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PURIFICATION AND BIOTECHNOLOGICAL APPLICATIONS OF LACCASE PRODUCED BY ASPERGILLUS NIGER UNDER SURFACE FERMENTATION TECHNIQUE

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Abstract

This study focuses on the production, purification, and biotechnological applications of laccase enzyme synthesized by Aspergillus niger under surface fermentation conditions. Laccases are multi-copper oxidases with wide substrate specificity, enabling their use in diverse industries such as textiles, food processing, and environmental remediation. A locally isolated strain of A. niger was cultivated, and fermentation parameters were systematically optimized using the one-factor-at-a-time (OFAT) approach. Carbon and nitrogen sources, pH, temperature, incubation period, and metal ion supplementation were evaluated to enhance enzyme yield. Sucrose (4 g/100 mL) and yeast extract (5 g/100 mL) were identified as optimal nutritional sources, while pH 6.0, incubation at 30 °C, and a 7-day fermentation period yielded maximum extracellular activity (0.416 ± 0.016 U/mL). Copper (0.5 g/100 mL) acted as a strong inducer, whereas higher concentrations of zinc and iron salts inhibited production. Biochemical characterization revealed maximum activity at 37 °C and pH 5.5, confirming the enzyme's moderate thermostability and acidic optimum. Partial purification by TLC and ammonium sulfate precipitation enriched enzymatic activity. The crude laccase displayed strong bioremediation potential: textile effluents were decolorized up to 50.78% after 10 days, while tannery wastewater exhibited rapid decolorization of 66.6% within 24 hours. These findings highlight the industrial significance of A. niger laccase as an eco-friendly biocatalyst and establish optimized conditions for its scalable production and application in wastewater treatment.

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INTRODUCTION

Laccases (benzenediol oxygen oxidoreductases, EC 1.10.3.2) represent a pivotal group of multi-copper oxidase enzymes that have garnered significant attention in the field of biotechnology due to their remarkable capacity to catalyze the oxidation of a broad spectrum of phenolic and non-phenolic compounds, concomitantly reducing molecular oxygen to water [1]. First identified in the Japanese lacquer tree Rhus vernicifera in 1883, laccases are ubiquitously produced by diverse organisms, including plants, insects, bacteria, and fungi[2]. Among these, fungal laccases, particularly from white-rot basidiomycetes and assomycetous fungi like Aspergillus niger, are of paramount industrial interest because of their role in lignin degradation and their high redox potential [3].The enzyme's versatility stems from its low substrate specificity, enabling its application in numerous sectors, including textile dye decolorization, pulp bleaching, food processing, beverage stabilization, biosensing, and bioremediation of environmental pollutants [4]. The ability of laccases to detoxify hazardous and recalcitrant compounds, such as synthetic dyes in industrial effluents, positions them as green catalysts crucial for advancing sustainable industrial processes [5].

The production of laccase on a commercially viable scale is predominantly reliant on microbial fermentation. Two primary techniques are employed: submerged fermentation (SmF) and solid-state fermentation (SSF) [6]. While SSF is often cited for its high productivity using agro-industrial wastes, SmF remains the most widely adopted method for industrial enzyme production due to superior control over parameters like pH, temperature, oxygen transfer, and nutrient availability [7].The filamentous fungus Aspergillus niger is a particularly promising candidate for laccase production. It is generally recognized as safe (GRAS) by the US FDA, possesses a robust secretory machinery, and can be cultivated on inexpensive substrates [8]. However, the inherent low yield of native laccase production in wild strains remains a major bottleneck for its widespread industrial adoption [9].

Consequently, significant research efforts have been directed toward optimizing the fermentation process to enhance laccase titers. Studies have systematically investigated the impact of various cultural and nutritional parameters [10]. The choice of carbon and nitrogen sources is critical; for instance, sucrose and glucose [11], are commonly used carbon sources, while complex organic nitrogen sources like yeast extract and peptone have been shown to significantly boost enzyme production compared to inorganic salts [12]. Furthermore, the presence of inducers, especially copper (Cu^{2+}), which is integral to the laccase active site, often dramatically enhances transcription and enzyme activity [13].Physical parameters such as initial pH, incubation temperature, incubation period, and inoculum size also profoundly influence fungal physiology and enzyme synthesis [14]. Most fungal laccases exhibit optimal activity under acidic conditions (pH 4.0–6.0) and moderate temperatures (30–37°C) [15]. The "one-factor-at-a-time" (OFAT) approach, despite its limitations, remains a practical and widely used method for establishing a foundational understanding of these complex variable interactions for a given microbial strain[16].

The successful application of laccase hinges not only on its production but also on its catalytic efficiency under specific conditions [12]. Therefore, the biochemical characterization of the crude or purified enzyme determining its optimal pH, temperature, thermal stability, and kinetic parameters (Km and Vmax) is an essential step in assessing its suitability for targeted industrial processes [17]. For instance, a laccase intended for wastewater treatment must be active and stable under the pH and temperature conditions typical of industrial effluents [18]. The ultimate validation of a produced laccase is its performance in real-world applications. Numerous studies have demonstrated the efficacy of fungal laccases in decolorizing dyes from textile and tannery wastewaters, often achieving degradation rates exceeding 50-80%, thereby offering an eco-friendly alternative to harsh chemical treatments [19].

Despite the extensive body of literature on laccase production, a significant research gap persists concerning the strain-specific optimization of Aspergillus niger under submerged fermentation conditions. While many studies report on laccases from white-rot fungi, the potential of assomycetes like A. niger is comparatively underexplored.

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Furthermore, many optimization studies focus on a limited set of parameters, and there is a lack of comprehensive research that integrates the optimization of nutritional components (carbon, nitrogen, metal inducers) with critical physical factors (pH, temperature, incubation time) for a local strain of A. niger. Moreover, a clear disconnect often exists between optimization for biomass growth and optimization for actual enzyme yield, necessitating a holistic approach. Finally, there is a need to not only produce the enzyme but also to directly demonstrate its efficacy in addressing a relevant environmental problem, such as the treatment of specific industrial effluents from local sources.

The existing literature lacks a comprehensive, integrated optimization strategy for a locally isolated strain of Aspergillus niger that simultaneously determines the optimal conditions for both its growth and extracellular laccase production using the OFAT method under submerged fermentation. Furthermore, a thorough biochemical characterization of the resulting crude enzyme and a direct demonstration of its application in bioremediating specific, locally sourced industrial wastewaters (textile and tannery effluents) remains underexplored.

The principal objectives of this research were to produce and optimize the yield of laccase enzyme from Aspergillus niger using submerged fermentation, and to explore its industrial applicability. This was achieved through a systematic optimization of critical fermentation parameters including carbon and nitrogen sources, metal ion inducers, pH, temperature, and incubation period via the one-factor-at-a-time (OFAT) approach to enhance extracellular laccase production. The crude enzyme was subsequently characterized for its functional activity under varying pH and temperature conditions and partially purified. The final objective was to evaluate the practical efficacy of the produced laccase in bioremediation by assessing its decolorization potential against industrial wastewaters from textile and tannery sources.

2 Materials and Methods

2.1 Procurement and Maintenance of Fungal Strain

The fungal strain Aspergillus niger was procured from the Department of Botany, GC University, Lahore. For routine culture maintenance, Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) media were prepared, adjusted to pH 7.0, and sterilized at 121 °C (15 psi) for 15–20 min. Approximately 5–6 mL of medium was dispensed aseptically into sterile test tubes and solidified in a slanting position to increase surface area. After inoculation with the fungal strain, slants were incubated and stored under aseptic conditions at 4 °C for further experimental use.

Figure 2.1: Slants for Aspergillus niger

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2.2 Inoculation of Fungal Strains
Pure Aspergillus niger cultures were aseptically transferred to PDA and MEA slants using an inoculating loop under a laminar airflow hood. Inoculated tubes were sealed, wrapped, and incubated at 27 °C for 7 days. Slants with vigorous growth were maintained as parent cultures, while others were sub-cultured to obtain fresh inoculum. Since PDA supported the best growth, it was used for preparing subsequent slants.

2.3 Fermentation Technique
Laccase production was carried out using a surface fermentation approach in Erlenmeyer flasks containing a defined medium. This technique was selected due to its cost-effectiveness and suitability for fungal enzyme production.

2.4 Preparation of Fermentation Medium
A self-modified medium was prepared using sucrose (5 g), yeast extract (5 g), KH_2PO_4 (2 g), $FeSO_4 \cdot 7H_2O$ (0.1 g), $ZnSO_4$ (0.1 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), $CuSO_4 \cdot 5H_2O$ (0.1 g), and $CaCl_2$ (0.1 g) dissolved in 100 mL distilled water. The pH was adjusted to 6.0 before sterilization at 121 °C for 15 min (15 psi). The medium was then inoculated with A. niger spore suspension for fermentation studies.

2.5 Preparation of Inoculum and Fermentation
A spore suspension of Aspergillus niger was prepared from actively growing PDA slants by adding sterile distilled water and vortexing to obtain ~10^6 spores/mL. The suspension was aseptically inoculated into the sterilized fermentation medium, and cultures were incubated at 30 °C for 10 days to maximize laccase production.

Figure 2.2: Spore suspension of autoclaved fermentation media

2.6 Determination of Laccase Enzyme
After 10 days of incubation, the fermented broth was processed to obtain both intracellular and extracellular enzyme fractions. Mycelia were separated from the medium by filtration, soaked in acetone, homogenized, and centrifuged at 4000 rpm for 20 min at 4 °C to extract intracellular laccase from the supernatant. The extracellular enzyme was obtained by centrifuging the culture filtrate under the same conditions, and the clear supernatant was collected. Both enzyme fractions were stored at 4 °C for subsequent laccase activity assays using guaiacol as substrate.

2.7 Enzyme Extraction and Assay
Laccase activity was determined from both extracellular and intracellular fractions. For extracellular enzyme, culture broth was centrifuged, and the clear supernatant was used directly for assay. For intracellular enzyme, fungal mycelia were homogenized in buffer, and the extract was centrifuged to obtain the enzyme fraction. Laccase activity in both extracts was quantified spectrophotometrically using guaiacol as the substrate, with absorbance measured at 465 nm.

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Figure 2.3: Stages of centrifugation of crude enzyme

2.8 Guaiacol Assay and Standard Curve

Laccase activity was determined spectrophotometrically using guaiacol as the substrate, with absorbance recorded at 465 nm. A standard curve was prepared from known guaiacol concentrations, which showed a strong linear relationship between absorbance and concentration confirming the reliability of the method for enzyme quantification. This calibration was applied to both extracellular and intracellular extracts of A. niger. The results indicated detectable laccase activity in both fractions, with extracellular extracts exhibiting comparatively higher activity, reflecting the secretion potential of the fungus under surface fermentation.

Table 2.1: Preparation of dilutions of Guaiacol

Concentration of solution (µg/100mL)	Volume of Sodium acetate buffer (mL)	Volume of stock solution (mL)
20	5.2	1
40	2.1	1
60	1.06	1
80	0.5	1
100	0.24	1
120	0.03	1

Table 2.2 outlines the preparation of guaiacol dilutions used to generate the standard curve for laccase activity determination. Different concentrations of guaiacol (20–120 µg/100 mL) were prepared by mixing appropriate volumes of sodium acetate buffer with varying amounts of the guaiacol stock solution. For example, a 20 µg/100 mL solution was obtained by mixing 5.2 mL of buffer with 1 mL of stock, while higher concentrations such as 100 and 120 µg/100 mL required smaller stock volumes (0.24 mL and 0.03 mL, respectively) diluted with 1 mL of buffer. This stepwise dilution ensured accuracy and consistency in the preparation of standards, which were later used to plot the calibration curve for quantifying laccase activity.

Table 2.2: Absorbance of Guaiacol

Serial No.	Concentrations (microgram)	Absorbance (mg/ml/min)
1	20	0.17
2	40	0.35
3	60	0.51
4	80	0.66
5	100	0.85
6	120	1.00

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Table 2.2 presents the relationship between guaiacol concentration (20–120 µg) and absorbance measured at 465 nm. A progressive increase in absorbance was observed with increasing substrate concentration, ranging from 0.17 mg/mL/min at 20 µg to 1.00 mg/mL/min at 120 µg. The linear correlation between concentration and absorbance confirms the accuracy of the standard curve, validating its use for calculating laccase activity in both extracellular and intracellular enzyme extracts.

2.9 Standard Curve:

1.00 One unit of laccase is released/produced as 1micromol of laccase (1U)/min/mL in fermentation medium.

The standard curve generated from guaiacol dilutions (20–120 µg) exhibited a strong linear relationship between concentration and absorbance at 465 nm. As guaiacol concentration increased, absorbance rose proportionally, confirming the accuracy and sensitivity of the assay method. The linear regression obtained from the curve provided a reliable equation for calculating laccase activity in unknown samples. This strong correlation validates guaiacol as a suitable chromogenic substrate for laccase quantification.

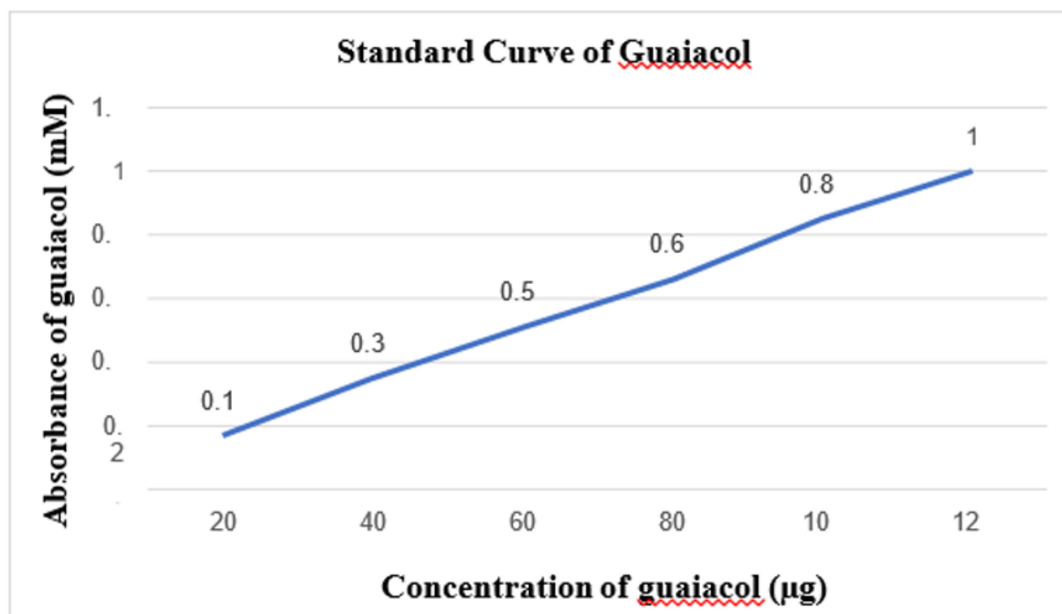

Figure 2.4: Standard curve of guaiacol oxidation showing the linear relationship between guaiacol concentration (20–120 µg) and absorbance at 465 nm, used for calculating laccase activity in enzyme extracts.

2.10 Assay of Laccase Enzyme

Laccase activity was determined using guaiacol as the substrate. Reaction mixtures contained sodium acetate buffer (10 mM, pH 5.5), guaiacol (2 mM), and enzyme extract, and were incubated at 30 °C for 15 min. Absorbance was measured at 450 nm against a control lacking enzyme. The assay was performed for both extracellular and intracellular extracts. Enzyme activity was expressed in international units (IU), where 1 IU corresponds to the amount of enzyme required to oxidize 1 µM guaiacol per minute, and calculated using the standard formula

$$\text{Enzyme activity (U/mL)} = \frac{A \times v}{t \times v \times \varepsilon}$$

where ***A*** = absorbance, ***V*** = total volume of mixture, ***t*** = incubation time, ***v*** = enzyme volume, and ***ε*** = extinction coefficient for guaiacol (0.6740 µM/cm).

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2.11 OPTIMIZATION OF FERMENTATION MEDIUM AND CONDITIONS FOR PRODUCTION OF LACCASE

Laccase production by Aspergillus niger was optimized using the one-factor-at-a-time (OFAT) approach, where individual parameters were varied while keeping others constant. Factors assessed included nutritional components such as different carbon and nitrogen sources, as well as varying concentrations of sucrose, yeast extract, and mineral salts (KH_2PO_4, $CaCl_2$, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $ZnSO_4$, and $CuSO_4$). Physicochemical conditions including pH (4–8), incubation temperature (20–40 °C), incubation period (up to 12 days), and inoculum size were also evaluated. The effect of each condition on enzyme yield was measured to identify the most favorable parameters for enhanced laccase production.

2.12 Effect of Carbon Sources, Nitrogen Sources, and pH on Laccase Production

The influence of nutritional and physicochemical factors on laccase yield was studied using a one-factor-at-a-time approach. Different carbon sources (sucrose, glucose, cellulose, fructose, lactose, maltose) were incorporated into the basal medium to assess their effect on enzyme production. Similarly, the role of nitrogen was evaluated using various sources, including urea, ammonium sulfate, ammonium nitrate, peptone, ammonia solution, and yeast extract. To determine the impact of medium acidity, pH was adjusted from 4 to 8 using 1N HCl or NaOH prior to sterilization. In all cases, media were autoclaved, inoculated, and incubated under identical conditions, and laccase activity was determined post-incubation using standard enzyme assay methods.

2.13 Effect of Medium Components and Environmental Factors on Laccase Production

To optimize laccase yield, different concentrations of medium components and environmental parameters were tested using the OFAT approach. Mineral salts including KH_2PO_4, $CaCl_2$, $FeSO_4 \cdot 7H_2O$, $ZnSO_4$, $CuSO_4 \cdot 5H_2O$, $MgSO_4 \cdot 7H_2O$, and yeast extract were individually varied within defined ranges (0.1–8 g/100 mL) while maintaining all other conditions constant. Similarly, sucrose, identified as the best carbon source, was further optimized at concentrations between 3–8 g/100 mL. Fermentation flasks were autoclaved, inoculated, and incubated under identical conditions, followed by laccase activity assays. In addition to medium composition, environmental factors such as incubation temperature (20–37 °C) and incubation period (up to 12 days) were also varied to evaluate their influence on enzyme production. The effect of each parameter was assessed individually, and results were used to identify optimal conditions for maximum laccase activity.

2.14 PRODUCTION OF LACCASE ENZYMEBY USING OPTIMUM INGREDIENTS

The laccase enzyme was produced by adding all the optimized fermentation ingredients to
100 mL of distilled water. The composition of the optimized medium. The medium contained sucrose (4 g/100 mL) as the primary carbon source, yeast extract (5 g/100 mL) as the nitrogen source, and mineral salts including KH_2PO_4 (2 g/100 mL), $FeSO_4 \cdot 7H_2O$ (0.1 g/100 mL), $ZnSO_4$ (0.1 g/100 mL), $MgSO_4 \cdot 7H_2O$ (0.5 g/100 mL), $CuSO_4 \cdot 5H_2O$ (0.5 g/100 mL), and $CaCl_2$ (2.5 g/100 mL). The pH was adjusted to 6.0 prior to sterilization at 121 °C for 15 min (15 psi). Inoculation was carried out with A. niger spore suspension under aseptic conditions, and cultures were incubated at 30 °C for 7 days, which supported maximum laccase production.

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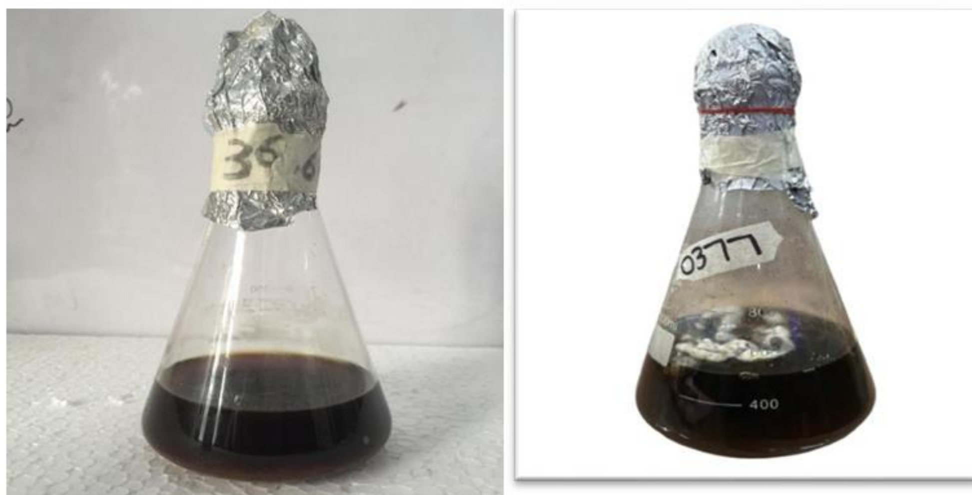

Figure 2.5: Fermentation media before and after inoculation

2.15 Characterization and Purification of Laccase

The crude laccase enzyme was characterized to determine its stability and activity under different conditions. Temperature effects were assessed by incubating enzyme–substrate mixtures at 25–60 °C, while pH dependence was evaluated across a range of 3.5–7.0 using sodium acetate buffer. Substrate concentration (1–50 mM guaiacol) was varied to examine enzyme kinetics. All assays were performed in triplicate, and activity was determined using standard spectrophotometric methods.

For purification, chloroform extraction was employed, where repeated partitioning separated the active enzyme fraction from impurities. Further analysis was performed using thin layer chromatography (TLC) on silica gel plates with a chloroform isopropanol water mobile phase. Separated enzyme components were visualized under UV light, and retention factor (Rf) values were calculated to confirm purification efficiency.

Figure 2.6: Adjustment of pH of fermentation media by pH meter at 6.5

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2.16 Statistical Analysis

Experimental data were analyzed using standard deviation and standard error to ensure accuracy and reliability, following the method of Steel and Torrie (1996). All experiments were performed in triplicate, and results are presented as mean ± SD.

2.17 Industrial Applications of Laccase

The potential of Aspergillus niger laccase in bioremediation was assessed through the decolonization of textile and tannery wastewaters. Textile wastewater, obtained from Nishat Mills Ltd. (Punjab, Pakistan), was characterized (pH 9.17, bluish tint, bleach-like odor) and treated in reaction mixtures containing sodium acetate buffer, effluent, and enzyme (ratio 5:4:1). Incubation was carried out at 30–35 °C, and decolonization was monitored visually and spectrophotometric ally at 480 nm. The percentage of decolonization was calculated by comparing absorbance values before and after treatment.

The laccase enzyme from Aspergillus niger demonstrated strong potential in the decolonization of both textile and tannery effluents. Textile wastewater obtained from Nishat Mills (pH 9.17, bluish tint) showed a gradual reduction in absorbance at 480 nm upon enzymatic treatment. Significant color loss was observed within the first 24 h, and the percentage decolorization increased progressively with time, confirming effective degradation of chromophoric compounds. These findings are in agreement with previous studies reporting fungal laccases as efficient agents for azo dye degradation and textile effluent detoxification [20].Tannery wastewater, which was initially dark blue with an objectionable odor (pH 9.15), also exhibited marked decolorization when incubated with laccase in sodium acetate buffer at pH 5.0. Absorbance at 470 nm decreased substantially, with more than half of the dye content degraded under optimal conditions. The enzyme's performance improved with extended incubation, consistent with earlier reports that fungal laccases require sufficient contact time for effective oxidation of recalcitrant phenolic and aromatic compounds [21].

3 Results and Discussion

3.1 Production and Systematic Optimization of Laccase by Aspergillus niger

The initial phase of this study confirmed the inherent capability of the locally sourced strain of Aspergillus niger to synthesize laccase enzyme under surface fermentation conditions. The baseline production, prior to any optimization, yielded promising results with extracellular laccase activity reaching 0.416 ± 0.020 U/mL and intracellular activity up to 0.484 ± 0.021 U/mL (Tables 3.1, 3.2). This established a strong foundation for further enhancement through media and process optimization.

A comprehensive One-Factor-At-A-Time (OFAT) strategy was employed to decipher the complex nutritional and environmental requirements for maximizing laccase yield. This involved a meticulous examination of macro/micronutrients and physical parameters. The capability of the fungal strain Aspergillus niger to synthesize and secrete laccase was first established under unoptimized surface fermentation conditions. Analysis of the extracellular culture supernatant revealed consistent production of the enzyme across three independent biological replicates. The measured laccase activities were 0.416 ± 0.020 U/mL, 0.361 ± 0.026 U/mL, and 0.323 ± 0.003 U/mL for replicates A, B, and C, respectively, yielding a mean baseline production of 0.367 ± 0.047 U/mL (mean ± SD, n=3). This initial quantification not only confirms the inherent laccase-producing potential of the isolate but also provides a critical benchmark against which the efficacy of subsequent fermentation optimization strategies could be rigorously evaluated. The observed variation between replicates is indicative of the natural biological variability inherent in fungal fermentation processes, while the relatively low standard deviation, particularly for replicate C, affirms the reliability of the assay methodology. This foundational data confirmed the suitability of Aspergillus niger for further investigation and medium enhancement to amplify laccase yield.

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Table 3.1: Production of Laccase in Extracellular extract from Aspergillus niger

Sr. No.	Sample	Enzyme activity (U/mL)
1	A	0.416 ± 0.020
2	B	0.361 ± 0.026
3	C	0.323 ± 0.003

Complementing the analysis of the extracellular fraction, the intracellular laccase activity of *Aspergillus niger* was also quantified under identical, unoptimized fermentation conditions. Enzyme assays performed on the mycelial extracts demonstrated a significant presence of laccase within the fungal cells. The specific activities for the biological replicates were determined to be **0.399 ± 0.007 U/mL**, **0.484 ± 0.021 U/mL**, and **0.414 ± 0.024 U/mL** for samples A, B, and C, respectively, resulting in a mean intracellular production of **0.432 ± 0.044 U/mL** (mean ± SD, n=3). Notably, the highest yield was observed in replicate B (0.484 U/mL), which exceeded the maximum activity found in the extracellular fraction. The relatively low standard deviations, particularly for sample A, indicate a high degree of precision in the analytical measurements for the intracellular extracts. This data confirms that a substantial portion of the total laccase synthesized by *Aspergillus niger* is retained intracellularly under the given conditions. The co-production of both extracellular and intracellular enzyme pools suggests a complex regulatory mechanism for laccase secretion in this strain, a factor that may be influenced by subsequent optimization of fermentation parameters. The robust intracellular activity further underscores the metabolic commitment of the fungus to laccase production and presents a potential target for extraction strategies to maximize the overall enzyme yield.

Table 3.2 Production of lacase in Intracellular extract of Aspergillus niger

Sr. No.	Samples	Enzyme activity (U/mL)
1	A	0.399 ± 0.007
2	B	0.484 ± 0.021
3	C	0.414 ± 0.024

3.2 Carbon and Nitrogen Source Optimization

The choice of carbon source is pivotal as it acts as the primary building block for both biomass and enzyme synthesis. Among the six carbon sources tested, sucrose emerged as the most superior, yielding the highest extracellular laccase activity (0.521 ± 0.024 U/mL at 4 g/100mL) (Table 3.3). This was closely followed by lactose and cellulose. Sucrose, a disaccharide, is easily hydrolyzed by fungi into glucose and fructose, providing a readily available and sustained energy source that efficiently drives metabolic pathways toward laccase production [22]. Conversely, fructose resulted in the lowest yield (0.199 ± 0.031 U/mL), suggesting a less efficient metabolic route for this monosaccharide in our specific strain.

Table 3.3: Effect of various sucrose concentration on the production of laccase extracellular extract

Sr. No.	Sucrose (g)	Laccase activity (U/mL)
1	3	0.298 ± 0.019
2	4	0.521 ± 0.024
3	5	0.341 ± 0.018
4	6	0.380 ± 0.009
5	7	0.342 ± 0.015
6	8	0.345 ± 0.022

The optimization of carbon source concentration revealed that sucrose levels significantly influenced extracellular laccase production by *Aspergillus niger*. The maximum enzyme activity of **0.521 ± 0.024 U/mL** was achieved at a concentration of **4 g/100mL sucrose**. A notable decrease in activity was

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observed at both higher and lower concentrations, with activity dropping to 0.341 U/mL at 5 g/100mL and remaining sub-optimal up to 8 g/100mL. This clear peak indicates that 4 g/100mL is the optimal sucrose concentration for laccase production under these conditions, suggesting the possibility of catabolite repression or inefficient metabolic routing at elevated sugar levels. This finding is critical for designing a cost-effective fermentation medium.

The relationship between sucrose concentration and the biomass yield of Aspergillus niger, measured as mycelial dry weight, is presented in Figure 3.1 Mycelial growth demonstrated a clear dependence on sucrose availability, increasing from 3.971 g at 3 g/100mL sucrose to a maximum of 6.196 g at 5 g/100mL sucrose. This indicates that 5 g/100mL is the optimal sucrose concentration for maximizing fungal biomass under the employed fermentation conditions.

Beyond this optimum, a further increase in sucrose concentration to 8 g/100mL resulted in a significant decline in growth to 2.973 g, suggesting the onset of catabolite repression or osmotic stress that inhibited fungal development.

However, a comparison with extracellular laccase activity data reveals a metabolic trade-off: the optimal sucrose level for biomass production (5 g/100mL) does not coincide with the optimum for laccase synthesis (4 g/100mL). This divergence suggests that while higher carbon availability up to 5 g/100mL supports increased fungal growth, the metabolic machinery for laccase production is most efficient at a slightly lower concentration of 4 g/100mL. This finding underscores the importance of optimizing carbon source levels specifically for the target metabolite rather than solely for biomass yield.

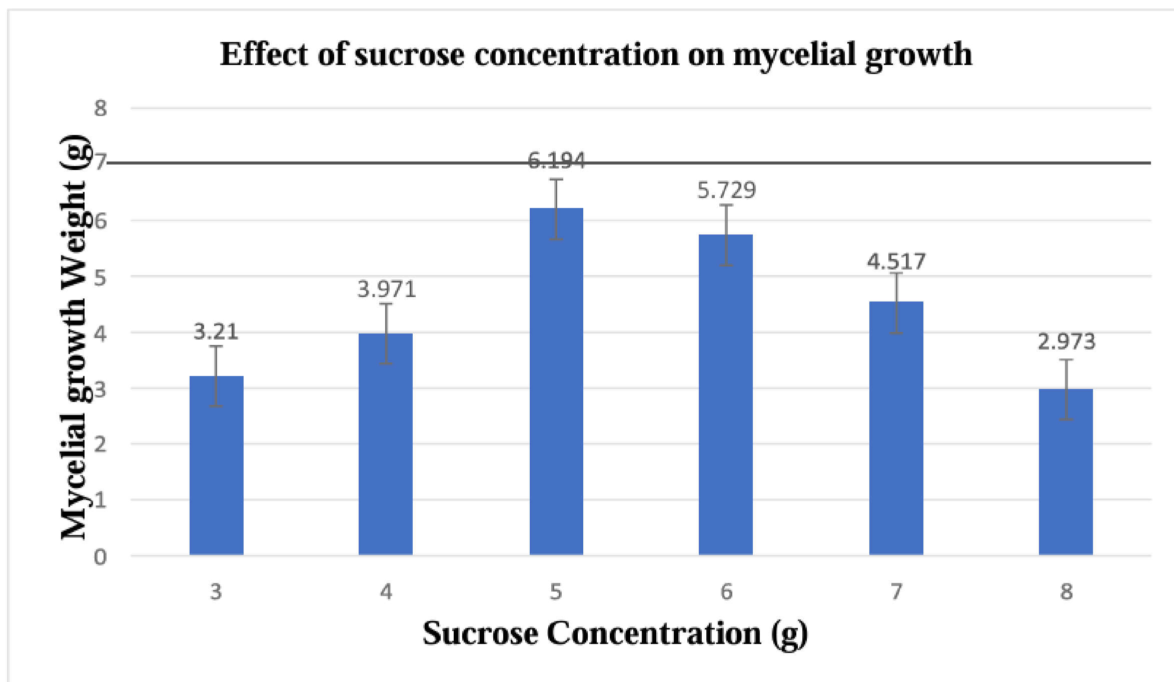

Figure 3.1: Effect of various sucrose concentration on mycelial growth.

Nitrogen sources profoundly influence fungal physiology and enzyme secretion. Our results demonstrated that yeast extract (a complex organic source) was unequivocally the best nitrogen source for extracellular laccase production, yielding a remarkable 1.450 ± 0.312 U/mL at 5 g/100mL

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(Table 3.4). Its complex composition of amino acids, peptides, vitamins, and trace minerals provides a complete nutritional package that likely induces the enzymatic machinery required for laccase synthesis. In contrast, simpler nitrogen sources like ammonium sulphate and peptone resulted in significantly lower activities (0.345 ± 0.442 U/mL and 0.312 ± 0.031 U/mL, respectively). Interestingly, for intracellular laccase, an inorganic source, ammonia solution, yielded the highest activity (0.602 ± 0.228 U/mL) (Table 3.5), hinting at a differential regulation between extracellular and intracellular enzyme pools based on nitrogen type.

The choice of nitrogen source profoundly influenced laccase production, with a distinct disparity observed between extracellular and intracellular enzyme activity (Tables 3.4 and 3.5). Yeast extract proved to be the superior nitrogen source for extracellular laccase secretion, yielding the highest activity of 1.450 ± 0.312 U/mL. In stark contrast, ammonium sulphate and peptone resulted in the lowest extracellular yields. Conversely, for intracellular laccase, ammonia solution supported the greatest activity (0.602 ± 0.228 U/mL), whereas urea was the least effective. This clear divergence indicates a strong influence of nitrogen source on the regulatory mechanisms governing laccase synthesis and secretion in Aspergillus niger. The results suggest that complex organic nitrogen sources like yeast extract are optimal for promoting the highest total laccase production, primarily by enhancing the extracellular yield, which is crucial for industrial enzyme recovery applications.

Table 3.4: Effect of various nitrogen sources the production of laccase extracellular extract

Sr. No.	Nitrogen sources	Laccase activity (U/mL)
1	Ammonia solution	0.802 ± 0.022
2	Yeast Extract	1.450 ± 0.312
3	Ammonium Nitrate	0.992 ± 0.414
4	Ammonium Sulphate	0.345 ± 0.442
5	Urea	0.933 ± 0.022
6	Peptone	0.312 ± 0.031

Table 3.5: Effect of various nitrogen sources the production of laccase intracellular

Sr. No.	Nitrogen sources	Laccase activity (U/mL)
1	Ammonia solution	0.602 ± 0.228
2	Yeast Extract	0.357 ± 0.013
3	Ammonium Nitrate	0.212 ± 0.009
4	Ammonium Sulphate	0.123 ± 0.011
5	Urea	0.112 ± 0.007
6	Peptone	0.132 ± 0.015

3 Physical Parameter Optimization

The physicochemical environment is a critical determinant of microbial growth and productivity. Our findings identified an optimum pH of 6.0 for laccase production (Extracellular: 0.391 ± 0.016 U/mL) (Table 3.6). This near-neutral pH is conducive for the growth of Aspergillus niger and likely stabilizes the enzyme during its secretion. A significant drop in yield was observed at pH 5 (0.219 ± 0.012 U/mL), indicating suboptimal fungal metabolism or enzyme instability under more acidic conditions. The initial pH of the fermentation medium was found to be a critical factor influencing extracellular laccase production by Aspergillus niger (Table 3.6). Enzyme activity was significantly affected across the tested pH range of 4 to 9. The optimum pH for laccase secretion was identified as 6.0, yielding the highest activity of 0.391 ± 0.016 U/mL. A sharp decrease in activity was observed at pH 5 (0.219 ± 0.012 U/mL), representing the lowest yield, while a more gradual decline occurred under alkaline conditions, with activity measuring 0.317 U/mL at

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pH 8. This narrow optimal range suggests that laccase production and/or stability is highly sensitive to the extracellular ionic environment, with a clear preference for a slightly acidic pH. These findings are essential for defining the precise cultural conditions required to maximize enzyme yield in subsequent fermentation processes.

Table 3.6: Effect of various pH values on the production of laccase extracellular extract

Sr. No.	pH	Laccase activity (U/mL)
1	4	0.367 ± 0.023
2	5	0.219 ± 0.012
3	6	0.391 ± 0.016
4	7	0.342 ± 0.011
5	8	0.317 ± 0.009
6	9	0.349 ± 0.016

The ideal incubation temperature was found to be 30°C (Extracellular: 0.299 ± 0.026 U/mL) (Table 3.7). This temperature represents a balance between efficient metabolic activity and the avoidance of thermal stress or denaturation of nascent enzymes. A fermentation period of 7 days was optimal for harvesting, yielding peak extracellular activity (0.377 ± 0.026 U/mL) (Table 3.7). This timeline corresponds with the late-log to early-stationary phase of fungal growth, where secondary metabolite production, including enzymes like laccase, is typically highest. The optimization of physical fermentation parameters revealed that both incubation temperature and duration are pivotal for maximizing extracellular laccase yield (Tables 3.6 and 3.7). A temperature of 30°C was identified as optimal, producing the highest extracellular laccase activity of 0.299 ± 0.026 U/mL. Activity was substantially lower at temperatures diverging from this optimum, particularly at the sub-optimal 20°C. Furthermore, the incubation period significantly influenced production, with a 7-day incubation yielding the peak activity of 0.377 ± 0.026 U/mL. Enzyme production markedly declined with extended incubation beyond this point, falling to its lowest level (0.141 U/mL) by day 13. This pattern suggests that laccase synthesis in Aspergillus niger is a secondary metabolic process that peaks during the late growth phase, after which enzyme degradation or inactivation likely occurs. The convergence of these findings establishes that cultivating the fungus at 30°C for 7 days provides the ideal conditions for extracellular laccase production under the applied fermentation system.

Table 3.6: Effect of various incubation temperature on the production of laccase extracellular extract

Sr. No.	Temperature (°C)	Laccase activity (U/mL)
1	20	0.149 ± 0.027
2	25	0.153 ± 0.022
3	27	0.247 ± 0.013
4	30	**0.299 ± 0.026**
5	37	0.165 ± 0.021

Table 3.7: Effect of various incubation period on the production of laccase extracellular extract

Sr. No.	Incubation period (Days)	Laccase activity (U/mL)
1	3	0.351 ± 0.016
2	5	0.262 ± 0.025
3	**7**	**0.377 ± 0.026**
4	9	0.259 ± 0.032
5	11	0.156 ± 0.021
6	13	0.141 ± 0.029

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3.4 Metal Ion Induction and Inhibition

The role of metal ions as enzyme co-factors was strikingly evident. Copper ($CuSO_4$), a core component of the laccase active site, showed a strong inductive effect. A concentration of 0.5 g/100mL yielded the highest extracellular activity (0.404 ± 0.052 U/mL) (Table 3.9). This aligns perfectly with the established literature where copper is known to upregulate laccase gene expression [2]. Similarly, Calcium ($CaCl_2$) at 2.5 g/100mL enhanced production (0.367 ± 0.062 U/mL) (Table 3.8), likely by stabilizing the enzyme structure and membrane integrity.

Conversely, Zinc ($ZnSO_4$) and higher concentrations of Iron ($FeSO_4$) appeared to be inhibitory. $ZnSO_4$ at 2.5 g/100mL resulted in the lowest extracellular activity (0.211 ± 0.019 U/mL) for that salt (Table 3.8), and $FeSO_4$ at 2 g/100mL was also strongly inhibitory (0.100 ± 0.022 U/mL). This inhibition could be due to metal toxicity at higher concentrations or competitive interference with the enzyme's copper centers. The supplementation of copper ($CuSO_4$) and zinc ($ZnSO_4$) salts in the fermentation medium had a distinct and contrasting impact on extracellular laccase production (Tables 3.9 and 3.8). The addition of $CuSO_4$ was highly stimulatory, with an optimal concentration of 0.5 g/100mL yielding the highest activity of 0.404 ± 0.052 U/mL. This aligns with the known role of copper as a central component of the laccase active site and its function as a transcriptional inducer of laccase genes. In stark contrast, $ZnSO_4$ demonstrated an inhibitory effect on production. While a low concentration of 0.1 g/100mL showed moderate activity (0.288 U/mL), any further increase resulted in a progressive decline in enzyme yield, with the lowest activity (0.211 U/mL) observed at the highest concentration of 2.5 g/100mL. This clear divergence underscores the metal ion specificity of laccase biosynthesis in Aspergillus niger; copper is essential for enhancing production, whereas zinc appears to be inhibitory at higher concentrations, potentially due to toxic effects or competitive inhibition. These results are critical for formulating a trace element composition that maximizes laccase yield by including optimal Cu^{2+} levels while avoiding Zn^{2+} over supplementation.

Table 3.8: Effect of various concentration ZnSO4 of on the production of laccase extracellular extract

Sr. No.	$ZnSO_4$ (g)	Laccase activity (U/mL)
1	**0.1**	**0.288 ± 0.019**
2	0.5	0.217 ± 0.009
3	1	0.264 ± 0.015
4	1.5	0.235 ± 0.000
5	2	0.221 ± 0.024
6	2.5	0.211 ± 0.019

Table 3.9: Effect of various concentration CuSO4 of on the production of laccase extracellular extract

Sr. No.	$CuSO_4$ (g)	Laccase activity (U/mL)
1	0.1	0.322 ± 0.021
2	0.5	0.404 ± 0.052
3	1	0.265 ± 0.031
4	1.5	0.341 ± 0.021
5	2	0.353 ± 0.024
6	2.5	0.329 ± 0.035

The integration of all these optimized parameters into a single fermentation medium culminated in a highly efficient production system, consistently yielding elevated extracellular laccase activity of 0.416 ± 0.016 U/mL. The

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culmination of the fermentation parameter optimization process was validated by cultivating Aspergillus niger in a medium composed of the identified optimum ingredients and conditions. As quantified in Table 3.10 and visually summarized in Figure 3.2, this optimized protocol successfully enhanced extracellular laccase production. The resulting enzyme activities were significantly high and consistent across replicates, with the highest yield reaching 0.416 ± 0.016 U/mL (Sample A). The low standard deviations associated with these measurements indicate excellent reproducibility and robustness of the optimized fermentation process. The convergence of these results confirms that the systematic, one-factor-at-a-time optimization of nutritional and physical parameters including carbon source, nitrogen source, metal ions, pH, temperature, and incubation period was effective in establishing a reliable and efficient protocol for maximizing laccase production from Aspergillus niger.

Table 3.10: Laccase enzyme production using optimum ingredients in extracellular extract

Sr. No.	Sample	Enzyme activity (U/mL)
1	A	0.416 ± 0.016
2	B	0.370 ± 0.019
3	C	0.359 ± 0.020

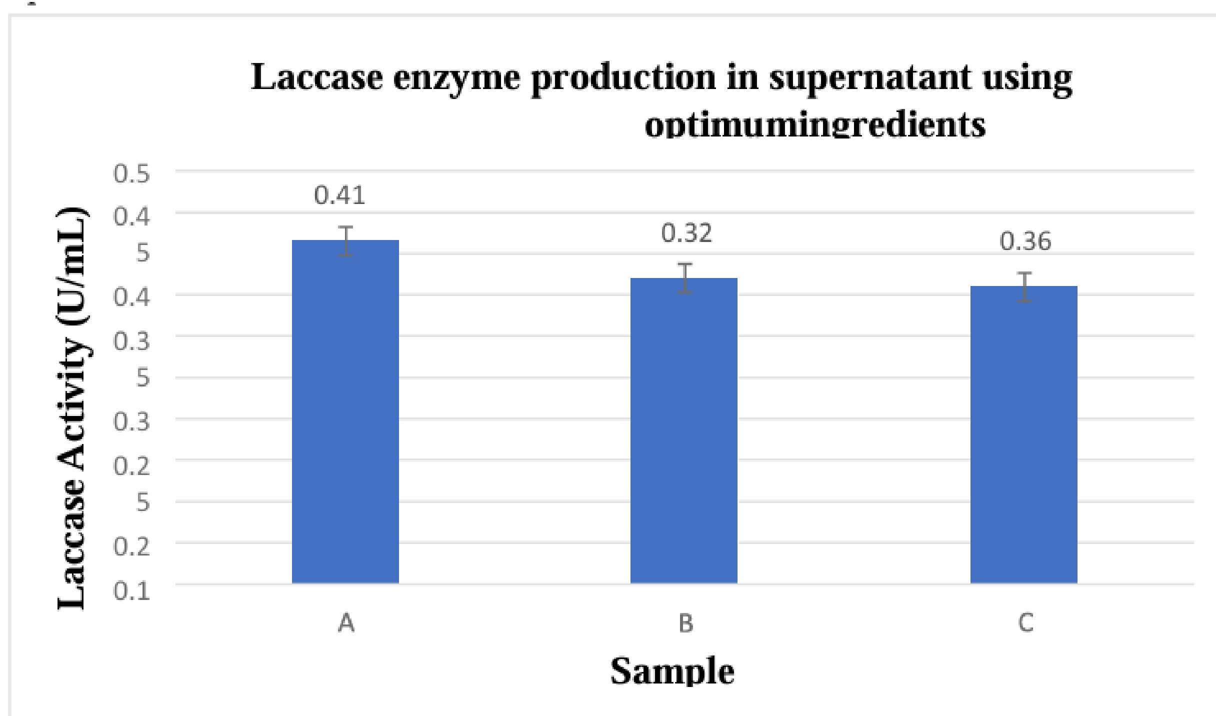

Figure 3.2: Laccase production in supernatant using optimum ingredients

The systematic optimization of fermentation parameters via the one-factor-at-a-time (OFAT) approach successfully identified the ideal conditions for maximizing laccase production by Aspergillus niger. The optimal medium formulation consisted of sucrose (4 g/100mL) as the carbon source and yeast extract (5 g/100mL) as the organic nitrogen source, supplemented with key inorganic salts including KH_2PO_4 (2 g/100mL), $CaCl_2$ (2.5 g/100mL), and $CuSO_4$ (0.5 g/100mL), the latter acting as a crucial inducer. Physical cultivation parameters were equally critical, with an initial pH of 6.0, an incubation temperature of 30°C, and a 7-day fermentation period yielding the highest enzyme titers. Validation experiments using this optimized recipe confirmed a significant enhancement in production, achieving a consistent and high extracellular laccase activity of up to 0.416 ± 0.016 U/mL. The low

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standard deviation across replicates underscores the robustness and reproducibility of the optimized process, establishing a reliable foundation for the efficient and scalable production of this industrially relevant enzyme.

Table 3.11: Optimized fermentation conditions for enhanced laccase production by *Aspergillus niger*.

Parameter	Optimal Condition	Laccase Activity (U/mL)	Significance / Proposed Role
Carbon Source	Sucrose, 4 g/100mL	0.521 ± 0.024	Preferred, easily metabolizable energy source
Nitrogen Source	Yeast Extract, 5 g/100mL	1.450 ± 0.312	Complex nutrition induces enzyme synthesis
pH	6.0	0.391 ± 0.016	Optimal for fungal growth and enzyme stability
Temperature	30°C	0.299 ± 0.026	Balances metabolic rate and avoids denaturation
Incubation Period	7 days	0.377 ± 0.026	Coincides with peak secondary metabolite production
Inducer ($CuSO_4$)	0.5 g/100mL	0.404 ± 0.052	Core component of laccase active site; gene upregulator
Stabilizer ($CaCl_2$)	2.5 g/100mL	0.367 ± 0.062	Enhances enzyme and membrane stability

3.5 Biochemical Characterization of the Crude Laccase Enzyme

Understanding the catalytic properties of the crude laccase is essential for determining its potential applications. The enzyme exhibited maximum activity at **37°C (0.399 ± 0.015 U/mL)** and retained over 90% of its activity at 40°C (**0.362 ± 0.016 U/mL**) (Table 3.12, Fig. 3.3). This moderate thermostability is advantageous for industrial processes that often operate at elevated temperatures. The characterization of the crude laccase enzyme's activity across a pH gradient revealed a distinct optimal range for catalytic function, as detailed in Table 4.13 and illustrated in Figure 3.3. Enzyme activity was significantly influenced by the reaction pH, demonstrating a clear peak at pH 5.5, which yielded the maximum activity of 0.319 ± 0.007 U/mL. Activity was substantially lower in more acidic conditions (e.g., 0.253 U/mL at pH 3.5) and declined gradually under neutral conditions (0.271 U/mL at pH 7.0). This narrow pH optimum is a characteristic feature of many fungal laccases and is crucial for defining the operational parameters for the enzyme's application. The sharp decline in activity outside this optimal range underscores the sensitivity of the enzyme's tertiary structure and the ionization state of its active site to the extracellular environment. These findings confirm that the laccase produced by Aspergillus niger under these conditions is an acidic laccase, informing the necessary pH control for its effective use in subsequent biotechnological processes.

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Table 3.12: Effect of temperature on the activity of laccase enzyme

Sr. No.	Temperature (°C)	Laccase activity (U/mL
1	30	0.121 ± 0.012
2	30	0.183 ± 0.014
3	35	0.275 ± 0.021
4	37	0.399 ± 0.015
5	40	0.362 ± 0.016
6	45	0.316 ± 0.018
7	50	0.281 ± 0.012
8	60	0.163 ± 0.015

The effect of pH on the activity of the crude laccase enzyme was determined across a range of pH values (3.5 to 7.0), as presented in Table 3.13. The results demonstrate that enzymatic activity is highly dependent on the pH of the reaction environment. The optimum pH for laccase activity was identified as 5.5, with the highest observed activity of 0.319 ± 0.007 U/mL. A sharp decline in activity was noted under more acidic conditions (pH 3.5–4.5), while a gradual decrease occurred as the pH approached neutral (pH 6.0–7.0). This pronounced peak at pH 5.5 indicates that the enzyme's catalytic efficiency is highly sensitive to the protonation state of key residues in its active site, a characteristic typical of many fungal laccases. These findings are critical for defining the optimal operational conditions for utilizing this laccase in subsequent applications, particularly in processes requiring slightly acidic environments.

Table 3.13: Effect of pH on activity of laccase

Sr. No.	pH	Laccase activity (U/mL
1	3.5	0.253± 0.024
2	4	0.248 ± 0.020
3	4.5	0.254 ± 0.016
4	5	0.281 ± 0. 001
5	5.5	0.319 ± 0.007
6	6	0.306 ± 0.019
7	6.5	0.301 ± 0.011
8	7	0.271 ± 0.015

The enzyme displayed a characteristic **acidic pH optimum of 5.5 (0.319 ± 0.007 U/mL)** (Table 3.13, Figure 3.3), which is a typical trait of many fungal laccases. This pH profile makes it particularly suitable for applications in the food and beverage industry (e.g., juice clarification) and for treating acidic industrial effluents [23].

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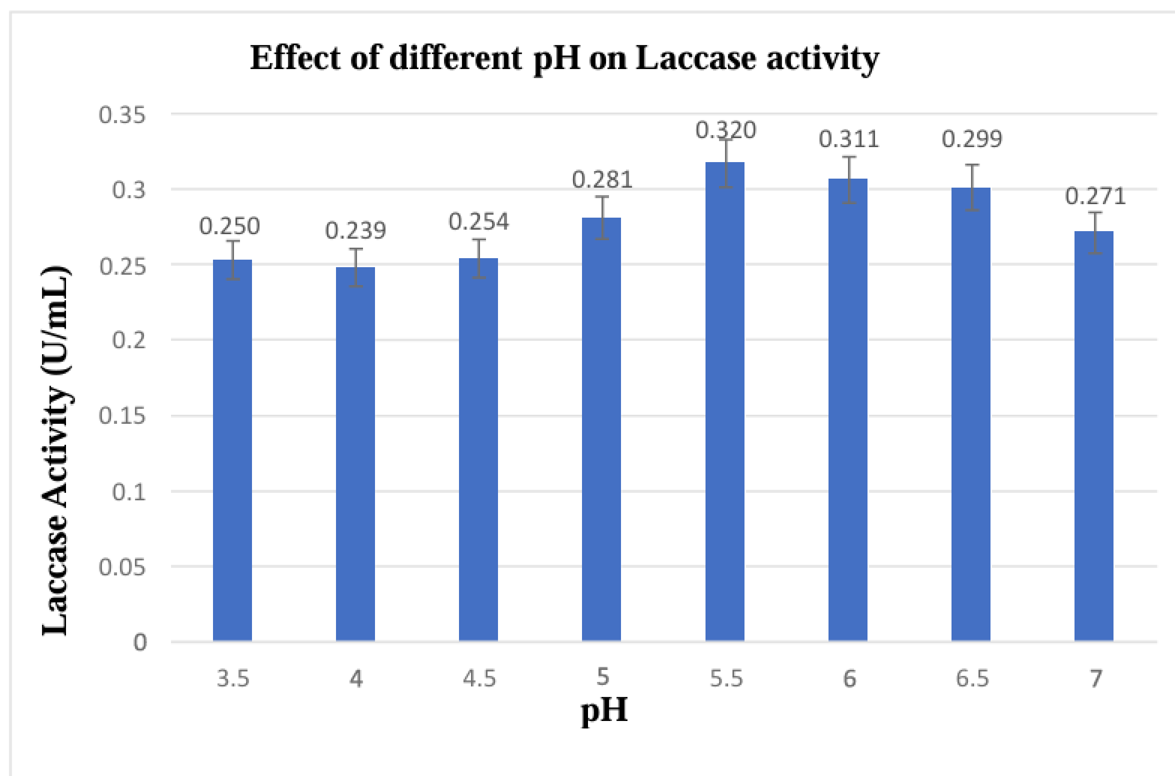

Figure 3.3: Effect of different pH on laccase activity

3.6 Effect of substrate concentration

Kinetic analysis using guaiacol as a substrate revealed a classic Michaelis-Menten relationship. The activity increased linearly with substrate concentration, reaching a **Vmax at approximately 30 mM (0.299 ± 0.015 U/mL)** and plateauing thereafter due to saturation of all available enzyme active sites. The influence of substrate concentration on laccase enzyme activity was evaluated using guaiacol as the substrate, with results detailed in Table 4.34 and graphically represented in Figure 4.18. Enzyme activity exhibited a strong dependence on substrate availability, increasing linearly with rising guaiacol concentrations from 1 mM to 30 mM. The maximum activity of 0.299 ± 0.015 U/mL was observed at 30 mM guaiacol, indicating saturation of the enzyme's active sites at this concentration. Beyond this point, further increases in substrate concentration (40 mM and 50 mM) resulted in a plateau and slight decline in activity, consistent with classical Michaelis-Menten kinetics where the enzyme becomes saturated and additional substrate does not enhance the reaction rate. This saturation kinetics pattern suggests that 30 mM guaiacol represents the approximate optimal concentration for achieving peak catalytic efficiency under the tested conditions. The clear saturation curve provides valuable insight into the enzyme's substrate affinity and is essential for determining kinetic parameters such as Km and Vmax, which are critical for evaluating the enzyme's applicability in industrial processes requiring high substrate conversion rates.

Table 3.14: Effect of substrate concentration

Sr. No.	Guaiacol concentration (mM)	Laccase activity (U/mL)
1	1	0.051 ± 0.023
2	3	0.083 ± 0.012
3	5	0.099 ± 0.001
4	10	0.183 ± 0.014

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5	20	0.204 ± 0.021
6	30	0.299 ± 0.015
7	40	0.291 ± 0.018
8	50	0.291 ± 0.025

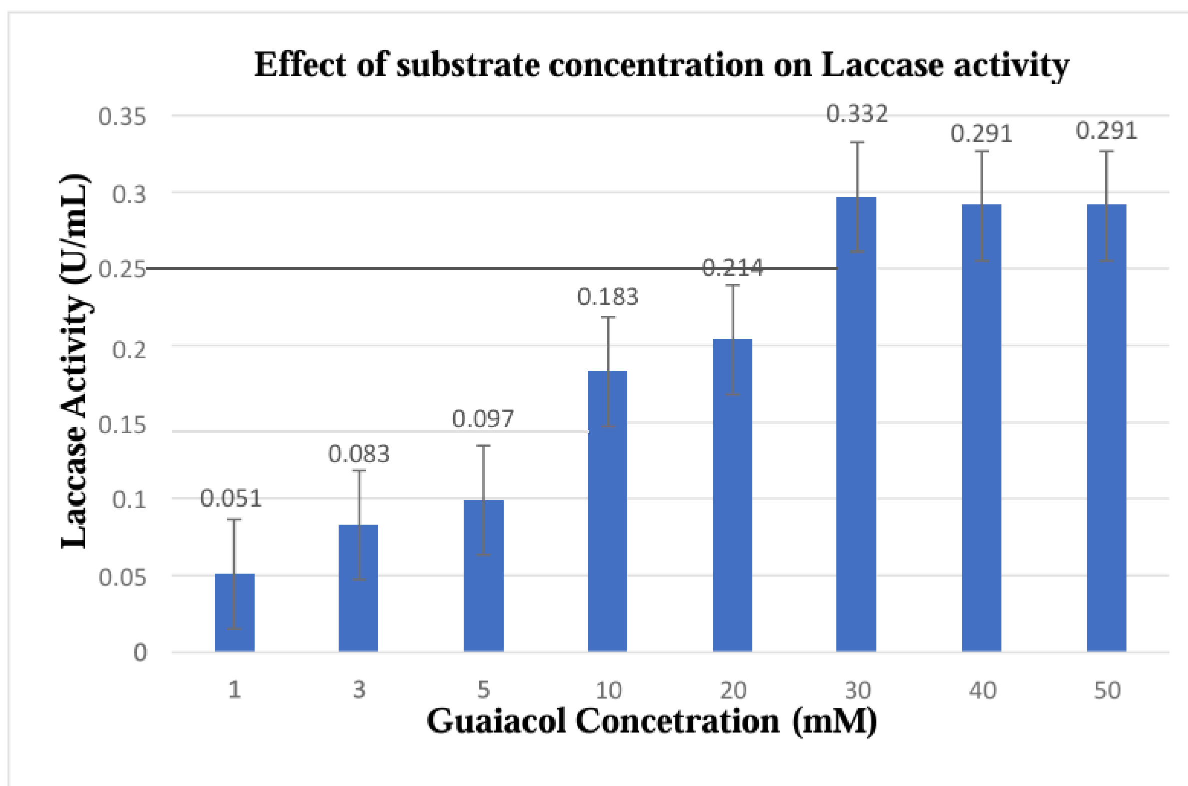

Figure 3.4: Effect of substrate concentration on laccase activity

3.7 Purification and Enrichment of Laccase

A two-step purification process was implemented. Initial separation using Thin Layer Chromatography (TLC) successfully resolved the enzyme mixture, with one fraction showing a significantly enriched activity of **0.755 ± 0.019 U/mL** (Table 3.15, Figure 3.5), demonstrating the effectiveness of TLC as a quick analytical tool. Subsequent protein precipitation using ammonium sulfate saturation and dialysis yielded a partially purified enzyme preparation with a final activity of **0.399 ± 0.019 U/mL** (Table 3.16, Figure 3.5). This process effectively concentrated the active enzyme while removing contaminants.

The purification of laccase enzyme from Aspergillus niger was systematically evaluated using thin-layer chromatography (TLC) and ammonium sulfate precipitation, as summarized in Table 3.15 and Table 3.16. TLC-based purification significantly enhanced laccase activity, with Sample B exhibiting the highest purified activity of 0.755 ± 0.019 U/mL (Table 3.15), confirming the effectiveness of this method in isolating active enzyme fractions. Subsequent purification via ammonium sulfate precipitation further refined the enzyme, yielding a maximum activity of 0.399 ± 0.019 U/mL in Sample B (Table 3.16). The comparative reduction in activity between TLC and ammonium sulfate purification steps suggests potential trade-offs between purity and enzyme stability or recovery efficiency. Notably, the successful concentration of laccase through these methods underscores the robustness of the purification pipeline. These results validate the combined use of TLC and ammonium sulfate precipitation for obtaining enzymatically active

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laccase, which is critical for downstream industrial applications requiring high-purity enzyme preparations. The reproducibility of these results across replicates (low standard deviations) further confirms the reliability of the purification strategy.

Table 3.15: TLC of laccase enzyme

Sr. No.	Samples	Laccase activity (U/mL)
1	A	0.534 ± 0.015
2	B	**0.755 ± 0.019**
3	C	0.657 ± 0.022

Table 3.16: Purification of laccase enzyme

Sr. No.	Samples	Laccase activity (U/mL)
1	A	0.275 ± 0.007
2	B	**0.399 ± 0.019**
3	C	0.324 ± 0.019

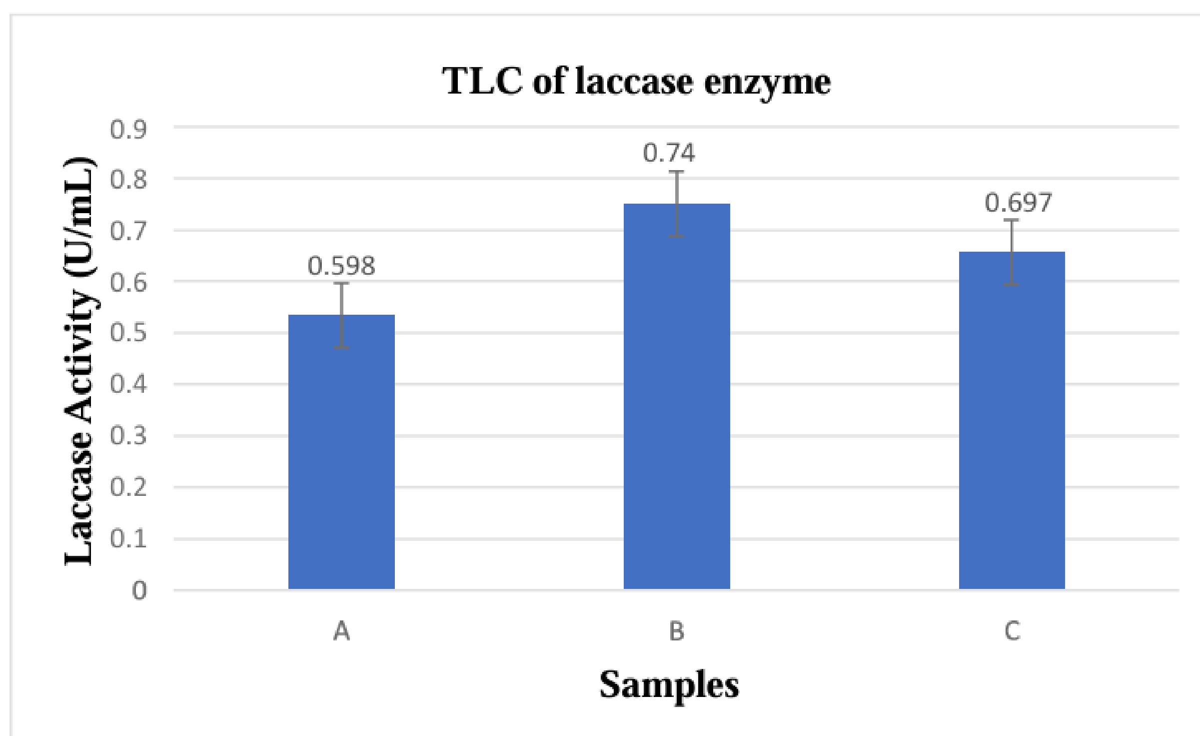

Figure 3.5: TLC of laccase enzyme

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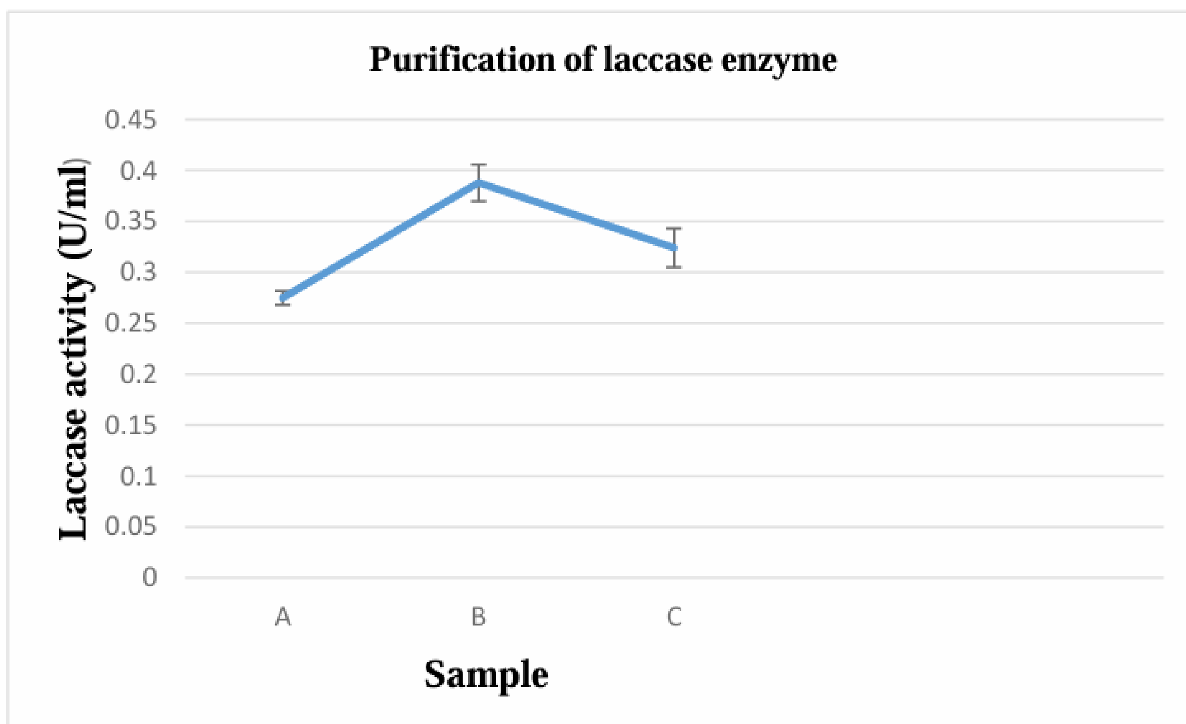

Figure 3.6: Graph showing purification of laccase enzyme

3.8 Evaluation of Biotechnological Potential in Wastewater Remediation

The ultimate objective of this work was to harness the produced laccase for an environmentally significant application: bioremediation of industrial wastewater.

3.8.1 Textile Wastewater Decolorization

The crude laccase preparation demonstrated a strong, time-dependent decolorization capability against recalcitrant textile dyes. The decolorization efficiency progressively increased from **1.69% at 24 hours** to **50.78% after 240 hours (10 days)** of treatment. This gradual increase suggests a continuous enzymatic breakdown of complex polymeric dyes into simpler, colorless molecules. The visual fading of the characteristic bluish tint of the wastewater corroborated the spectrophotometric data.

The efficacy of Aspergillus niger-derived laccase in decolorizing industrial textile wastewater was quantitatively assessed over a 10-day period, as detailed in Table 3.17 and visualized in Figure 3.7. The enzyme exhibited a time-dependent decolorization profile, with degradation efficiency progressively increasing from 1.69% at 24 hours to 50.78% by day 10. The most significant incremental increase occurred between days 2 and 4 (5.50% to 24.49%), suggesting accelerated enzymatic activity during this phase, likely due to optimal enzyme-substrate interaction. The subsequent gradual decline in the rate of decolorization beyond day 7 may indicate saturation of reactive sites or partial enzyme inactivation over extended incubation. These results demonstrate the potential of A. niger laccase as a biocatalytic agent for textile effluent treatment, achieving substantial decolorization through oxidative degradation of complex dyes. The progressive nature of the decolorization underscores the importance of retention time in designing bioremediation processes, while the >50% removal efficiency highlights the enzyme's practical relevance for sustainable wastewater management strategies in the textile industry.

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Table 3.17: Discoloration of textile industrial waste water during 10 days by laccase enzyme extracted from Aspergillus niger Absorbance of control (dye + medium, i.e initial absorbance) at 480 nm was 2.830 ± 0.0515.

Sr. No	Time (days)	Percentage of decolourization (%)
1	24	01.69 %
2	48	05.50 %
3	72	24.49 %
4	96	27.98 %
5	120	36.47 %
6	144	43.34 %
7	168	45.06 %
8	192	45.82 %
9	216	48.25 %
10	240	50.78 %

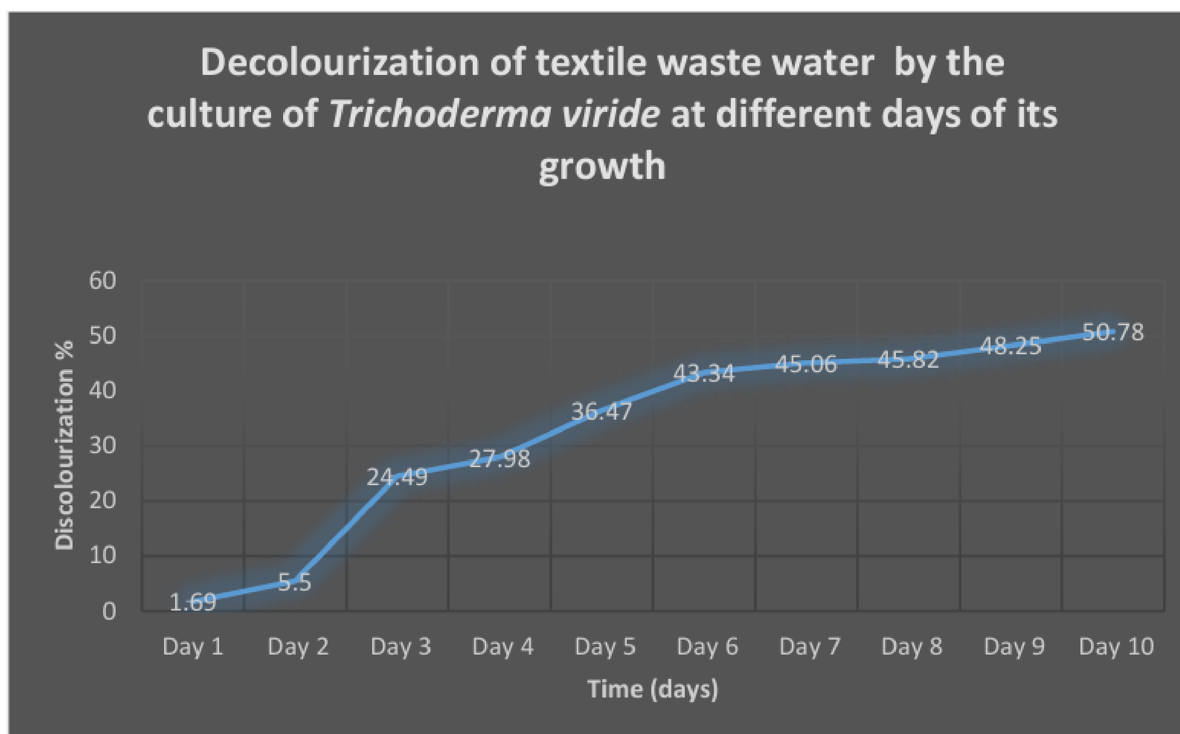

Figure 3.7: Decolourization of textile waste water by the culture of Aspergillus niger at different days of its growth

3.8.2 Tannery Effluent Decolorization

The enzyme was remarkably effective against the highly polluted and toxic tannery effluent. A rapid decolorization profile was observed, reaching a substantial **66.6 ± 3.08% reduction in absorbance at 470 nm within just 24 hours** (Table 3.18, Figure 3.8). The efficiency of the *crude* extract is particularly noteworthy, as it suggests that expensive and time-consuming purification may not be necessary for large-scale bioremediation applications, significantly improving economic feasibility.

The efficiency of crude laccase from Aspergillus niger in decolorizing tannery effluent was evaluated over a 24-hour period, as quantitatively detailed in Table

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3.18 and graphically represented in Figure 3.8. The enzyme exhibited a time-dependent increase in decolorization efficiency, with the extent of degradation rising progressively from 13.0 ± 1.00% at 1 hour to 66.6 ± 3.08% at 24 hours. The most notable acceleration in decolorization occurred between 5 and 6 hours, with activity increasing from 35.4% to 40.0%, suggesting sustained catalytic performance during this period. The highest decolorization rate was achieved at 24 hours, demonstrating the enzyme's capacity for prolonged and effective action against complex industrial dyes.

These results underscore the potential of A. niger-derived laccase as a biocatalyst for tannery wastewater treatment, with significant decolorization achieved within a practical timeframe. The efficiency of the crude extract highlights its suitability for cost-effective and environmentally friendly bioremediation applications, reducing the need for extensive purification processes. This supports the feasibility of using fungal laccase in large-scale industrial effluent treatment systems.

Table 3.18: Effect of Time on soluble laccase for tannery effluent decolonization

Sr. No.	Time in hours	Percentage decolorization By crude extract (%)
1	1	13.0 ± 1.00
2	2	18.0 ± 1.09
3	3	24.0 ± 2.00
4	4	30.0 ± 2.60
5	5	35.4 ± 2.60
6	6	40.0 ± 2.70
7	21	53.6 ± 2.18
8	24	66.6 ± 3.08

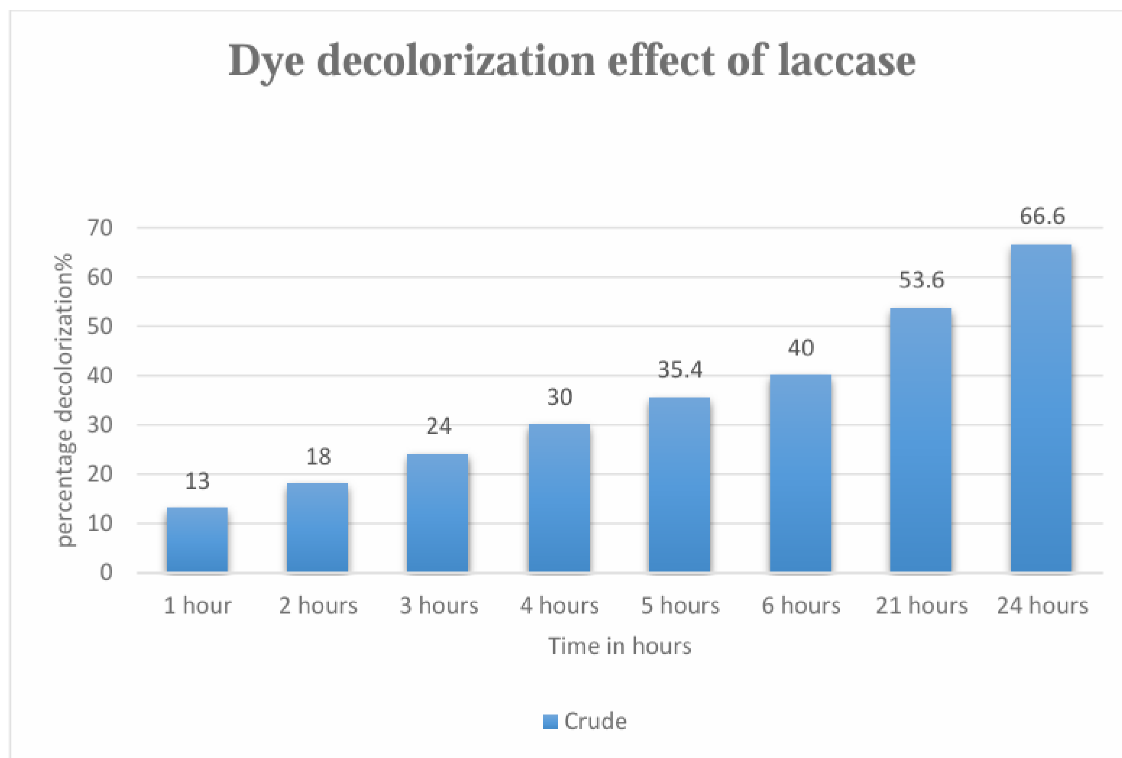

Figure 3.8: Dye decolorization effect of laccase

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The systematic optimization undertaken in this study successfully enhanced laccase production from *Aspergillus niger* by identifying strain-specific preferences for sucrose and yeast extract, and by fine-tuning critical parameters like pH, temperature, and metal ion concentration. The resulting enzyme possesses desirable biochemical characteristics moderate thermostability and acidic pH optimum that are compatible with many industrial settings [24].

Most significantly, the research conclusively proves the high efficacy of this locally produced laccase in treating two of the most challenging industrial waste streams: textile and tannery effluents [25]. The ability of a crude enzyme extract to achieve over 50-66% decolorization underscores its potent catalytic power and immense potential for developing cost-effective and eco-friendly bioremediation technologies. This aligns with the global pursuit of sustainable "green chemistry" solutions to replace harsh chemical treatment processes Future work will focus on immobilizing the enzyme to enhance its reusability and stability in continuous flow systems for real-world wastewater treatment plants [26].

Conclusion and Future perspective

This research successfully established Aspergillus niger as a valuable source of laccase enzyme produced under surface fermentation conditions and systematically optimized for enhanced yield. By employing the one-factor-at-a-time (OFAT) approach, critical nutritional and environmental factors influencing enzyme synthesis were identified, with sucrose (4 g/100 mL) and yeast extract (5 g/100 mL) recognized as the most effective carbon and nitrogen sources, respectively. The supplementation of copper ions served as a strong inducer of laccase production, whereas excessive concentrations of zinc and iron salts showed inhibitory effects, emphasizing the importance of metal ion balance in the fermentation process. Biochemical characterization further revealed that the crude enzyme exhibited moderate thermostability and an acidic optimum (pH 5.5, 37 °C), properties that align well with industrial requirements. Partial purification enhanced enzymatic activity, while the crude laccase demonstrated significant bioremediation potential by achieving over 50% decolorization of textile wastewater within 10 days and more than 66% decolorization of tannery effluents within 24 hours, underscoring its efficiency in degrading complex industrial dyes. Collectively, these findings highlight the dual industrial significance of A. niger laccase in both cost-effective enzyme production and eco-friendly wastewater treatment. Looking ahead, future research should prioritize scaling up enzyme production through statistical optimization techniques, genetic modifications, and metabolic engineering to further boost yields. The immobilization of laccase onto suitable supports could improve its reusability, stability, and adaptability for continuous flow systems, making it more practical for large-scale industrial applications. Additionally, exploring synergistic interactions between laccase and other oxidative enzymes may enhance its catalytic efficiency against a broader range of pollutants. Expanding investigations into the degradation of pharmaceuticals, pesticides, and other emerging contaminants will further strengthen the role of fungal laccases in environmental biotechnology, supporting the development of sustainable green chemistry solutions for industrial and environmental challenges.

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