Punjab University Journal of Zoology

39(2): 141-149 (2024)
https://dx.doi.org/10.17582/journal.pujz/2024/39.2.141.149

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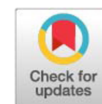

Research Article

Optimization of Laccase Production Parameters by *Aspergillus niger* from Different Eco-Logical Sites of Lahore

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Article History
Received: August 04, 2023
Revised: April 05, 2024
Accepted: April 29, 2024
Published: August 06, 2024

Authors' Contributions
SRZ perfromed all the researcha as part of her PhD studies, compiled the results and wrote the manuscript. SAM supervised the project, performed statistics and graphs, helped the main author in research.

Keywords
Laccases, Guaiacol, *Aspergillus niger*, Industrial waste water, Lahore

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Abstract | The present study comprises of isolation and screening of fungal isolates for laccase enzyme production from different environmental samples of soil, water and air obtained from selected areas of Lahore. A total 248 strains of microscopic fungi were cultured by using serial dilution method and plate assay method. Pure cultures of these strains were further inoculated on PDA (Potato Dextrose Agar) plates supplemented with indicator compounds guaiacol or tannic acid to check the laccase producing ability. Among 08 isolated strains belonging to *Aspergillus* species of microscopic fungi, only 04 strains were screened for laccase production. In order to select most potential laccase enzyme producing strain among different isolated strains, quantitative analysis was carried out. Results clearly indicated that *Aspergillus niger* isolated from soil sample collected from agricultural area produced remarkable zone diameter and culture colony diameter. The remaining strains showed variable results for enzyme production. Laccase enzyme production was carried out by screened strain of *Aspergillus niger* under submerged fermentation technique. Optimization of different cultural parameters that influence laccase enzyme biosynthesis by *A.niger* strain was done. To measure enzyme activity indicator compound guaiacol and acetate buffer were used. It has been found that laccase activity was maximum on day 9 of incubation when operated under following cultural conditions, 2 pH, 35°C, 3.0 % wheat straw as carbon source among other synthetic (glucose and maltose), and agricultural sources (sugarcane bagasse, rice bran), 1.5% urea as nitrogen source among other sources that include peptone, yeast extract, ammonium sulpate and ammonium chloride. Abench top fermenter was used in order to carry out scaled up studies). All the optimized cultural conditions were adjusted to culture *Aspergillus niger* for enhanced production of laccase enzyme. Concentrated crude laccase enzyme extract was obtained by filtration and centrifugation. Partial purification was carried out by ammonium sulphate precipitation method and further purification was done by column chromatography and gel filteration method. It has been concluded that locally isolated strain *A. niger* was one of the potential and promising fungal isolate, screened for laccase enzyme activity, from soil sample collected from agricultural area near Harbans Pura, Lahore.

Novelty Statement | A novel strain of filamentous fungus *Aspergillus niger* i.e., SAN5 was isolated from soil sample collected from agricultural area Harbans Pura of District, Lahore Pakistan and screened for laccase enzyme production with promising potential.

To cite this article: Zaidi, S.R. and Mirza, S.A., 2024. Optimization of laccase production parameters by *Aspergillus niger* from different eco-logical sites of Lahore. *Punjab Univ. J. Zool.*, **39**(2): 141-149. https://dx.doi.org/10.17582/journal.pujz/2024/39.2.141.149

Introduction

Laccases are oxido-reductases as they catalyse oxidation (of phenolic and non-phenolic compounds) and

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reduction (of water) reactions at the same time. Laccases are extracellular and catalyzes oxidation of various phenolic compounds, diamines, aromatic amines, non-phenolic compounds like lignin degradation and detoxification. Because of its most important ability to oxidize phenolic and non-phenolic lignin compound, this enzyme finds application in the field of biotechnology and thus gained interest of biotechnologists from all over the globe. Its commercial applications include different industries including textile, paper and pulp and food industry. The potential applicable fields are cosmetics, bioremediation, biodegradation of environmental pollutants, and removal of endocrine disruptors. In recent years, laccases have been efficiently applied to nano-biotechnology because of their ability to catalyse electron transfer reactions without using any additional co-factor (Patel *et al*., 2009). Industrial waste contains synthetic dyes that are toxic to the environment. Using laccases degradation of theses toxic materials can be carried out without using physical or chemical method. Detoxification of environmental pollutants can also be done by using laccases. Major part of agricultural waste consists of lignocellulose present in abundance as natural material that remained unutilized. This material is rich source of organic compounds including lignin, cellulose and hemicellulose. Thus its utilization as bioconversion substrate into value added products i.e., enzymes. The enzyme released can also be used to cope with the environmental imbalance created by continuous release of phenolic compounds. Because of substrate verstality laccases reacts with variety of substrate and various indicator compounds can be used for its production. Guaiacol, tannic acid, 2,2 ABTS (azinibis-3 ethylbenzthiazoline, 6 sulphonic acid), syringaldazine and polymeric dyes including RBB-R (remazol brilliant blue-R) are the compounds commonly used as indicators. Laccases are present widely in nature including bacteria, fungi and higher plants. Laccases that are obtained from the fungi are more stable, having wide substrate specificity and ability of oxidation of various phenolic compounds. Thus fungal laccases are advantageous as compared to bacterial or plant laccases. Fungi have ability to produce a wide variety of extracellular enzymes as they are able to break down all kinds of organic matter thereby regulating the balance of carbon and nutrients. It has been reported that fungi successfully inhabit soil as they possess remarkable capacity to assume various morphologies while responding unfavourable or challenging extreme environmental conditions. They contributed to preserve carbon and nutrient balance because of their ability to secrete extracellular enzyme that involves in breakdown of organic matter and decomposition of soil components. Thus, fungal activity is influenced by number of biotic and abiotic factors such as temperature and soil pH. Laccase production found in various fungi in abroad range of taxa. Fungi belonging to different families are known to be laccase positive including ascomycete, deuteromycetes and basidiomycetes. Laccase synthesis has been reported in many ascomycetes but not in complete family of this phylum has been described. Presence of complex group of MCO coding genes in genome of filamentous fungi makes them good source for laccases having potential biotechnological applications. *Aspergillus niger* are reported to produce laccase. *A. niger* found to be good source of new laccases. Substrate versatility noticed in plate assay makes them interesting for purification and biochemical comparison. *A. niger* is widely used as tool for laccase producers and various oxidoreductases including laccase from black aspergilla have been industrially commercialized. Laccases are produced as a result of secondary metabolism and its production is directly proportional to various limiting factors including type of culture, medium composition, time of incubation, presence of inducers as they contains regulatory sites for example MREs (metal responsive elements and XREs xenobiotics responsive elements. Submerged fermentation (SF) technique has been the most commonly used for production of most enzymes including laccases. Submerged or liquid state fermentation method results in homogeneous distribution of nutrients due to which cultured micro-organisms would be able to get full contact and absorption of nutrients. Because of substrate verstality, high catalytic efficiency environmentally friendly etc. properties of laccases, these are used in application in various industrial, medical, biotechnological and environmental fields for energy exploitation and water and solid waste management. The main drawback of industrial scale production and usage of this enzyme is high cost. Thus, it is need of an hour to find out cheap resources in order to produce laccases on commercial scale at minimum cost. Keeping in view such justifications, this research work was designed to isolate local fungal strains belonging to filamentous fungi from different environmental sites of Lahore, having potential to produce laccase enzymes. The main focus of this work was to investigate improved production of laccase enzyme by locally isolated filamentous fungi.

Materials and Methods

The current research work was carried out in the Institute of Agriculture Sciences, University of the Punjab and Pakistan Council of Scientific and Industrial Research, Laboratories Feroz Pur Road, Lahore. Isolation of laccase positive fungal strain, production and optimization of cultural conditions for the production of laccase enzyme from isolated, screened and identified potential filamentous fungi from different samples collected from selected sites was observed. Following materials and methods were used to carry out this process.

Sample collection

In order to isolate filamentous fungi, different soil and water samples from selected environmental sites of Lahore

and nearby areas were collected in sterilized bottles and plastic bags, brought to the laboratory and stored at 4°C.

Isolation and identification of fungi

To isolate filamentous fungi from soil samples, serial dilution and plating method, by Harley *et al*. (1993) were used. The species were morphologically identified by the help of 'Compendium of Soil Fungi' and *Aspergillus* spp. were identified by using Doyle and Doyle (1987) on molecular basis.

Screening for laccase producing filamentous fungal species

To screen laccase positive fungal strains, both qualitative and quantitative analysis were applied.

Qualitative analysis: (Plate assay method) To test laccase activity, all the isolated fungal strains from purified colonies were inoculated on PDA medium containing petri plates supplemented with 0.2% guaiacol compound. Laccase enzyme activity was visualized by the appearance of prominant reddish- brown zones beneath and around the colony. This zone formation is due to oxidative polymerization of used indicator compound i.e., guaiacol, by the laccase produced.

Quantitative analysis

Selected strains of different fungal species that showed positive results for laccase activity were picked up for quantitative analysis using submerged fermentation method. The experiment was carried out by using PDB medium.

Screening of culture medium

The selected fungal isolates were cultured on different synthetic liquid media for determination of laccase activity.

Optimization of culture conditions for enzyme production

Various parameters were employed to determine the optimum cultural conditions for laccase synthesis and fungal dry mycelial mass. Sivakumar *et al*. (2010) from isolated strain *Aspergillus niger.*

Effect of temperature

In order to find optimum temperature experiment was performed employing temperatures such as 20°C, 25°C, 30°C, 35°C and 40°C .

Effect of pH

To monitor the effects of initial pH the reaction was carried out at different pH levels that ranges from 2 to 8, after incubation of 7 days.

Inoculum size

The laccase enzyme activity was calculated by inoculating inoculum discs of different numbers such as 1, 2, 3, 4 and 5 discs, each of which was 0.5 cm.

Incubation time

For the determination of the effects of incubation period on improved laccase yield.

Effect of nitrogen source

To investigate effects of nitrogen on laccase production, five different synthetic nitrogen sources that include yeast extract, peptone, urea, ammonium sulphate and ammonium chloride were tested. Laccase activity was also assessed using different concentrations (0.5%, 1%, 1.5%, 2%, 2.5%, 3%) of selected source of nitrogen.

Upscale studies

A bench top fermenter was used to carry out upscale studies. All previously optimized cultural conditions were adjusted to isolated, screened and identified strain of *Aspergillus niger* for enhanced production of laccase enzyme.

Extraction of crude enzyme

After optimum incubation period fungal culture was harvested by centrifugation at 10,000 rpm at 4 °C for 10 min. The supernatant obtained was crude enzyme and was stored for further experiments.

Enzyme activity assay

Laccase activity was calculated at 30 using 3 ml of 10 mM guaiacol in 100 mM acetate buffer pH 5 having 10% v/v acetone and 1 ml of culture filtrate and incubated for 15 min. The changes in absorbance was monitored at 470 nm using UV spectrophotometer. A blank was also prepared that contains 1 ml of distilled water in place of culture filtrate. Enzyme activity can be defined as amount of enzyme required to oxidize 1 mole of substrate per minute and is expressed as international units denoted by IU.

Determination of dry mycelial mass

To calculate the dry mycelial mass of fungal culture, broth was filterated using filter paper Whatman no. 1. The fungal mycelial mass was then oven dry at 80 °C for 1 h.

Results and Discussion

Isolation and identification of filamentous fungi and phylogenetic analysis of Aspergillus niger (TKM010)

Newly generated sequence in the present study was BLAST searched at NCBI https://blast.ncbi.nlm.nih.gov/Blast.cgi. The BLAST result suggest that local strain (TKM010) has a close affinity with *Aspergillus flavus*. Initial BLAST analysis of the ITS nucleotide sequences revealed that the local strain (strain code) showed 99.98 % identity with *Aspergillus niger* (OL629242-OL629244). Closely related *Aspergillus* section *flavi* sequences were

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retrieved from NCBI along with *Neopetromyces muricatus* (EF661434), which was chosen as outgroup. All the sequences were aligned using online MUSCLE tool at https://www.ebi.ac.uk/Tools/msa/muscle/. The aligned data set was subjected to MEGA 6.0 for constructing phylogenetic tree. The aligned data set after trimming from both ends consisted of 607 characters of which 463 were conserved, 130 were variable and parsimony informative and 125 were parsimony un-informative. In the phylogram the local strain clustered with *A. niger* (OL629242—OL629244, MN788109 and MN788114) with a strong bootstrap support of 100 (Figure 1). The closely allies of the local strain *A. leporis* (FJ491467 and AF257796), *A. alliaceus* (EF661543 and EF661556) and *A. coremiiform* (FJ491474) also clustered in a separate subclade.

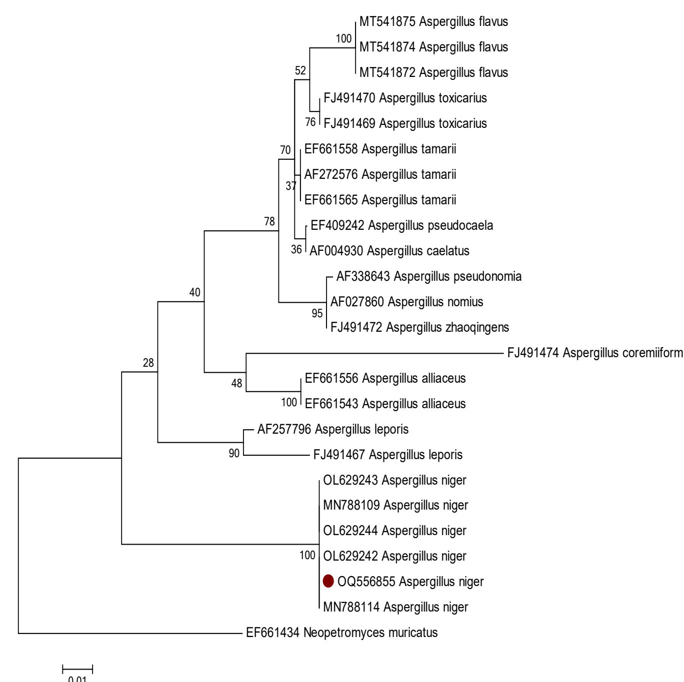

Figure 1: The evolutionary history was inferred by using the maximum likelihood method based on the Tamura 3-parameter model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter= 0.2474)). The analysis involved 25 nucleotide sequences. Evolutionary analyses were conducted in MEGA6. The sequence generated in the present study is marked with.

Screening for potential laccase producing filamentous fungal species

Qualitative studies by plate assay method: Purified strains were grown on solid medium plates supplemented with indicator compounds guaiacol and tannic acid for this purpose. The reddish brown zone appeared be neat or around the colony showed laccase production.

Quantitative assay

The strains showing zone formation were selected for quantitative analysis by employing liquid state fermentation technique using potato dextrose broth. Highest laccase activity was shown by *A. niger* strain i.e 0.097 isolated from agricultural soil (Table 1).

Table 1: The strains showing laccase production.

Strains	Sources of material	Laccase activity (U/ml)
SAN_3	Rose root	0.076
SAN_4	Potato tuber	0.071
SAN_5	Agricultural soil	0.097
SAN_6	Polluted water	0.088
SAN_8	Industrial waste water	0.083

Screening of culture medium

Results indicated that among different media used Medium of Olgas *et al*. (1998) proved best for laccae enzyme production by isolated strain of *A. niger* (Table 2).

Table 2: Screening of synthetic medium for liquid state fermentation.

Medium	Reference	Enzyme activity (U/ml)
M1	Jonthan and Faisidi, 2001	0.082
M2	Chawachart *et al*., 2004	0.095
M3	Olgas *et al*., 1998	0.155
M4	Coll *et al*., 1993	0.149
M5	Couto *et al*., 2006	0.101

Optimization of cultural conditions for laccase production in shake flask

Improved laccase production was achieved by adjusting physical conditions such as pH, temperature and inoculum size etc. and nutritional conditions which includes carbon, nitrogen sources.

Temperature

In our results laccase activity increase with the increase in temperature from 20°C to 25°C and 30°C and maximum at 35°C i.e. 0.167 Uml^{-1}. Fungal dry mycelial mass rise from 20°C to 25°C and 30°C and reached maximum at 35°C i.e 1.68g (Figure 2).

Initial pH

In our results enzyme activity was found to be maximum at pH 4. *A. niger* strain showed minimum enzyme synthesis at high acidic and high alkaline conditions. So the least enzyme activity was pH 2 i.e., 0.121 U/ml. It has been noticed that laccase activity decrease with the increase of initial pH. Fungal mycelial mass increase from pH 2 and become maximum at pH 4 i.e 0.236 U/ml. Above this decrease is noted. Maximum fungal mycelial mass was recorded at pH 5 (Figure 3).

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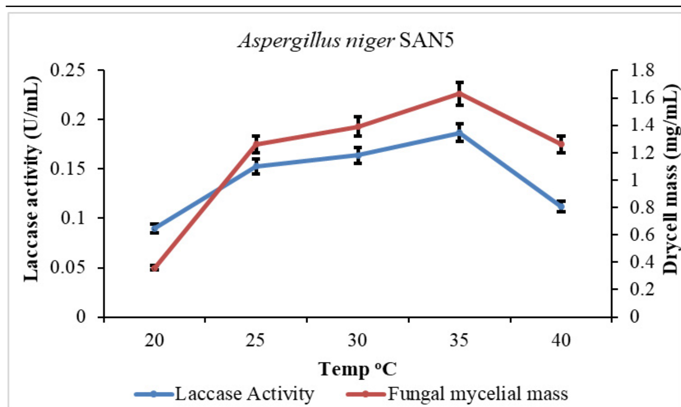

Figure 2: Optimization of temperature for laccase produce from *Aspergillus niger* (SAN$_5$).

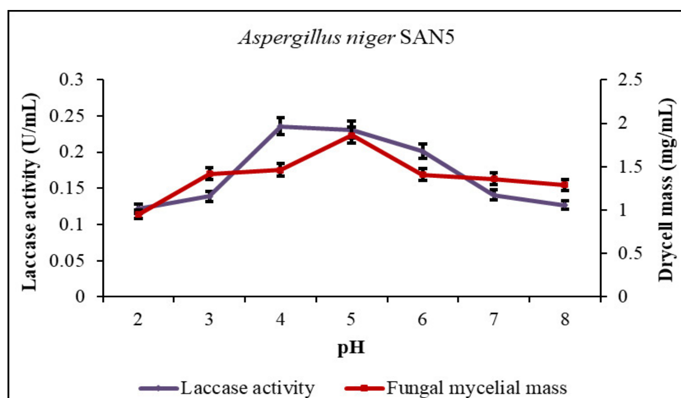

Figure 3: Optimization of pH for laccase production from *Aspergillus niger* (SAN$_5$).

Inoculum size

In present study, highest enzyme production was calculated in reaction mixture containing three inoculum discs each of which 0.5 cm. Thus the optimum inoculum size was recorded to be 1.5 cm. It has been also noted that as the inoculum size increase, enzyme activity also increased but up to specific limit. Above optimum level of inoculum size, enzyme production decreased may be due to depletion of nutrients. The fungal dry mass was also calculated maximum in reaction mixture containing three inoculum discs and least in reaction mixture containing two inoculum discs (Figure 4).

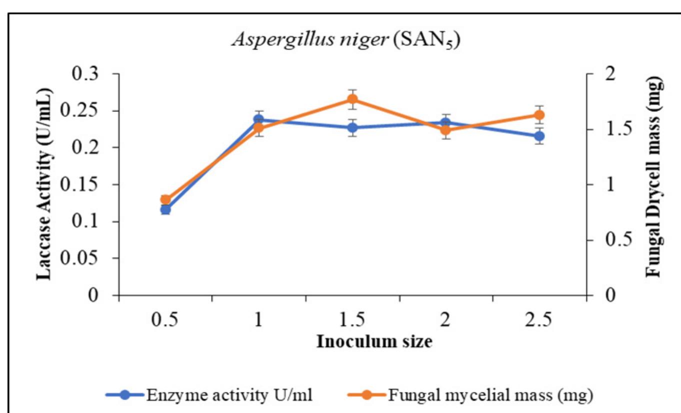

Figure 4: Optimization of size of inoculum for enzyme production from *Aspergilluus niger* (SAN$_5$).

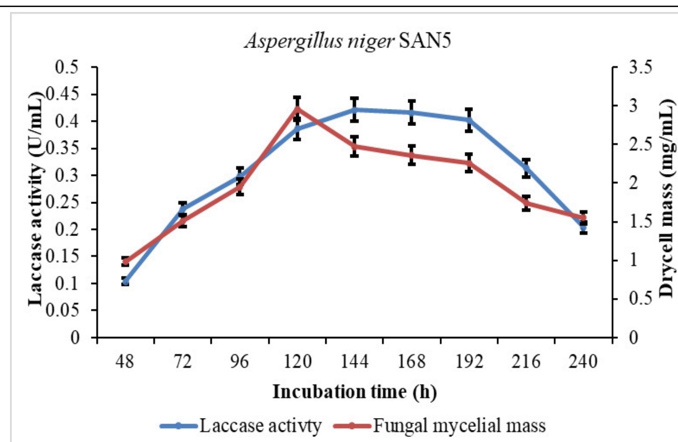

Figure 5: Effect of incubation time on the production of laccase and fungal mycelial mass of *Aspergillus niger* (SAN$_5$).

Incubation time

Laccase enzyme production in selected strain of *Aspergillus niger* was also found to be highest on sixth day of incubation. Fungal mycelial mass increased with the passage of time and maximum mass was observed on ninth day of incubation. On tenth day it has been observed that there was decrease in mass, because of depletion of nutrients (Figure 5).

Carbon sources

The strain *Aspergillus niger* showed results in which maximum laccase activity was recorded with wheat straw i.e., 0.986 followed by sugarcane bagasse 0.638 and with maltose. The fungal mycelial mass was observed maximum with wheat straw i.e., 2.91 g followed by sugarcane bagasse. Minimum enzyme induction was observed with glucose. Results showed that all carbon sources act as inducers and helped in improving enzyme production. All carbon sources act as substrates for mycelial mass improvement (Figures 6, 7, 8 and 9) Results also clearly indicated that agricultural wastes act as good substrate comparatively synthetic carbon sources.

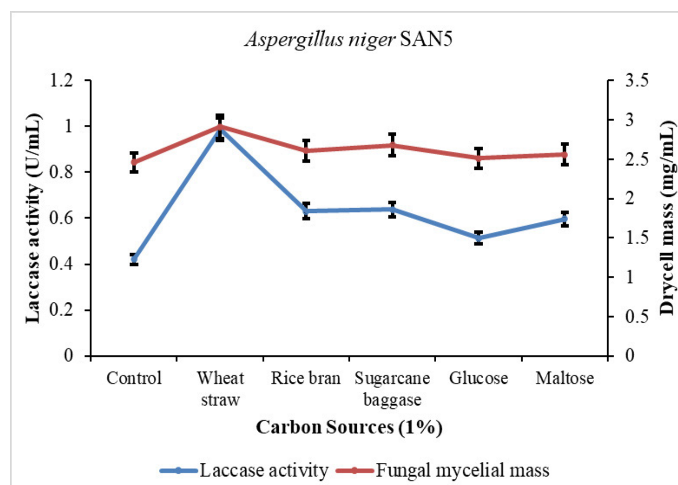

Figure 6: Carbon source optimization (1%) for production of laccase through *Aspergillus niger* (SAN$_5$).

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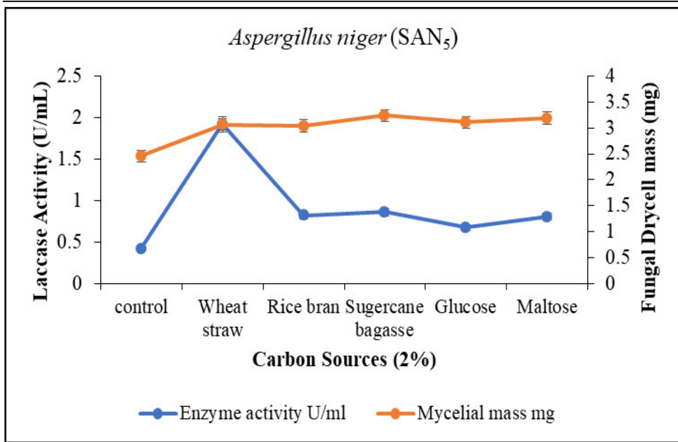


Figure 7: Carbon source optimization (2%) for production of laccase through *Aspergillus niger* (SAN_5).

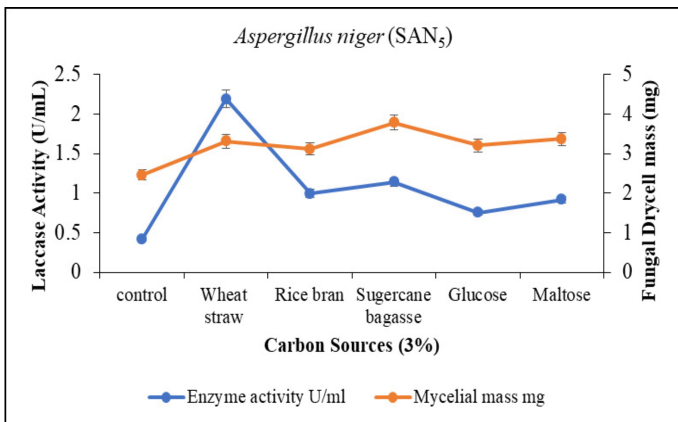


Figure 8: Carbon source optimization (3%) for production of laccase through *Aspergillus niger* (SAN_5)

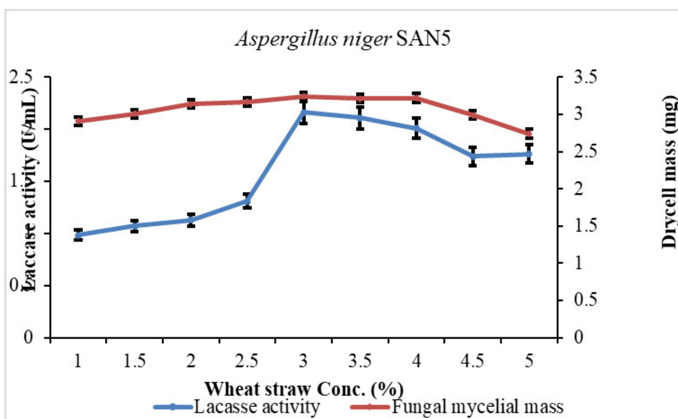


Figure 9: Wheat straw concentration optimization for laccase production through *Aspergillus niger* (SAN_5).

Nitrogen source

Results also showed that in *Aspergillus niger* strain maximum enzyme production and fungal mycelial mass was calculated using urea as nitrogen source among other nitrogen sources. This study also showed that by increasing Urea concentration fungal mycelial mass also increases and maximum was recorded at 1.5% i.e., 3.93 afterwards it started declining. Minimum mycelial mass was observed at 0.5% i.e., 1.46. All concentrations showed increase in mycelial mass shown in Figures 10, 11, 12 and 13.

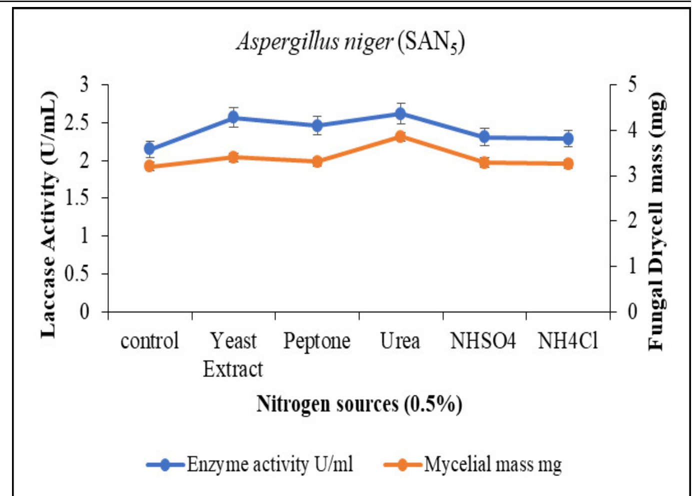


Figure 10: Nitrogen source optimization (0.5%) for laccase production through *Aspergillus flavus* (SAN_5).

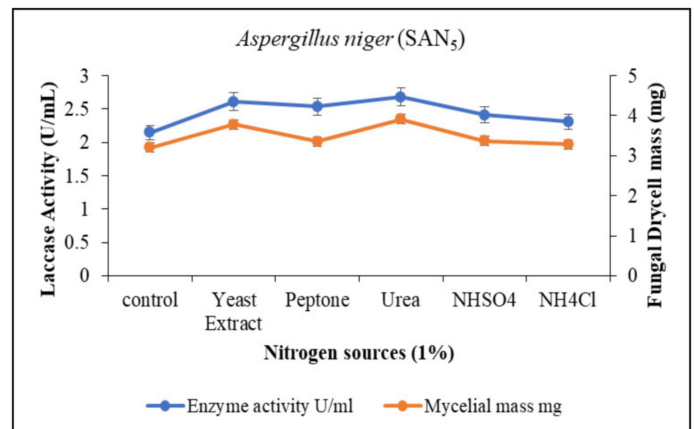


Figure 11: Nitrogen source optimization (1%) for production of laccase through *Aspergillus niger* (SAN_5).

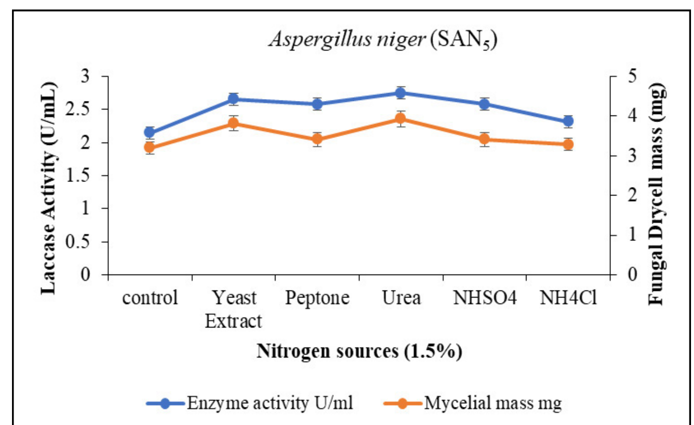


Figure 12: Nitrogen source optimization (1.5%) for production of laccase through *Aspergillus niger* (SAN_5).

Upscale studies

In a bench top fermenter, all optimized cultural conditions were adjusted to *A. niger* strain for maximum laccase enzyme production. It includes temperature: 35 C, pH: 2, carbon source: 3.0% wheat straw, nitrogen source: 1.5% urea, incubation time: 9 days. Laccase enzyme production was found to be increased from 0.097 to 4.25 Uml^- (Table 3).

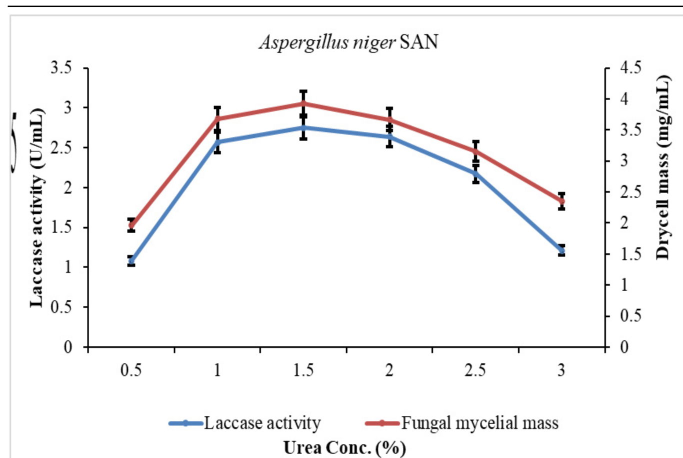


Figure 13: Optimization of urea concentration for laccase production from *Aspergillus niger* (SAN_5).

Table 3: Improved enzyme production using bench top fermenter under optimized conditions.

Strains	Enzyme activity (U/ml)	Fungal mycelial mass (mg)
Aspergillus niger SAN_5	4.256	5.45

Purification of laccase enzyme

Ammonium sulphate different percentages were employed and at 80% saturation complete precipitation of enzyme was recorded. Maximum activity of enzyme was also calculated at 80% saturation. Partially purified enzyme was subjected to further purification using gel filtration and column chromatography techniques (Table 4).

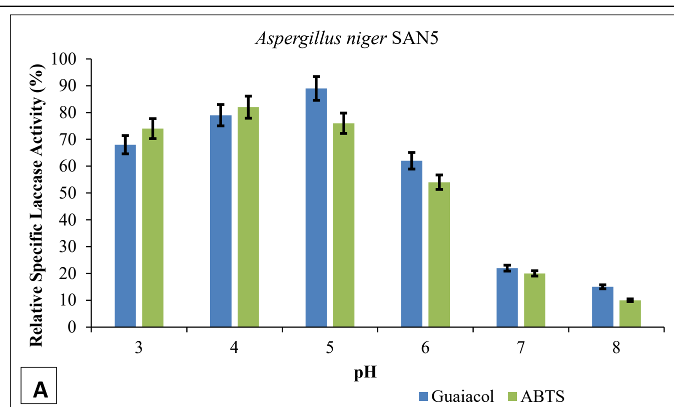
Table 4: Purification of fungal laccase by *Aspergillus niger*.

Purification steps	Total protein (mg)	Total enzyme activity (U)	Specific activity	Purifi-cation factor
Crude laccase	212	7139	33	1
Ammonium sulphate precipitation method	126	5611	44	1.33
DEAE-cellulose	78	2512	32	0.9
Sephadex G -100	18	1232	68	2

Total protein (mg)= Total volume (ml). Protein concentration (mg/ml) Specific Activity= No of moles of product/ mg of protein min Total Activity= Total protein. specific activity Purification Factor= Sp. Activity of each sample/ Sp. Activity of crude enzyme.

Characterization of crude and purified enzyme from Aspergillus niger

Relative Laccase Activity= It is calculated as total enzyme activity of each fraction divided by total enzyme activity of all fractions, multiplied by 100. To investigate the properties of enzyme, Characterization of enzyme against various treatments pH and temperature was carried out. Results obtained were represented in Figures 14A, B and 15A, B, respectively.



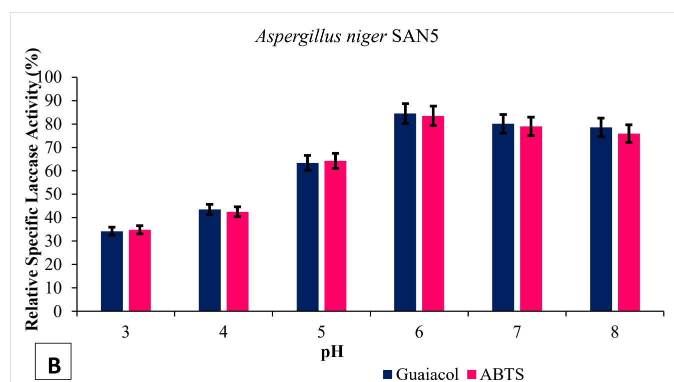
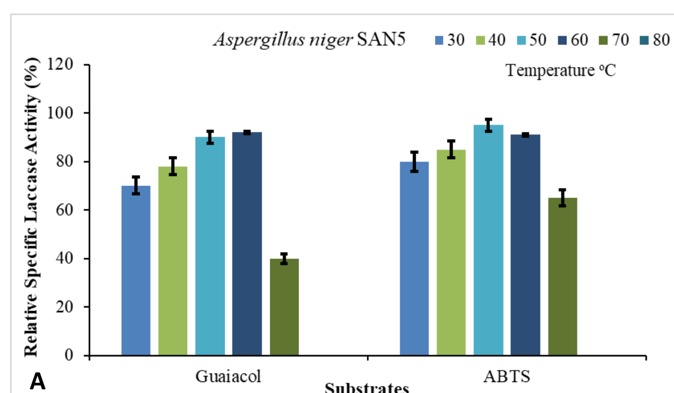


Figure 14: Effect of pH on crude enzyme (A), purified enzyme (B) produced by *Aspergillus niger*.



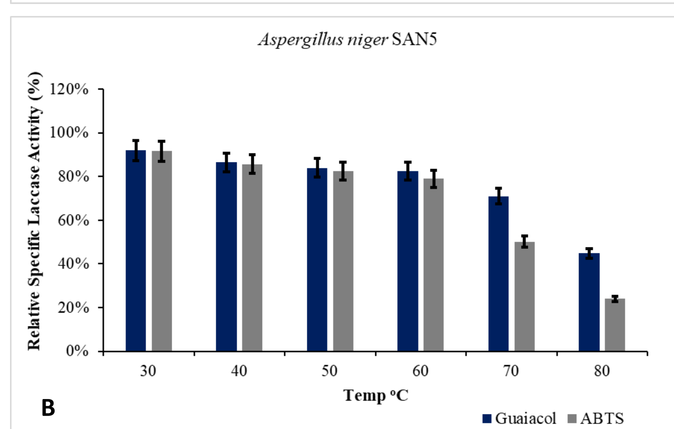


Figure 15: Effect of temperature on activity of crude enzyme (A), purified enzyme (B), produced by *Aspergillus niger*.

Enzyme production by filamentous fungi was proved to be highly productive, environmental friendly, cost

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effective and the most efficient method Kumar *et al.* (2011). The main aim of the current research work was the evaluation of laccase production potentials of locally isolated indigenous fungi belonging to filamentous species like *Trichoderma*, *Aspergillus*, *Fusarium* and *Penicillium* purified from various samples of water, soil and plant materials collected from different areas in and around Lahore city. As there is variations occur among biological aspects of fungi so identification of different strains was required for accurate management. Similar to our results reported isolation and screening of laccase positive fungal strains of. *Stereum ostrea* and *Phanerochaete chrysosporium* using 0.02% guaiacol as an indicator. Aftab and Ahmed (2015) isolated and screened laccase producing fungal species from various environmental samples and showed that 07 out of 29 isolates belong to *Trichoderma* species were found to be laccase producers. The results of preliminary screening indicated that pH of the fermentation medium is important for growth of culture and influence the metabolic activities of micro-organisms. In their research studies it was mentioned that low pH favored increased activity and production of secondary metabolites and isolated 11 fungal colonies from compost soil, using ABTS as indicator compound, 5 out of 11 colonies were found to be laccase positive. Results indicated that isolated strains were belonged to group of filamentous fungi i.e., *Fusarium* specie and *Aspergillus* specie. They isolated 37 colonies of fungi and only 8 of them found laccase producers, using indicator compound guaiacol. The most potential strain was isolated and identified and found to belong from *Apophysomyces* specie that belongs to group of filamentous fungi, commonly present in soil.

In present research work different parameters such as, incubation temperature, initial pH, carbon and nitrogen sources and their optimum concentrations were optimized in shake flask by applying liquid state fermentation method for enhanced enzyme production by isolated strain *A. niger* SAN_5. Results of earlier studies proved that all the mentioned parameters considered to be vital for enhanced laccase production. Similar to our results concluded that 30°C as optimum temperature for improved laccase synthesis with 172 IU/ml which showed that by increasing temperature enzyme activity increased but up to certain limits and at high temperature gradual decrease was observed and optimum temperature range for production of laccase enzyme using strain of *Pycnoporus sanguineus* was between 25°C and 30°C. occurred and reduced growth was obtained at 35 °C and reported maximum laccase production at pH 5. One of the important factors for culture growth and morphology is pH. as they were sensitive to the hydrogen ion concentration present in the medium. The optimal value of pH changes according to the substrate used because different compounds used as substrate cause different reactions for laccase enzyme. Patel *et al.* (2009) and Elshafei *et al.* (2012) findings are similar to our results that increasing inoculum size laccase production also increases but up to certain limits. Utilization of agro-wastes as natural inducer for laccase production proved to be an efficient way to reduce production cost and replace costly aromatic chemical substrates and synthetic inducers. Contrary to our results, rice bran as a carbon source was found to be the most effective substrate for laccase production by *Coriolus versicolor* strain compared to glucose, wheat bran and rice straw. Elsayed *et al.* (2012) used various lignocellulosic wastes including wheat bran, wheat straw, rice husk, rice straw, corn cob stalk, saw dust and sugar cane bagasse, in place of glucose in the medium for laccase production, and found that the maximum activity was recorded with glucose which is again opposite to our findings.

Conclusion

It can be concluded that *A. niger* strain SAN_5 isolated from agricultural soil was found to be most significant one. Its production can be improved by optimizing cultural conditions. Enhanced production can be achieved by addition of natural and synthetic compounds like 3.0% wheat straw, 1.5% urea at 35°C temperature under 5 pH. The use of guaiacol as indicator compound seems to be preferable than tannic acid. As there are many environmental and industrial applications of laccase producing fungi thus, isolated fungal strain can be used for further research work.

Acknowledgement

Authors are thankful to Higher Education Commission, Islamabad, Pakistan for financial support.

Conflict of interest

The authors have declared no conflict of interest.

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