, S. J., Mohammed, K. M., Abedd, M.H., Muhamad, and H. A. Hasan. 2023. Activity of laccase enzyme extracted from *Malva parviflora* and its potential for degradation of reactive dyes in aqueous solution. Biocatalysis and Agricultural Biotechnology Journal. 50: 102671

6. Bao, W., R., Peng, Z., Zhang, Y., Tian, W., Zhao, Y., Xue, J., Gao, and Q., Yao. 2012. Expression, characterization and 2,4,6-

trichlorophenol degradation of laccase from *Monilinia fructigena*. Molecular Biology Reports Journal. 39(4):3871–3877

7. Barker, S. A. and J. A. Shirley. 1980. Microbial enzyme and bioconversion. In: Economic Microbiology. (ed. Rose,A.H.). 5: 173–186. Academic Press. London

8. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Analytical Biochemistry Journal. 72: 248-254

9. Chesworth, J. M., T., Stuchbury, and J. R., Scaif. 1998. An Introduction to agricultural biochemistry. Chapman and Hall, London. 5(2): 215-223

10. Cliff, J. B., K. H., Jarman, N. B.,Valentine, S. L., Golledge, D. J., Gaspar,. D. S., Wunschel, and K. L., Wahl. 2005. Differentiation of spores of *Bacillus subtilis* grown in different media by elemental characterization using time-of-flight secondary ion mass spectrometry. Applied and Environmental Microbiology Journal. 71(11): 6524–6530

11. Clive, D. 2002. Aguide to Protein Isolation. Kluwer Academic Publisher.NewYork

12. Coll, P. M., J. M., Abalos, J. R., Villanueva, R., Santamaria, and P., Perez. 1993. Purification and characterization of a phenoloxidase (Laccase) from the lignin degrading basidiomycete PM1 (CECT 2971). Applied and Environmental Microbiology Journal. 59(8): 2607-2613.

13. Ezike, T. C., A. L., Ezugwu, J. O., Udeh, S. O. O., Eze, and F. C., Chilaka. 2020. Purification and characterisation of new Laccase from *Trametes polyzona* WRF03. Biotechnology Reports Journal. 28: e00566.

14. Farnet, A.M., G., Gil, and E., Ferre. 2008. Effects of pollutants on laccase activities of *Marasmius quercophilus*, a white-rot fungus isolated from a Mediterranean schlerophyllous litter. Chemosphere Journal. 70(5):895-900

15. Gawlik-Dziki, U., U., Złotek, and M., Świeca. 2007. Characterization of polyphenol oxidase from butter lettuce (*Luctuca sativa* var. capitata L.). Food Chemistry 107: 129-135

16. Hussein, S. I. 2019. .Effeciency of immobilized polyphenol oxidase on some textile dyes degradation using batch operation system by packed bed bioreactor, Iraqi Journal of Agricultural Sciences, 50(3). doi: 10.36103/ijas.v50i3.711

17. Hussein, S.I , A. F. Kaluf, M. A. Sameh, M.A. and M. T. Salah. 2021. Determination of the optimum conditions for urease inhibition extracted from some local plants." Iraqi Journal of Agricultural Sciences, 52(4): 802–809. doi: 10.36103/ijas.v52i4.1389

18. Issa, S. N., A. J., Hashim, and W. M., Abood. 2022. Biodegradation of some environmental pollutants by Laccase produced from *Trichodrme Harziauum* using solid state fermentation. Iraqi Journal of Agricultural Sciences, 53(3):522–533. doi: 10.36103/ijas.v53i3.1560

19. Kalral, K., R., Chauhan, M., Shavez, and S., Sachdeva. 2013. Isolation of laccase producing *Trichoderma Spp*. and effect of pH and temperature on its activity. International Journal of Chemistry and Technology Research. 5(5): 2229-2235

20. Karmali, K., A., Karmali, A., Teixeira, and M.J., Marcelo Curto. 2002. The use of Fourier transform infrared spectroscopy to assay for urease from *Pseudomonas aeruginosa* and *Canavalia ensiformis*. Analytical Biochemistry Journal. 331(1): 115-121

21. Khalil-ur-Rehman, M. Y., M. A., Sheikh, and M., Arshad. 1999. Extraction and Evaluation of Peroxidase from Vegetable Sources. International Journal of Agriculture and Biology. 3: 170-173

22. Kim, Y., S., Yeo, M. K., Kim, and H. T., Choi. 2008. Removal of estrogenic activity from endocrine-disrupting chemicals by purified laccase of *Phlebia tremellosa*, FEMS Microbiology Letters Journal. 284(2): 172-175

23. Levine, W. G, 1965. Laccase, A review, In: The Biochemistry of Copper, Academic Press Inc., New York, pp. 371-385

24. Li, A., Y. Zhu, L. Xu, W. Zhu and X. Tian. 2008. Comparative study on the determination of assay for laccase of *Trametes* sp. African Journal of Biochemistry Research. 2 (8): 181-183

25. Ma, J., Z. S., Xu, F., Wang, and AS., Xiong. 2015. Purification and characterization of two laccases from carrot (*Daucus carota* L.) and their response to abiotic and metal ions stresses. Protein Journal. 34(6):444-52.

26. Maher, H. R. and E. A.,Cordes. 1971. Biochemistry of Bacterial Growth. 3rd ed. Blackwell Scientific Publication, Oxford

27. Mester, T., and M. Tien, 2000. Oxidation mechanism of ligninolytic enzymes involved in the degradation of environmental pollutants. International Journal of Biodeterioration & Biodegradation. 46:51-59

28. Muro, T., Y., Tominaga, and S., Okada. 1984. Purification and some properties of a protease from *Streptomyces cellulosae* . Agricultural and Biological Chemistry Journal.48(5):1223-1230

29. Niladevi, K. and P., Prema. 2008. Effect of inducers and process parameters on laccase production by *Streptomyces psammoticus* and its application in dye decolourization, Bioresource Technology Journal. 99: 4583-4589

30. Nilsang, S., S., Lertsiri, M., Suphantharika, and A., Assavanig. 2005. Optimization of enzymatic hydrolysis of fish soluble concentrate by commercial proteases. Journal of Food Engineering. 70: 571-578

31. Predescu, N.C., C., Papuc, V., Nicorescu, I., Gajaila, G.V., Goran, C.D., Petcu, and G., Stefan. 2016. The influence of solid-to-solvent ratio and extraction method on total phenolic content, flavonoid content and antioxidant properties of some ethanolic plant extracts. Revista De Chimie Journal. 67: 1922–1927

32. Rittstieg, K., A., Suurnakki, T., Suortti, T., Kruus, GM., Guebitz, and J., Buchert. 2003. Polymerization of guaiacol and a phenolic beta-O-4-substructure by *Trametes hirsuta* laccase in the presence of ABTS. Biotechnology Progress Journal. 19:1505–1509

33. Safary, A., R., Moniri, M., Hamzeh-Mivehroud, and S., Dastmalchi. 2016. A strategy for soluble overexpression and biochemical characterization of halo-thermotolerant *Bacillus* laccase in modified *E. coli*. Journal of Biotechnology. 227:56–63

34. Salih, H. N. and S. I. Hussein. 2022. Assessment of purified collagenase inhibition activity from *Staphylococcus aureus* HN77 by some local plants. Iraqi Journal of Agricultural Sciences, 53(5):1035–1047. doi: 10.36103/ijas. v53i5.1617.

35. Segel, I. H. 1976. Biochemical Calculation. John Wiley and Sons. pp.441

36. Telke, A. A., C. D., Kalyani, U.U, Jadhav, K. G., Parshetti, and S. P., Govindwar. 2009. Purification and characterization of an extracellular laccase from *Pseudomonas* sp. LBC1 and its application for the removal of bisphenol A. Journal of Molecular Catalysis B : Enzymatic. 61:252-260

37. Thurston, C.F. 1994. The structure and function of fungal laccases. Journal of Microbiology. 140 (1): 19–26

38. Tortora G. J., B. R., Funke and C. L., Case. "Microbiology". (8th ed.). Pearson Education, Inc. San Francisco, New York, 2004

39. Win, D.T., M.M., Than, and S. Tun. 2003. Lead removal from industrial waters by water hyacinth. Assumption University Journal of Technology. 6: 187-192

40. Xu, F.,1999. Recent progress in laccase study: properties, enzymology, production, and applications. In: The encyclopedia of bioprocessing technology: fermentation, biocatalysis, and bioseparation. eds Flickinger MC, Drew SW. John Wiley and Sons, Inc.: New York: 1545–1554

41. Youn, H.-D., K.-J., Kim, J.-S., Maeng, Y.-H., Han, I.-B., Jeong, G., Jeong, S.-O., Kang, and Y.C., Hah. 1995. Single electron transfer by an extracellular laccase from the while rot fungus *Pleurotus ostereatus*. Journal of Microbiology.141: 393-398.