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RESEARCH ARTICLE

Laccase production by White-Rot fungi of Arunachal Pradesh under Solid-State fermentation

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Abstract

Laccase is one of the major enzymes used for various industrial, bioremediation and biotechnological purposes. It is mainly produced by fungi belonging to ascomycetes and basidiomycetes. In this study, 37 wood-rotting basidiomycetes collected from Arunachal Pradesh were screened for laccase activity using both qualitative (guaiacol plate assay) and quantitative (solid-state fermentation on wheat bran) methods. Out of these, 25 fungi exhibited laccase activity, with significant variability observed among species and strains. Notably, *Pycnoporus sanguineus* and *Trametes hirsuta* ARFR303 appeared to be the most promising, producing 431 IU/L and 370 IU/L of laccase, respectively, under unoptimized conditions. Further optimization of fermentation parameters may further enhance their laccase production.

Key words: Wood-rotting basidiomycota; *Pycnoporus*; *Trametes*; Laccase; Time course study

1. Introduction

Laccases (EC 1.10.3.2) are versatile oxidoreductases capable of oxidizing a broad spectrum of substrates thereby making them highly valuable across multiple industries including paper, textiles, cosmetics, medical diagnostics, biosensors, food and beverages, agrochemicals, biofuel production, and also in the bioremediation of textile dyes and pesticides (Couto and Herrera, 2006; Shraddha et al., 2011; Ai et al., 2015; Upadhyay et al., 2016; Zerva et al., 2019). Given their diverse applications, large-scale laccase production at a cheaper cost is highly sought after. However, obtaining high laccase yields continues to be a major challenge (Liu et al., 2010), driving ongoing efforts to identify prolific laccase sources (Gassara et al., 2011; Yao et al., 2013).

White rot fungi (WRF) of phylum basidiomycota are natural degraders of woods. They degrade wood-lignin very efficiently by secreting several peroxidases and laccases and have been exploited for large-scale laccase production (Arora and Sharma, 2010; Dashora et al., 2023). However, laccase-producing ability varies significantly among WRF species and even different strains of the same species due to the inherent genetic make-up and also various environmental factors (Janusz et al., 2013; Yang et al., 2017; Han et al., 2020; An et al., 2021).

The source organism is considered the most crucial factor for laccase production (Brugnari et al., 2021; Das et al., 2024b). Therefore, exploration of various WRF of diverse ecological and geographical origins can be instrumental in identifying potential species. The biodiversity-rich tropical and temperate forests of Arunachal Pradesh (Indo-Burma biological hotspot) showcase a very high diversity of wood-rotting basidiomycetes due to abundant rainfall, high humidity, and a wide variety of host trees present. In the present study, several wood-rotting basidiomycetes were collected from different forest types of this region and screened for laccase production under solid-state fermentation (SSF) in order to identify efficient producers.

2. Material and method

2.1. Chemicals and agricultural lignocellulosic substrate

All the chemicals used were of analytical grade and were procured from HiMedia (India), Sigma-Aldrich Pvt. Ltd. (USA) and Merck (USA). Wheat Bran was chosen as an agricultural lignocellulosic substrate for SSF and was sourced from the local market.

2.2. Sample Collection and Isolation

Basidiocarps of 37 wood-rotting basidiomycetes were collected from their natural habitats, including trees, decaying wood, trunks, branches, and stumps, from 3 districts of Arunachal Pradesh: Lepa Rada (6), Papum Pare (24), and West Kameng (8). The samples collected were placed in clean paper bags and brought to the laboratory for further processing.

Fresh basidiocarps were thoroughly washed under running tap water and surface-sterilized with 70% ethanol cut into small pieces and inoculated onto potato dextrose agar (PDA) plates. The plates were incubated at 25°C in BOD incubator and subcultured until pure cultures were obtained. The pure cultures were maintained on PDA plates at 4°C for further studies.

2.3. Identification of fungi

The fungi were identified using stereo zoom microscope (Stemi58) and compound microscope (Zeiss A1). Identification of specimens was made by referring to taxonomic literatures. Additionally, the identification of some species was confirmed through ITS1-5.8S-ITS2 rDNA sequence analysis.

2.4. Qualitative screening of laccase-producing fungi

Agar plate assay containing 0.02% guaiacol was performed (Kiiskinen et al., 2004) to test the laccase activity of the fungus and development of a brick-red color under the fungal colony resulting from oxidation of guaiacol was considered as a positive indication.

Sl. No.	Isolate No.	Fungi	GenBank accession number	Family	District	Laccase activity (IU/L)
1	ARFR33	*Trametes gibbosa*	PV162548	Polyporaceae	Lepa Rada	50
2	ARFR34	*Ischnoderma resinosum*		Fomitopsidaceae	Papum Pare	-ve
3	ARFR35	*Trametes hirsuta*	PV123950	Polyporaceae	Papum Pare	30
4	ARFR36	*Ganoderma* sp. 1		Polyporaceae	Papum Pare	23
5	ARFR37	*Rigidoprus microporus*		Meripilaceae	Papum Pare	8
6	ARFR38	*Ganoderma* sp. 2		Polyporaceae	Papum Pare	21
7	ARFR39	*Rhodofomitopsis feeii*		Fomitopsidaceae	Papum Pare	-ve
8	ARFR43	*Trametes cubensis*		Polyporaceae	Lepa Rada	33
9	ARFR46	*Hexagonia tenuis*		Meripilaceae	Papum Pare	-ve
10	ARFR50	*Trametes vespacea*		Polyporaceae	Papum Pare	31
11	ARFR52	*Xylobolus subpileatus*		Stereaceae	Papum Pare	11
12	ARFR55	*Pycnoporus sanguineus*	PV124361	Polyporaceae	Lepa Rada	153
13	ARFR62	*Earliella scabrosa*		Polyporaceae	Papum Pare	21
14	ARFR68	*Flavodon flavus*		Irpicaceae	Papum Pare	-ve
15	ARFR72	*Cubamyces flavidus*	PV124353	Polyporaceae	Papum Pare	64
16	ARFR301	*Cellulariella warnieri*	PV124723	Polyporaceae	West Kameng	42
17	ARFR303	*Trametes hirsuta*	PV174240	Polyporaceae	West Kameng	71
18	ARFR319	*Xylobolus frustulatus*		Stereaceae	West Kameng	-ve
19	ARFR324	*Trametes ochracea*	PV133106	Polyporaceae	West Kameng	51
20	ARFR326	*Fomitopsis pinicola*		Fomitopsidaceae	West Kameng	-ve
21	ARFR329	*Cerrena zonata*	PV124445	Cerrenaceae	West Kameng	29
22	ARFR365	*Fomitopsis* sp.		Fomitopsidaceae	West Kameng	-ve
23	ARFR369	*Fomes fomentarius*		Polyporaceae	West Kameng	-ve
24	ARFR394	*Ganoderma* sp. 3		Polyporaceae	Papum Pare	22
25	ARFR425	*Vanderbylia fraxinea*	PV123958	Polyporaceae	Papum Pare	31
26	ARFR426	*Lentinus* sp		Polyporaceae	Papum Pare	26
27	ARFR427	*Laetiporus sulphureus*	PV036451	Polyporaceae	Papum Pare	-ve
28	ARFR428	*Ganoderma* sp. 4		Polyporaceae	Lepa Rada	11
29	ARFR429	*Ganoderma* sp. 5		Polyporaceae	Papum Pare	34
30	ARFR431	*Lentinus strigosus*		Polyporaceae	Papum Pare	7
31	ARFR433	*Bjerkandera adusta*		Phanerochaetaceae	Papum Pare	1
32	ARFR434	*Pleurotus eous*		Pleurotaceae	Papum Pare	6
33	ARFR435	*Amauroderma* sp.		Polyporaceae	Papum Pare	8
34	ARFR436	*Microporus affinis*		Polyporaceae	Lepa Rada	-ve
35	ARFR439	*Gyrodontium saccharii*		Coniophoraceae	Papum Pare	5
36	ARFR440	*Microporus* sp.		Polyporaceae	Lepa Rada	-ve
37	ARFR441	*Gloeophyllum sepiarium*		Gloeophyllaceae	Papum Pare	-ve

Table 1. Collected wood-rotting basidiomycetes and their laccase activity.

2.5. Quantitative laccase production

Laccase production for each fungus was quantified under SSF. Wheat bran was selected as the lignocellulosic substrate because of its low cost, ready availability, and rich nutrient profile, which supports efficient laccase synthesis (Bagewadi et al., 2017). The SSF was performed in 250 mL Erlenmeyer flask containing 5 g of wheat bran supplemented with 15 mL mineral salt solution (MSS) comprising 0.05% $(NH_4)_2SO_4$, KH_2PO_4, and $MgSO_4$; the pH was adjusted to 5.0 using either 1 N HCl or 1 M NaOH (Sharma et al., 2005). The substrate was then inoculated with four mycelial discs, each 10 mm in diameter, obtained from a 7- to 10-day-old pure culture of respective fungus, and left for incubation for 11 days.

2.6. Laccase extraction

Laccase was extracted from the fermentation medium using 100 mM citrate phosphate buffer (pH 5.0) at 1:10 w/v. The mixture was agitated at 150 rpm for an hour in a shaker and filtered through muslin cloth. The filtrate was centrifuged at 10,000 rpm for 12 minutes at 4°C, and the resulting supernatant was used for enzyme assay.

2.7. Laccase assay

Laccase production was quantified using substrate solution containing 10 mM guaiacol in 100 mM citrate-phosphate buffer (pH 5.0). For the assay, 900 µL of the substrate solution was added with 100 µL of appropriately diluted culture supernatant and incubated at room temperature for 30 minutes. Absorbance was then measured at 470 nm using a UV–visible spectrophotometer. Enzyme activity was expressed in IU/L, where one IU corresponds to the amount of laccase needed to oxidize 1.0 µM of substrate per minute (Baltierra-Trejo et al., 2015).

$$\frac{IU}{L} = \frac{\Delta A \times V_t \times D_f \times 10^6}{\varepsilon \times t \times d \times V_s}$$

Here,

ΔA = change in absorbance,
Vt = total reaction volume (ml),
Df = dilution factor,
10^6 = correction factor (µmoL mol^{-1}),
ε = molar extinction coefficient (26,600 M^{-1} cm^{-1}),
Vs = sample volume (ml), d = path length (1 cm),
t = reaction time (min.)

2.8. Time course study of laccase production

Laccase production of five superior WRF found from this study was recorded from 8 to 20 days at every three days interval to further identify the best WRF and their optimal incubation period.

3. Result

3.1. Identification of wood-rotting basidiomycetes and their laccase activity

Based on morphological and molecular characteristics 37 wood-rotting basidiomycetes were identified. Their name, place of collection, and laccase activity have been given in Table 1. Based on plate assay, only 25 fungi showed laccase activity as shown in Figure 1(a-d).

3.2. Laccase production under solid-state fermentation

Based on the results obtained from SSF to quantify laccase production by 25 WRF for 11 days on wheat bran, *Pycnoporus sanguineus* (formerly *Trametes sanguineus*) appeared as the most efficient fungus producing 153 IU/L of laccase. It was followed by *T. hirsuta* ARFR303 (71 IU/L), *C. flavidus* (64 IU/L), *T. ochracea* (51 IU/L) and *T. gibbosa* (50 IU/L) and. The least amount of laccases was produced by *B. adusta* (1 IU/L) (Table 1). It was also observed that two different *T. hirsuta*, ARFR35 and ARFR303, differed widely in their laccase production, which was 2.4 times more in the case of the former (30 IU/L) than the latter. In general, *Trametes* spp. appeared to be decent laccase producer among all the WRF screened in this study.

3.3. Time course study

Time course study on wheat bran was performed under SSF (8-20 days) for the top five laccase-producing WRF to find out the most efficient fungi and the day of maximum enzyme production (Figure 2). The obtained results showed that among all WRF, *P. sanguineus* always produced the higher amount of laccase throughout the study period. It showed an exponential increase in production from day 8 onwards, reaching a peak value of 431 IU/L on the 20th day. A similar trend was observed for *T. hirsuta* ARFR303 and *T. gibbosa*. On the 8th and 20th day, the laccase production by the former and the latter species respectively was 65 IU/L and 370 IU/L, and 33 IU/L and 71 IU/L. In contrast, *C. flavidus* and *T. ochracea* attained their peak production early, i.e., day 8 (77 IU/L) and day 11 (51 IU/L), respectively (Figure 2). Their laccase production declined more or less gradually afterwards.

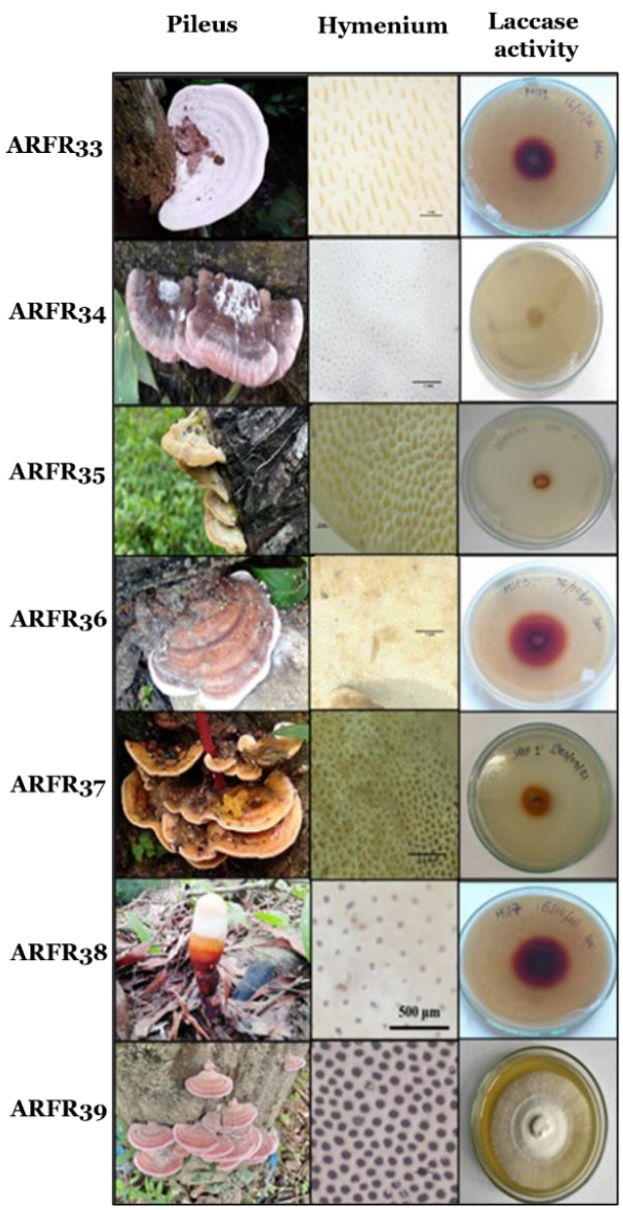

Figure 1a. Surface features of the fruit bodies of test fungi and their qualitative laccase assay (Guaiacol method). Formation of brick-red color under the fungal colony indicates positive laccase activity

4. Discussion

Laccases are highly regarded for their extensive industrial applications. However, their large-scale production continues to be a challenge due to low yields, prompting ongoing research to identify more efficient sources particularly from amongst the WRF. Among various laccase producers, basidiomycetes that cause white rot in wood are recognized as the most effective (Baldrian, 2006; Toca-Herrera et al., 2007; Arora and Sharma, 2010). In this context, we screened 37 wood-rotting basidiomycetes from three distinct ecological regions of Arunachal Pradesh and found 25 fungi displaying laccase activity. Quantitative estimation of their laccase production during SSF on wheat bran at 11 days of incubation showed a significant variation with amounts ranging from 1-153 IU/L. Among

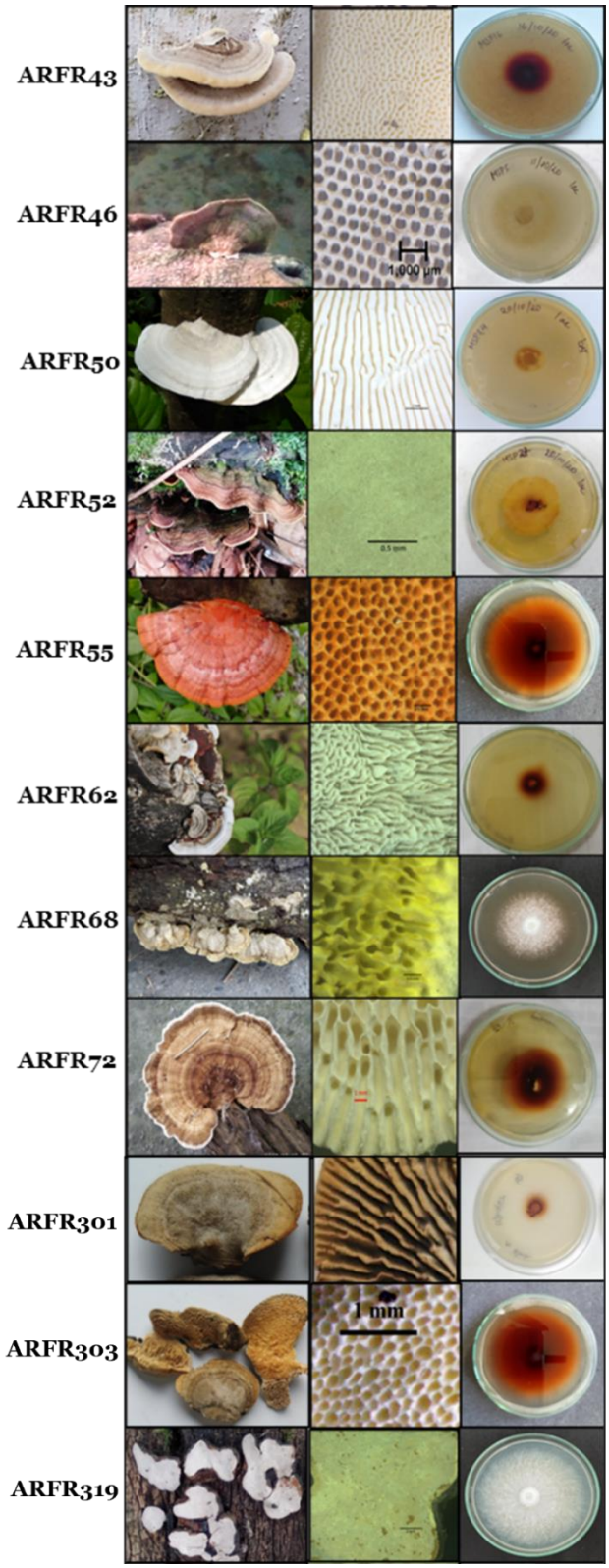

Figure 1b. Surface features of the fruit bodies of test fungi and their qualitative laccase assay (Guaiacol method). Formation of brick-red color under the fungal colony indicates positive laccase activity.

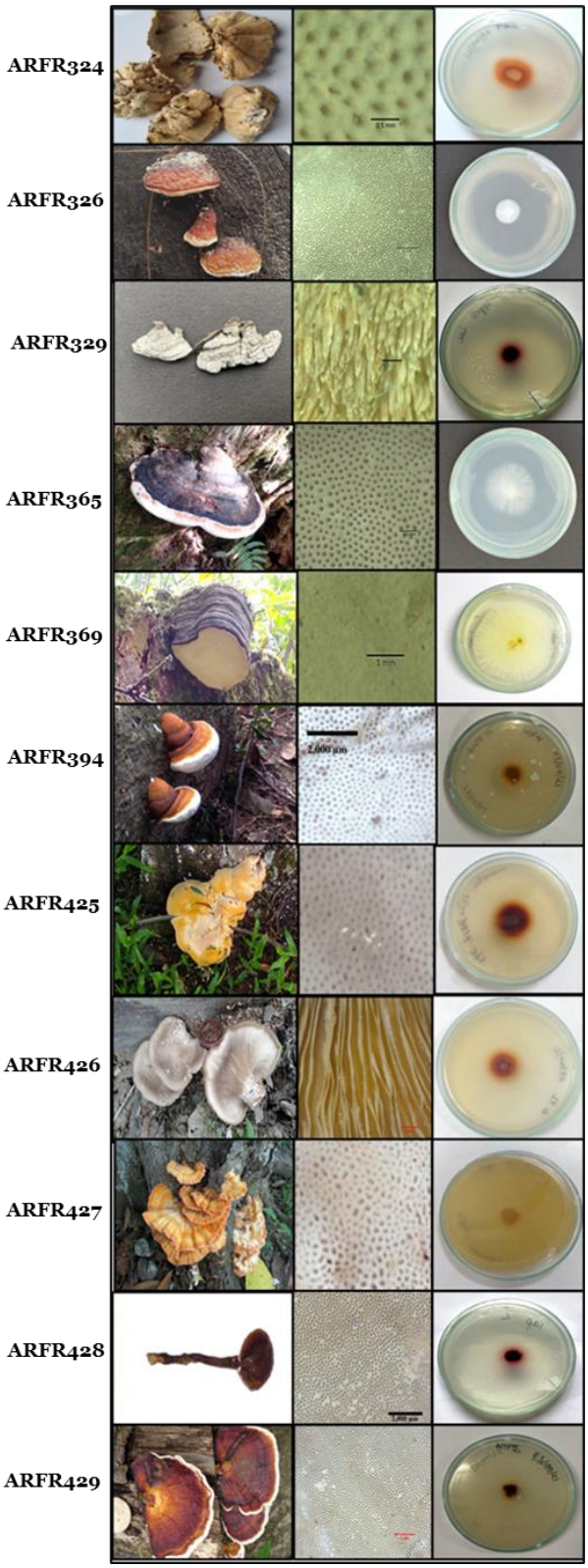

Figure 1c. Surface features of the fruit bodies of test fungi and their qualitative laccase assay (Guaiacol method). Formation of brick-red color under the fungal colony indicates positive laccase activity.

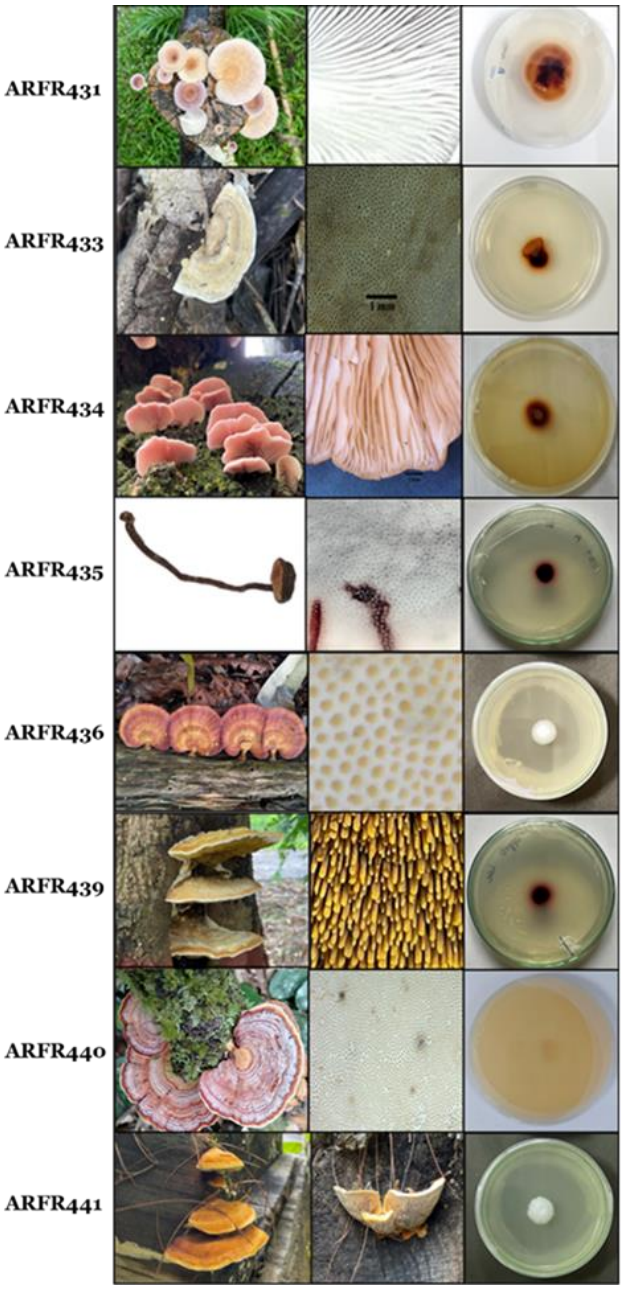

Figure 1d. Surface features of the fruit bodies of test fungi and their qualitative laccase assay (Guaiacol method). Formation of brick-red color under the fungal colony indicates positive laccase activity.

25 laccase-producing WRF, the top five were *P. sanguineus, T. hirsuta* ARFR303, *C. flavidus, T. gibbosa* and *T. ochracea*. Several studies have reported a wide variation in laccase production among genera and species of wood-rotting basidiomycetes (Herpoël et al., 2000; Stajić et al., 2006; Elisashvili and Kachlishvili, 2009; An et al., 2020) and have recognized both *Trametes* and *Pycnoporus* as high laccase-producing genera (Wang et al., 2019; Cheute et al., 2024). This might be the plausible reason for the intraspecific variation in laccase production on 11th day as observed in the present study between *T. hirsuta* ARFR303 (71 IU/L) and *T. hirsuta* ARFR35 (30 IU/L).

Incubation period is regarded as one of the major factors significantly influencing laccase production by WRF (Abd El et al., 2016; Hasan et al., 2023). Additionally, fermentation conditions like temperature, pH, moisture content, and kind and amount of substrate, are the other major influencing factors (Dhakar et al., 2013; Boran and Yesilada, 2022; Han et al., 2022; Umar and Ahmed, 2022; Ibarra-Islas et al., 2023; Das et al., 2024a). In the present study, when laccase production of top five WRF was monitored under the same SSF conditions for 20 days, they exhibited a significant variation with respect to time and yield. There was a steady increase in laccase production by *P. sanguineus, T. hirsuta* ARFR303 and *T. gibbosa* from 8th day to 20th day whereas *C. flavidus* and *T. ochracea* exhibited an early peak on 8th day and 11th day respectively followed by a gradual decline afterward.

In the present study conducted under unoptimized SSF conditions without adding any laccase inducers, *P. sanguineus* produced 84 IU/L and 431 IU/L laccase, whereas *T. hirsuta* ARFR303 produced 65 IU/L and 370 IU/L, on the 8th and 20th day respectively. It was also observed that the laccase yield of the former species was always much higher than the latter species throughout the time period. Other studies have reported laccase production by *P. sanguineus* on various substrates varying between 22 U/L to 6000 U/L from day 7 to day 20 day under unoptimized and optimized SSF conditions (Pointing, 2000; Dantán-González et al., 2008; Hernández et al. 2016). Likewise for *T. hirsuta*, the maximum laccase production is reported to vary between 10 to 406 IU/L during 5 to 12 days incubation period (Dhakar et al., 2013; Krumova et al., 2018; Anita et al., 2021; Liu et al., 2022). In case of other WRF, an enhancement in laccase production by 8 to 200-fold has been reported on optimization of SSF conditions with adequate supplementation with suitable additives and inducers (Arora and Gill, 2000; Bagewadi et al., 2017). Such difference in enzyme production dynamics suggests that each fungal species and even strains may have different regulatory mechanisms governing laccase biosynthesis, potentially influenced by growth phases, substrate availability, and culture conditions (Janusz et al., 2013; An et al., 2020).

The present study established both species as promising laccase producers whose level of production may further increase on further optimization of physical and nutritional factors for SSF together with supplementation of certain inducers like guaiacol and CuSO4 etc. in appropriate amount.

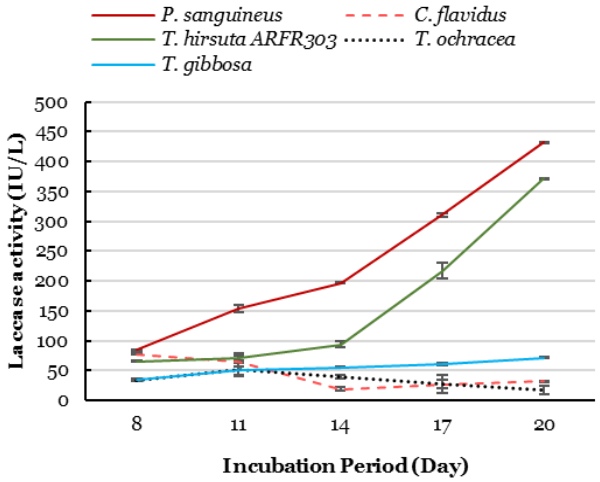

Figure 2. Time course of laccase production *by T. gibbosa, P. sanguineus, C. flavidus, T. hirsuta* ARFR303, and *T. ochracea* (mean±SD).

6. Conclusion

The selection of high-yielding WRFs is crucial to satisfy the growing demand for laccases in various industrial and biotechnological applications. In this study, 37 wood-rotting basidiomycetes were screened for laccase activity, out of which 25 species/strains produced the enzyme in varying amounts, thus indicating a significant variability among species and strains. The study identified two WRF, namely *P. sanguineus* and *T. hirsuta* ARFR303 as the most promising WRF providing a good amount of laccase under unoptimized SSF conditions. Further optimization of cultural and physical parameters may further enhance their laccase production.

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Authors' contribution

Marjum Badak: Concept development, methodology design, experiment execution, data collection and analysis, original draft writing. Junmoni Das: Draft writing, validation, review, and editing. Rajiv Kumar Singh: Project funding, Work supervision, validation, review, and editing. All authors contributed to the article and approved the final version.

Conflict of interest

The authors declare no conflict of interest.

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