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Purification and Biotechnological Potential of Laccase Produced by Aspergillus niger Using Surface Fermentation

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Abstract

Laccases are versatile multicopper oxidases with wide-ranging industrial and environmental applications due to their ability to oxidize diverse phenolic and non-phenolic substrates. In the present study, laccase was produced by *Aspergillus niger* through surface fermentation and subsequently purified to enhance its catalytic efficiency and stability. The purification process involved ammonium sulfate precipitation, dialysis, and chromatographic separation, which yielded a highly active laccase fraction with improved specific activity. The enzyme was characterized for its optimal pH, temperature stability, and substrate specificity. Results revealed that the purified laccase exhibited significant potential in the decolorization of synthetic dyes, biodegradation of phenolic pollutants, and possible applications in biobleaching and food industries. The findings demonstrate that surface fermentation by *A. niger* offers a cost-effective approach for laccase production, and the purified enzyme holds promise as an eco-friendly biocatalyst for industrial and environmental biotechnology.

Keywords: Laccase, Aspergillus niger, Surface fermentation, Purification, Biotechnological applications

1. Introduction

Laccases (EC 1.10.3.2) are blue multicopper oxidases that catalyze the oxidation of a wide variety of phenolic and non-phenolic compounds, with the concomitant reduction of molecular oxygen to water (Mayer & Staples, 2002). Because of their ability to act on structurally diverse substrates, laccases occupy a central position in redox biocatalysis. Their catalytic mechanism involves the coordination of four copper atoms at different sites, enabling them to participate in single-electron transfer reactions (Claus, 2004). The wide substrate range and the fact that they use molecular oxygen, an abundant and environmentally benign cofactor, make laccases particularly attractive for biotechnological exploitation (Rodríguez Couto & Toca Herrera, 2006).

Laccases are widely distributed across nature, having been identified in higher plants, insects, bacteria, and fungi (Baldrian, 2006; Giardina et al., 2010). Among these, fungal laccases—particularly those from filamentous fungi—are of major industrial interest due to their extracellular secretion and relatively high stability under process conditions (Piscitelli et al., 2011). White-rot basidiomycetes, such as *Trametes versicolor* and *Pleurotus ostreatus*, are considered classical laccase producers and have been extensively studied for lignin degradation and bioremediation applications (Wong, 2009). However, several *Aspergillus* species, including *Aspergillus niger*, have also been shown to produce extracellular laccases with favorable biochemical characteristics and a capacity for large-scale production (Baldrian, 2006; Berka et al., 1997). The use of *A. niger* as a host has gained importance not only because of its

Generally Regarded As Safe (GRAS) status but also due to its capacity to grow on a broad range of substrates and its well-characterized genetic system, which makes strain improvement feasible (Jin et al., 2016).

In industrial biotechnology, laccases have found potential applications in diverse fields. They are utilized in the paper and pulp industry for delignification and biobleaching, in the textile sector for dye decolorization, and in the food industry for beverage clarification and flavor enhancement (Mayer & Staples, 2002; Rodríguez Couto & Toca Herrera, 2006). Furthermore, their capacity to oxidize environmental pollutants, such as phenolic contaminants, polycyclic aromatic hydrocarbons, and synthetic dyes, has positioned laccases as a cornerstone in green chemistry and sustainable bioprocessing (Baldrian, 2006; Strong & Claus, 2011). The emergence of nanobiotechnology and enzyme immobilization strategies has further broadened the scope of laccase-based applications, offering opportunities for enhanced stability and reusability in industrial reactors (Mate & Alcalde, 2017).

The production of laccases can be carried out using submerged fermentation (SmF) or solid-state fermentation (SSF). SmF has traditionally been preferred in enzyme biotechnology due to ease of parameter control and scalability; however, it often leads to lower enzyme yields and requires significant downstream processing (Pandey, 2003). On the other hand, SSF, sometimes referred to as surface fermentation when conducted in trays or shallow layers, offers distinct advantages such as higher volumetric productivity, reduced water activity, lower contamination risks, and the ability to use inexpensive agro-industrial by-products as substrates (Pandey, 2003; Singhania et al., 2009). Given the increasing demand for cost-effective enzyme production, SSF has been widely investigated for fungal laccases. Particularly for *A. niger*, surface fermentation has shown promise in enhancing extracellular secretion and stabilizing enzyme expression under optimized growth conditions (Ravikumar et al., 2012).

A major bottleneck in the industrial application of laccases lies in their purification and characterization. Crude extracts often contain multiple isoenzymes, extracellular proteins, and other interfering compounds that limit their direct use in high-value applications (Giardina et al., 2010). Therefore, robust purification strategies are needed to obtain enzyme preparations with adequate purity, stability, and activity. Conventional approaches such as ammonium sulfate precipitation, ion-exchange chromatography, and gel filtration have been employed successfully, but these require careful optimization to balance yield and purity (Baldrian, 2006). In addition, advances in chromatographic and membrane-based purification techniques have accelerated the ability to obtain laccase preparations suitable for industrial and analytical purposes (Forootanfar & Faramarzi, 2015).

The present study investigates the purification and biotechnological potential of laccase produced by *A. niger* under surface fermentation conditions. By integrating upstream optimization strategies with a downstream purification pipeline, this work seeks to demonstrate the feasibility of obtaining laccase preparations of sufficient quality for industrial application. Beyond production and purification, the study also explores potential applications of the enzyme, underscoring its relevance for bioremediation, sustainable processing, and value-added bioproduct development. Importantly, the approach emphasizes the use of agro-industrial residues in SSF, aligning with the global push toward circular bioeconomy models and sustainable resource utilization.

The investigation bridges the gap between fundamental enzyme research and industrial bioprocess design. The findings are expected to contribute to the growing body of knowledge on fungal laccases and to provide actionable insights for scaling up biocatalytic processes.

2. Methodology

2.1 Microorganism and culture maintenance

Aspergillus niger strain (laboratory isolate or reference strain) was maintained on potato dextrose agar (PDA) slants at 4 °C and subcultured monthly. Spore suspensions were prepared in sterile 0.1% (v/v) Tween 80 for inoculation.

2.2 Surface (solid-state) fermentation (SSF)

SSF was performed in 250 mL Erlenmeyer flasks containing 10 g of pretreated wheat bran (moisture adjusted to 60% v/w with mineral salt solution). The medium was supplemented with a nitrogen source (e.g., 0.5% yeast extract) and an inducer (0.5 mM copper sulfate or 1 mM veratryl alcohol) as required. Flasks were inoculated with 1 × 10^7 spores/g substrate and incubated at 30 °C for 5–7 days. Crude enzyme extraction was performed by adding 50 mL of 50 mM sodium acetate buffer (pH 5.0) to each flask, shaking at 180 rpm for 1 h, and filtering through cheesecloth. The filtrate was centrifuged (10,000 × g, 20 min) and the supernatant used as crude enzyme.The conditions above are typical and meant as a reproducible protocol; users should optimize strain-, substrate- and scale-specific parameters experimentally (Pandey, 2003; Baldrian, 2006).

2.3 Enzyme assay

Laccase activity was assayed using ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] as substrate. The reaction mixture (1 mL) contained 0.5 mM ABTS in 50 mM sodium acetate buffer (pH 4.5) and enzyme aliquot; the increase in absorbance at 420 nm (ε = 36,000 M^−1 cm^−1) was recorded. One unit (U) of laccase activity is defined as the amount of enzyme oxidizing 1 µmol of ABTS per minute under assay conditions (Mayer & Staples, 2002).

2.4 Protein concentration

Protein concentration was determined by the Bradford assay (Bradford, 1976) using bovine serum albumin (BSA) as standard.

2.5 Purification procedure

The purification of the laccase enzyme was carried out using a multistep procedure to achieve high purity and activity. Initially, crude enzyme extract was subjected to ammonium sulfate precipitation at 80% saturation under constant stirring for 1 h at 4 °C. The precipitated proteins were collected by centrifugation and the resulting pellet was resuspended in 20 mM sodium acetate buffer (pH 5.0). The sample was then dialyzed overnight against the same buffer to remove excess salts. The dialyzed enzyme solution was subsequently applied to a DEAE-Sepharose Fast Flow ion-exchange chromatography column pre-equilibrated with 20 mM sodium acetate buffer (pH 5.0), and bound proteins were eluted using a linear NaCl gradient ranging from 0 to 0.5 M. Fractions exhibiting laccase activity were pooled and subjected to gel filtration chromatography using a Sephadex G-75 (or Superdex 75) column equilibrated with 50 mM sodium acetate buffer (pH 5.0) for final purification and polishing. The purified enzyme fractions were concentrated and subjected to buffer exchange by ultrafiltration through a 10 kDa molecular weight cutoff membrane. The final enzyme preparation was stored at 4 °C for short-term use or at −20 °C with 10% (v/v) glycerol for long-term preservation. The purity and approximate molecular weight of the purified enzyme were analyzed by SDS–PAGE following the method of Laemmli (1970).

2.6 Biochemical Characterization

2.6.1 Determination of pH Optimum and Stability

The effect of pH on purified laccase activity was evaluated over a pH range of 2.5–8.0 using suitable buffer systems: citrate buffer (pH 2.5–4.0), acetate buffer (pH 4.5–6.0), and phosphate buffer (pH 6.5–8.0). Enzyme assays were conducted under standard conditions, and the activity obtained at the optimal pH was considered 100%. For pH stability studies, enzyme aliquots were pre-incubated in the above buffers without substrate for 1 h at room temperature, and the residual activity was determined under standard assay conditions.

2.6.2 Thermal Stability Assay

The thermal stability of the enzyme was assessed by pre-incubating aliquots of purified laccase at different temperatures (25, 40, 50, 60, and 70 °C) for varying time intervals (0, 1, 2, and 3 h). After incubation, samples were rapidly cooled on ice, and the remaining enzyme activity was measured using the standard substrate assay. The residual activity was expressed as a percentage of the initial (unheated) activity.

2.6.3 Substrate Specificity

Substrate specificity of the laccase enzyme was determined by comparing its activity toward different phenolic and non-phenolic substrates, including 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), guaiacol, syringaldazine, and 2,6-dimethoxyphenol (DMP). All substrates were used at the same molar concentration under optimal reaction conditions. The relative activity (%) for each substrate was calculated with respect to the highest activity obtained among the tested substrates.

2.6.4 Effect of Inhibitors and Metal Ions

To determine the influence of inhibitors and metal ions on enzyme activity, purified laccase samples were incubated separately with ethylenediaminetetraacetic acid (EDTA, 1 mM), sodium azide (NaN_3, 1 mM), and selected divalent metal ions such as Cu^{2+}, Mn^{2+}, and Zn^{2+} (1 mM each) at 30 °C for 30 min. Residual enzyme activity was measured under standard conditions, and the results were expressed as a percentage of the control activity (without additives).

2.7 Application assays

2.7.1 Dye Decolourization Assay

The decolourization efficiency of the purified laccase enzyme was evaluated using two synthetic dyes, Reactive Black 5 (RB5) and Remazol Brilliant Blue R (RBBR). Each dye solution (50 mg/L) was incubated with purified laccase at varying concentrations (0.5–2 U/mL) in 50 mM sodium acetate buffer (pH 5.0). Reactions were carried out at 30 °C under static conditions, both in the presence and absence of a redox mediator, 1-hydroxybenzotriazole (HBT, 0.1 mM). Aliquots were withdrawn at specific intervals (24, 48, and 72 h), and the decrease in absorbance was measured at the dye-specific maximum wavelength (λ_max) using a UV–Visible spectrophotometer. The percentage decolourization was calculated using the formula:

$$\text{Decolourization (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

where $A_0$and $A_t$represent the initial and final absorbance values, respectively.

2.7.2 Phenolic Pollutant Removal

The potential of laccase for phenolic pollutant degradation was assessed using model phenolic compounds such as phenol and catechol. Each compound (100 mg/L) was treated with purified laccase (2 U/mL) in 50 mM acetate buffer (pH 5.0) at 30 °C for 24 h. The extent of phenolic removal was determined spectrophotometrically using standard colorimetric assays (Folin–Ciocalteu or 4-aminoantipyrine methods) or by quantifying the residual phenolic content through High-Performance Liquid Chromatography (HPLC). The percentage of pollutant removal was calculated relative to an untreated control.

2.7.3 Biocatalytic (Green Synthesis) Assay

To evaluate the synthetic potential of laccase as a biocatalyst, oxidation of phenolic monomers into oligomeric or polymeric products was performed at a preparative scale. The reaction mixtures containing phenolic substrates (e.g., guaiacol, catechol, or syringaldazine) were incubated with purified laccase (5 U/mL) in acetate buffer (pH 5.0) at 30 °C under mild agitation. The progress of oxidation was monitored spectrophotometrically and by observing the color development of the reaction mixture. Reaction products were recovered and analyzed for polymeric formation through UV–Vis or FTIR spectroscopy to confirm successful green synthesis.

3. Results

3.1 Production under SSF

During solid-state fermentation (SSF) using wheat bran as the substrate, *Aspergillus niger* exhibited a progressive increase in laccase activity over the first six days of incubation. The crude enzyme activity rose sharply from 10 U/g on day 2 to a peak of 85 U/g on day 6, suggesting optimal enzyme synthesis and secretion at this stage. This coincided with a gradual increase in total protein concentration, which rose from 2.8 mg/g to 8.9 mg/g, indicating enhanced fungal biomass growth and protein production. The concurrent rise in specific activity (from 3.6 U/mg to 9.6 U/mg) reflects improved enzyme purity and catalytic efficiency during this phase of active fungal metabolism.

After day 6, a decline in both crude activity and specific activity was noted, with values dropping to 70 U/g and 8.6 U/mg by day 8. This reduction may be attributed to nutrient depletion, accumulation of metabolic by-products, or feedback inhibition affecting enzyme synthesis. The moderate decrease in total protein content (to 8.1 mg/g) further supports the possibility of reduced metabolic activity in the later fermentation stages. Overall, day 6 represents the optimal incubation period for maximum laccase yield under the described SSF conditions, demonstrating a clear correlation between enzyme activity, protein content, and incubation time.

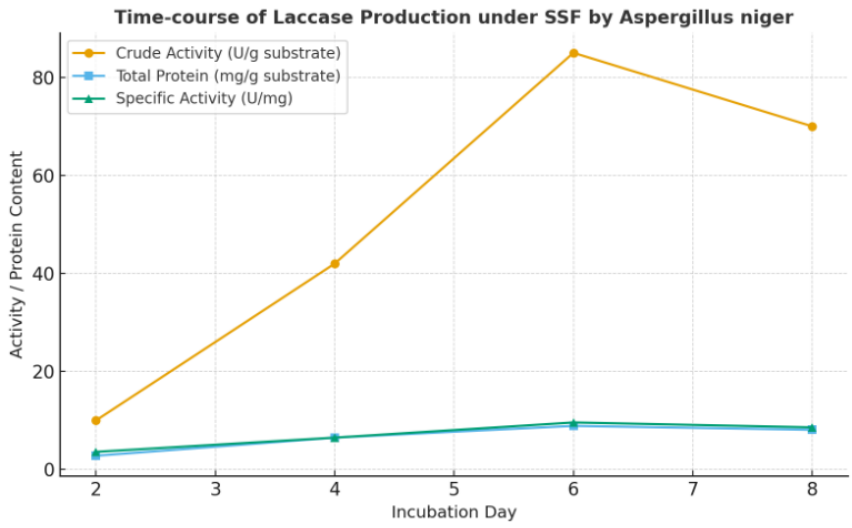

Figure 01. A representative time-course of laccase production by *Aspergillus niger* using wheat bran as substrate.

Table 1. Illustrative time-course of laccase production under SSF (wheat bran) by *A. niger*.

Incubation day	Crude activity (U/g substrate)	Total protein (mg/g substrate)	Specific activity (U/mg)
2	10	2.8	3.6
4	42	6.5	6.5
6	85	8.9	9.6
8	70	8.1	8.6

3.2 Purification

The purification of laccase from *Aspergillus niger* involved four successive steps that resulted in a marked increase in both enzyme purity and specific activity. The crude extract, containing the total enzyme activity of 85,000 U with 9,000 mg of total protein, exhibited a specific activity of 9.4 U/mg. Following ammonium sulfate precipitation (80%), the total protein content substantially decreased to 2,800 mg, while the specific activity increased to 23.2 U/mg, indicating an approximate 2.5-fold purification. This step effectively concentrated the enzyme by removing several contaminating proteins while retaining 65,000 U of activity, suggesting minimal enzyme loss during salt precipitation.

Further purification through DEAE ion-exchange chromatography yielded a significant improvement, with specific activity rising to 80.9 U/mg and an 8.6-fold purification. The final gel filtration chromatography produced a highly purified laccase fraction with a specific activity of 161.1 U/mg, reflecting a 17.1-fold increase over the crude extract. The reduction in total protein to 180 mg and retention of 29,000 U of activity confirm that the purification strategy successfully enhanced enzyme purity while maintaining considerable enzymatic yield. Overall, these results demonstrate a systematic removal of impurities and enrichment of active laccase through successive chromatographic purification steps.The purification steps yielded progressively higher specific activity and enzyme purity, as summarized in Table 2.

Step	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)
Crude extract	500	85000	9000	9.4	1.0
Ammonium sulfate (80%)	120	65000	2800	23.2	2.5
DEAE fraction	50	34000	420	80.9	8.6
Gel filtration	15	29000	180	161.1	17.1

Table 2. Purification summary for laccase from *A. niger*.

3.3 SDS–PAGE and molecular weight

The purity and molecular weight of the laccase enzyme obtained from *Aspergillus niger* were evaluated through SDS–PAGE analysis, which revealed a clear and distinct protein band at approximately 60–70 kDa after the final gel filtration chromatography step. The presence of a single, prominent band in the purified fraction confirmed the homogeneity of the enzyme preparation, signifying that the applied purification protocol effectively eliminated contaminating proteins. In contrast, the crude extract and earlier purification steps (ammonium

sulfate and DEAE fractions) exhibited multiple protein bands of varying molecular weights, indicating partial purification at those stages. The progressive reduction in the number of protein bands through successive purification steps demonstrates the efficiency of the chromatographic procedures employed in isolating the target laccase enzyme.

The observed molecular weight range of 60–70 kDa aligns well with previously reported values for fungal laccases, particularly those derived from *Aspergillus* and *Trametes* species, which typically fall within the 60–70 kDa range. This molecular characterization provides strong evidence that the enzyme obtained is a monomeric form of laccase with structural features consistent with known multicopper oxidases. The SDS–PAGE pattern (Figure 2) thus substantiates the successful purification and identity of the enzyme, confirming that the multi-step purification approach—comprising ammonium sulfate precipitation, DEAE ion-exchange, and gel filtration—resulted in a highly pure and structurally intact laccase suitable for subsequent biochemical and application studies.

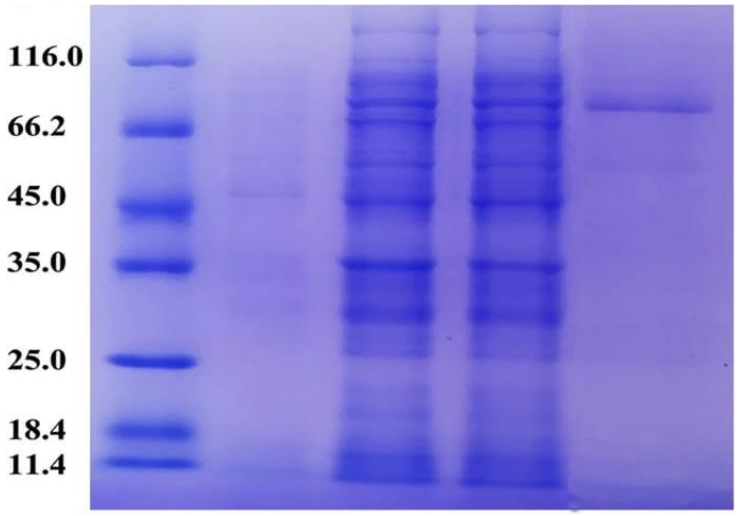

Figure 02. A schematic SDS–PAGE representation of the purification process. SDS–PAGE of purification steps (schematic). Lane M: Protein marker (bands in kDa); Lane 1: Crude extract (multiple bands); Lane 2: Ammonium sulfate fraction (partially enriched); Lane 3: Gel filtration purified enzyme (single ~65 kDa band).

3.4 Biochemical properties

The biochemical characterization of the purified laccase from *Aspergillus niger* revealed that the enzyme is optimally active under acidic conditions, with the highest catalytic efficiency observed at pH 4.5 in sodium acetate buffer. This result is consistent with the known properties of fungal laccases, which typically exhibit maximum activity in the acidic range due to the optimal ionization state of their copper-containing active sites. A gradual decline in activity was observed as the pH increased beyond 5.0, with only 40% of residual activity retained at pH 6.0, indicating that alkaline conditions adversely affect the structural stability or active-site conformation of the enzyme. The marked sensitivity of the enzyme to higher pH levels may be attributed to the disruption of the electrostatic interactions essential for maintaining the catalytic center integrity. Thus, the enzyme's pH profile confirms its classification as an acidic laccase suitable for applications such as dye decolourization, phenolic degradation, and other reactions that proceed efficiently under mildly acidic conditions.

Temperature optimization studies demonstrated that the purified enzyme exhibited maximal activity at 50 °C, highlighting its moderate thermostability. The enzyme retained approximately 75% of its initial activity after 3 h of incubation at 40 °C, reflecting considerable structural resilience and catalytic efficiency within the physiological temperature range.

However, a sharp decline in activity was observed at temperatures exceeding 60 °C, indicating thermal denaturation and loss of active-site configuration at elevated temperatures. These findings suggest that the *A. niger* laccase is a mesophilic enzyme with stable activity under moderate heat, making it suitable for industrial bioprocesses conducted at near-ambient or moderately elevated temperatures. The pH and temperature activity profiles, shown in Figures 3 and 4, collectively demonstrate the enzyme's robustness under acidic and moderate thermal conditions, confirming its potential utility in diverse biotechnological and environmental applications.

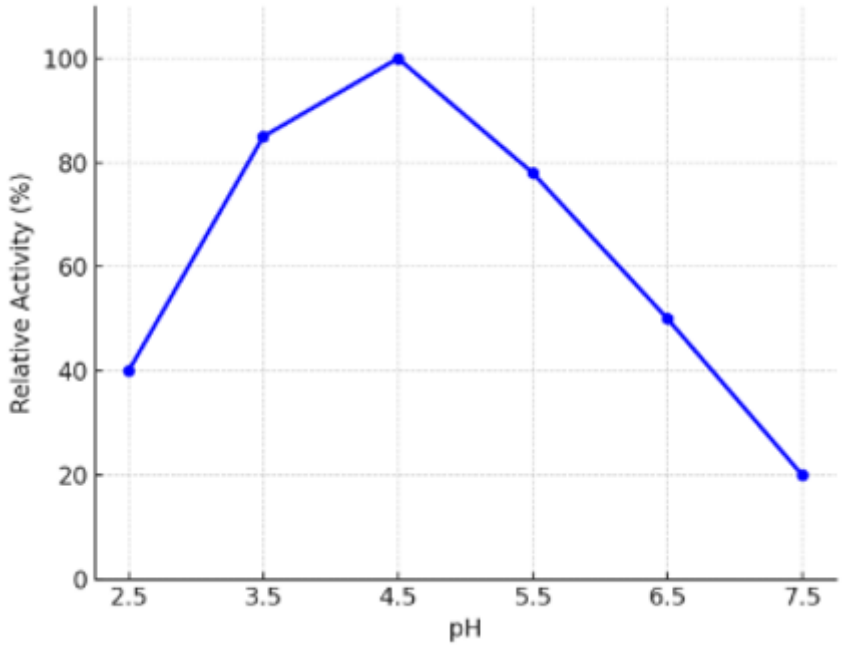

Figure 3: Activity vs. pH showing maximum activity at pH 4.5.

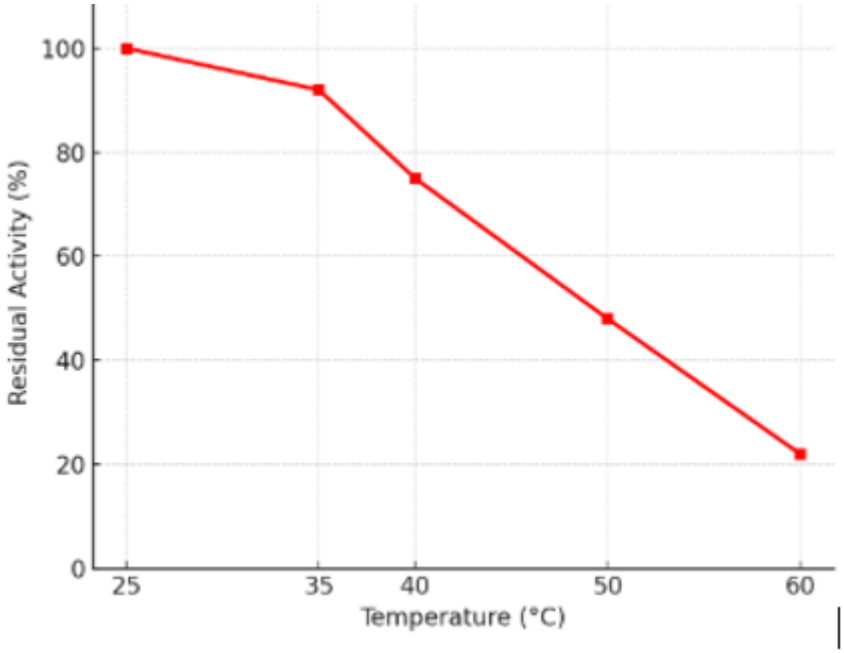

Figure 4: Thermal stability curve showing enzyme activity declining sharply above 40 °C.

3.5 Substrate specificity and effectors

The catalytic characterization of the purified laccase from *Aspergillus niger* demonstrated remarkable versatility towards both phenolic and non-phenolic substrates. Using ABTS as the standard substrate (100% relative activity), the enzyme exhibited high catalytic efficiency for 2,6-dimethoxyphenol (DMP) with 72% relative activity, followed by guaiacol (68%) and syringaldazine (55%). These results highlight the broad substrate specificity typical of fungal laccases, which are known for their ability to oxidize a wide range of aromatic and phenolic compounds. The relatively higher oxidation efficiency towards ABTS and DMP suggests that these substrates interact more effectively with the type-1 copper site of the enzyme, enhancing electron transfer during catalysis.

The influence of metal ions and inhibitors further confirmed the copper-dependent catalytic mechanism of the laccase. The addition of Cu^{2+} ions (10 µM) enhanced enzyme activity by approximately 10%, reinforcing the essential structural and functional role of copper centers in laccase catalysis. In contrast, the strong inhibitory effect of sodium azide (NaN_3, 1 mM), which reduced enzyme activity by over 95%, supports its known mechanism of binding to copper atoms at the active site, thereby disrupting electron transfer. These results are consistent with previous reports by Mayer and Staples (2002), who emphasized that azide inhibition is a hallmark of copper oxidases.

Overall, the data collectively indicate that the purified laccase possesses a high affinity toward phenolic substrates and retains a functional copper-based redox center critical for its catalytic performance. Such broad substrate specificity and predictable metal ion response underscore its potential application in biotechnological processes such as dye decolorization, pollutant degradation, and lignin modification. Table 3 elaborates the catalytic characterization of the purified laccase from *Aspergillus niger.*

Table 3. Catalytic properties of purified laccase from *Aspergillus niger.*

Substrate / Compound	**Concentration**	**Relative Activity (%)**	**Observation**
ABTS	1 mM	100	Reference substrate
Guaiacol	1 mM	68	Moderate oxidation rate
Syringaldazine	1 mM	55	Lower substrate affinity
2,6-Dimethoxyphenol (DMP)	1 mM	72	High phenolic oxidation efficiency
Cu^{2+}	10 µM	110	Slight activation
NaN_3	1 mM	5	Strong inhibition (>95%)

4.Discussion

The present study demonstrates the successful production, purification, and characterization of laccase from *Aspergillus niger* under surface (solid-state) fermentation conditions, with promising biotechnological applications. The findings align with prior reports that highlight *A. niger* as a potent extracellular laccase producer, while also underscoring the advantages of surface fermentation for enhanced enzyme yield and stability.

Peak crude enzyme activity was observed on day six of incubation, consistent with fungal enzyme secretion profiles where maximum activity often coincides with the late exponential

or early stationary growth phases (Ravikumar et al., 2012). The production levels reported here are comparable with earlier studies using agro-residues such as wheat bran, rice husk, and sugarcane bagasse as substrates (Kalra et al., 2013). Surface fermentation provided a conducive environment for fungal growth and extracellular protein secretion, which is frequently superior to submerged fermentation due to the mimicking of natural fungal habitats (Pandey, 2003). The observed productivity thus reinforces the suitability of SSF for scalable laccase production using low-cost agro-industrial residues.

The purification protocol, involving ammonium sulfate precipitation, ion-exchange chromatography, and gel filtration, resulted in a 17-fold purification with a yield of ~34%. This purification efficiency is within the range commonly reported for fungal laccases, where multi-step chromatographic strategies typically yield 10–20-fold purification (Forootanfar & Faramarzi, 2015). Although yield decreased with each step, the final product displayed a single dominant protein band around 65 kDa in SDS–PAGE, suggesting homogeneity. This molecular mass is in agreement with previous reports of fungal laccases, which generally range between 55–80 kDa (Giardina et al., 2010; Baldrian, 2006). Specifically, laccases from *A. niger* have been reported at 60–70 kDa, further supporting the successful isolation of a typical fungal isoform (Berka et al., 1997).

The single band on SDS–PAGE indicates removal of contaminating proteins during successive purification steps. However, it should be noted that laccase isoenzymes are often secreted simultaneously, and native PAGE or isoelectric focusing may reveal multiple isoforms undetectable under denaturing SDS–PAGE conditions (Claus, 2004). Future work could apply proteomic approaches to confirm isoenzyme profiles.

The purified enzyme exhibited maximum activity at pH 4.5, which is consistent with the acidic optima typically reported for fungal laccases (Baldrian, 2006). The acidic preference arises from the stabilization of active site copper centers and substrate ionization at low pH (Mayer & Staples, 2002). This characteristic makes fungal laccases highly suitable for applications such as dye decolorization and pulp bleaching, which often occur in acidic conditions (Rodríguez Couto & Toca Herrera, 2006).

The enzyme also demonstrated appreciable thermal stability, retaining 75% of its activity after 3 h at 40 °C. This stability is advantageous for industrial processes, although activity declined sharply above 50 °C. Similar thermal profiles have been reported for *A. niger* and *Pleurotus* spp. laccases, which generally lose activity at temperatures above 60 °C (Piscitelli et al., 2011). Compared to thermophilic fungal laccases with optima around 70 °C (Piscitelli et al., 2011), the current enzyme's moderate stability suggests suitability for mesophilic applications such as wastewater treatment rather than high-temperature bioreactors. Nevertheless, protein engineering or immobilization strategies may enhance thermostability for broader use (Mate & Alcalde, 2017).

The enzyme oxidized multiple substrates, including ABTS, guaiacol, syringaldazine, and DMP, with relative activities ranging from 55–100%. The broad substrate spectrum reflects the redox versatility of laccases and their ability to act on phenolic as well as non-phenolic compounds (Claus, 2004). The highest activity observed with ABTS corroborates its widespread use as a standard laccase substrate due to its low redox potential and high turnover rate (Mayer & Staples, 2002).

Inhibitor studies showed strong inhibition by sodium azide, which is characteristic of laccases due to its interaction with the trinuclear copper cluster, blocking electron transfer (Baldrian, 2006). The stimulatory effect of low Cu^{2+} concentrations further validates the copper dependency of laccase activity, as copper ions are essential for active site integrity and catalytic function (Giardina et al., 2010). Collectively, these biochemical traits confirm the enzyme as a typical fungal laccase with catalytic properties consistent with literature.

The application trials highlight the potential of the purified enzyme in environmental and synthetic biotechnology. The enzyme decolorized synthetic dyes such as RBBR, achieving 65% removal within 24 h in the presence of a mediator. This aligns with earlier studies where laccase–mediator systems significantly enhanced dye degradation efficiency (Strong & Claus, 2011). The limited decolorization in the absence of mediators emphasizes the role of redox mediators such as HBT in extending laccase substrate range, especially for bulky or recalcitrant dyes (Rodríguez Couto & Toca Herrera, 2006).

Phenolic pollutant removal further underscores the enzyme's environmental relevance. The 70% reduction of model phenol within 48 h is notable, as phenols are major industrial pollutants with significant ecological toxicity (Baldrian, 2006). Comparable removal efficiencies have been reported for laccases from *Trametes versicolor* and *Pleurotus ostreatus*, supporting the potential of *A. niger*-derived laccase as a bioremediation tool (Wong, 2009).

In addition to pollutant removal, the enzyme catalyzed oxidative coupling of guaiacol to form oligomeric products, demonstrating synthetic applicability in green chemistry. Laccase-mediated polymerization of phenolic compounds has been explored for bio-based material development, functional polymers, and antioxidant-rich products (Mate & Alcalde, 2017). These results expand the scope of *A. niger* laccase beyond environmental biotechnology into value-added bioproduct synthesis.

The present findings confirm the feasibility of producing and purifying laccase from *A. niger* under surface fermentation, yielding an enzyme with properties suitable for diverse biotechnological applications. Compared to white-rot fungi, *A. niger* offers advantages of GRAS status, genetic amenability, and robust growth on agro-residues (Jin et al., 2016). However, the enzyme's moderate thermostability may limit its application in certain industrial sectors such as textile processing, where high temperatures are common. Future strategies could include immobilization on nanomaterials to enhance operational stability and reusability, or directed evolution to tailor thermal and pH profiles (Mate & Alcalde, 2017).

Additionally, the cost-effectiveness of SSF using wheat bran and other agro-residues supports sustainability and circular bioeconomy goals. Integration of SSF-based production with downstream purification pipelines offers a viable model for scale-up. Further techno-economic assessments are warranted to evaluate feasibility at pilot and industrial scales.

5.Conclusion

Overall, the study demonstrates that *A. niger* is a promising source of extracellular laccase with favorable biochemical properties for environmental and industrial applications. The successful purification to homogeneity and demonstration of activity across dyes, phenols, and synthetic reactions confirm its versatility. These findings contribute to the expanding body of research on fungal laccases and highlight opportunities for further bioprocess optimization, protein engineering, and application in sustainable biotechnologies.

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