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PURIFICATION AND CHARACTERIZATION OF A LACCASE ENZYME PRODUCED BY CO-CULTURING OF LOCALLY ISOLATED *ASPERGILLUS NIGER* AND *ASPERGILLUS ORYZAE*

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Abstract

Present study aimed to assess the potential of co-culturing of two locally isolated fungal strains, Aspergillus niger and Aspergillus oryzae, for enhanced production of laccase enzyme for possible use in industrial applications. After identification and confirmation, both strains were investigated individually and together for laccase enzyme production under optimal conditions. Maximum production of laccase enzyme (35.5±0.025 U/mL) was observed when both strains were co-cultured at 28°C using 10% wheat bran as substrate having pH 6 after 72 hours. The enzyme was purified to homogeneity through ammonium sulfate precipitation and gel filtration chromatography. The molecular mass of purified laccase enzyme from both fungal strains was observed as almost 65 kDa using SDS-PAGE. Purified laccase enzyme showed great stability (60%) upto 70°C for 4 hours and pH 9.0 for 3 hours. The enzyme exhibited stability even in the presence of highest concentrations of solvents (30%) and diverse inhibitors (3%). The presence of divalent ions also seemed to enhance the enzymatic activity whereas EDTA showed a negative impact on it. All the characteristics recorded in this research proved laccase enzyme a capable nominee for industrial utilization.

Key words: Laccase; Co-culturing; Production; Purification; Characterization.

Introduction

Many industries depend on oxidation processes, but conventional methods often face restrictions due to environmental concerns, nonspecific reactions, and unintended consequences. So, researchers are very interested in the possibility of studying new oxidation techniques to biological systems, such as enzymatic oxidation (Taoufik *et al.*, 2021). Polyphenols oxidases, of which laccase ("benzenediol": "oxygen oxidoreductase", EC 1.10.3.2) is a member, are often denoted to as multi-copper oxidases due to the presence of copper ions in their catalytic domains. The substrate is typically oxidized when oxygen is condensed to water via laccase- "mediated catalysis" (Baldrian, 2006). Laccases can oxidize an extensive series of substrates, such as methoxy-substituted phenols, aromatic diamines, polyphenols, and more (Brugnari *et al.*, 2021).

This growing interest in enzymatic oxidation aligns with the increasing focus on enzyme production. Submerged fermentation (SmF) has long been the primary method for enzyme production (Thakur *et al.*, 2023), but in recent years, solid-state fermentation (SSF) has gained attention. SSF is recognized for its ability to mimic natural microbial processes like composting and ensiling, making it an appealing approach for producing a broader range of enzymes (Costa *et al.*, 2018). Several studies have reported successful laccase production using SSF with various lignocellulosic substrates such as agricultural residues, sawdust, and wheat bran. These substrates provide an inexpensive and renewable source of carbon and induce the production of laccase enzymes by fungi, including Aspergillus species and white-rot fungi (Jaramillo *et al.*, 2017). It's also been demonstrated that raising the SSF media pH, aeration, temperature, and substrate composition may increase laccase production. The efficiency and scalability of laccase production by SSF have been further enhanced by developments in bioprocess engineering, including the construction of innovative fermentation techniques and the utilization of bioreactors (Tišma *et al.*, 2012; Chen *et al.*, 2011). Overall, recent advances in laccase production, with an emphasis on SSF, highlight the enzyme's potential for a variety of commercial applications, including wastewater treatment, bioremediation, textile dye decolorization, and biofuel generation (Wang *et al.*, 2019). Fungal enzymes produced through SSF are reported to be more stable in terms of pH and temperature, have higher volumetric efficiency, and are less likely to face substrate inhibition challenges (Hasan *et al.*, 2023). There is less risk of undesirable proteases destroying the produced enzymes, and fermentation proceeds more quickly as a result (Hölker, 2004).

Laccases are abundantly found in a wide variety of organisms, including insects, bacteria, higher plants, and fungus (Messerschmidt & Huber, 1990). Laccases occuring in the basidiomycetes, deuteromycetes, and ascomycetes fungal kingdoms, exhibit a particularly high prevalence within numerous white-rot fungi that play a crucial role in lignin metabolism (Bourbonnais *et al.*, 1995; Leontievsky *et al.*, 1997). Fungal laccases have been variously applied, primarily in the breakdown of lignin, owing to their elevated redox potential (+800 mV) in comparison to laccases derived from bacteria or plants (Thurston, 1994). *A. niger* and *A. oryzae* belonging to the division ascomycota have a great potential for industrial enzyme production (Kong *et al.*, 2021). These industrial species are frequently utilized to produce enzymes, food components, and medicinal components due to their production of a wide variety of enzymes that contributes significantly to the breakdown of various plant polysaccharides, including xyloglucan, pectin, xylan, cellulose and galactomannan (Hu *et al.*, 2011).

To further enhance the production and understanding of these enzymes and their associated metabolites, co-culturing is employed as a biotechnological technique. Co-culturing helps to replicate the natural living conditions of macrofungi, which promotes the production of diverse extracellular metabolites and enzymes. Additionally, this technique reveals crucial processes involved in interspecific fungal relationships and novel gene functions (Yu *et al.*, 2021). Co-culturing technique of macrofungi and other microorganisms has revealed potential medical uses, and this can be exploited to enhance the production of functional enzymes and for better nutritional value of fruit bodies (Peláez *et al.*, 2021).

Lignocellulose is a more reasonable choice as an alternative complex nutrient source in SSF systems for laccase synthesis (Huang *et al.*, 2017). Lignocelluloses contain three primary polymers including cellulose, hemicellulose, and lignin. Laccase has the ability to indirectly affect lignocellulose by contributing to the depolymerization of lignin, which can enhance the accessibility of cellulose and hemicellulose for further degradation by other enzymes. In addition to provide nutrients, some lignocellulosic wastes have naturally occurring stimulating components, containing phenolic compounds and flavonoids, that can be directly utilized in SSF to enhance the production of fungal laccase (Wang *et al.*, 2019).

The effectiveness of enzyme application is further supported by advancements in enzyme purification and characterization. The enzymes which are purified and characterized from various organisms, such as *A. flavus*, *A. niger*, *Pleurotus ostreatus*, *Bacillus* sp., and *A. sydowii*, have shown important potential for industrial usage (Bakshi *et al.*, 2021). The process of enzyme purification was done through precipitation, chromatography, and gel filtration, that led to substantial increase in enzyme purity and specific activity, making them appropriate for industrial applications (Bolivar *et al.*, 2016). Furthermore, the characterization of these purified enzymes gives fundamental information on their optimal conditions, stability, and kinetic properties, which are important for their efficient utilization in different industries (Westphal *et al.*, 2021).

Despite the known benefits of laccase enzymes in industrial applications, current research often lacks focus on optimizing co-culturing techniques for enhanced enzyme production. While individual fungal strains such as *A. niger* and *A. oryzae* have been studied separately, there is limited exploration of their combined potential. This study addresses this gap by investigating the co-culturing of these locally isolated strains to enhance laccase production, aiming to fill the void in understanding how such combinations can improve enzyme yield and stability for practical industrial applications.

Material and Methods

Selection of strains of microorganisms and chemicals: For the isolation of fungal strains, fruits (lemon, ginger, apple, and onion bulb) were collected from various sources in Lahore, Pakistan, and subjected to serial dilution. *A. niger* was identified from lemon samples while rice and wheat samples were utilized for the isolation of *A. oryzae*. Serial diluted samples were spread on PDA media and incubated at 28°C for 72 hours. Further both strains were purified and maintained on PDA media for further processing. All reagents and chemicals utilized in the study were of analytical grade.

Confirmation of *A. niger* and *A. oryzae*: Confirmation of fungal strains (*A. niger* and *A. oryzae*) was done through morphological studies as well as by 18SrRNA sequencing. After the purification of both fungal strains, various morphological characteristics including shape, spore color, spore size and hyphae were observed microscopically. Further confirmation of both fungal strains was achieved by 18SrRNA sequence analysis. Amplification of 18SrRNA sequence was carried out using genomic DNA as a template and 18S universal primers. After the amplification, the nucleotide sequencing was done by 1st Base-Malayshia and then analysis was performed by nucleotide BLAST studies. (Altschul *et al.*, 1990).

Lignocellulosic substrates preparation: Agro-industrial waste material was utilized as substrate to produce laccase enzyme from *A. niger* and *A. oryzae*. Different substrates such as wheat straw, rice bran, sugarcane bagasse and sawdust were obtained from the local market and after washing and drying, these were converted into fine particles (0.2-2 mm) by using a lab grinding mill. These agro-based materials were utilized as substrates for enzyme production after sterilization.

Fungal sporulation and spore collection: For laccase enzyme production, fungal spores were collected from mature cultures of *A. niger* and *A. oryzae* after 4 days of incubation. These mature fungal strains were used to prepare aqueous spore suspensions. Hyphae of both strains were carefully scraped with a wire loop in the presence of autoclaved distilled water to liberate the spore in the sterile solution for inoculation into the production flasks. A hemocytometer was used to count the number of spores.

Laccase enzyme production: Solid-state fermentation technique was employed to produce the laccase enzyme using rice bran as a substrate and carbon source. In a 250 mL Erlenmeyer flask, 6.0 g of air-dried, chopped rice bran and 15 mL of Reese mineral medium was added to moisten the substrate. Reese mineral medium was prepared by using 1 g potassium nitrate, 2 g potassium phosphate monobasic, 0.3 g magnesium sulphate, 1 g ammonium sulphate, 0.3 g calcium chloride, 0.002 g manganese sulphate, 0.002 g zinc and 0.002 g cobalt chloride and raised the volume upto 1 L by using distilled water (Baig *et al.*, 2003). Culture medium was sterilized by autoclaving and allowed to cool at room temperature. Inoculation of sterilized medium was carried out by 5 mL of fungal spore suspension. For laccase enzyme production, a total of three flasks were employed. Two flasks were inoculated with each separate strain, while in one flask, a spore suspension containing both strains was utilized. After inoculation, flasks were incubated for 72 hours in a static incubator at 28°C. From 72 hours grown culture, laccase enzyme was extracted using 100 mM sodium citrate buffer (pH 5.0). For enzyme extraction, 50 mL of sodium acetate buffer was added aseptically in each flask and placed at 4°C in shaking incubator with shaking speed of 165 rpm. The liquid was then filtered out by using cotton wool and

subsequently subjected to centrifugation for 10 minutes at the speed of 10000 rpm to acquire the clear supernatant for further use in enzymatic assay. All experiments were performed in triplicates.

Enzyme assay: For laccase enzyme, the activity assay was performed by following the protocol stated by Adak *et al*., (2016), A 5.0 mM solution of 2,2-azino-bis-3-ethyl benzothiazoline-6-sulfonic acid was used as a substrate. For the determination of enzyme activity, reaction mixture was prepared containing 1 mL of crude enzyme along with 1 mL of ABTS solution (5 mM) and incubated in a water bath incubator for 30 minutes at 30°C. Following the incubation period, the reaction was diluted two times using a citrate phosphate buffer and at 436 nm the absorbance was recorded using a double beam Spectrophotometer. In a control experiment, the addition of enzyme was carried out after incubation. The standard curve of trolox was used to determine enzyme activity. Unit of laccase was defined as the amount of enzyme required to oxidize 1 micromole of substrate (ABTS,) per minute under specified conditions.

Total protein estimation: The quantification of total proteins was performed using the Bradford reagent. A reaction mixture of 6 mL was prepared by using 5 mL of Bradford reagent, 100 mL of the enzyme sample, and 900 mL of phosphate buffer. In the control experiment, the enzyme was replaced by phosphate buffer. The absorbance of sample was measured through spectrophotometer at the wavelength of 595 nm to estimate the total amount of protein.

Optimization of enzyme production: For maximum laccase enzyme yield through co-culturing of *A. niger* and *A. oryzae*, several parameters especially media optimization, pH of media, temperature of incubation, time of incubation, and inoculum sizes were optimized. Strains were also cultured individually parallel to compare the production of laccase enzyme. For the optimization of pH of media to get the best production of laccase enzyme through co-culturing of *A. niger* and *A. oryzae*, a range of pH (3-9) of Reese mineral medium was used, and enzyme activity was observed under specific conditions. In the same way, the incubation temperature was optimized by incubating the inoculated culture at different temperatures (20°C to 45°C) in a static incubator for 72 hours, and after the optimization, enzyme activity was determined under specified conditions. To estimate the best incubation period for maximum production of laccase enzyme through co-culturing of *A. niger* and *A. oryzae*, inoculated cultures were incubated for numerous time periods ranging from 24 to 120 hours. The size of inoculum was optimized by using several inoculum sizes for inoculating the medium ranging from 2% to 10%, and the maximum production of laccase enzyme was estimated by calculating the enzyme activity. To get the maximum yield of laccase enzyme, several culture media including wheat straw, rice bran, sugarcane bagasse, and sawdust were used, and highest enzyme production was evaluated by calculating the enzyme activity under specific conditions. All experiments were performed in triplicates.

Purification of laccase enzyme: To purify the laccase enzyme produced through coculturing of *A. niger* and *A. oryzae*, two steps purification method was used. Partial purification was attained with the help of ammonium sulphate precipitation method as explained by Moraes & Kalil (2009). For the final purification of laccase enzyme, the ion exchange chromatography technique was utilized by following the protocol described by Zafar *et al.* (2022a). The purified laccase enzyme after final purification step was tested by SDS-PAGE following the protocol of Zafar *et al*., (2022b).

Characterization of laccase enzyme: For the characterization of purified laccase enzyme, different parameters were analyzed including its stability at different temperatures ranging from 30°C to 80°C for 1 to 4 hours. Likewise, enzyme's pH stability was also observed at different pH range (4-9) for 1 to 3 hours. Stabilized enzymatic activity of laccase enzyme was also analyzed in combination with several organic solvents including methanol, acetone, methanol, isopropanol, and n-butanol in different concentrations (10% - 30%) at 25°C for about 1 hour. Effect of metal and divalent ions was also observed on the purified laccase enzyme activity by incubating the purified enzyme with 1mM of many ions including Ca^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+}, Na^{1+}, K^{1+}, NH_4^{1+}, and Ni^{2+} for 1 hour at 25°C and under the specific conditions the residual activity of enzyme was determined. Effects of inhibitors like SDS, β-mercaptoethanol, DMSO, Tween 80, sodium azide and dithiothreitol (DTT) were determined using inhibitors at varying concentrations (ranging from 1% to 3%) for a duration of 1 hour at room temperature. Subsequently, the residual enzyme activity was determined. The substrate specificity of laccase enzyme produced by coculturing of both fungal strains was also determined by using 5 mM of different substrate such as 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), hydroquinone (Van *et al.*, 2023), guaiacol, syringaldazine (Eichlerová *et al.*, 2012), benzidine (Cárdenas-Moreno *et al.*, 2019) under standard assay conditions. All experiments were performed in triplicates.

Results

Isolation of *A. niger* and *A. oryzae*: Using the serial dilution technique and spreading the resulting culture on potato dextrose agar plates, *A. niger* was successfully isolated and purified from a lemon sample. A greenish-black fungal growth after 72 hours was observed when incubated at 30°C as shown in Fig. 1. The second fungal strain *A. oryzae* was also isolated and purified from rice grains using same method as above. After 72 hours of incubation at 30°C, greenish-white fungal growth was observed on the petri plate as shown in 1B.

Strain identification and confirmation: The isolated and purified fungal strains, *A. niger* and *A. oryzae*, were identified and confirmed through morphological characterization and molecular analyses. The spores of *A. niger* measured between 3 and 5 mm in diameter and were blackish brown in color, while the spores of *A.* The 18SrRNA of both strains was amplified through PCR. Agarose gel electrophoresis was used to an examination depicted the size of amplified fragments of both strains

around 2000 bp as shown in Fig. 2. Nucleotide BLAST results indicated the one strain as *A. niger* (Accession no. A0A9W6ACJ1) with 98% similarity index with previous strains and the second strain was confirmed as *A. oryzae* (Accession no. I8U4Z0) due to the 92% similarly index with previously reported *A. oryzae* strains.

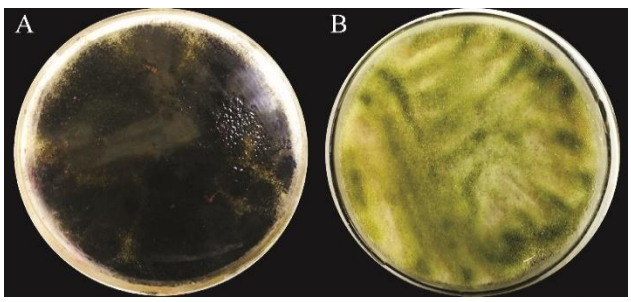

Fig. 1. (A) Isolation of purified culture of *A. niger* from lemon sample; (B) Isolation of purified culture of *A. oryzae* from rice sample.

Optimization of laccase enzyme production: To obtain the maximum yield of laccase by coculturing of *A. niger* and *A. oryzae*, following parameters were optimized.

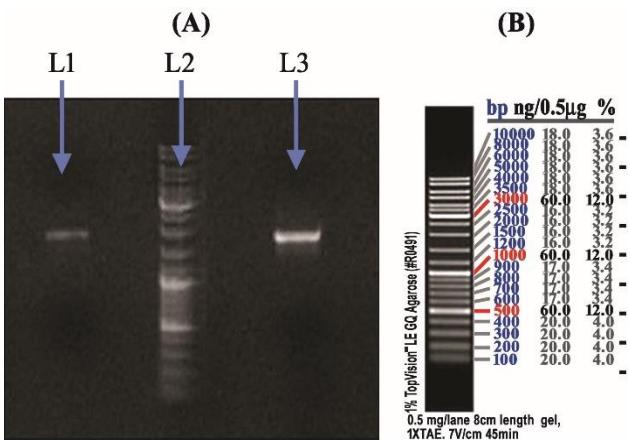

Fig. 2. (A) The amplified products of the 18SrRNA of *A. niger* and *A. oryzae*. In L1 amplified 18SrRNA from *A. niger* of 2000 bp. The DNA ladder in Lane 2, whereas the *A. oryzae* band, which is also 2000 bp in size, is shown in Lane 3; (B) 1kb DNA ladder used in this study.

pH and temperature of incubation: To get the optimum pH of laccase enzyme production from coculturing of *A. niger* and *A. oryzae*, pH of moistening agent (Reese mineral medium) was set ranging from 3–9 using rice straw as a substrate at 30°C for 72 hours. Culturing of individual strains was also carried out parallel under same conditions for comparison. Maximum production of enzyme (21.18±1.04 U/mL) was achieved at pH 6.0 by coculturing of both strains. While individually maximum production of laccase enzyme (10.5±0.40 U/mL) from *A. niger* was obtained at pH 6.0 but for *A. oryzae* the optimum pH to produce laccase enzyme was 5.0 (Fig. 3A). Similarly, temperature of incubation for best production of laccase enzyme (24.7±0.37 U/mL) from coculturing of both fungal strain was observed as 28°C**.** Maximum individual production of enzyme from both strains was also achieved at 28°C. Maximum production of laccase enzyme was 12.74±0.22 U/mL and 10.49±0.21 U/mL from *A. niger* and *A. oryzae*, respectively, when these were grown individually (Fig. 3B).

Effect of incubation time and inoculum size: To determine the effect of incubation time on laccase production by coculturing of *A. niger* and *A. oryzae*, a range of incubation time (24 – 120 hours) was used and best production of laccase enzyme (25.22±0.18 U/mL) was obtained after 72 hours of incubation from cocultured strains. Individual production of enzyme was also maximum after 72 hours of incubation for both fungal strains (Fig. 3C). Maximum enzyme production after 72 hours of incubation was 13.25±0.22 U/mL and 10.51±0.19 U/mL for *A. niger* and *A. oryzae*, respectively. Similarly, maximum production of laccase enzyme (30.25±0.22 U/mL) was achieved with 6% inoculum for cocultivation as shown in Fig. 3E. Enzyme production was slightly decreased with higher concentration of inoculum sizes i.e., 8% and 10%.

Media optimization: For best production of laccase enzyme by coculturing of *A. niger* and *A. oryzae*, lignocellulosic biomass such as rice straw, wheat straw, sugarcane bagasse and sawdust were utilized as substrate for solid-state fermentation. From the results obtained, it was analyzed that maximum enzyme (35.59±0.13 U/mL) was produced when wheat straw was utilized as fermentation substrate (Fig. 3D). Wheat straw also gave maximum results when fugal strains were cultivated individually. Laccase production obtained from rice straw and sugarcane bagasse was 30.87±0.28 U/mL and 24.37±0.32 U/mL while 16.56±0.26 U/mL enzyme production was obtained from sawdust as shown in Fig. 3D.

Purification of laccase enzyme: Purification of laccase enzyme was attained through ammonium sulfate precipitation in the range of 50-70%. The fractions that precipitated and displayed the highest laccase activity were pooled and utilized for subsequent purification steps. Subsequently, the partially purified enzyme was subjected to anion exchange chromatography by using Bioscale Mini Unosphere pre-packed column. The fractions displaying the highest laccase activity were combined and utilized for SDS-PAGE analysis (Fig. 4).

Physicochemical characterization of purified laccase enzyme: To check the industrial potential of laccase enzyme produced by co-culturing of *A. niger* and *A. oryzae*, its physicochemical characterization was performed by studying the following parameters.

pH stability: To determine the pH stability of purified laccase enzyme a wide range of pH (4 - 9) was used in this study. Purified enzyme was incubated for 1 to 3 hours in buffers with different pH at ambient temperature and enzyme activity was calculated under specific conditions. Results indicated that the enzyme in this study was very much stable at wide pH range as shown in figure 4A. Purified laccase enzyme exhibited almost 44% activity after 3 hours of incubation at pH 4.0 and up to 42% activity after 3 hours of incubation at pH 9.0. At pH 6.0 enzyme showed almost 100% for long time duration (up to 3 hours) (Fig. 5A). Almost 90% enzyme activity was noticed after the incubation period of 1 hour at pH 5.0 which was slightly reduced (up to 76%) when time was prolonged up to 3 hours.

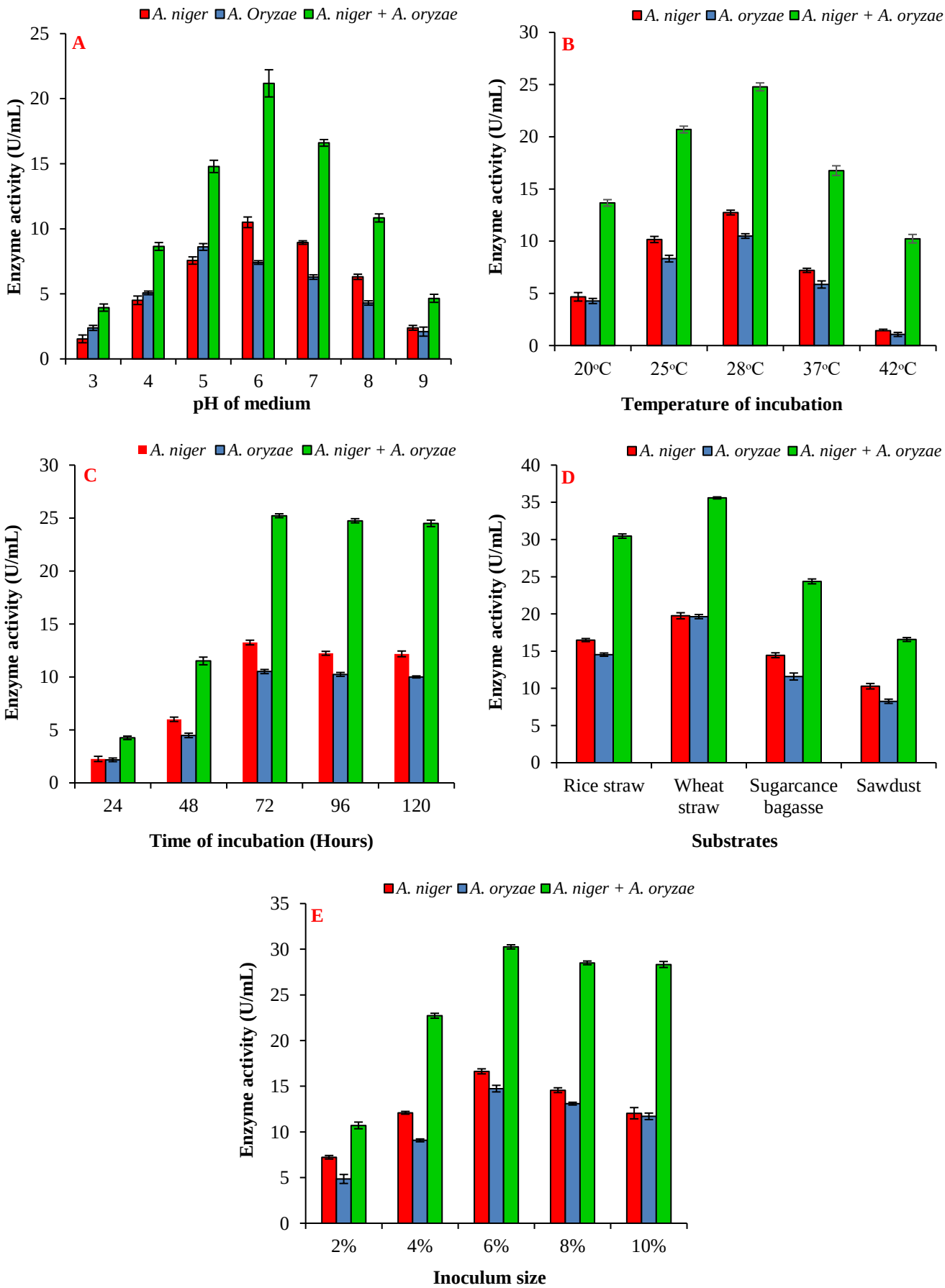

Fig. 3. Optimization of laccase enzyme production by co-culturing of *A. niger* and *A. oryzae*. (A) Optimization of pH of culture medium; (B) Optimization of temperature of incubation; (C) Optimization of time of incubation; (D) Substrate optimization; (E) Optimization of inoculum size.

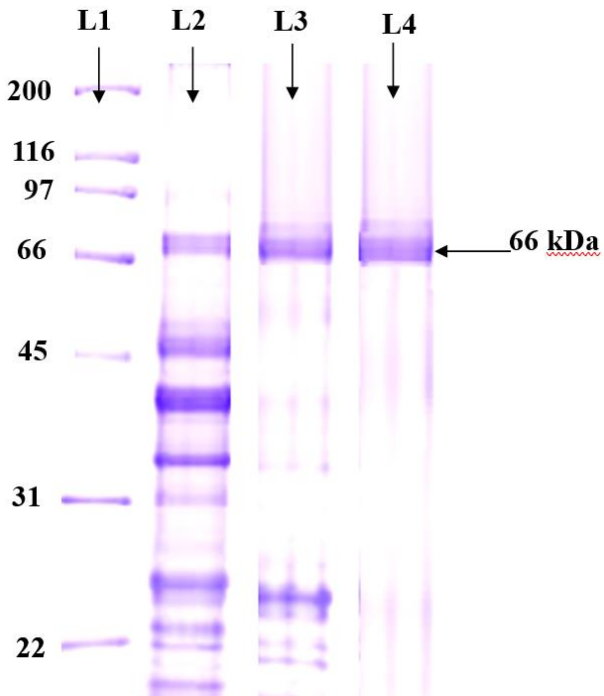

Fig. 4. SDS-PAGE of laccase enzyme produced by co-culturing. L1 represents the protein ladder, L2 represents the crude laccase enzyme, L3 represents the partially purified laccase enzyme and L4 represents the final purified laccase enzyme. A very clear single protein band of almost 66 kDa represents the successful purification of laccase enzyme produced by co-culturing.

Thermostability: Thermal stability of purified laccase enzyme was assessed by incubating the purified enzyme at a wide temperature range (30 to 80°C) for 1 to 4 hours. Residual enzymatic activity was checked to assess the stability of enzyme after each experiment. It was observed from the obtained results that purified laccase was very stable at a wide range of temperature for long period as shown in figure 4B. Results indicated that the enzyme retained almost 42% activity after 4 hours of incubation at 80°C. No change in activity of enzyme was observed at 30°C, 40°C and 50°C for up to 4 hours at room temperature (Fig. 5B). Whereas, a slight reduction in the activity of laccase enzyme (70% & 60%) was noticed at the temperature ranges of 60°C and 70°C after the incubation period of 4 hours.

Effect of divalent ions: To assess the effect of divalent ions on the stability and activity of laccase enzyme, it was incubated with 1 mM of different ions (Ca^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+}, Na^{1+}, K^{1+}, NH_4^{1+}, and Ni^{2+}) and EDTA at room temperature for 1 hour and later the residual enzyme activity was measured. Observations revealed that most of the ions did not showed any effect on the enzyme activity but some metal ions such as Ca^{2+}, Mg^{2+} and Cu^{2+} enhanced the enzymatic activity up to 142%, 116% and 164% respectively as provided in figure 4C. Minimal reduction in activity of enzyme was noticed in the presence of other metal ions but EDTA influenced strongly on the enzyme activity and reduced it up to 23% (Fig. 5C). Calculated enzyme activity was 91%, 86%, 80%, 73% and 74% with 1 mM Co^{2+}, Na^{1+}, K^{1+}, NH_4^{1+}, and Ni^{2+}.

Effect of organic solvents: The incubation of purified laccase was carried out for 1 hour at room temperature with different solvents (10 – 30%) to check their influence on enzyme activity. The observed residual enzyme activity indicated that purified laccase enzyme was efficiently stable even with higher concentrations (30%) of organic solvents as shown in Fig. 5D. With 10% of all used organic solvents, the enzyme possessed more than 90% activity after 1 hour. The enzyme activity was calculated as 85%, 81%, 79%, 76% and 80% in the presence of 30% ethanol, methanol, acetone, n-butanol and isopropanol as shown in figure 4D. Similarly, in the presence of 20% methanol, ethanol, isopropanol, acetone and n-butanol the activity of enzyme was recorded as 86%, 85%,86% and 84%, 83% respectively.

Effect of inhibitors: To determine the effect of different enzyme inhibitors such as SDS, β-mercaptoethanol, sodium azide, Tween 80, DMSO, and dithiothreitol (DTT) on the activity and stability of purified laccase, incubation was carried out for 1 hour with varying concentrations (1 – 3%). Results indicated that enzyme retained very good activity in the presence of 1% of all used inhibitors in the study (Fig. 5E). Whereas mild reduction in enzyme activity was noticed with higher concentration (3%) of inhibitors. The calculated enzyme activity was 41%, 61%, 85%, 53%, 75% and 52% in the presence of 3% SDS, DMSO, NaN3. BME, Tween 80 and DTT, respectively (Fig. 5E).

Substrate specificity: The determination of substrate specificity of purified laccase enzyme was carried out by using 5 mM of various substrates like ABTS, hydroquinone, guaiacol, benzidine and syringaldazine under standard assay conditions. The maximum enzyme activity (35.85±1.07 U/mL) of laccase enzyme produced from co-culturing of *A. niger* and *A. oryzae* was obtained when ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) was used as a substrate as shown in figure 5F. With other used substrates such as hydroquinone, guaiacol, benzidine and syringaldazine the enzyme activity was 10.73±0.19 U/mL, 9.25±0.22 U/mL, 14.81±0.40 U/mL and 13.12±0.63 U/mL, respectively (Fig. 5F).

Discussion

Co-cultivation of microorganisms is an efficient method for large scale production of enzymes due to high yield, less input of materials and shorter production time (Benoit-Gelber *et al.*, 2017; Flores *et al.*, 2010). Previously, co-cultivation of *Pleurotus ostreatus* and *A. niger* resulted in increased activity of enzymes like laccase and carboxymethyl cellulase during the breakdown of powdered bamboo shoot shell (Wang *et al.*, 2021). Present study dealt with enhanced production of laccase enzyme (35.59±0.13 U/mL) by co-cultivation of *A. niger* and *A. oryzae* by using solid state fermentation process. The increased laccase activity and yield achieved through co-cultivation can potentially lower the production costs associated with enzymatic processes as both strains were co-cultivated instead of two separate reactions.

The optimization of laccase enzyme production through co-culturing of *A. niger* and *A. oryzae* was conducted by determining the optimal conditions, including pH, temperature of incubation, incubation time, inoculum size, and media composition. It has been reported that most fungal species perform well in acidic or neutral pH environments but lose functionality in alkaline conditions (Zou *et al.*, 2012). Iyer & Chattoo (2003) also demonstrated that maximum laccase production from the rice blast fungus, *Magnaporthe grisea*, occurred within a pH range of 3-9 using syringaldazine as a substrate. The best production of laccase enzyme in this study was obtained after 72 hours of incubation from co-cultured strains. However, Vandana *et al*., (2014) reported the best production of laccase after 120 hours from *A. niger*. In another study, Dhakar *et al*., (2015) reported that the highest yield of laccase from *A. niger* was obtained after 21 days of incubation. The results obtained in this study suggested that the time of incubation was reduced (72 h), and enzyme production was increased (25.22±0.18 U/mL) by co-culturing of *A. niger* and *A. oryzae*. Due to the temperature sensitivity of both fungal strains, incubation temperature showed a significant impact on the fermentation process in SSF. High temperatures have a detrimental impact on the metabolic processes of microorganisms ultimately resulting in the denaturation of essential enzymes. Šnajdr and Baldrian (2007) reported the best production of laccase enzyme at 20 to 30°C from *T. versicolor*. In another study it is reported that the laccase enzyme production begins to reduce above 28°C (Elsayed, 2012).

The purified laccase enzyme produced by co-culturing of *A. niger* and *A. oryzae* has a molecular mass of 66 kDa as shown in Figure 4 that is like the molecular masses of most other fungal laccases, which have been reported to be between 60 kDa to 80 kDa (Thurston, 1994). Songulashvili *et al*., (2016) reported molecular mass of 66 kDa isolated from *Coriolopsis gallica*. Umar & Ahmed (2022) determined the molecular weight of isolated laccase from *Ganoderma leucocontextum* was 65.0 kDa by using SDS-PAGE.

Purification of laccase enzyme produced in this study by co-culturing of *A. niger* and *A. oryzae* was achieved by ammonium sulphate precipitation method as well by ion exchange chromatography. Ammonium sulfate is frequently used for salting out of proteins due to its great solubility, which enables solutions to have extremely strong ionic charge, low cost, and the accessibility of the original product (Duong-Ly *et al.*, 2014). Ion exchange chromatography can be utilized as a fundamental technique to distinguish different ionic species within a given sample. (Ko *et al.*, 2007) This method proves valuable in separating both inorganic and organic ions, including proteins, amino acids, and oligonucleotides (Isenberg *et al.*, 1995). Previously, many heat sensitive enzymes were purified by ammonium sulphate precipitation method (Zafar *et al.*, 2022a; Zafar *et al.*, 2022b; Hamid *et al.*, 2021; Zafar *et al.*, 2019; Zafar *et al.*, 2018).

Effect of divalent ions on the stability and activity of purified laccase enzyme was examined in this study and it was observed that the presence of Cu^{2+} ion increased laccase activity to 164%, which might be due to the addition of copper helping in laccase folding correctly that resulted in a more efficient transportation of copper ion to the golgi apparatus (Uldschmid & Marbach, 2003), whereas EDTA resulted in reduced laccase activity up to 23%. These results are in accordance with previous studies. Kumar *et al*., (2012) also reported the increased laccase activity in the presence of Cu^{2+} and Mg^{2+} ions from *Cereus pterogonus*. Similarly, Vivekanandan *et al*., (2014) reported that from *Aspergillus nidulans* addition of Mg^{2+} and Cu^{2+} ions increased the laccase activity. The impact of different inhibitors on purified laccase activity was also investigated by using SDS, DMSO, NaN3**.** BME, Tween 80 and DTT in this study. The purified laccase enzyme showed a good stability in the presence of all inhibitors as shown in figure 5E even at higher concentrations. Previously reported laccase enzymes were shown to be inhibited in the presence of certain inhibitors. Atalla *et al*., (2013) reported that laccase activity was completely inhibited by sodium azide at a maximum amount of 2 mM. The interaction of sodium azide to the types 2 and 3 copper sites impacts internal electron transfer, consequently inhibiting the activity of *Trichoderma harzianum* laccase (Sadhasivam *et al.*, 2008).

The stability of the catalytic hydrophobic core residues of laccase and its capacity to maintain hydration characteristics can be affected differently by the kind of organic solvent used (Jafari *et al.*, 2020). In this research it was observed that the activity of the laccase enzyme remained stable in the presence of different organic solvents even at higher concentration. This observation was similar to previous research done by Rasekh *et al*., (2014) on bacterial laccase organic-solvent tolerance, which revealed that alcohols with short hydrocarbon chains might influence the stability and function of the enzyme. Furthermore, the presence of organic solvents is also essential for the use of enzyme membrane immobilization (Liu *et al.*, 2019). In another report, it was described that in the presence of 10% organic solvents, the catalytic activity of the laccase from *S. carnis* was reduced (Olajuyigbe & Fatokun, 2017).

The maximum enzyme activity of laccase enzyme produced by co-culturing of *A. niger* and *A. oryzae* was gained when ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) was used as a substrate as shown in figure 5F. This is because of the position, structure, and sequences on the active site which makes the enzyme specific to its substrate. Similar results were reported by Vana *et al*., (2023) study. Ezike *et al*., (2021) also claimed that the laccase enzyme had greater substrate specificity towards ABTS than other substrates used in the experiment.

In the light of all these studies, the laccase enzyme produced through co-cultivation of *A. niger* and *A. oryzae* could be a competent candidate for industrial usage, particularly in processes requiring high thermal stability, solvent tolerance, and resistance to inhibitors.

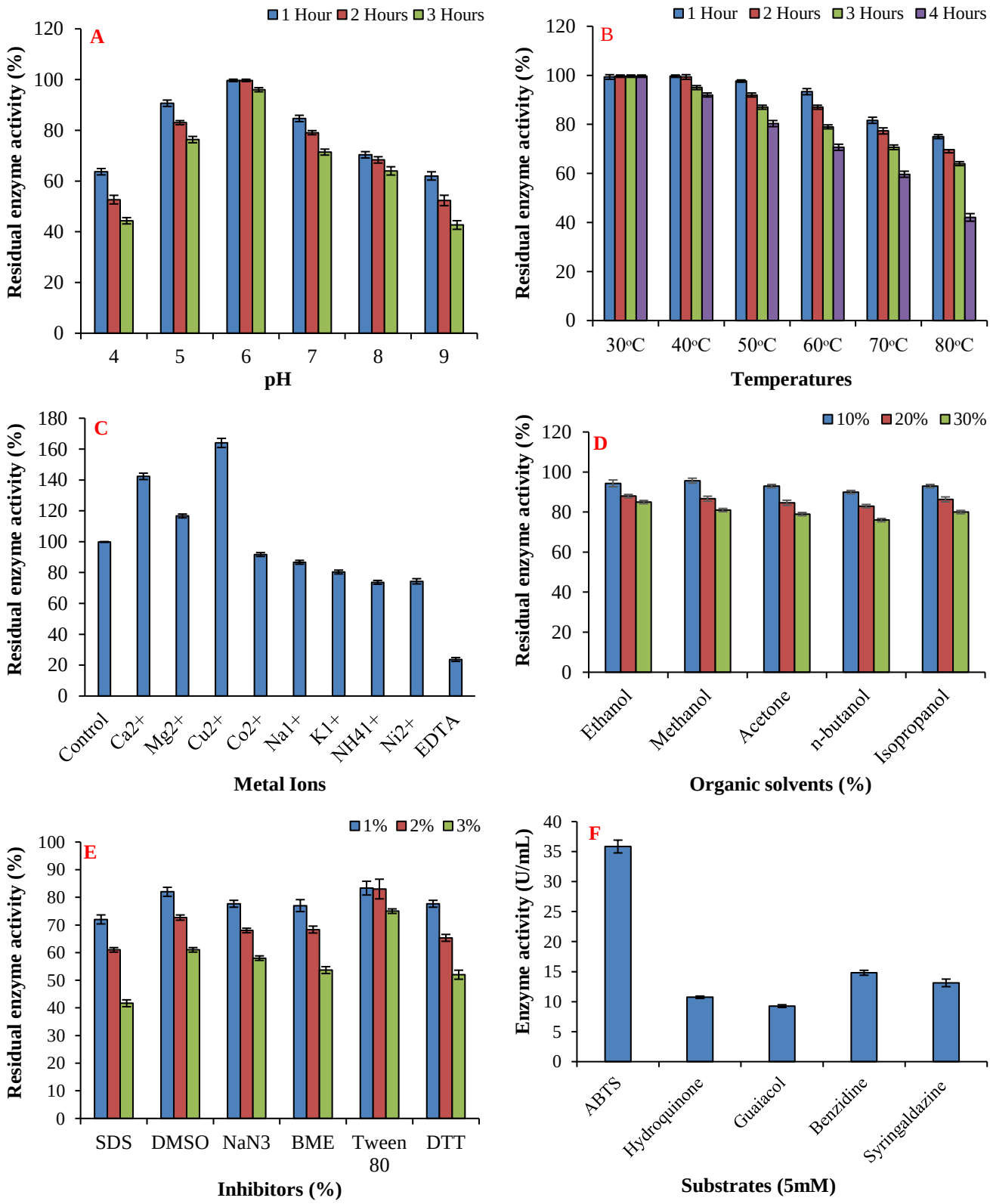

Fig. 5. Characterization of purified laccase enzyme. (A) pH Stability (B) Thermostability (C) Effect of metal ions (D) Effect of organic solvents (E) Effect of inhibitors (F) Substrate specificity.

Conclusion

The co-culturing of *A. niger* and *A. oryzae* for laccase enzyme production shows great promise. Through their combined metabolic pathways, this synergistic collaboration has exhibited enhanced laccase production, reflecting a potential increase of enzymatic activity. As a part of characterization, enzymatic characteristics like substrate specificity, optimum temperature, pH and molecular weight through SDS-PAGE were determined. Successful characterization clarifies the functional characteristics of the enzyme and provides an effective and sustainable way to use laccase for a variety of purposes, including industrial and environmental ones.

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