ARTICLE

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Production and Purification of Laccase enzyme by *Trichoderma viride*

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إنتاج وتنقية إنزيم Laccase بواسطة *Trichoderma viride*

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Abstract

Background

The ability of *Trichoderma viride* to synthesize extracellular enzymes (Lacase enzyme) using a substrate that contains media was examined. This isolate generated laccase at its greatest level (0.166U/ml) in liquid medium. This study was demonstrated the production, purification, and characterisation of the laccase enzyme from *T.viride*. The results showed that 96 hours was the optimum period of time for Laccase to incubate from this fungus. Laccase displayed the maximum level of activity at pH 4.5 and temperature 30 °C. The results revealed that the best ratio for laccase precipitation was 90% by using ammonium sulphate. In addition, for the purification of laccase enzyme, one peak of Laccase was appeared in gel filtration purified from *T.viride*, while 2 peaks of Laccase were seen in ion exchange was roughly 66KD under denaturation conditions.

Keywords: Laccase enzyme, purification, Ion Exchange chromatography, SDS.

الخلاصة

تم فحص قدرة ***Trichoderma viride*** على تصنيع إنزيمات خارج الخلية (إنزيم **Laccase**) باستخدام وسط يحتوي على المادة الاساس للأنزيم. أنتجت هذه العزلة اللاكيز عند أعلى مستوى لها (0.166 وحدة / مل) في الوسط سائل. اوضحت هذه الدراسة إنتاج وتنقية وتوصيف إنزيم اللاكيز من ***T.viride***. أظهرت النتائج أن 96 ساعة هي الفترة الزمنية المثلى لحضانة **Laccase** من هذا الفطر. أظهر **Laccase** الحد الأقصى لمستوى النشاط عند درجة الحموضة 4.5 ودرجة حرارة 30 درجة مئوية. اثبتت النتائج أن أفضل نسبة لترسيب **laccase** كانت 90٪ باستخدام كبريتات الأمونيوم. بالإضافة الى ذلك, لأجل تنقية انزيم اللاكيز ظهرت قمة واحدة من **Laccase** في ترشيح الهلام المنقى من ***T.viride*** ، بينما شوهدت قمتان من **Laccase** في كروماتوغرافيا التبادل الأيوني. وفقًا للنتائج، كان الوزن الجزيئي لـ ***T. viride* Laccase** باستخدام **SDS–PAGE** حوالي 66 كيلو دالتون تحت الظروف الماسخة.

الكلمات المفتاحية :إنزيم لاكيز ، تنقية ، كروماتوغرافيا التبادل الأيوني ، **SDS**.

Introduction

Enzymes are used as natural catalysts in biocatalysis to change the chemical composition of organic molecules. In biocatalysis, enzymes produced by living cells in their entirety or in isolation are employed. The biocatalyst's specificity, which may lead to high yields of a particular product, is the primary benefit of biocatalysts [1]. The development of novel, useful enzymes is the foundation for new biocatalytic processes. These enzymes are typically discovered by looking for bacteria that can perform the necessary target reaction. Exploration of severe settings can result in special collections of microbial cultures that can be exploited to find the best enzymes to carry out a specified biocatalytic process. Then, in industrially important bioprocesses, these enzymes could be utilized as biocatalysts [2]. The polyphenol oxidase family includes Laccases, which are multicopper-containing enzymes that convert molecular oxygen to water [3]. This enzyme is categorized as a blue copper protein because it uses molecular oxygen as the electron acceptor to catalyze the oxidation of a wide range of organic and inorganic substances [4]. Diphenols, polyphenols, diamines, and aromatic amines are only a few examples of aromatic molecules with hydroxyl and amine groups that Laccase choose as substrates [5]. Syringaladazine [4-hydroxy-3,5-dimetehoxy benzaldehyde azine] is thought to be the only substrate that the Laccase enzyme can oxidize [6]. Four different sorts of living things, including bacteria, insects, higher plants, and fungi, create Laccases. Few Laccase enzymes of bacterial origin have been isolated and described. The prokaryotic Laccase, which was generated from the rhizospheric bacterium *Azospirillium lipoferum*, is the focus of the first investigation on this topic [7]. Bacterial Laccases have attracted more interest recently since they have overcome the limitations of instability when compared to fungal Laccases. When the temperature increases and pH levels increased, they are substantially more stable and extremely active. Bacterial Laccases have developed into an important industrial enzyme that is used in a variety of processes, including the detoxification of industrial effluents, primarily from the paper and pulp, textile, and petrochemical industries, as well as in the production of anticancer drugs and as a cleaning factor for certain water purification systems. Lack of sufficient enzyme stocks, high production costs for this biocatalyst, and chemical enzyme modification to produce more robust and active enzymes were the main barriers to the commercialization of bacterial Laccase [8]. According to those who were mentioned above, the goal of this study was to examine *Trichoderma viride* Laccase enzyme production and determine the best conditions and purification of this enzyme.

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Materials and Methods

This fungus was obtained from unit of mycology/ college of science / university.

Biochemical analysis

Preparation of *Trichoderma viride* Inoculum

A pure culture of recently isolated *Trichoderma viride* was cultured on PDA(potato dextrose agar) that prepared according Himiedia companay. To inoculate flasks (25o ml in size) with fresh mycelium from each *Trichoderma viride* growing in slant culture(that kept at 4°C for further use), 10 mm agar medium plugs were inserted into the medium after it had been autoclave sterilized for 15 minutes. Flasks were rotated at 28 °C for five days while being shaken at 150 rpm.

Laccase production medium

This medium was used that composed of (g/l): urea, 0.14; sucrose, 2.0; yeast extract, 0.34; MggSO4 7H2O, 0.07; CCaCl2 2H2O, 0.004; NNiSO4 7H2O, 0.003; KKH2PO4, 0.1; and Na2HPO4, 0.3. Sequential optimization studies for the production of laccase have been carried out using a variety of organic nitrogen sources inorganic nitrogen sources , and carbon sources .This medium was sterilized by using autoclave. [9].

Screening for laccase-producing fungi

Producing laccase enzyme in liquid media

These reactions were established by inoculating each of the examined fungal isolates with a 5mm agar disc plug after adding 50 mL of enzyme production media to 250 mL Erlenmeyer flasks. following a seven-day incubation period at 28 °C with a 150-rpm rotary shaker. The medium containing substrate and fungus mycelium and then was extracted using filter paper, and the fungus was then centrifuged for 15 minutes at 6000 rpm. This method was described to assess the enzyme activity [10].

Determination of laccase activity

The oxidation of Guaiacol was monitored for three minutes at 525 nm to confirm the enzymatic activity. 1 ml crude enzyme filtrate, 1.5 ml of 1 mM guaiacol, and 2 ml of 0.1 mM sodium phosphate buffer (pH 7.5) were used to compute laccase activity using the formula:

$$\text{Enzyme activity U/ml} = \frac{OD}{0.01*time*volume(crude)}$$

The effect of some culture conditionon the synthesis of Laccase enzyme

Incubation period: Four flasks was used ,each flask with a volume of 250 mL, and filled with the preparation media. To inoculate the production media, a 10mm disc was taken from the fungi's

borders *Trichoderma viride*. It was then incubated for 5 days at 27 °C (120 hours). Enzyme activity was assessed in samples every 24 hours.

pH. The production media were put in 24 flasks (twelve flasks for each isolate, volume 250 ml). Medium with various pH values (3, 3.5, 4, 4.5, 5, and 5.5) was injected and incubated at 27°C for 96 hours by extracting a disc (10 mm) from the borders of colonies (2 replicates for each isolate). Enzyme activity was calculated based on samples that were obtained.

Temperature:24 flasks were used to prepare the production media (twelve flasks for isolate, volume 250 ml).Media were containing (pH 4.5 for *Trichoderma viride*) for different temperatures (20,25,30,35,40 and 45) disc (10mm) taken from the edges of the isolate and used the same method that was used in PH.

Purification of laccase

$(NH4)_2SO_4$ Precipitation

Various percentage of $(NH4)_2SO_4$ (20, 30, 40, 50, 60, 70, 80, and 90%) were applied to precipitate the crude laccase solution by using cooled conditions. After that, separated by centrifugation at 10,000 rpm for 10 minutes, dissolved in a small amount of sodium phosphate buffer (00.1 M, pH 7.5), and their laccase activity was measured. Dialyzing the protein solution for 12 hours, and changing the buffer every 6 hours, was done using sodium phosphate buffer (0. 1 M, pH 7.5).

Ion-exchange chromatography (IEC)

A DEAEE cellulose column (2–300 cm) that had previously been equilibrated with sodium phosphate buffer received a volume (10 ml) of the dialysate (0. 1 M, pH 7.5). Following a 50 ml wash with the same buffer, the bound proteins were gradually eluted from the column using a NaCl solution (00.1 - 00.5 M) produced with the used buffer. The laccase activity of each chromatographic fraction was measured after it was collected in 5 ml fractions at a flow rate of 2.5 ml/min. For the subsequent purification phase, laccase-active fractions were mixed and concentrated.

Gel filtration chromatography

After ion exchange chromatography, a volume (10 ml) of the concentrated enzyme was added gradually to a Sephadex G-200 membrane (2-76 cm) that had been pre-equilibrated with sodium phosphate buffer (00.1 M, pH 7.5). Laccase activity was measured after fractions of 5 ml were collected at a flow rate of 0.25 ml/min. At 280 nm, the amount of protein in each fraction was calculated.

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SDS–Polyacrylamide Gel Electrophoresis (PAGE)

The enzyme's homogeneity on a 10% and 15% gel was examined using SDS-PAGE, and the enzyme's molecular weight was calculated using a common protein marker [11]. Coomassie brilliant blue R-250 was used to stain the gel in order to make the protein bands visible.

Results

Figure 1 showed the Screening of laccase enzyme produced via fungi after growing on liquid media containing 0.02% guaiacol as an indicator compound after 4 days of incubation at 30°C. where the first flask shows the *T.viride* after growing on the media, while another flask shows the media only as control.

Figure 1. Determining of laccase-producing fungi using liquid media containing 0.02% guaiacol as indicator compound after 4 days of incubation at 30°C. A: *T.viride* with the substrate, B: *T.viride* as control negative.

Determination of the optimum conditions for laccase production

Determination of the optimum hydrogen concentration (pH)

T.viride was incubated on a submerged liquid medium with various pH values to explore the initial medium pH which affects laccase synthesis (3, 3.5, 4,4.5, and 5.5). Results in figure (2) demonstrated that laccase activity was at its highest level (1.036U/ml) when the medium's pH was equal to 4.5.

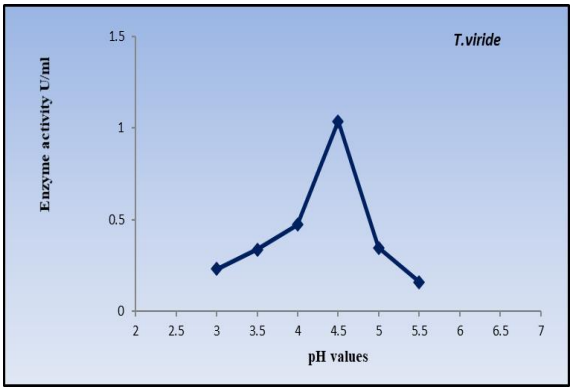

Figure 2. The Effect of pH in laccase production from *T.viride* using liquid medium after incubation at 30˚C for 96hours and 150rpm.

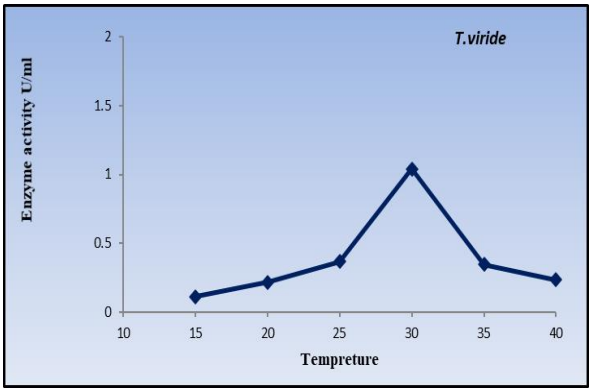

Figure 3. The Effect of temperature in laccase production from *T.viride* using liquid medium after incubation at 30 ˚C for 96hours and 150rpm.

Determination of the optimum temperature

Different incubation temperatures were revelaed (15, 20, 25, 30, 35, and 40°C) to determine the impact of temperature for laccase production from this fungus. Temperature is thought to be a critical and effective component in the growth of microorganisms and their metabolism. The findings of this experiment (Figure 3) proved how different temperatures impacted the formation of laccase. At 30 C, *T. viride's* laccase enzyme displayed its highest level of activity (1.0406 U/ml).

Determination of the optimum incubation period

T.viride was cultivated on a laccase production medium for various incubation times in an effort to determine the ideal incubation time for laccase synthesis (24,48,72,96,120, and 144 hr). According to the findings in figure (4), the laccase activity at (1.0177U/ml) during the ideal incubation time of 96 hours. After 120 hours, however, there was a dramatic fall in enzyme activity that reached (0.46875U/ml).

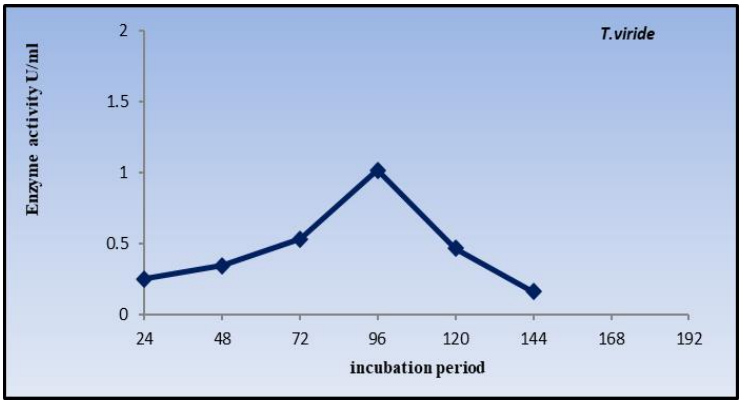

Figure 4. The Effect of the incubation period in laccase production from *T.viride* using liquid medium after incubation at 30˚C , 96 hours and 150rpm.

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Purification of Laccase enzyme

The crude enzyme from *T.viride* was precipitated with ammonium sulphate, then the enzyme was subjected to an ion exchange procedure using DEAE-cellulose, and finally, the enzyme was purified using Sephadex G-200 gel filtration. When the enzyme activity reached 20.7U/ml, the 90% saturation ratio for *T.viride* was chosen as the perfect percentage for precipitating the crude extract of the enzyme. In addition, in accordance with the purification steps shown in table (1), *T.viride* was shown specific activity of 0.2700U/mg, with a purification fold of 1.279 and yield of 65.1.

Table 1: Purification steps of laccase enzyme purified from *T.viride*.

Purification steps	Volume (ml)	Total activity (U)	Enzyme activity	Protein concentration (mg/ml)	Specific activity U/mg	Purification fold	Yield %
Crude Enzyme	10	254	25.4	120	0.211	1	100
$(NH4)_2SO_4$ precipitation (90%)	8	165.6	20.7	76.666	0.2700	1.279	65.1
Ion exchange (DEAEE-cellulose)	4.5	87.7	19.5	10.7	1.8	6.66	52.9
Gelfiltration (Sephadex G-200)	3	25.5	8.5	0.666	12.762	7.09	29.076

After the ammonium sulfate crystallized, the wash fraction's wavelength at 280 nm was recorded by passing laccase solution over a DEAE-cellulose matrix that had recently reached equilibrium with sodium phosphate buffer (0.1 M, pH 7.5). The elution stage for binding protein was shown to have two peaks of protein and enzyme activity apparent in the fractions from *T.viride* (that carry a negative charge). The solution enzyme was eluted in this stage using a gradient of 0.1-0.5M NaCl solutions. These findings demonstrated that the anionic ion exchange protein that the laccase enzyme binds to has a negative net charge (DEAE-cellulose). This protein peak has a specific activity of 1.8 U/mg, a purification fold of 6.66, and a yield of 52.9%, these data were showed in figure 5. The enzyme solution was run through a Sephadex G-200 column (2-76 cm) that had been pre-equilibrated with sodium phosphate buffer (0.1 M, pH 7.5). The fraction was gathered and its absorbency at 280 nm was tested. As illustrated in figure 6, a protein peak and an enzyme activity peak both developed. According to the results in table 1, this peak of protein has a specific activity of 12.762U/mg, a purification fold of 7.09, and a yield of 29.076%. Denaturing polyacrylamide gel electrophoresis was carried out to verify the purity of laccase that was purified from *T.viride*. When the gel was submerged in Coomassie Brilliant Blue R-250, several protein bands with different molecular weights appeared along the gel in the crude

enzyme extract sample, while only one band appeared in the other sample produced from ion exchange and gel filtration. The electrophoresis involved three samples: the first sample was crude enzyme extract, while the other samples were produced from ion- exchange (peak A) and one peak (peak B) from gel filtration. These samples have a 66KD molecular weight.

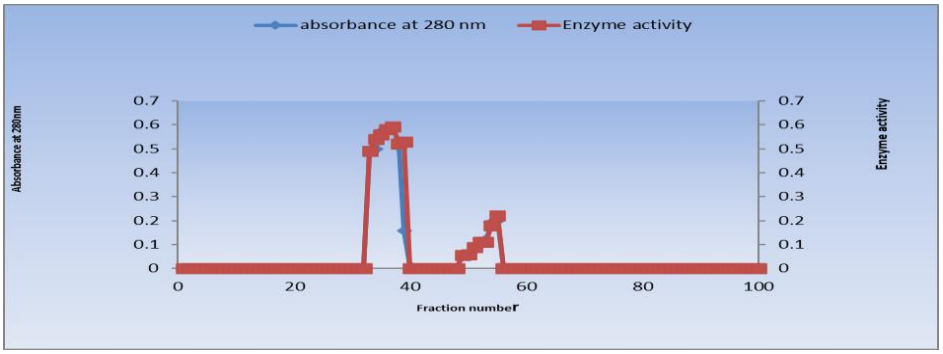

Figure 5. Ion exchange chromatography uses a DEAEE-cellulose membrane (2 -30) cm with sodium phosphate buffer (0. 1 M, pH 7.5), for purification of laccase enzyme from *T.viride*.

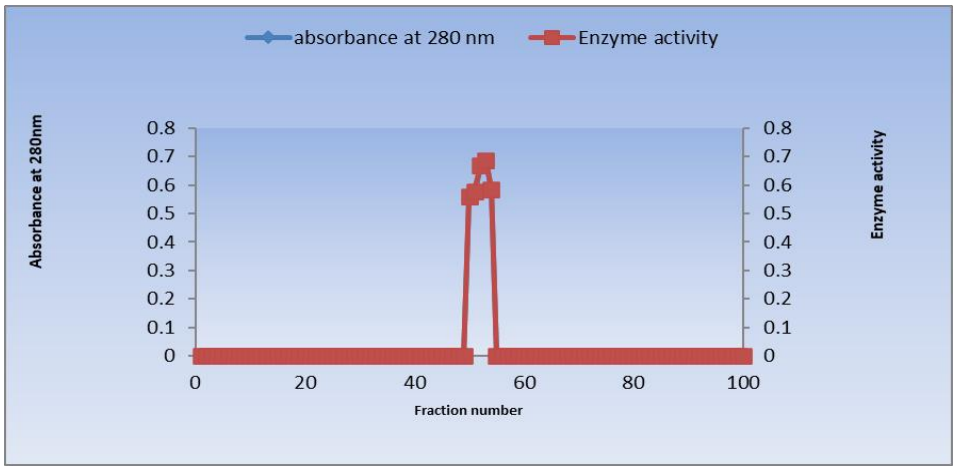

Figure 6. Gel filtration uses Sephadexx G-200 membrane (2-76 cm) with sodium phosphate buffer (0.1M, pH 7.5), for purification of laccase enzyme from *T.viride*.

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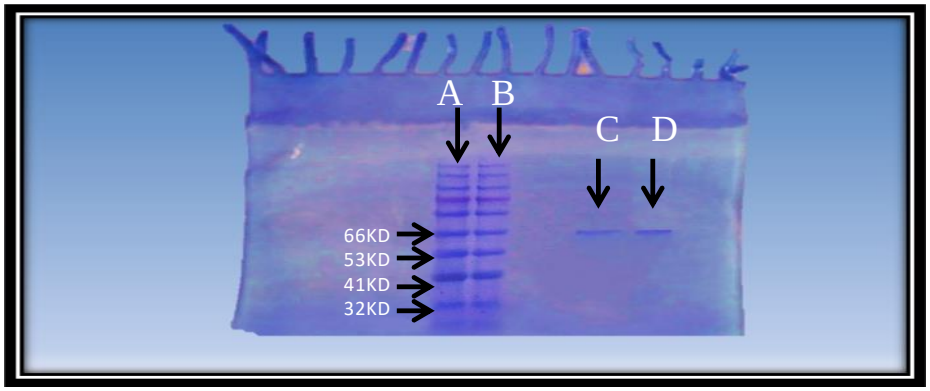

Figure 7. PAGE of the laccase from *T. viride* under (SDS condition), A: molecular weight marker, B: crude extract enzyme, C: represents the purified enzyme produced from Ion exchange chromatography and D: a purified enzyme produced from gel filtration

DISCUSSION

The oxidative polymerization of ABTS caused a change in color, which verified the formation of extracellular laccase by *Trichoderma viride*. For more research, *T.viride* was chosen. Without hydrogen peroxidase, the ABTS molecule served as a special laccase substrate. As a result, the enzymes generated were genuine laccases. The oxidative polymerization of guaiacol caused the medium to appear reddish brown [13]. 12 types of white rot fungus generated a reddish-brown color on a solid media containing 0.5% guaiacol, according to Artiningish et al [14].'s findings. In contrast, 6 isolates of brown rot fungi formed a very faint reddish-brown color, and 7 isolates gave a negative reaction. Using 0.02% guaiacol, 12 types of white rot fungus were assayed. Only six of the isolates showed positive guaiacol oxidation results [15] Submerged culture was used to verify this fungal isolate's capacity to produce laccase. Guaiacol was used as a substrate to measure enzyme activity. According to these findings, *T.viride* had the greatest laccase production at (0.266U/ml). These observations have led to the choice of *T.viride* for use in enhancing laccase production. The *Basidiomycota* species P. sapidus, which grows on a submerged liquid medium, was shown by Linke et al [16] to be the most active laccase producer. These findings support our research shown in Figure (1). The impact of pH can be attributed to two things: its capacity to influence the characteristics of the medium, such as nutrient solubility and transportation, and consequently affects the availability of nutrients to the developing microorganisms; and its impact on enzyme ionizable group, which

influences the stability. In addition, a change in pH was associated with a reduction in enzyme yield. This might have been caused by the limited mycelial growth at a higher pH, which may also have reduced the output of the laccase. Similar findings were made by Flores et al [17], who found that *Pycnoporus* sp. produced the most enzymes at pH 4.5. On the other hand, Kuntal et al [18] reported that the perfect beginning pH for laccase activity was between 4.5 and 5.5. However, the optimal pH value varies depending on the substrate since various substrates result in various laccase production processes [19]. There is a defined pH range where enzymes are active. This may be explained by the possibility that a pH change could modify the enzymes' three-dimensional structure [20].Through its effects on the solubility of oxygen in the medium, kinetic energy, and the speed of enzymatic reactions, the temperature has a significant role in the production of the enzyme from microorganisms. Given that both fungal growth and the synthesis of enzymes are temperature-sensitive processes, the incubation temperature has a significant impact on how the fermentation process in SmF plays out. According to Shraddhaa *et al* [19] the influence of temperature on laccase synthesis is constrained, and the ideal temperature for laccase varies greatly depending on the strain. *Pycnoporus sanguineus* produced laccase best when the temperature was between 25 and 30 °C, while *Trichoderma harzianum* produced laccase best when the temperature was 35 °C [21]. Similar findings were made by Medeiros *et al* [22], who discovered that laccase production for *M. palmivorus* reached its peak after 96 hours but that laccase production for the same strain was at its peak after 72 hours [23]. According to earlier research, *T. harzianum* and *T. viride* produce laccase at their highest levels between 2 and 4 days after being incubated. Furthermore, in a fully sporulated culture, the maximal protein produced at day 7 resulted in an increase in the particular laccase activity [24,25]. This might be because laccase activity increased throughout vegetative growth (matching the mycelial mass), but it rapidly decreased just before the fruit body formed[6].

Figure (4) presented these findings. The Japanese lacquer tree *Rhus vernicifera* provided the first laccases to be reported in 1883 [26]. Since that time, a number of laccases have been investigated in terms of their biological activity, substrate selectivity, copper binding structure, and industrial applications [27]. The enzyme's extracellular makeup and the fact that it is the only lignin lytic enzyme that *T. viride* visibly produces made the purification procedure easier. According to Chaurasia *et al* [28]. Research for laccase from Trameetes hirsute MTCCC-1171,

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90 % ammonium sulphate saturation is necessary for the greatest precipitation of laccase from solution in this investigation. However, laccase from *Russuula viresecens*, *Trameetes troogii*, and *Sporoothrix carinis* CPF-05 was reported to have 80% ammonium sulphate saturation by Zhuu et al [29], Olajuyiigbe and Fatokun [30], respectively. Few laccases from fungi that decompose trash were documented, despite the fact that many fungal laccases were purified and examined [31]. Ion Exchange chromatography and gel filtration are used to purify T. Versicolor laccase, with a specific activity of 101 L1 and a purity factor of 34.8. [32]. The current laccase from *T.viride* is a monomeric protein with an MW of 66 kDa, indicating that both its molecular weight (about 50-90 kDa) and molecular composition (monomeric) were similar to those of normal fungal laccases [33]. All of the *Marasmiaceae laccases* that have been compared are monomeric, have MWs between 53 and 75 kDa, and exhibit variable-specific activity, recovery of activity, and purification fold. The *M. scorodonius* laccase had the lowest recovery rate (5%), but the highest specific activity (432. 8 U/mg) [34]. Another laccase from *Marasmius sp.* BBKAV79 was purified with a final yield of 13.5% and the lowest specific activity of 0.226 U/mg [35-36]. It was revealed that laccases from the *Marasmiaiceae* family had a high degree of MW and specific activity variability.

Conclusion

We have come to the conclusion that the copper-containing laccase enzyme has a wide range of potential uses in the food, pharmaceutical, and textile industries when purified by the best way. Trichoderma viride was extracted from agricultural soil, bio control agent and can be used to purify enzyme by good method . The optimization of culture conditions was done for PH, Temperature, and incubation period to find the best environmental conditions for laccase production.

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Conflict of interests.

The authors have declared no conflict of interest.

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