**Benha Journal of Applied Sciences (BJAS)** **print: ISSN 2356–9751**
**Vol. (10) Issue (2) (2025), (121-126)** **online: ISSN 2356–976x**
https://bjas.journals.ekb.eg/
**Applied and Basic Science**

# Optimization of Laccase Production and Purification from *Penicillium chrysogenum*

**Asmaa A.Badawy, Mervat G.Hassan, Radwan kh Radwan and Hamed M El-Sora**
Botany and Microbiology Dept., faculty of science ,Benha University
Botany and Microbiology Dept.,faculty of science ,Mansoura University
**E-mail:**asmaabadawy800@gmail.com

**Abstract**
Laccase {Enzyme commission (EC) 1.10.3.2} is a multicopper-containing enzyme essential for oxidizing a broad range of aromatic compounds, particularly phenolic substrates. It utilizes molecular oxygen, which is reduced to water during oxidation. Laccase facilitates electron transfer via oxygen as an electron acceptor, leading to reactive free radical intermediates that trigger nonenzymatic reactions to break down substrates. The enzyme used in this study was derived from *Penicillium chrysogenum*. After 5 days of incubation at 35°C and pH 7.0, the highest laccase production was achieved. The enzyme catalyzed oxidation reactions with various phenolic compounds, with ABTS being the most efficient substrate. After purification through ammonium sulfate fractionation, Sephadex G-200, and DEAE-Sepharose chromatography, the final specific activity was 113.5 units per milligram of protein, with a separation factor of 103.2-fold and a recovery rate of 26.8% . the optimized culture and purification condtions considweably improved enzyme yield ,activity, and stability. These finding highlight the high catalytic potential of P.chrysogenum laccase in various industrial and biotechnological applications.

**Keywords:**Enzyme , penicillium , chrysogenum ,optimization , purification , enzyme activity, Laccase enzyme

## Introduction

Enzymes are often hired in business operations as an alternative to chemical catalysts due to the fact that they are both highly impactive and beneficial to the environment. Among all enzymes, laccase is distinguished by its remarkable versatility and wide range of potential applications. [1]

Laccase (EC 1.10.3.2) is an extracellular enzyme from the blue multi-copper oxidase family, known for its capability to oxidize a variation of aromatic compounds, especially those containing phenolic groups. It operates through a radical-based mechanism, which facilitates the reduction of oxygen to water [2]. Laccase is a crucial enzyme in various practical applications because of its capability to catalyze a wide range of enzymatic reactions with both natural and synthetic organic substrates. Its versatility makes it an essential tool in many industries, including those focemployed on environmental management, bioremediation, and the production of biofuels, textiles, and food products. The enzyme's capability to oxidize a variety of phenolic and non-phenolic compounds makes it particularly valuable for industrial processes that require eco-friendly and efficient catalytic solutions [3].

As a results, including complex aromatic compounds. Its capability to oxidize various substances, including both phenolic and non-phenolic compounds, and challenging environmental pollutants, has led to significouldt interest from researchers. This enzyme's versatile catalytic properties make it an attractive option for tackling complex environmental issues, such as the degradation of hazardous chemicals and the cleanup of polluted sites [4].

There have been hundreds of investigations that have been conducted to date, and laccases from every kingdom have been created and documented. Laccase could be exhibited in many different organisms, ranging from higher plants [5], bacteria [6,7], and predominantly in fungi [8,1].

The substrate specificity of laccase varies among different species, and its capability to oxidize a broader range of substrates is significouldtly enhanced when appropriate redox mediators are present. These mediators assist in electron transfer, expanding the enzyme's catalytic capacity beyond its natural substrates. By facilitating the oxidation of otherwise non-reactive compounds, redox mediators enable laccase to efficiently break down a wider variety of complex organic and environmental pollutants. This increased substrate versatility enhances the enzyme's applicability in diverse industrial and environmental processes [9].

Bacterial laccase production is affected by several environmental factors, such as the presence of carbon and nitrogen sources, pH, temperature, and oxygen levels. Studies have shown that each of these factors is crucial in regulating gene expression and enhancing the production of laccase in bacterial strains. Optimizing these conditions could significouldtly improve enzyme yield and activity. The nature and concentration of carbon and nitrogen sources could greatly impact enzyme yield, while pH and temperature play key roles in determining the stability and activity of the enzyme. Furthermore, oxygen levels are essential for the aerobic processes necessary for effective laccase synthesis. By carefully controlling these parameters, bacterial laccase production could be maximized for industrial and biotechnological applications.[10]

Laccases are integral to a diversity of biotechnological applications, including the detoxification of pollutants, wastewater treatment, textile dyes decolorization, pulp bleaching in the paper industries, organic synthesis, and biosensors development for detecting polyphenols within juice and wine. Their broad substrate specificity and catalytic properties make them versatile for various uses [11,12]. In addition, laccases are heavily researched for constructing oxygen reduction biocathodes for biofuel cells, and for their role in biosensors, immunoassay labeling, and biocatalysis in organic synthesis. These diverse applications are driven by the enzyme's flexible catalytic capabilities [13]. The present work aimed to isolate laccase and optimize its production from *Penicillium chrysogenum (P. chrysogenum)* and purify it with suitable procedure.

**Materials and Methods**

**Chemicals**: Sigma was the supplier of all of the chemicals, and they were all of the analytical grade.

**Microorganism media and growth conditions**

The experimental microbe involved in the complex synthesis of the enzyme laccase was *P. chrysogenum* Thom AUMC 14100 gb (Accession No. MN219732), which was carefully acquired from the renowned Mubasher Mycological Center (AUMMC) at Assiut University, located in Egypt, providing a reliable source for microbial strains. The synthesis and isolation of laccase were meticulously conducted via a modified ABTS-glucose liquid medium, which was specially prepared and carefully adjusted to meet the specific experimental conditions. This medium was prepared in accordance with the method described by Saxena and Sinha (1981) [14].

**Enzyme assay**

Laccase enzyme activity was thoroughly and meticulously evaluated by carefully incubating an appropriate enzyme sample in a well-prepared reaction mixture, which had a total volume of exactly 2.0 milliliters. This reaction mixture consisted of precisely 0.5 milliliters of 0.3 millimolar ABTS substrate, carefully combined with 0.1 millimolar sodium citrate buffer at pH 4.5, ensuring that the optimal conditions were fully met for the reaction to efficiently occur. The oxidation process of ABTS was continuously and precisely monitored at 420 nm ($\varepsilon420 = 36$ mM$^{-1}$ cm$^{-1}$) via a highly accurate and reliable Cary-100 Agilent UV-Vis Spectrophotometer, which was manufactured with great precision in Germany, for a specific duration of one full minute. The enzyme activity was then carefully quantified and expressed as the exact amount of enzyme required to oxidize one micromole of substrate per minute, providing a precise and clear measure of the enzyme's performance and efficiency in the reaction [11].

**Optimum conditions**

Experiments were carefully and systematically conducted to determine the most ideal and impactive cultural conditions for the efficient production of the enzyme laccase. Various parameters, including different incubation times, temperatures, and pH values, were thoroughly evaluated for their potential impact on the overall production process, and the most favourable and optimal conditions were then selected based on the detailed results obtained from these experiments. The impacts of various incubation times, specifically 24, 48, 72, and 96 hours, were carefully considered, along with a range of temperatures, including 25°C, 30°C, 35°C, 40°C, 45°C, and up to 50°C. Additionally, pH values, which included 4, 5, 6, 7, 8, and 9, were precisely adjusted via measured amounts of HCl or NaOH to ensure the ideal conditions for enzyme production were achieved. After the completion of each experiment, the crude laccase (supernatant) from the *P. chrysogenum* culture flasks was carefully filtered to remove any residual solids or impurities and then utilized to accurately and reliably determine the laccase activity, ensuring consistent and reliable results for further analysis and comparison..

**Purification of laccase**

At a temperature of 4 degrees Celsius and a saturation level of 80%, the crude enzyme was precipitated by the use of ammonium sulfate fractionation. Ten minutes were spent centrifuging the mixture at a temperature of four degrees Celsius and a force of 10000 rpm. The pellets were dialyzed for twenty-four h at four degrees Celsius against the same buffer after being dissolved in sodium phosphate buffer with a concentration of 0.1 M and a pH of 6.0. After the enzyme was dialyzed, it was placed on a Sephadex G-200 column that had been pre-equilibrated with sodium phosphate buffer (0.1 M, pH 6.0). The column was 2x50 cm in length. At a rate of one milliliter per minute, fractions of two milliliters were collectedIn accordance with the findings of Othman and Wollenberger (2020) [13], a detailed analysis and quantification of both laccase activity and protein concentration were performed on the collected fractions. The active fractions were carefully pooled together, concentrated, and subsequently loaded onto a 2x30 cm DEAE-Sepharose column that had been thoroughly equilibrated with a 0.1 M Tris buffer at pH 6.0 to ensure optimal conditions for protein separation. After an initial wash step with the same buffer, the bound proteins were progressively eluted from the column by applying NaCl gradients ranging from 0.0 to 0.4 M, in the equilibration buffer, over a series of minutes while maintaining a consistent flow rate of 1 ml per minute. Once the 3.0 mL chromatographic fractions were collected, the laccase activity and protein content in each fraction were accurately measured and quantified, following well-established protocols to ensure reliable results.

**Protein determination**

For the purpose of determining the amount of protein present, the Bradford (1976)[15] technique was employed, with bovine serum albumin serving as the standard reference material.

**Statistical analysis**.

The tests were all performed three times to ensure accuracy. The data is represented by utilizing the averages of the values obtained, with a standard error of ±.

**Results**

Research was conducted to determine how factors such as incubation time, temperature, and pH of the growth medium affected the production and activity of laccase enzyme. The ultimate goal was to optimize these conditions to boost laccase production in *P. chrysogenum*. According to the findings which are shown in Table 1, *P. chrysogenum* demonstrated the capacity to synthesize the laccase enzyme on each and every day of the studies, although with varying degrees of enzymatic activity. The fifth day of the studies, on the other hand, produced the maximum amount of enzymes, with an enzyme activity of 15.0 units per milliliter.

Table 1: Impact of varying incubation duration on laccase production by *P. chrysogenum*.

| Incubation period (days) | Enzyme activity (U/ml) |
|---|---|
| 1 | 3.5±0.2 |
| 2 | 7.3±0.3 |
| 3 | 10.4±0.4 |
| 4 | 12.4±0.3 |
| 5 | 15.0±0.5 |
| 6 | 13.6±0.4 |

The observations of the investigation examining the impact of pH levels on laccase enzyme synthesis by *P. chrysogenum* are presented in Table 2. The data indicates that a growth medium with a pH of 7, which resulted in an enzymatic activity of 14.6 units/ml, produced the highest laccase yield. Further details could be observed in the following table:

Table 2: Impact of varying pH values on laccase production by *P. chrysogenum*

| pH | Enzyme activity (U/ml) |
|---|---|
| 4 | 2.2±0.2 |
| 5 | 5.8±0.3 |
| 6 | 9.0±0.3 |
| 7 | 14.6±0.6 |
| 8 | 11.2±0.4 |
| 9 | 4.7±0.2 |

It has been shown that *P. chrysogenum* had the maximum capacity to manufacture the laccase enzyme at both 30°C and 35°C. However, the most impactive synthesis of laccase was exhibited at 35°C, with an enzyme activity of 14.0 units/ml. A representation of the findings of the research is shown in Table 2, which depicts the influence that temperature has on the synthesis of the laccase enzyme. The fact that the synthesis of enzymes is so low at temperatures of 25 and 45 degrees Celsius makes it abundantly evident that these temperatures are not optimum for laccase activity.

Table 3: Impact of varying temperatures on laccase production by *P. chrysogenum*.

| Temperature (°C) | Enzyme activity (U/ml) |
|---|---|
| 25 | 4.7±0.2 |
| 30 | 8.3±0.3 |
| 35 | 14.0±0.5 |
| 40 | 10.5±0.4 |
| 45 | 8.3±0.3 |
| 50 | 6.0±0.3 |

The activity of the enzyme laccase was investigated via *P. chrysogenum* in order to determine the impact that various substrates had on the enzyme's performance. ABTS, 2, 2,6-Dimethylphenol (6-DMP), syringaldazine, guaiacol, catechol, and pyrogallol are the compounds that are included in the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) compound.

A few examples of the substrates that were employed in the procedure for this. The reaction media was employed to investigate them, and the concentration of the reaction medium was 5 mM. According to the data that are provided in Table 4, ABTS was exhibited to be the most efficient substrate for laccase. It had an activity level of 14.0 units/ml, which further demonstrates its impactiveness.

Table 4: Impact of different substrates on laccase activity from *P. chrysogenum*.

| Substrate (5 mM) | Enzyme activity (U/ml) |
|---|---|
| ABTS | 14.0±0.5 |
| 2, 6-DMP | 10.3±0.4 |
| Syringaldazine | 7.7±0.3 |
| Guaiacol | 5.5±0.3 |
| Catechol | 3.7±0.2 |
| Pyrogallol | 2.9±0.2 |

Table 5: Purification of laccase from *P. chrysogenum*.

| Purification step | Total protein (mg) | Total activity (U) | Specific activity (U $mg^{-1}$ protein) | Recovery (%) | Fold of Purification |
|---|---|---|---|---|---|
| Crude extract | 77.9 | 85.0 | 1.1 | 100 | 1 |
| Ammonium sulphate ppt | 19.0 | 69.5 | 3.7 | 81.8 | 3.7 |
| Sephadex G-200 | 2.4 | 48.9 | 20.4 | 57.5 | 18.5 |
| DEAE-Sepharose | 0.2 | 22.7 | 113.5 | 26.8 | 103.2 |

**Discussion**

Laccases are highly versatile and efficient biocatalysts that are widely employed for various industrial applications, owing to their capability to catalyze a wide range of reactions. The techniques and variables that significouldtly contribute to the enhanced activity of laccase enzymes, particularly those generated by bacteria, have been the subject of a substantial and ongoing investigations that has been done in the past years. These extensive investigations primarily focus on determining the substrate specificity of the enzymes, which is crucial for understanding how they interact with different substrates. Additionally, considerable attention has been given to optimizing the pH and temperature conditions under which the laccase enzymes exhibit their highest performance and efficiency, leading to improvements in their overall catalytic activity and their application potential in various industrial processes [10].

Laccase enzymes could be produced by various species, including fungi, bacteria, and cyanobacteria, utilizing different types of substrates [10,16] Among these, bacterial laccases are particularly advantageous due to their superior thermal and pH stability when compared to those derived from fungi and plants, which makes them significouldtly more efficient and reliable for various industrial applications [17] . Moreover, bacterial laccases exhibit a notably broad substrate specificity, allowing them to catalyze a wide variety of reactions. This versatility, combined with their economic efficiency, significouldtly enhances their industrial potential. Their capability to work with diverse substrates makes them valuable for applications in industries such as bioremediation, textiles, food, and biofuel production. Additionally, their relatively low cost of production compared to chemical catalysts further boosts their appeal for large-scale processes, making bacterial laccases a highly attractive tool in sustainable and cost-impactive industrial applications [18].

The research exhibited that *P. chrysogenum* produced the highest levels of laccase after five days of incubation at 35°C, with the growth medium adjusted to a neutral pH of 7. This result is consistent with several other studies that have examined different methods to enhance laccase enzyme production in microorganisms. These studies consistently show that by fine-tuning culture conditions, such as incubating the culture for five days at temperatures between 30°C and 35°C and adjusting the pH of the medium to a range of 7 to 8, laccase productivity could be significouldtly maximized. Such optimization strategies are crucial for improving enzyme yield and efficiency in industrial applications [19,20].

The laccase enzyme from *P. chrysogenum* was purified with a 103.2-fold increase in activity. In contrast, laccase from *Trichoderma harzianum* strain HZN10 achieved a 25-fold purification via ammonium sulfate (70%), ultrafiltration, DEAE-Sepharose, and Sephadex G-100 chromatography [21] . Ion exchange and gel filtration chromatography methods were also employed for laccase purification, with DEAE-cellulose being commonly employed. Other researchers have utilized CM-Cellulose, Sepharose, and DEAE-Sepharose CL-6B for similar purposes [22]. Additionally, various forms of Sephadex, including Sephadex G-25, G-75, G-100, and G-200, are widely applied in gel filtration techniques for purifying laccase enzymes [23].

In conclusion: Laccase production reached its maximum following six days of incubation at 35°C, via a growth medium with a pH of 7. The findings indicated that ABTS was the most efficient substrate for the laccase produced by *P. chrysogenum*. Due to their broad substrate specificity, laccases offer substantial potential for various biotechnological applications, such as in biochemical pulping, dye degradation, and the detoxification of xenobiotics. The enzyme was purified by 103.2-fold with a recovery of 26.8%. Consequently, this purified laccase enzyme shows promise for use in industrial processes.

**References**

[1] El-Shora, Hamed M., Mohamed H. Yaseen, Ryad M. El-Sharkawy, and Rehab A. Abdel Aziz (2021). Thermostcapability of fungal laccase and the influence of amino acids. Egyptian Journal of Experimental Biology (Botany). 17(2): 167-172. https://doi.org/10.5455/egyjebb.20211117034103

[2] Elsayed, A. M., Mahmoud, M., Abdel Karim, G. S., Abdelraof, M., and Othman, A. M. (2023). Purification and biochemical characterization of two laccase isoenzymes isolated from *Trichoderma harzianum* S7113 and its application for bisphenol A degradation. Microbial Cell Factories, 22(1), 1. https://doi.org/10.1186/s12934-022-02011-z

[3] Pannu JS and Kapoor RK. (2014). Microbial Laccases: A Mini-Review on Their Production, Purification and Applications. Int J Pharm Arch. 2014; 3(12): 528-536. https://doi.org/10.1016/j.biortech.2022.128487

[4] Sheikhi F, Roayaei Ardakani M, Enayatizamir N, and Rodriguez-Couto S. (2012). The determination of assay for laccase of *Bacillus subtilis* WPI with two classes of chemical Compounds as substrates. Indian J Microbiol; 52(4): 701–07. https://doi.org/10.1007/s12088-012-0298-3

[5] Liu, M., Dong, H., Wang, M., and Liu, Q. (2020). Evolutionary divergence of function and expression of laccase genes in plants. Journal of Genetics, 99: 1-16. https://doi.org/10.1007/s12041-020-1184-0

[6] Neifar, M., Chouchane, H., Mahjoubi, M., Jaouani, A., and Cherif, A. (2016). *Pseudomonas extremorientalis* BU118: a new salt-tolerant laccase-secreting bacterium with biotechnological potential in textile azo dye decolourization. 3 Biotech, 6, 1-9. https://doi.org/10.1007/s13205-016-0425-7

[7] Guan, Z. B., Luo, Q., Wang, H. R., Chen, Y., and Liao, X. R. (2018). Bacterial laccases: promising biological green tools for industrial applications. Cellular and Molecular Life Sciences, 75: 3569-3592. https://doi.org/10.1007/s00018-018-2883-z

[8] El-Shora, H. M. (2008). Induction and efficacy of immobilized laccase from *Bacillus lichniformis* for removal of phenolics in aqueous solution. Journal of Biotechnology, 136, S682. https://doi.org/10.1016/j.jbiotec.2008.07.1580

[9] Sadhasivam S, Savitha S, Swaminathan K, and Lin F-H. (2008). Production, purification and characterization of mid-redox potential laccase from a newly isolated *Trichoderma harzianum* WL1. Process Biochem. 43:736–42. https://doi.org/10.1016/j.procbio.2008.02.017

[10] Chauhan PS, Goradia B, and Saxena A. (2017). Bacterial laccase: recent update on production, properties and industrial applications. 3 Biotech. 7(5): 323. https://doi.org/10.1007/s13205-017-0955-7

[11] Othman AM, Elsayed MA, Elshafei AM, and Hassan MM (2018). Purification and biochemical characterization of two isolated laccase isoforms from *Agaricus bisporus* CU13 and their potency in dye decolorization. Int J Biol Macromol. 113:1142–8. https://doi.org/10.1016/j.ijbiomac.2018.03.043

[12] Sun Y, Liu Z-L, Hu B-Y, Chen Q-J, Yang A-Z, Wang Q-Y, et al.(2021). Purification and characterization of a thermo- and pH-stable laccase from the litter decomposing fungus *Gymnopus luxurians* and laccase mediator systems for dye decolorization. Front Microbiol.12:1746. https://doi.org/10.3389/fmicb.2021.672620

[13] Othman AM and Wollenberger U. (2020). Amperometric biosensor based on coupling aminated laccase to functionalized carbon nanotubes for phenolics detection. Int J Biol Macromol. 153:855–64.

https://doi.org/10.1016/j.ijbiomac.2020.03.049

[14] Saxena, R. K., and Sinha, U. (1981). L-asparaginase and glutaminase activities in the culture filtrates of *Aspergillus nidulans*. Current Science, Corpus ID:89805472. https://doi.org/10.1007/s12010-015-1917-3

[15] Bradford MM. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72: 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

[16] Dhiman K, and Shirkot P. (2015). Bioprospecting and molecular characterization of laccase producing bacteria from paper mills of Himachal Pradesh. Proc Natl Acad Sci India Sect B Biol Sci. 85(4): 1095-103. https://doi.org/10.1007/s40011-015-0541-x

[17] Lu R, and Miyakoshi T. (2012). Studies on acetone powder and purified rhus laccase immobilized on zirconium chloride for oxidation of phenols. Enzyme Res. 2012. https://doi.org/10.1155/2012/375309

[18] Sharma V, Ayothiraman S, and Dhakshinamoorthy V. (2019). Production of highly thermo-tolerant laccase from novel thermophilic bacterium Bacillus sp. PC-3 and its application in functionalization of chitosan film. J Biosci Bioeng. 127(6): 672-78. https://doi.org/10.1016/j.jbiosc.2018.11.008

[19] Mishra SK, and Srivastava SK. (2016). Production of extracellular laccase from bacterial strain *Bacillus subtilis* MTCC 1039 via different parameter. Biosciences Biotechnology Research Asia. 13(3): 1645-50. http://dx.doi.org/10.13005/bbra/2312

[20] Srinivasan P, Selvankumar T, Kamala-Kannan S, et al. (2019). Production and purification of laccase by *Bacillus sp*. via millet husks and its pesticide degradation application. 3 Biotech. 9(11): 396. https://doi.org/10.1007/s13205-019-1900-8

[21] Bagewadi Z, Mulla S, and Ninnekar H. (2017). Purification and immobilization of laccase from *Trichoderma harzianum* strain HZN10 and its application in dye decolorization. J Genet Eng Biotechnol. 15:150. https://doi.org/10.1016/j.jgeb.2017.01.007

[22] Afreen S, Shamsi TN, Baig MA, et al. (2017). A novel multicopper oxidase (laccase) from cyanobacteria: Purification, characterization with potential in the decolorization of anthraquinonic dye. PLoS One. 12(4): e0175144. https://doi.org/10.1371/journal.pone.0175144

[23] Aziz GM, Hussein SI, M-Ridha MJ, Mohammed SJ, Abed KM, Muhamad MH, and Hasan HA. (2023). Activity of laccase enzyme extracted from *Malva parviflora* and its potential for degradation of reactive dyes in aqueous solution. Biocatal Agric Biotechnol. 50: 102671. https://doi.org/10.1016/j.bcab.2023.102671