Isolation and Screening of Bilirubin Oxidase Production in *Trichothecium roseum* F20 Strain: A Novel Source for Medical Diagnostics and Bioremediation

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Background: Bilirubin oxidase (BOX) is a key enzyme with important applications in medical diagnostics, particularly jaundice detection, and environmental bioremediation, such as azo dye degradation. This study aimed to isolate and screen fungi for BOX production, focusing on identifying new fungal strains capable of producing this enzyme.
Methods: A total of 28 fungal isolates were screened for BOX activity across multiple batches. The most promising isolate, F20, was obtained from decayed wood mixed with soil in Baramulla, Gulmarg, Jammu and Kashmir. The enzyme was partially purified using ammonium sulfate precipitation and dialysis. Electrophoresis and zymogram analysis were performed to determine enzyme activity and molecular weight. Additionally, the strain was identified through 18S rRNA gene sequencing.
Results: Isolate F20 consistently exhibited the highest BOX activity, achieving an average of 49.35%, equivalent to 10.63 units in 25 ml of culture filtrate. The recovery rate after partial purification was 73.21%, with increased enzyme purity. Electrophoresis and zymogram analysis confirmed BOX enzyme activity with a molecular weight of 68 kDa. The isolate was identified as *Trichothecium roseum*, marking the first documented report of BOX activity in this species.
Conclusions: This study demonstrates the potential of *T. roseum* as a novel source of BOX, with significant enzyme activity and molecular characterization. These findings offer new insights into the microbial production of BOX, suggesting future optimization for industrial applications.

Introduction

Background and Context

Bilirubin oxidase (BOX) is a multicopper oxidase enzyme that catalyzes the oxidation of bilirubin to biliverdin, playing a critical role in the medical diagnosis of jaundice and the bioremediation of environmental pollutants such as azo dyes[1,2,3]. The enzyme's unique ability to reduce oxygen to water during the oxidation of bilirubin makes it highly valuable for a variety of applications, including medical diagnostics, biosensors, and biofuel cells[4,5]. BOX is a monomeric protein, similar to other multicopper oxidases, with a molecular mass of 64 kDa and consisting of 538 amino acids[6]. The enzyme features three copper-binding domains types 1, 2, and 3 responsible for substrate binding and electron transfer, which are necessary for the oxidation of bilirubin to biliverdin:

$$2\text{bilirubin} + O_2 \rightarrow 2\text{biliverdin} + 2H_2O$$

This reaction underscores the enzyme's relevance in diagnosing jaundice, as bilirubin levels in the serum can be accurately measured based on BOX activity[5]. Microbial sources of enzymes, especially fungi, have long been favored for therapeutic applications due to their ease of production, higher yields, and overall performance[7]. *Myrothecium verrucaria* MT-1 was the first fungal source reported to produce BOX, and its enzyme demonstrated the ability to lower plasma bilirubin levels in jaundiced rats to normal[8,7,7,9]. BOXs and laccases which are produced extracellular belong to the family of MCOs, have great potential in electrochemical applications because of their ability to catalyze the four-electron reduction of O_2 to water[10]. The exclusive extracellular activity of laccases is a result of the fungal secretion mechanisms, which involve a highly coordinated process of protein synthesis, transport, and release. Mature laccase proteins are packaged into vesicles and transported via the secretory pathway to the plasma membrane. These vesicles fuse with the membrane, releasing the enzymes into the extracellular space. Once secreted, laccases either remain in the extracellular matrix or diffuse into the surrounding medium, where they perform their catalytic functions effectively[11]. BOX production has been explored using bacteria, yeast, and fungi, with fungi showing higher yields and simpler growth requirements. Among fungal strains, *M. verrucaria* MT-1 demonstrated the highest activity (19 U/mL), outperforming *M. verrucaria* IFO 6113 (3.3

U/mL) and *M. cinctum* IFO 9950 (0.02 U/mL)[8]. *Schizophyllum commune K-17* produced 6.5 U/mL[12]. *Bacillus licheniformis* achieved 14.5 U/L[13], while recombinant production in *Pichia pastoris* reached 5 U/mL[14]. Fungi are advantageous for BOX production due to their cost-effective media requirements, higher yields, and efficient secretion of extracellular enzymes. The production of BOX at an industrial scale is not extensively documented, and detailed information is limited in the public domain. However, available literature suggests that BOX is primarily produced using submerged fermentation methods using Recombinant technologies. Genetically engineered *Pichia pastoris* and Aspergillus oryzae, have been employed in recent years to enhance BOX production. These systems offer significant advantages, including higher yields, improved enzyme stability, and streamlined purification processes compared to native strains[14,15]. Despite these promising results, there remains limited research exploring fungal sources for large-scale BOX production, particularly for industrial and therapeutic purposes. The limited number of studies on production of BOX for industrial and therapeutic applications can be attributed to challenges such as low yield, stability issues, and the complexities of producing and purifying the enzyme in large quantities. This emphasizes the need for further investigation into alternative fungal strains that could offer enhanced enzyme production for broader biotechnological applications.

Problem Statement and Rationale

While fungal sources have been identified for BOX production, including *M. verrucaria*, research on alternative fungal strains remains insufficient. This lack of diversity in sourcing BOX limits its biotechnological applications. Specifically, the fungal strain *T. roseum* has not been associated with BOX production despite its known metabolic capabilities. Exploring new strains for BOX production could offer increased yields and broaden its application in health and environmental biotechnology.

Significance and Purpose

The significance of this study lies in its potential to identify novel fungal sources for BOX, contributing to more efficient enzyme production for medical and environmental applications. BOX can address critical health challenges, such as jaundice detection, and environmental concerns like the degradation of azo dyes. This study seeks to explore the BOX-producing potential of *T. roseum* and improve the current understanding of its biochemical characteristics.

Objectives

The main objectives of the study are:

1. To isolate and screen fungal strains for BOX production.
2. To identify the new fungal strain showing consistent BOX activity using 18 s rRNA sequencing
3. To partially purify the enzyme and find the molecular weight and activity on zymogram
4. To optimize the fermentation time for optimal enzyme production.

Scope and Limitations

This study focuses on the isolation and characterization of BOX from *T. roseum*, a fungal strain not previously associated with BOX activity. While it aims to identify and optimize enzyme production, the study is limited by the need for further exploration of other environmental and industrial applications beyond the scope of this research.

Methodology Overview

A total of 28 fungal isolates were screened for BOX activity, with a focus on identifying high-yielding strains. The strain *T. roseum* was selected based on its BOX activity, and the enzyme was partially purified using ammonium sulfate precipitation and dialysis. Electrophoresis and zymogram analysis were performed to confirm the enzyme's activity and molecular weight. Additionally, the strain was identified through 18S rRNA gene sequencing to confirm its taxonomy.

Materials and methods

Media, Chemicals, and Instruments

- **Samples:** Rotten wood samples were sourced from regions in Ravugodlu, Kashmir, and Uttarakhand for microbial isolation.
- **Media and Chemicals:** The media used included Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB), alongside bilirubin and Remazol Brilliant Blue R (RBBR), which were used in enzyme assays.
- **Analytical Instruments:** Experiments were carried out using a UV-Vis spectrophotometer (Shimadzu UV-1601) and ultrasonicator (Trans-O-sonic).
- **Laboratory Apparatus:** Standard laboratory tools such as an analytical balance, petri dishes, hot plate stirrer, incubator, refrigerator, centrifuge, and laminar airflow were employed throughout the study.

Media Preparation

Fungal strains were maintained on PDA or PDB media supplemented with 1% glucose. For the production of BOX, the cultures were grown in a liquid medium composed of 1% glucose and 40% potato extract, adjusted to a pH of 7.0, and incubated at 30 $^{\circ}C$ with constant shaking at 150 rpm for 72 hours. The media were sterilized at 121 $^{\circ}C$ and 15 psi for 20 minutes before use.

Enzyme Assays

BOX activity was determined by incubating 1 ml of enzyme (culture filtrate or supernatant) with 2 ml of 0.002 % bilirubin in 0.2 M Tris-HCl buffer (pH 7) at 37 $^{\circ}C$ for 5 minutes. The change in absorbance at 440 nm was recorded using a UV-Vis spectrophotometer, measuring the oxidation of bilirubin to biliverdin2. One unit of BOX activity was defined as the amount of enzyme required to oxidize 1 micromole of bilirubin per minute at pH 7.0 and 37 $^{\circ}C$. Protein concentration was determined using the Bradford assay, and specific enzyme activity was expressed as units per milligram of protein.

Fig. 1 Rotten wood samples collected from the Suvarnamukhi river dried bed, Jammu and Kashmir, and Uttarakhand regions.

Fig. 2 Students aseptically isolating fungal strains and conducting BOX assay

Isolation and Screening of Microbes for BOX Production

Sampling: Rotten wood samples were collected from the Suvarnamukhi River dried bed (Ravugodlu, Karnataka), Baramulla (Gulmarg, Kashmir), and New Tehri (Garhwal District, Uttarakhand) regions, stored in sterile plastic bags, and kept at 4 $^{\circ}C$ (Fig. 1). Sampling locations were selected based on visual evidence of fungal growth on decayed wood. The chosen regions Baramulla, Gulmarg, and the Suvarnamukhi River bed offer unique biodiversity and ecological niches, rich in decayed organic matter, providing favorable conditions for fungi with enzymatic capabilities. The cold climates of Gulmarg and Garhwal, along with the diverse vegetation and decayed plant material near the Suvarnamukhi River bed, create ideal environments for isolating novel fungal species capable of producing bilirubin oxidase (BOX). Rotten wood was specifically chosen to isolate BOX-producing microbes due to the association of wood-rotting fungi with laccase production, a group of multicopper oxidases that degrade lignin[16]. As BOX also belongs to this enzyme group, decayed wood serves as a promising substrate for identifying fungi with these enzymatic properties. This strategic sampling approach enhances the likelihood of discovering novel fungal strains with potential industrial and therapeutic applications.

Isolation Protocol: A 10 g portion of each wood sample was homogenized in 90 ml of sterile saline (0.85%) and shaken for 1 minute. Serial dilutions up to 10^{-3} were prepared, and 1 ml aliquots were inoculated onto PDA media containing 0.05% chloramphenicol to prevent bacterial contamination (Fig. 2). Media were supplemented with 500 mg/ml RBBR to select for BOX-producing fungi, as BOX belongs to the laccase enzyme group. Plates were incubated at 25 $^{\circ}C$ for 5-7 days. Laccase and bilirubin oxidase, both belonging to the multicopper oxidase family, share the ability to degrade RBBR dye[17,18]. RBBR decolorization was employed as a screening tool to identify colonies with oxidative enzymatic activity, indicative of potential BOX production. Given the substrate specificity overlap among multicopper oxidases, RBBR decolorization serves as a reliable indicator of BOX activity. Strains producing visible zones of RBBR decolorization were identified as potential BOX producers.

Fermentation and Enzyme Production

Fungal isolates exhibiting positive RBBR degradation were cultivated in 10 ml of PDB for 5 days at 25 $^{\circ}C$ with agitation at 150 rpm. Both intracellular and extracellular enzyme activities were evaluated. To assess intracellular activity, the fungal biomass was subjected to ultrasonication using a tip sonicator (Trans-O-Sonic), which lysed the cells and released the intracellular enzymes. For extracellular enzyme activity, the culture filtrate was directly analyzed. For time-course studies, 8 ml of the selected isolate culture (demonstrating BOX activity) was used as inoculum for 200 ml of production medium containing 1% glucose and 40% potato extract. The cultures were incubated under identical conditions for 5 days. Enzyme activity, both intracellular and extracellular, was measured at regular intervals.

Partial Purification of BOX

The enzyme was produced through submerged fermentation. After incubation, the culture medium was filtered through muslin cloth, and the filtrate was centrifuged at 5000 rpm for 10 minutes to remove cell debris. The proteins were precipitated by adding 80% ammonium sulphate to the filtrate and kept at 4 $^{\circ}C$ for five hours. The precipitated protein was separated via centrifugation at 5000 rpm and 4 $^{\circ}C$ and resuspended in 0.01 M phosphate buffer (pH 7.0). After dialysis to remove excess salts, the purified enzyme was stored at 4 $^{\circ}C$ for further analysis.

Identification of Fungal Strain F20

The identification of the unknown fungal strain was conducted at Neuberg Diagnostics in Bangalore, Karnataka, employing a combination of molecular and morphological techniques. The isolate F20 was cultivated under sterile conditions, and the cultures were meticulously preserved before being submitted for analysis. The identification process commenced with the amplification of fungal DNA using polymerase chain reaction (PCR), focusing on the highly conserved 18S ribosomal RNA (rRNA) gene region, which is widely utilized for fungal species identification due to its variability across different taxa. Following PCR amplification, the 18S rRNA gene sequence was directly sequenced. The resulting sequence data were analyzed and compared against reference databases to accurately determine the species identity of the fungal isolate.

Concurrently, a detailed morphological examination of the fungal spores and mycelium was performed using microscopy. This analysis provided insights into key morphological characteristics, such as spore structure and arrangement, hyphal features, and other distinguishing traits. The morphological findings were then cross-referenced with the sequencing results to confirm the identity of the fungal strain. By integrating both molecular and morphological methodologies, a thorough and reliable identification of the fungal isolate F20 was achieved, ensuring accurate characterization of the strain.

Electrophoresis and Zymogram Analysis

Electrophoresis was performed using a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel. The culture filtrate was precipitated with 80% ammonium sulfate, and the resulting protein was dialyzed against 0.05 M Tris-HCl buffer (pH 7). A total of 10 μg of protein was loaded onto the 10% SDS-polyacrylamide gel, separated, and stained with Coomassie Brilliant Blue R250 for 1 hour. The gel was then destained until distinct blue bands became visible. For activity staining (zymogram), 10 mg of protein was separated on the 10% SDS-polyacrylamide gel without boiling. The gel was incubated in 0.05 M Tris-HCl buffer (pH 7) at 37 $^{\circ}C$ for 1 hour to renature the proteins. The gel was then further incubated in the same buffer containing 0.007% bilirubin at 37 $^{\circ}C$, where zones of clearance appeared against the yellow background, indicating enzyme activity.

Results and Discussion

Isolation and Preliminary Screening of Laccase-Producing Strains

In the current study, the microbial candidates were explored for BOX production, focusing on laccase-producing bacteria and fungi isolated from decayed wood samples. The appearance of RBBR decolorization zones around the colonies indicated the presence of laccase activity. A total of 28 fungal strains were isolated from two distinct batches of wood samples, with batch 1 collected from Karnataka and batch 2 from Jammu and Kashmir regions (Fig. 3). The isolates were subjected to preliminary screening for BOX production using BOX enzymatic assay. Bilirubin oxidation was used to verify the production of BOX enzyme. The initial screening of fungal isolates relied on the presence of RBBR decolorization zones around colonies, indicating potential laccase production. However, the subsequent selection criteria were more robust, involving quantitative assessment of BOX activity using bilirubin as a substrate. The reproducibility of enzyme activity across all selected isolates was ensured through triplicate experiments to validate the results. Similar approaches for isolating laccase-producing strains from environmental samples have been widely reported in recent studies. For instance, Ganoderma lucidum was isolated from decayed wood and shown to exhibit significant laccase production[9]. Similarly, Phanerochaete chrysosporium has been used to degrade lignin and produce oxidative enzymes like laccase and BOX[19]. These findings align with the methodology of targeting decayed wood to isolate laccase-producing microbes.

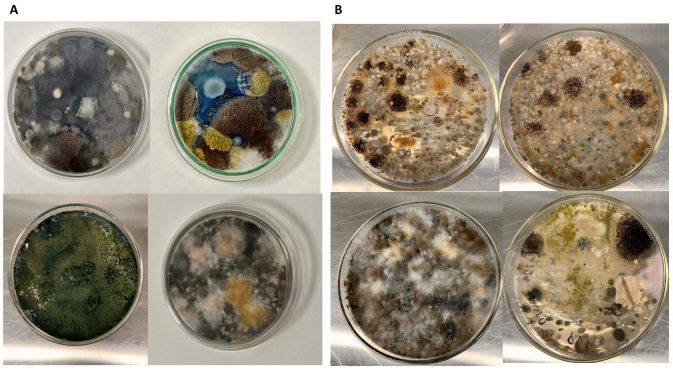

Fig. 3 Screening of fungal isolates showing zone of decolorization from batch 1 (A) and batch 2 (B)

Intra- and Extracellular BOX Assay

Out of the initial 28 fungal isolates, only four strains, two from batch 1 and two from batch 2, showed significant BOX activity during the screening process, with activity levels exceeding 20% (Table 1). Notably, BOX activity was exclusively found in the extracellular samples, with no detectable activity in the intracellular fractions. Three isolates (F10, F12 and F16) out of four isolates demonstrated inconsistent activity, making them unsuitable for further experimentation (Table 1). The inconsistent activity observed in isolates is attributed to significant standard deviations in enzyme activity levels across triplicates. These variations likely stem from factors such as the genetic instability of the strains, which may lead to inconsistent enzyme expression, and the inherent instability of the enzyme produced under the experimental conditions. These findings align with reports highlighting the impact of genetic and environmental factors on microbial enzyme production[20]. However, isolate F20, derived from the Jammu and Kashmir region, exhibited the highest and most consistent BOX activity, with a rate of 49.35%, making it a promising candidate for continued study (Table 1). These observations are consistent with other studies that highlight extracellular BOX activity in fungi. For example, Chauhan et al reported extracellular BOX production in Pleurotus ostreatus strains. The lack of intracellular BOX activity is consistent with the known secretion mechanisms of extracellular laccases in fungi[21]. The extracellular BOX production offers multiple ecological advantages to fungi in their natural habitats. This enzyme catalyzes oxidation reactions, enabling fungi to detoxify harmful substances such as bilirubin or aromatic compounds in their environment. By doing so, BOX allows fungi to utilize these compounds as alternative carbon or nitrogen sources, enhancing their adaptability and resource utilization[10]. Additionally, the reactive oxygen species (ROS) generated by BOX activity can inhibit the growth of competing microorganisms, providing the producing fungus a competitive edge. Furthermore, BOX contributes to the breakdown of complex organic materials like decaying wood and plays a significant role in lignin degradation, reinforcing the fungus's ecological role as a decomposer. In this way, BOX production is not merely a survival mechanism but also underlines the integral role of fungi in nutrient cycling and ecosystem sustainability[11].

Results are presented as mean ± standard deviation. Mean was taken as the average of three readings of each experiment.

Table 1 Extracellular BOX activity of fungal isolates from batch 1 and batch 2

Batch 1		**Batch 2**	
Isolate no.	**Extracellular BOX activity (%)**	**Isolate no.**	**Extracellular BOX activity (%)**
Isolate F1	6.81 ± 3.96	Isolate F16	21.90 ± 7.51
Isolate F2	9.54 ± 5.54	Isolate F17	0
Isolate F3	5.90 ± 3.68	Isolate F18	2.80 ± 1.14
Isolate F4	0	Isolate F19	0
Isolate F5	6.81 ± 3.35	Isolate F20	49.35 ± 3.68
Isolate F6	2.54 ± 1.22	Isolate F21	0
Isolate F7	0	Isolate F22	0
Isolate F8	5.45 ± 3.68	Isolate F23	8.44 ± 3.25
Isolate F9	0	Isolate F24	5.19 ± 2.63
Isolate F10	20.06 ± 13.48	Isolate F25	0
Isolate F11	0	Isolate F26	15.58
Isolate F12	25.45 ± 12.84	Isolate F27	0
Isolate F13	0	Isolate F28	2.28 ± 1.82
Isolate F14	2.72 ± 2.14		
Isolate F15	0		

Partial Purification of BOX Enzyme

Isolate F20 was cultured in 600 ml of PDB medium to obtain the culture filtrate required for partial purification of the BOX enzyme (Fig. 4). The purification involved ammonium sulfate precipitation (80%) and subsequent dialysis. The protein concentration and enzyme activity were analyzed at each stage of purification, including the crude extract, ammonium sulfate

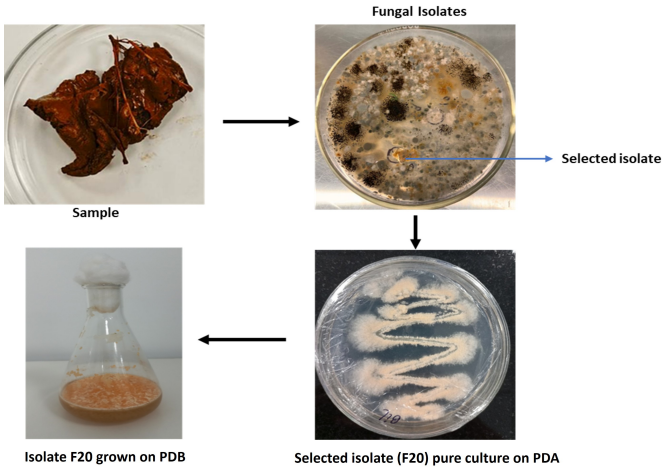

Fig. 4 Isolation of fungal strain F20 from Batch 2, Jammu region sample, followed by streaking on PDA and cultivation in PDB for growth analysis

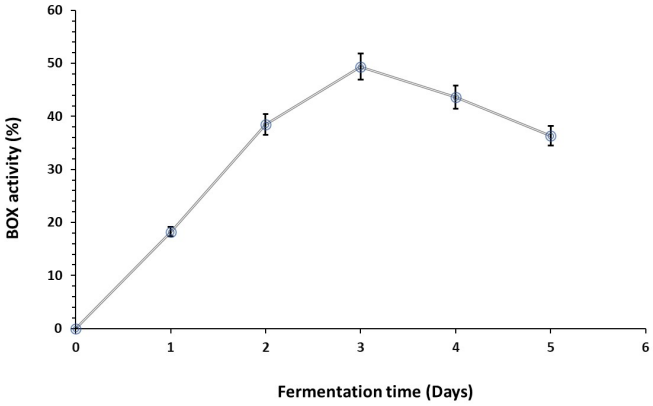

Fig. 5 Graph depicting BOX production by the isolated F20 strain in PDB glucose media across various fermentation time points. Results are presented as mean ± standard deviation. Mean was taken as the average of three readings of each assay.

precipitation, and dialysis. The specific enzyme activity was monitored using the Bradford assay to quantify protein concentration. A recovery rate of 73.21% was achieved, with an increase in specific activity at each stage of purification, indicating that ammonium sulfate precipitation effectively enhanced the purity of the BOX enzyme (Table 2). The use of ammonium sulfate for enzyme precipitation has been widely documented in the literature as an effective method for purifying extracellular enzymes like laccase and BOX[22].

Table 2 Purification of BOX from the grown culture of isolate F20

S. No.	Recovery step	Volume	Total activity (U)	Specific activity (U/mg of protein)	Recovery (%)
1	Culture filtrate (Crude enzyme)	600 ml	255.12 U	31.51	100
2	Precipitation by ammonium sulphate	32 ml	195.29 U	98.34	76.54
3	Dialysis	57 ml	186.77 U	104.23	73.21

Recovery (%) = (Total activity of purified enzyme/ Total activity of crude enzyme) × 100

Time Course Studies: Production of BOX in Submerged Fermentation

Time course studies on submerged fermentation using glucose and potato extract media revealed that isolate F20 reached peak BOX production on day 3 (Fig. 5). Beyond this point, BOX activity gradually decreased, likely due to changes in medium pH and the accumulation of secondary metabolites, which may have inhibited enzyme activity. This trend emphasizes the importance of optimizing media components and fermentation parameters to enhance enzyme production efficiency. Similar findings have been observed in other fungi, such as Pleurotus species, which exhibited optimal laccase activity at 72 hours of fermentation[23,24]. The decline in enzyme activity after peak production is consistent with observations in other studies. The production of secondary metabolites and changes in the pH of the fermentation medium have been reported to affect the stability of BOX and laccase enzymes[25]. Optimization of fermentation conditions can therefore play a crucial role in enhancing BOX production[26].

The statistical analysis was conducted to evaluate significant differences between BOX activity and fermentation time. BOX activity was compared with the activity observed on the 3rd day, as it was found to be the highest on that day and decreased by the 5th day. The student's t-test was used for analysis. The p-values were as follows: 1 vs. 3: 0.000012, 2 vs. 3: 0.0017, 3 vs. 4: 0.02685, and 3 vs. 5: 0.000787. The values indicated significant differences; however, the increase from the 2nd to the 3rd day was less pronounced. Similarly, between the 3rd and 4th days, a lower significant difference was observed, with only a slight decrease in value.

Additional experiments were conducted to monitor pH changes during BOX production, and the results offer insight into the observed trends. On Day 1, the pH started at 6.7 and showed a slight decline to 6.4 by Day 2. From Day 3 to Day 5, a significant drop in pH was observed (pH 6.1 to 4.2), likely due to the secretion of organic acids and acidic intermediates as *T. roseum* metabolized carbon and nitrogen sources. By Day 6 and 7, the pH stabilized with a slight increase (pH 4.6), which could be attributed to reduced acid production and the utilization of previously secreted organic acids. *T. roseum* is known

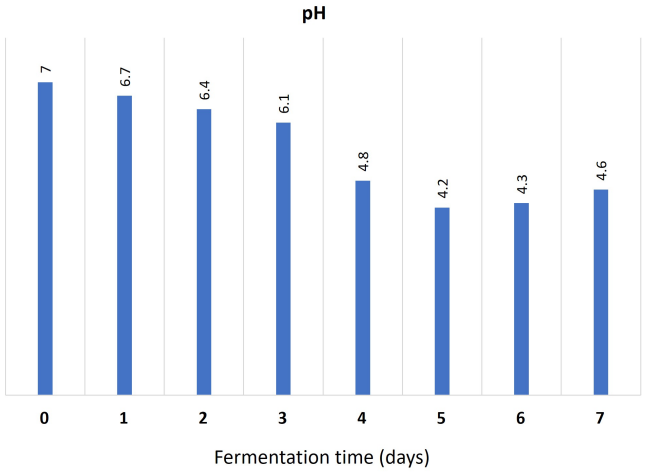

Fig. 6 pH variations during fermentation of *T. roseum* over time

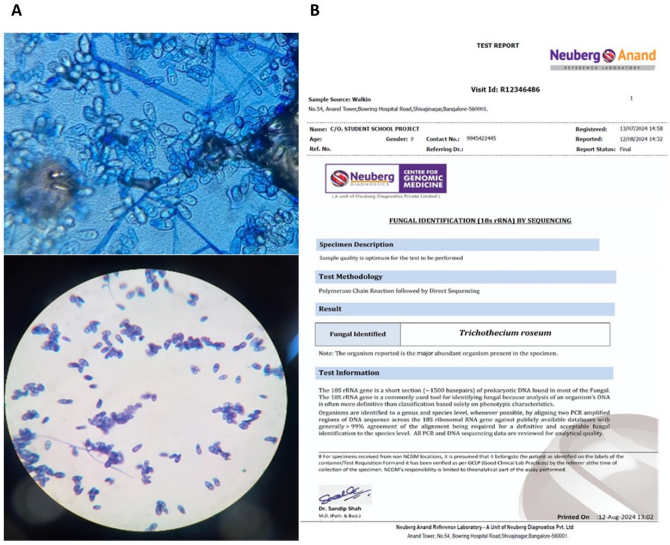

Fig. 7 Microscopic examination (A) and 18S rRNA sequencing analysis (B) identifying the isolated F20 strain as *T. roseum*

to produce a diverse range of secondary metabolites, including toxins, organic acids, antibiotics, diterpenoids, sesquiterpenoids, and other biologically active compounds, as reported in existing literature[27]. These metabolites likely contribute to the decreased activity from the 3rd day of incubation.

Identification of F20 Strain Using rRNA Sequencing and Morphological Features

The identification of the high-performing isolate F20 was carried out using direct sequencing of the 18S rRNA gene, which identified the fungus as *Trichothecium roseum* (Fig. 7B). The cultured colonies displayed flat, granular, and powdery characteristics, initially white and turning light orange to peach, typical of the Trichothecium genus. Microscopic examination revealed septate conidiophores (Fig. 7A), consistent with known features of *T. roseum*[28]. While *T. roseum* is primarily known as a plant pathogen, this is the first documented report of BOX production by this species. *T. roseum* has been reported to produce a wide range of secondary metabolites, including trichothecenes and roseotoxins, which are mycotoxins that impact fruit crops. Recent studies have highlighted the broader metabolic potential of *T. roseum*, including its use in biotransformations[24]. However, the discovery of BOX production in this species is novel and expands the understanding of the potential industrial and biotechnological applications of *T. roseum*.

Molecular Weight and Activity Staining

SDS-PAGE analysis of the crude enzyme, ammonium sulfate-precipitated enzyme, and enzyme after dialysis from isolate F20 revealed a molecular weight of approximately 68 kDa for the BOX enzyme (Fig. 8, Lane F). Multiple bands appeared in the crude enzyme, indicating impurities before purification (Fig. 8, Lane D). The 80% ammonium sulfate-precipitated enzyme showed a single band, indicating the highest purity (Fig. 8, Lane E). The BOX activity was confirmed by the appearance of clearance zones on the native gel (Fig. 8, Lanes B and C) using zymogram staining, while the negative control (heat-inactivated enzyme) showed no activity (Fig. 8, Lane A). The molecular weight is consistent with previously reported BOX enzymes from fungi *M. verrucaria*[2]. Similar molecular weights have been reported for BOX enzymes in related fungal species, such as Phanerochaete chrysosporium and Ganoderma lucidum[19], further supporting the potential of isolate F20 for industrial enzyme applications.

Discussion

Restatement of Key Findings

The present study successfully isolated and screened laccase-producing fungi, with *T. roseum* (isolate F20) identified as a promising candidate for BOX production. Of the 28 fungal strains isolated from decayed wood samples, isolate F20 exhibited the highest and most consistent BOX activity (44.35%), demonstrating its potential as an efficient extracellular enzyme producer. Time course studies indicated that the optimal BOX production occurred on day 3 of submerged fermentation, after which enzyme activity began to decline. Partial purification using ammonium sulfate precipitation achieved a recovery rate of 73.21%, with an increase in specific activity, highlighting the effectiveness of the purification process. Molecular identification through 18S rRNA sequencing confirmed the strain as *T. roseum*, marking the first documented report of BOX production

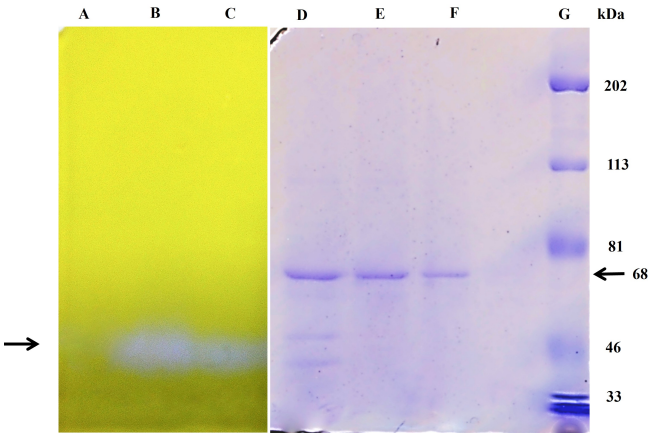

Fig. 8 Electrophoretic Analysis of BOX Activity: The zymogram displays BOX activity (B and C, indicated by arrow), while the heat-inactivated enzyme shows no activity (A). Crude BOX (D, indicated by an arrow), BOX following 80% ammonium sulfate precipitation (E), and BOX after dialysis (F) are compared alongside molecular weight standards (G) on a 10% SDS-PAGE gel.

by this species.

Implications and Significance

The discovery of BOX production by *T. roseum* has significant implications for both industrial and biomedical fields. BOX is widely used in biosensors, bioremediation, and as a biocatalyst in various redox reactions. Its role in bioelectronics, particularly in the development of biofuel cells, further underscores its industrial importance. The consistent extracellular production of BOX by *T. roseum* makes it a viable candidate for large-scale enzyme production, offering a novel and cost-effective source of this enzyme. The ability of *T. roseum* to produce BOX expands the known metabolic capabilities of this fungus, opening new avenues for its application in enzyme technology.

Connection to Objectives

The study's primary objective was to isolate potent microbial strains capable of producing BOX, focusing on decayed wood samples as a microbial reservoir. The successful isolation of *T. roseum* and its ability to produce high levels of extracellular BOX align directly with this objective. Furthermore, the secondary goal of optimizing and partially purifying the enzyme was achieved through ammonium sulfate precipitation, which enhanced the specific activity of the enzyme. The identification of *T. roseum* as a novel BOX producer also fulfills the study's aim to contribute new insights into microbial enzyme production.

Recommendations

Given the promising results of this study, several recommendations can be made for future research and industrial applications. First, the optimization of fermentation parameters, such as pH, temperature, and nutrient composition, should be further explored to enhance BOX production and reduce the fermentation time. Additionally, large-scale fermentation studies are needed to evaluate the economic feasibility of producing BOX from *T. roseum* for commercial purposes. Genetic engineering approaches could also be considered to enhance the yield and stability of BOX production in this strain. Finally, exploring other environmental samples, such as decayed wood from different geographic regions, may lead to the discovery of additional novel BOX-producing strains.

Limitations

While the study successfully identified *T. roseum* as a BOX producer, several limitations should be noted. While partial purification of the enzyme was achieved, further purification and characterization are needed to fully understand the biochemical properties like stability and functionality of the BOX enzyme produced by *T. roseum*. Additionally, while the strain exhibited consistent enzyme activity under lab-scale conditions, larger-scale industrial trials are required to validate its commercial viability.

Closing Thought

This study represents a significant step forward in the discovery of *T. roseum* as a potent BOX producer expands the understanding of the metabolic diversity of fungi. Continued research in this area holds great promise for advancing biocatalysis and industrial biotechnology.

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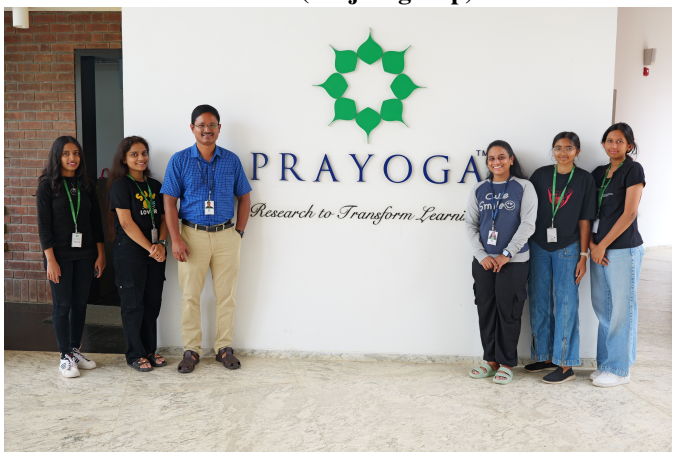
Authors (Project group)



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