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Qualitative Assays and Quantitative Determinations of Laccases of White Rot Fungi from Plantation and Natural Forests of Arsi Forest Enterprise, Ethiopia

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ABSTRACT

The white rot fungi (WRF) isolated from the plantation and natural forests of Arsi forest enterprise (Dagaga and Gambo sites) were screened for their ligninolytic potentials and their Laccases were quantitatively determined. The fungi were grown in both submerged fermentation (SmF) and solid state fermentation (SSF) of wheat bran (WB) and sawdust of *Eucalyptus grandis* (sawdust). The activities of Laccases of the fungal isolates were quantified and their productions were optimized. Quantification of Laccases indicated that the fungal isolates secreted their highest enzymes on the 8th day of SmF and on 12th day of SSF in WB and sawdust substrates. Laccase activities of SSF were higher than Laccase activities of SmF in both growth substrates. The highest Laccase activities of 0.345 U/ml and 0.379 U/ml were obtained during SmF and SSF of WB, respectively, by isolate 003-2G. Laccase activities of 0.430 U/ml and 0.446 U/ml were also obtained while SSF of sawdust by the same isolate. Laccase quantification data indicated sawdust to be more suitable than WB for Laccase production. 45^{o}C was most optimum for the Laccase activities and the enzyme was stable in the temperature range of 40-45^{o}C. It was also found that the Laccases of the fungi were active and stable at pH 5.0. Optimization experiments also revealed that production of Laccases from the fungal isolates can be maximized by adjusting the fungi cultivation conditions and supplementations of the growth media.

***Keywords*:** WRF, Laccase, Arsi, Dagaga, Gambo

1. INTRODUCTION

Lignocellulosic substrates are mainly composed of cellulose, hemicellulose, and lignin [1]. These substrates are naturally recalcitrant to microbial degradation due to the lignin barrier. White rot fungi (WRF) are among the potential microorganisms that can enzymatically modify lignin and degrade lignocellulosic plant materials through ligninolytic enzymes secretions such as Laccase [2]. Laccases are important in biodegradation and bioremediation processes [3]. The enzyme can be employed for many biotechnological purposes, like in bioconversion of lignocellulosic substrates [4].

Recent studies have shown that ligninolytic enzymes can be produced using plant raw materials as substrates [5]. Production of ligninolytic enzymes, particularly production of Laccases should be enhanced for optimum utilization. Optimization of Laccase production involves searching for new microbial strains and modifying the physico-chemical requirements of the potential microbial strains like WRF [6]. In the present study, the WRF which were previously isolated and identified from Dagaga-Gambo natural and plantation forests [7] were qualitatively assayed for laccase productions and then laccases of the potential WRF were characterized and their productions were optimized.

2. MATERIALS AND METHODS

2. 1. Fungal cultures and substrates

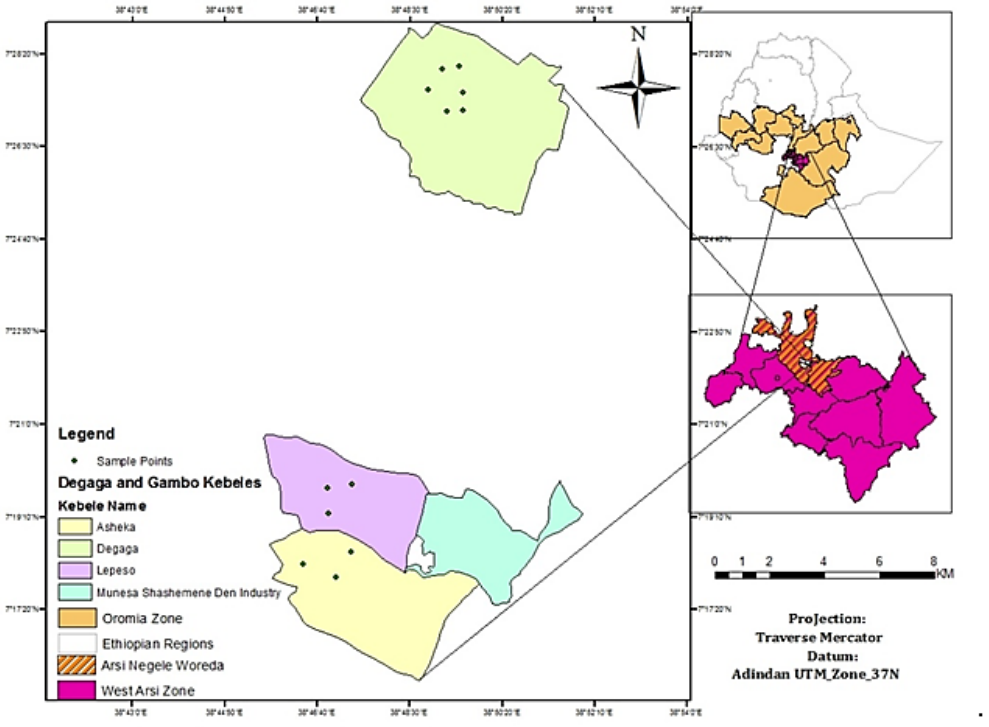

Fig. 1. Site map of the study area, Arsi forest enterprise, Oromia, Ethiopia

Wood rot fungi which were maintained at the microbiology laboratory of Wood Technology Research Center (WTRC) of the Ethiopian Environment and Forest Research Institute (EEFRI) in Addis Ababa were used for this research. The cultures were previously collected from the natural and plantation forests of Arsi forest enterprise, Oromia, Ethiopia [7] (Fig. 1). WB was obtained from Fafa food processing factory in Addis Ababa and fresh sawdust was obtained from sawmill of Arsi forest enterprise.

2. 2. Qualitative assays of WRF for Laccase production potentials

WRF were qualitatively screened using the lignin modifying fungal enzymes basal medium (LBM) supplemented with tannic acid [8,9]. Ligninolytic activity was examined for 10 days and appearance of brown oxidation zone below and around cultures was recorded. The colored zone and culture diameter were measured and enzymatic index (EI) was calculated for each culture as follows.

$$\mathrm{EI} = \frac{diameter\ of\ hydrolysis\ zone}{diameter\ of\ colony}$$

2. 3. Quantitative determination of Laccases of WRF

2. 3. 1. Inoculum preparation

Inocula of the selected ligninolytic fungi were prepared using standard medium of Altaf *et al*. [10]. Four disks (Ø 0.5 mm) of each isolate were inoculated and grown in 100 ml of the standard media in 250 ml flask at 150 rpm and at room temperature (Fig. 2). After six days of incubation, the mycelial pellets were harvested, homogenized and used as inocula for submerged and solid state fermentations in quantification of Laccases

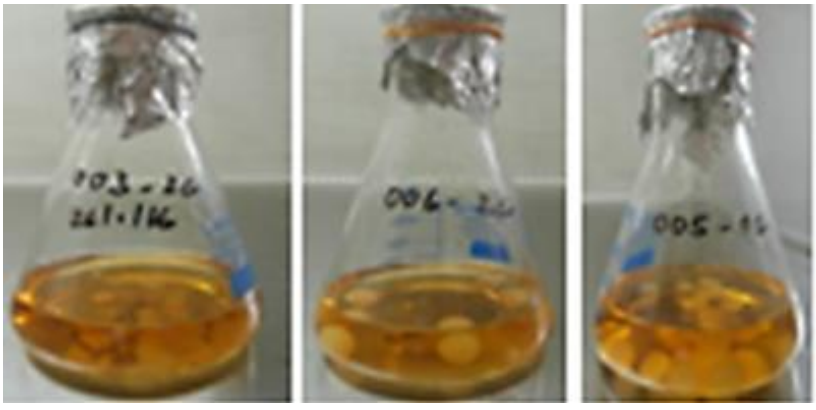

Fig. 2. Some of the flasks containing culture inocula of the fungal isolates

2. 3. 2. Submerged fermentation (SmF)

SmF was carried out replacing glucose of the standard media with four grams of wheat bran (WB) and *E. grandis* sawdust. WB and sawdust were ground and sieved with 4.0 mm

pore size sieve before use. 50 ml of each medium was added into 250 ml flasks. The initial pH of the medium was adjusted to 6.0 prior to sterilization using 2M NaOH. Each flask was inoculated with 3ml of mycelial homogenate and incubated on a rotary shaker at 150 rpm and room temperature (Fig. 3). After five, eight and twelve days of growth, solids were separated by filtration through nylon cloth followed by centrifugation at 4000 rpm for 15 minutes.

Fig. 3. Some flasks during SmF of the fungal isolates on WB and sawdust

2. 3. 3. Solid state fermentation (SSF)

Five grams WB and ten grams sawdust were moistened with 12 ml of the standard medium in 250 ml flasks. The flasks were autoclaved, inoculated with 3ml of mycelial homogenate and incubated at room temperature (Fig. 4). After 7, 12 and 15 days of cultivation, the extracellular enzymes were extracted from the whole biomass twice with 25 ml of distilled water (total volume 50 ml). The solids were separated by filtration through nylon cloth followed by centrifugation at 4000 rpm for 15 minutes.

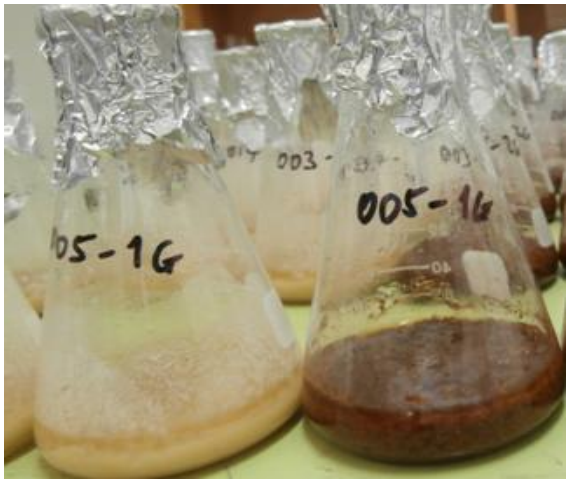

Fig. 4. Some flasks during SSF of the fungal isolates on WB and sawdust

2. 3. 4. Determination of Laccase activity

The filtered extract (both SmF and SSF) was analyzed for the Laccase activity using 2,6-dimethoxyphenol (DMP) as a substrate [11]. The absorbance was immediately measured at 468 nm (ε_{4960} $LM^{-1}cm^{-1}$) at 37 °C. One unit (U) of Laccase activity was defined as activity of an enzyme that catalyzed the conversion of one μmole of DMP per minute under the specified assay condition. The enzyme activity was reported as U/ml ($\mu molmin^{-1}ml^{-1}$).
Laccase activity was calculated using Beer-Lambert equation:

$$A = \varepsilon dC \quad \Rightarrow \quad C = \frac{A}{\varepsilon d}$$

where A = Absorbance/min, ε = Molar absorptivity (L mol^{-1} cm^{-1}), d = Path length of the cuvette containing the sample (cm), C = Concentration of the compound in the solution (mol L^{-1}), U = μmol/ml min (U/ml).

2. 4. Partial characterization of Laccase

2. 4. 1. Effect of temperature on the activity and stability of the Laccase

To determine the effect incubation temperature on laccase activity, the enzyme substrate mixtures were incubated at 5 °C intervals (30-70 °C) for 30 minutes in a digital incubator [12]. After 30 minutes of incubation, absorbance was measured using UV-Vis spectrophotometer. Heat stability study of the enzyme was performed by pre-incubating the crude enzyme in 0.05 M acetate buffer (pH 4.5) at 5 °C intervals (35-70 °C) for 120 minutes absorbance was measured.

2. 4. 2. Effect of pH on the activity and stability of Laccase

pH optimum of Laccase was determined by incubating enzyme substrate mixture at different pH values (3.0-9.0) for 30 minutes. The buffers used for adjusting the pH values were citrate buffer (pH 3.0-6.0), acetate buffer (pH 4.0-6.0), and phosphate buffer (pH 7.0-8.0). Finally absorbance was measured. For the pH stability experiments, the crude extract without substrate was pre-incubated under initial conditions (pH values 3.0-8.0) for 120 minutes at 30^{o}C. Finally absorbance was measured.

2. 5. Optimization of culture conditions for Laccase production

2. 5. 1. Effect of temperature on production of Laccase

SmF of the standard medium was carried out in an incubator at 5 °C intervals in the range of 20-45 °C for 8 days. Afterwards, the cell free extract from each flask was analyzed for Laccase activity.

2. 5. 2. Effect of pH on production of Laccase

SmF of the standard medium was carried at different pH ranges of 3.0-9.0. The pH of the medium was adjusted by using 1N HCl or 1N NaOH. The flasks were placed in an incubator adjusted at 30 °C for 8 days of cultivation. Afterwards, the cell free extract from each flask was analyzed for Laccase activity.

2. 5. 3. Effect of carbon sources on production of Laccase

SmF of the standard medium was carried out by replacing glucose of the standard medium with 0.6% dextrose, maltose, lactose, sucrose or starch as carbon sources. The flasks were placed in an incubator adjusted at 30 °C. Afterwards, the cell free extract from each flask was analyzed for Laccase activity.

2. 5. 4. Effect of nitrogen sources on production of Laccase

SmF of the fungal isolates was conducted by supplementing the nitrogen source (yeast extract, peptone, $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$ or NH_4Cl) at 0.3% rate into the standard medium. The flasks were placed in an incubator adjusted at 30 °C for 8 days. Afterwards, the cell free extract from each flask was analyzed for Laccase activity.

2. 5. 5. Effect of metallic ions on production of Laccase

The effect of different metallic ions on the Laccase production was studied by replacing the metallic ion of the standard medium with $CaCl_2$, $CuSO_4$, $MgSO_4$, $FeSO_4$, $MnSO_4$ and $ZnSO_4$ at concentration of 0.05%. Laccase activity was determined after 8 day of incubation period at 30 °C.

2. 6. Statistical Analysis

All experiments were performed in triplicates. Means of three replicate values for all data obtained were tested in a one way ANOVA at P = 0.05 using SPSS software and Tukey's test was used to evaluate differences between treatments.

3. RESULTS AND DISCUSSION

3. 1. Qualitative assays of the Laccase

Of the 56 fungal cultures screened many showed positive ligninolytic activities with different oxidation zone round cultures (Picture 1). Those cultures which showed significant colored zone round the growing mycelia were particularly considered potential ligninolytic enzyme producers [13] and selected for quantitative determination of the Laccases (Table 1).

Picture 1. Colored region around ligninolytic fungi grown on tannic acid agar medium

Table 1. WRF isolates selected for Laccase determination based on qualitative screening.

No	Fungal isolates	Fungal species	[a] Tannic acid activity scale
1	010-1D	*Postia stiptica* (Polyporales, Fomitopsidaceae)	+++
2	005-1G	*Polyporus giganteus* (Polyporales, meripilaceae)	++++
3	024-1G	*Macrolepiota procera* (Agaricales, Lepiotaceae)	+++
4	001-2D	*Termitomyces eurrhizus* (Agaricales, Lyophyllaceae)	++
5	003-2G	*Pholiota squarrosa* (Agaricales, Strophariaceae)	++++
6	006-2G	*Ganoderma aplanatum* (Polyporales, Polyporaceae)	++++
7	014-2G	*Inonotus hispidus* (Hymenochaetales, Hymenochaetaceae)	+++
8	016-2G	Suillus luteus (Boletales, Suillaceae)	++
9	019-2G	*Leucopaxillus albissimus* (Agaricales, Tricholomataceae)	++
10	022-2G	*Fomitopsis pinicola (*Polyporales, Fomitopsidaceae*)*	+++
11	029-2G	*Trichaptum biforme* (Polyporales, Polyporaceae)	+++

[a] oxidation scale (EI calculated on the 7th day of growth): + = EI<1.5, ++ = 1.5<EI<2.5, +++ = 2.5<EI<3.5, ++++ = 3.5<EI<4.5, +++++ = EI>4.5

Many of the fungal cultures tested in this experiment showed detectable oxidation zones under and round the culture zones of the tannic acid supplemented LBM. The oxidation zone round the culture colony was considerably different indicating that the fungi have got different ability of secreting ligninolytic enzymes. Size of colored zone round culture colony is a good indication of secreting high amount of ligninolytic enzymes [13]. Enzyme production by WRF also depends mainly on fungal species and fungal strains [6]. Eleven isolates (001-1D, 005-1G, 024-1G, 001-2D, 003-2G, 006-2G, 014-2G, 016-2G, 019-2G, 022-2G, 029-2G) were selected for quantitative determination of laccases.

3. 2. Quantitative determination of Laccase

3. 2. 1. Determination of Laccase activities using SmF and SSF of WB

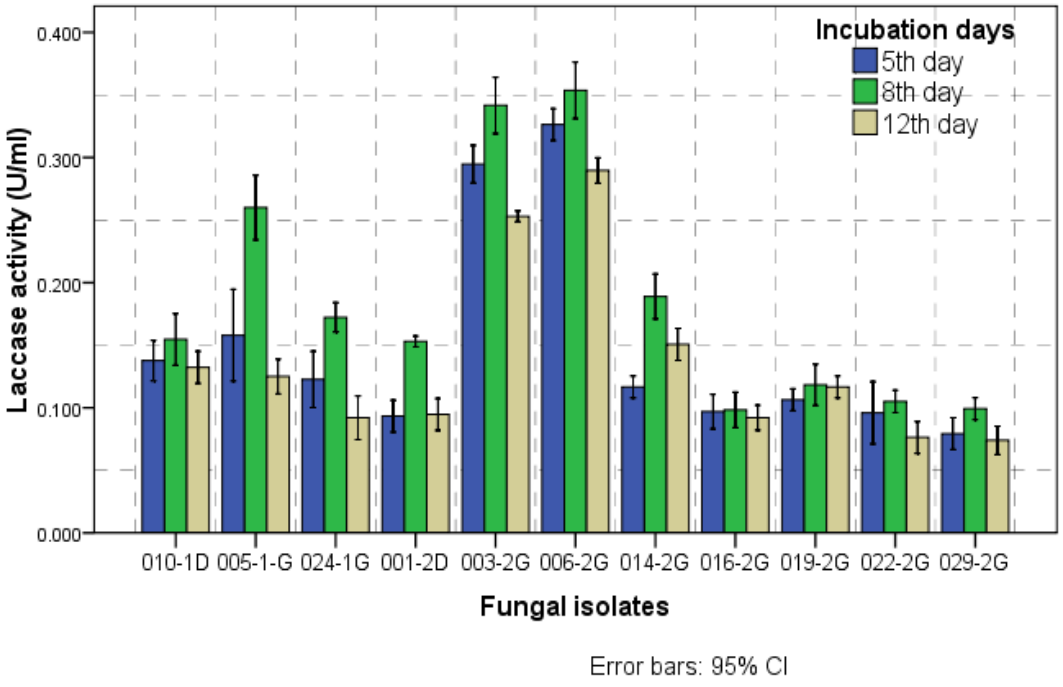

Fig. 5. Laccase activities of the fungal isolates grown in SmF condition of wheat bran.

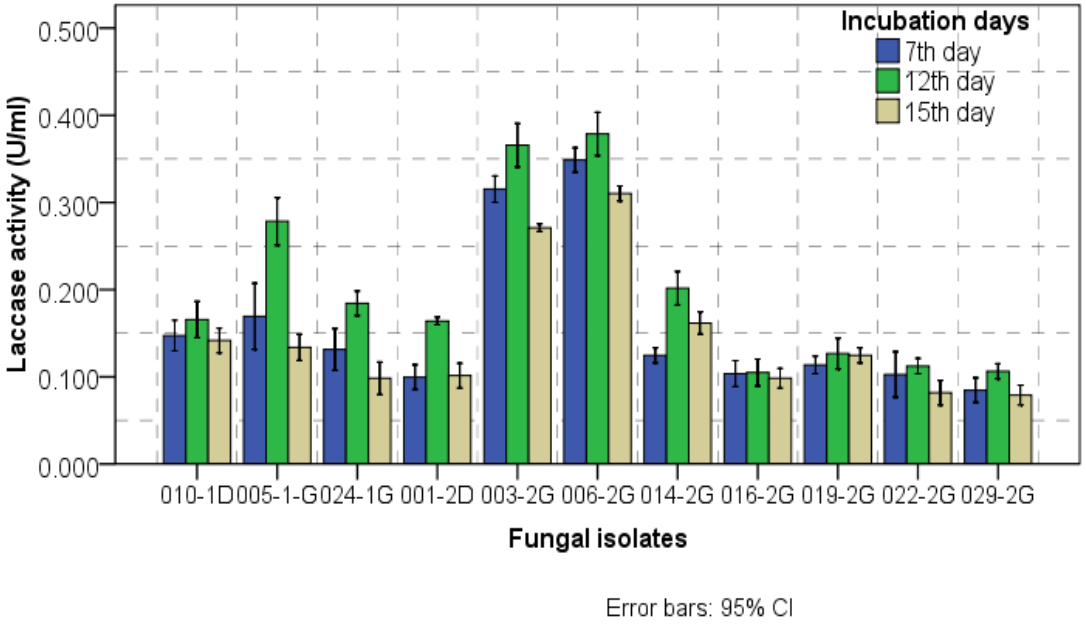

Fig. 6. Activities of Laccase of the fungal isolates grown in SSF condition of wheat bran.

Laccase activities of the WRF isolates were checked after the 5th, 8th and 12th days of SmF and 7th, 12th and 15th days of SSF growth conditions. They displayed their highest Laccase secretions on the 8th day of SmF and 12th day of SSF using WB substrate. Isolates 003-2G (*Pholiota squarrosa*), 006-2G (*Ganoderma aplanatum*) and 005-1G (*Polyporus giganteus*) were found to be the three top efficient Laccase producers under both fermentations (Fig. 5 and Fig. 6). The highest Laccase activities of 0.354 U/ml, 0.342 U/ml and 0.260 U/ml during SmF and 0.379 U/ml, 0.366 U/ml and 0.278 U/ml during SSF were displayed by the isolates, respectively. Though required longer incubation period, an increased Laccase activity was observed in SSF than in SmF indicating higher production of Laccase in SSF than in SmF of WB.

This is in line with the reports of different authors [14-16] who indicated that ligninolytic enzymes are better produced in SSF than in SmF. Compared to reports made by other authors, some isolates displayed better Laccase activities. Poojary *et al.* [17] screened the WRF from natural environment and reported the highest Laccase activity (0.247 U/ml) on the 6th day using *Pleurotus* sp (hpF-16) and 0.254 U/ml) on 4th day using *Trametes* sp. (hpF-35) in SmF of LMM medium. On the other hand, Hong *et al*. [18] reported the highest Laccase activity by *P. ostreatus* and *T. albidus* on 15th day in SmF condition using paper mill sludge.

Different authors have also reported different Laccase activities on different fermentation days and on different substrates. Asgher *et al*. [19] cultivated *Pleurotus sapidus* WC 529 in SSF condition (pH 4.5 and 30 °C) using wheat straw and reported the highest Laccase activity of 0.199 U/ml on 8th day. Similarly, the authors reported Laccase activity of 0.190 U/ml on 4th day for rice straw. Boran and Yesilada [20] have grown two WRF *Funalia trogii* ATCC200800 and *Trametes versicolor* ATCC200801 in SSF condition of WB moistened with olive oil mill wastewater (25%) and obtained highest Laccase activity of 0.346 U/ml and 0.497 U/ml, respectively, on the 10th day. Osma *et al*. [21] also cultivated *T. pubescens* and *Trametes versicolor* in SSF condition of WB and reported the highest Laccase activities of 0.214 U/ml and 0.264 U/ml on the 10th and on 13th days, respectively.

3. 2. 2. Determination of Laccase activities using SmF and SSF of sawdust

Like in SmF and SSF of WB, similar Laccase secretion pattern was observed when sawdust used (Fig. 7 and Fig. 8). The highest Laccase activities of 0.430 U/ml, 0.400 U/ml and 0.310 U/ml in SmF were displayed by isolates 003-2G, 006-2G and 005-1G. Laccase activity obtained in SmF of sawdust was significantly higher than that were obtained in both SmF and SSF of WB (Fig. 7). But the amount of Laccase produced by each isolate was significantly different. The highest Laccase activities of 0.446 U/ml, 0.419 U/ml and 0.323 U/ml were obtained from isolate 003-2G, 006-2G and 005-1G, respectively, on 12th day of SSF.

Different authors also reported different Laccase activities for different WRF. Botto *et al.* [22] cultivated a WRF, *Dichostereum sordulentum*, on *Eucalyptus dunnii* wood sawdust and found the maximum Laccase activity of 0.720 U/ml on 16th day of SmF which is of course higher than Laccase activity reported in this work. On the other hand, Sukarta and Sastrawidana [23] reported the highest Laccase activity of 0.190 U/ml by cultivating *Polyporus* sp. on sawdust in SSF condition on the 7th day incubation. Hashim [24] obtained the highest level of Laccase activity (0.395 U/ml) from *P. ostreatus* in SSF of sawdust on 15th day at room temperature. Mikiashvili *et al*. [25] cultivated *G. frondosa* MBFBL 596 on oak

wood sawdust in SSF condition and reported the highest Laccase activity of 0.703 U/ml 15th day.

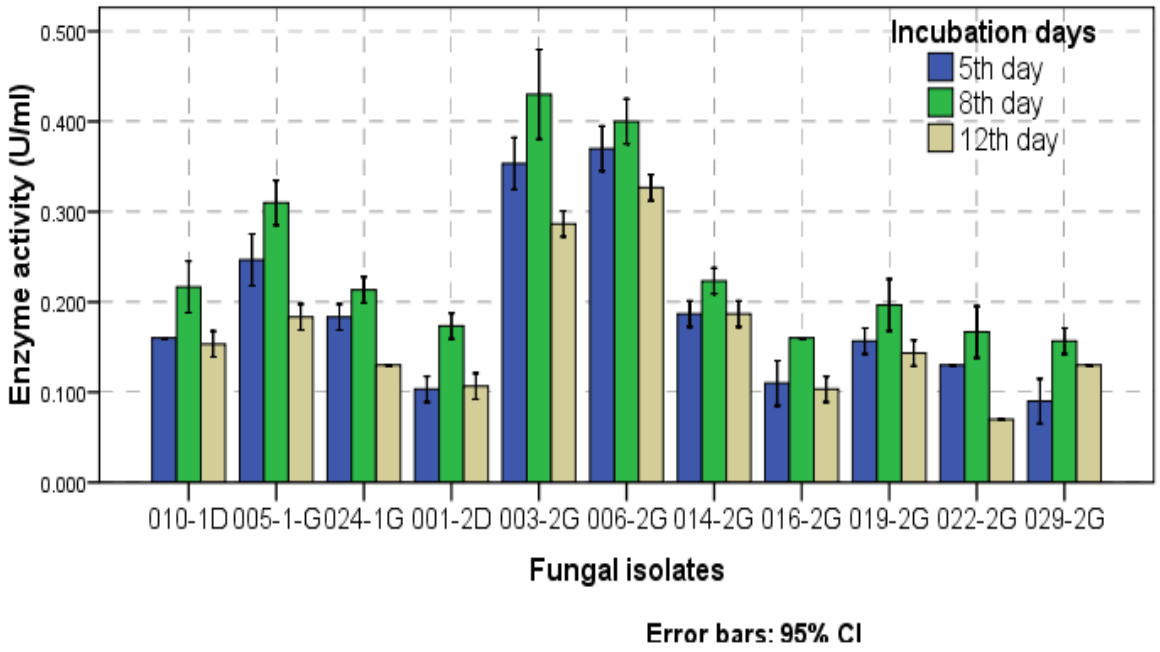

Fig. 7. Laccase activities of the fungal isolates grown in SmF condition of *E. grandis* sawdust

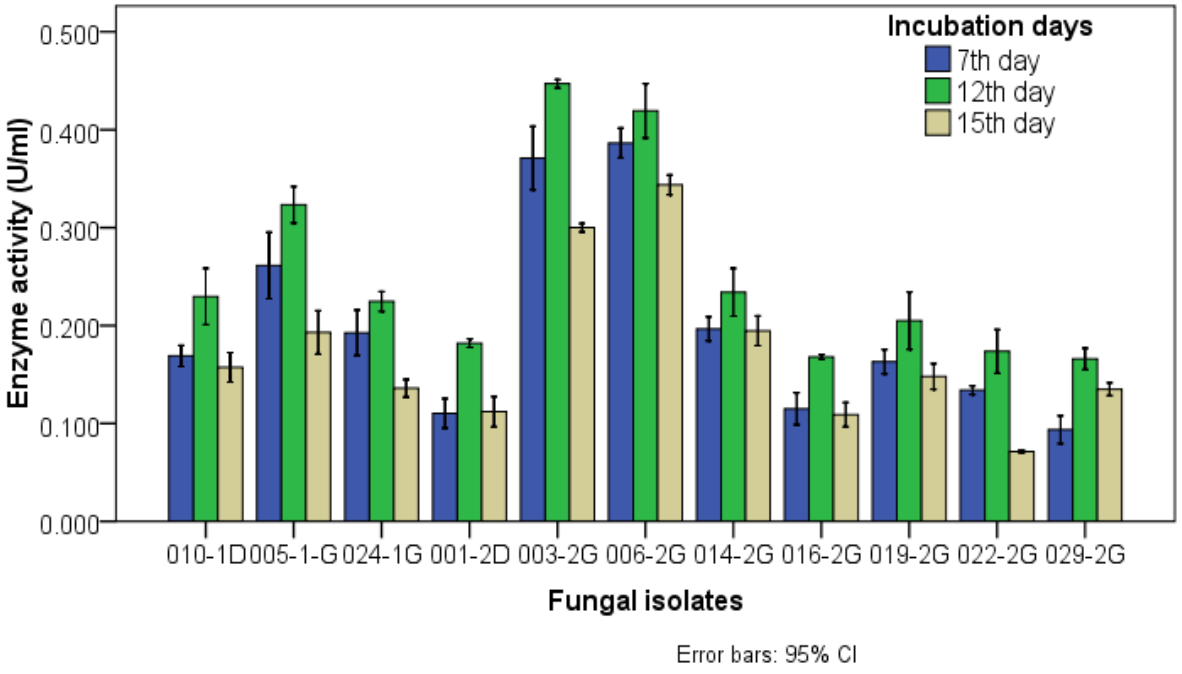

Fig. 8. Laccase activities of the fungal isolates grown in SSF condition of *E. grandis* sawdust

3. 3. Partial characterization of Laccase

For the partial characterization experiments, enzyme extracts of the fungal isolates grown in SSF of sawdust for 12 days was used.

3. 3. 1. Effect of temperature on the activity and stability of Laccase

With some exceptions, temperature range of 40-45 °C was most optimum for Laccase activities of the isolates (Fig. 9). Laccase of isolate 006-2G was most active at 45°C displaying the highest activity of 0.651 U/ml. Isolate 003-2G also displayed its highest Laccase activity (0.617 U/ml) at the same temperature. Thermal stabilities of the Laccase of the fungal isolates were summarized (Fig. 10). Isolates 006-2G, 003-2G and 005-1G displayed their most stable Laccase activities of 0.625 U/ml, 0.592 U/ml and 0.523 U/ml, respectively, at 45 °C. It was well observed that all Laccases of all fungal isolates were not stable at 70 °C showing very low enzyme activities.

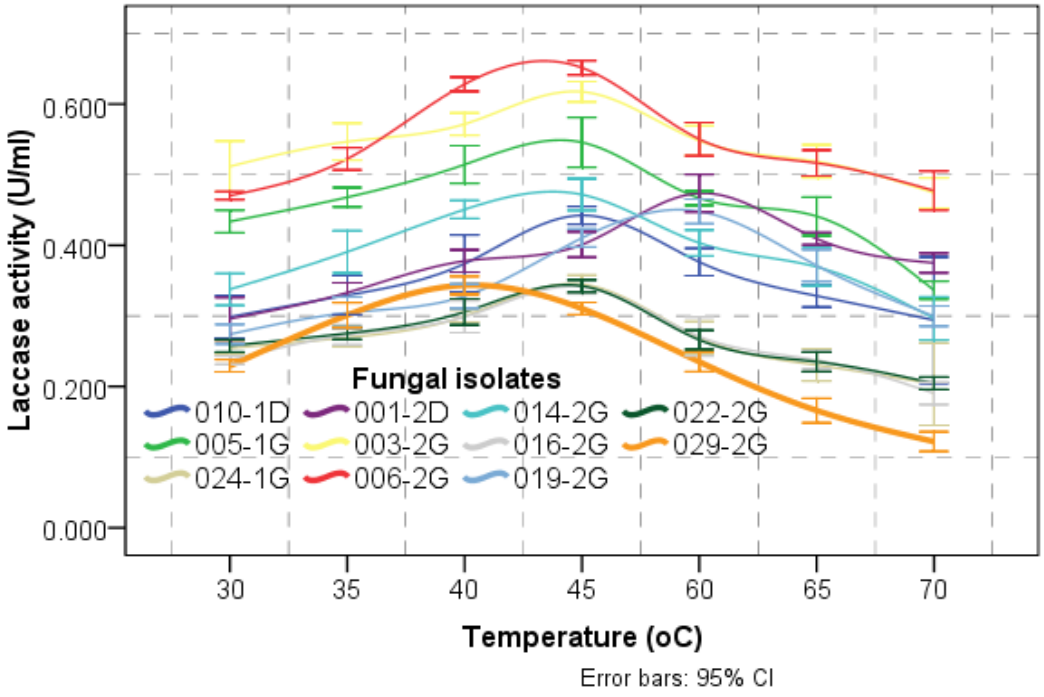

Fig. 9. Temperature optima of Laccase of the fungal isolates grwon on SSF condition of E. grandis sawdust for 12 days.

Temperature optimum and stability for fungal ligninolytic enzymes differ from one fungal strain to another [26,27]. The temperature profile of extracellular ligninolytic enzymes usually tend to be between 25 °C and 60 °C [28]. Aslam and Asgher [29] evaluated the effect of different temperatures on Laccase activity of *Pleurotus ostreatus* and reported optimum temperature of 30 °C. Singh *et al.* [28] also tested the Laccase activity of *Pycnoporous sanguineus* over a temperature range of 10-70 °C and reported optimum temperature of 30 °C.

The temperature stabilities of fungal ligninolytic enzymes also varied. Singh *et al*. [28], pre-incubated the enzyme of *Pycnoporus sanguineus* (SCC 108) in 50 mM citric acid buffer (pH 3.0) at 10-70 °C, for thermal stability studies, and obtained maximal Lac activity at 35 °C though it grew at the elevated temperature (47 °C).

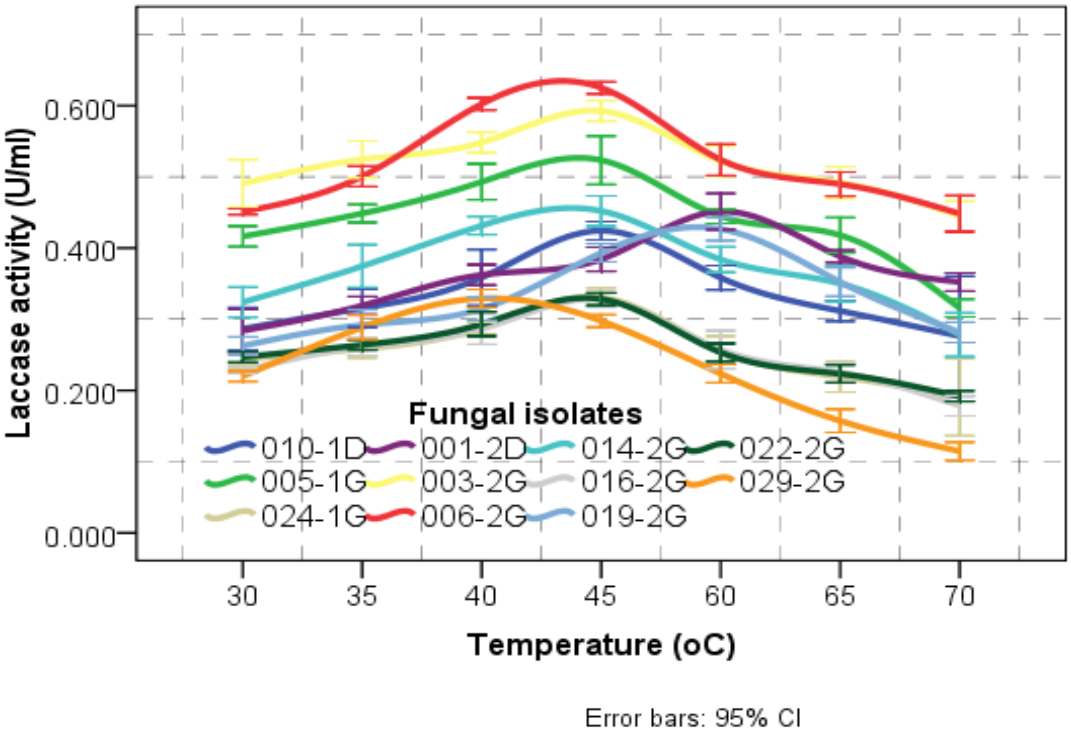

Fig. 10. Temperature stabilities of Laccases of the fungal isolates grown in SSF condition of the *E. grandis* sawdust for 12 days**.**

3. 3. 2. Effect of pH on the activity and stability of Laccase

The fungal isolates exhibited Laccase activity optima at different pH values (Fig. 11). While isolate 006-2G and 005-1G showed the most stable Laccase activities of 0.631 U/ml and 0.586 U/ml, respectively, at pH 5.0, isolate 003-2G displayed the highest Laccase activity (0.657 U/ml) at pH 4.0. Laccase of isolate 006-2G was most stable at pH 5.0 (0.534 U/ml) while isolate 003-2G showed the highest activities of Laccase (0.556 U/ml) at pH 4.0 (Fig. 12). Isolate 005-1G showed the highest Laccase activity (0.496 U/ml) at pH value of 5.0.

Enzyme activity is very dependent on the pH of fermentation medium [30]. The WRF isolates tested displayed their highest Laccase activities in the pH range of 3-6 indicating that this pH range is optimum for the enzyme. Particularly, pH 4 and 5 were most optima for the efficient Laccase producing isolates. Laccase of the isolates was also found stable within the mentioned pH range. Kunamneni *et al*. [31] obtained the pH optima of Laccase activity in the range 3.0-5.0 using ABTS as a substrate. Similarly, Singh *et al*. [28] measured over a pH range of 2.0-7.0 using ABTS as substrate, and found the most optimum Laccase activity at pH 4.5.

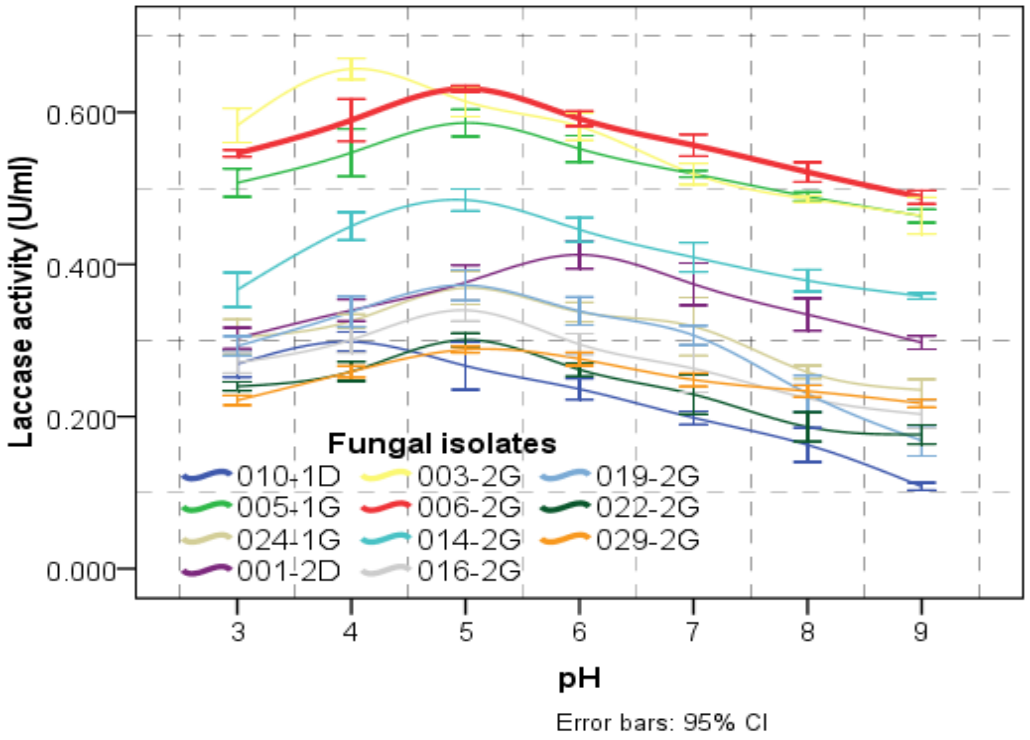

Fig. 11. pH optima of Laccase of the fungal isolates grown on SSF condition of *E. grandis* sawdust for 12 days

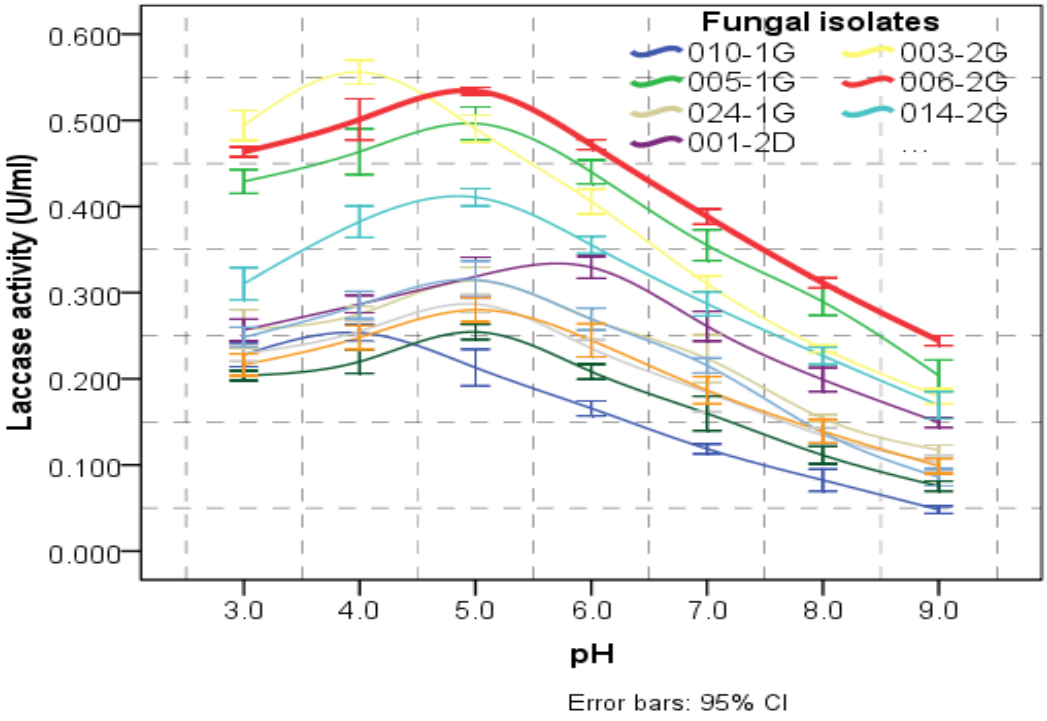

Fig. 12. pH stabilities of Laccases of the fungal isolates grown on SSF condition of *E. grandis* for 12 days

3. 4. Optimization of culture conditions for Laccase production

3. 4. 1. Effect of temperature on production of Laccase

Temperature is one of the most important factor affecting fungal growths though there is no universal condition [32]. All isolates secreted their highest Laccases at 30 °C (Fig. 13). The highest amounts of Laccase produced by isolate 003-2G (0.501 U/ml), 006-2G (0.455 U/ml) and isolate 005-1G (0.345 U/ml) at 30 °C were comparable to amount produced in other works. Hashim [24] reported 25 °C as optimum temperature for Laccase production by growing *P. ostreatus* on sawdust (0.690 U/ml).

Dhakar and Pandey [33] reported an optimum production of Laccase (0.410 U/ml) at 35 °C on 12th day in SmF of Kirk and Farrell medium. Nadeem *et al*. [34] incubated *Pleurotus ostreatus* at different temperatures ranged from 10-50 °C and reported the maximum Laccase production (0.562 U/ml) at 30 °C and they observed a declined Laccase production at temperature higher than 30 °C.

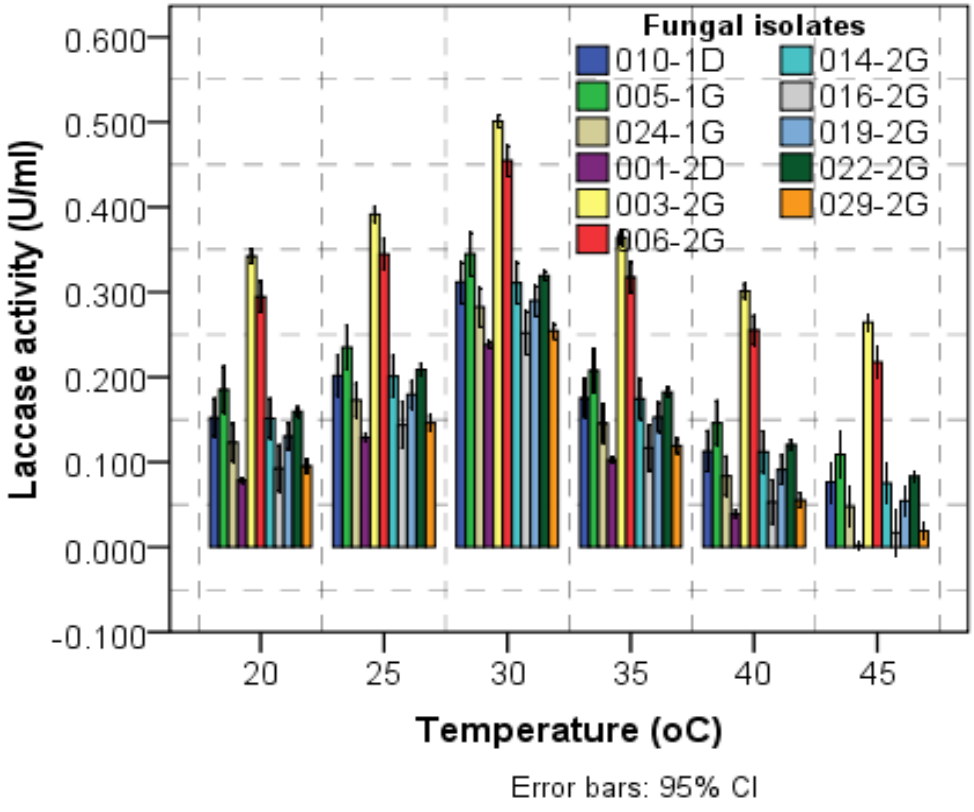

Fig. 13. Effect of temperature on the production of Laccases of the ligninolytic fungal isolates

3. 4. 2. Effect of pH on production of Laccase

pH ranges of 4-6 was found optimum for Laccase production by the test WRF isolates and the highest amounts were observed when grown in media initially adjusted at pH 5.0 (Fig. 14).

The highest amount of Laccase was obtained from isolate 003-2G (0.477 U/ml) and this was followed by amount of Laccase produced by isolate 006-2G (0.431 U/ml) and isolate 005-1G (0.321 U/ml). Nadeem *et al*. [34] reported an optimum production of Laccase (5.58 U/ml) at pH 5.5 by cultivating *Pleurotus ostreatus* at a series of pH values ranged from 3.5-9.5 and then a declining trend. Dhakar and Pandey [33] reported an optimum production of Laccase (0.300 U/ml) at pH 5.5 for on 8th day in SmF of Kirk and Farrell medium.

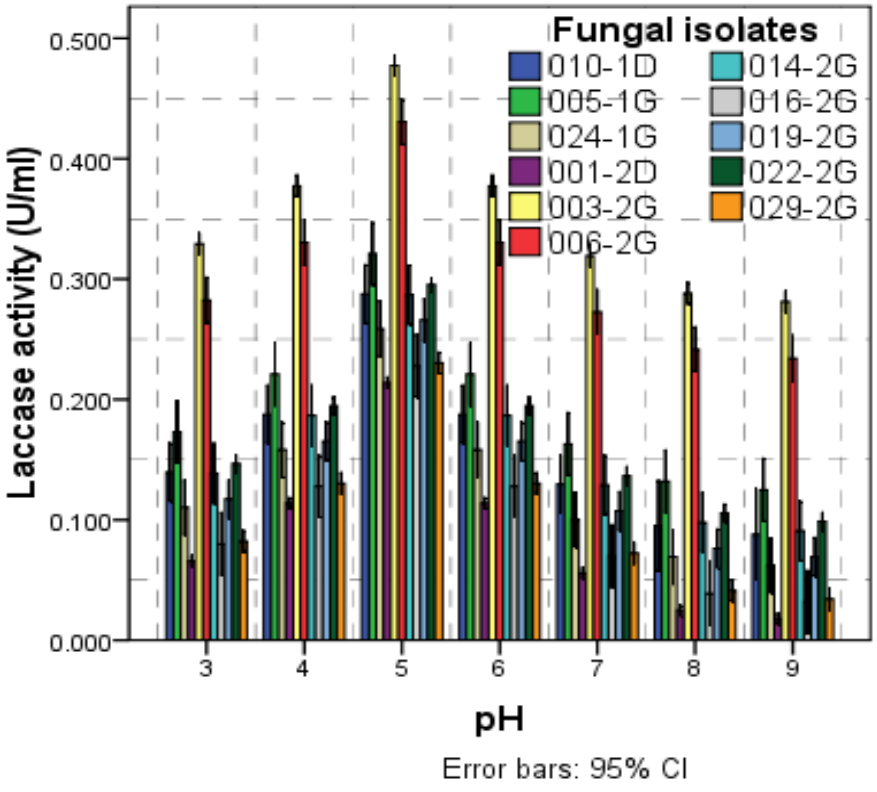

Fig. 14. Effect of pH on the production of Laccases of the ligninolytic fungal isolates

3. 4. 3. Effect of carbon sources on production of Laccase

Supplementation of the growth media with different carbon sources had different effects on Laccase productions (Fig. 15). Sucrose resulted in the highest Laccase productions by isolates 003-2G (0.438 U/ml), 006-2G (0.396 U/ml) and 005-1G (0.298 U/ml). Glucose and dextrose had similar effects on the amount of Laccase produced.

Different authors reported different carbon sources to be used for Laccase productions. Elisashvili and Kachlishvili [4] reported higher Laccase production with glucose in the growth medium by *Trametes pubescens*. Similarly, Dhakar and Pandey [33] reported an optimum production of Laccase (0.435 U/ml) using fructose at concentration of 0.4% in SmF.

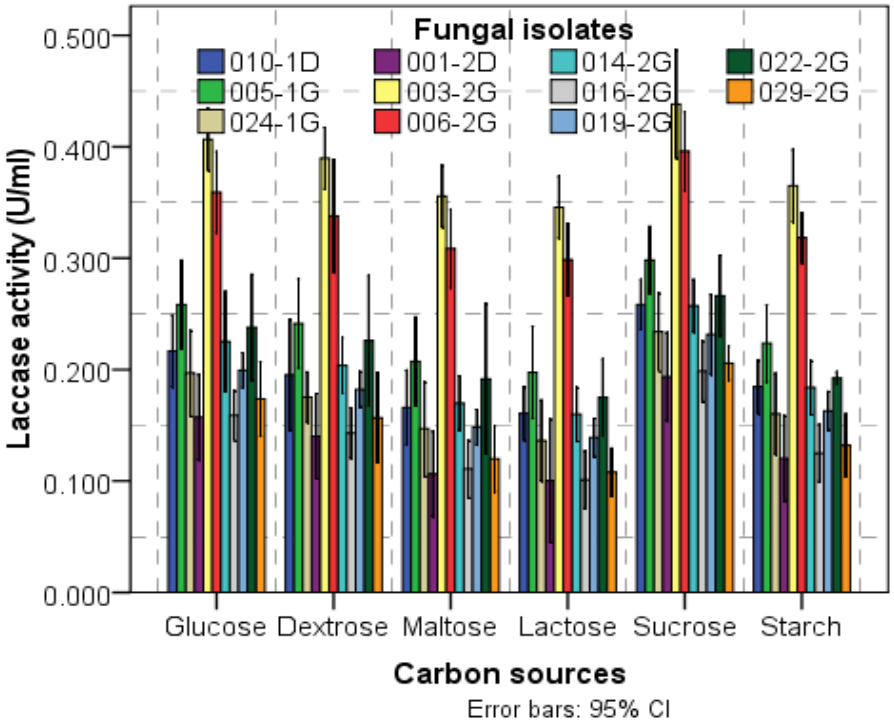

Fig. 15. Effect of carbon source on the production of Laccase of the ligninolytic fungal isolates

3. 4. 4. Effect of nitrogen sources on production of Laccase

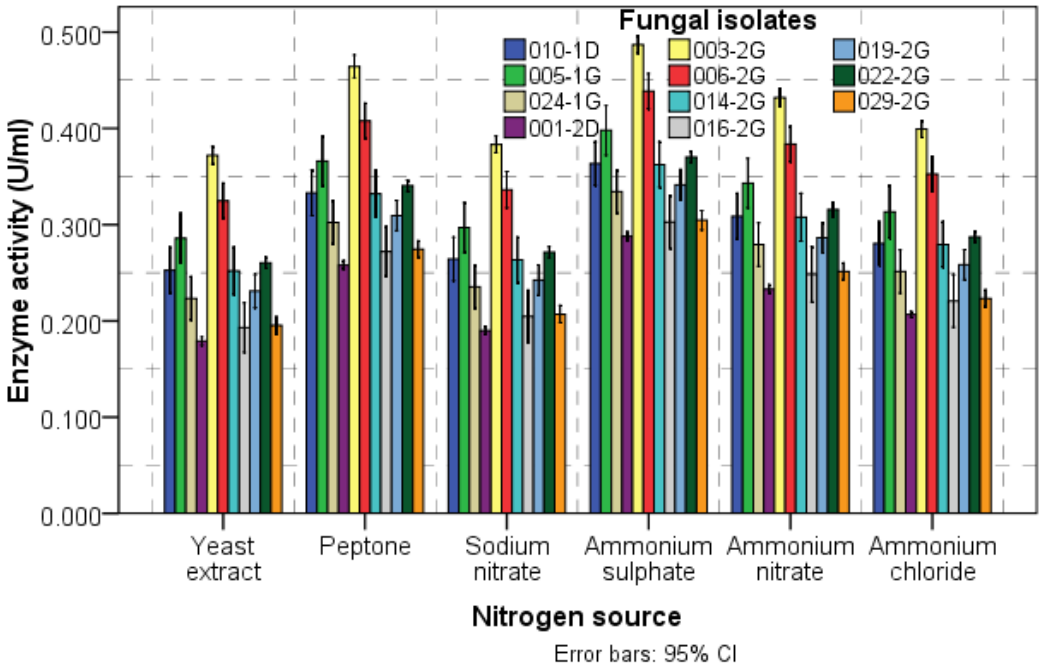

Fig. 16. Effect of nitrogen source on the production of Laccase of the ligninolytic fungal isolates.

Both the nature and concentration of nitrogen sources in the culture media regulate the fungal ligninolytic enzyme secretions [35]. Similarly, different effects were observed while the effects of ammonium sulphate, peptone and ammonium nitrate on the Laccase productions were particularly significant (Fig. 16). Three top Laccase amounts (0.475 U/ml, 0.453 U/ml and 0.448 U/ml) were secreted by isolate 003-2G in media supplemented with ammonium sulphate, peptone and ammonium nitrate, respectively.

Stajic *et al*. [36] noted the highest Laccase activity (0.181 U/ml) in NH_4NO_3 enriched medium at an initial medium pH of 5.0 compared to the control medium (0.076 U/ml) where wheat straw was the unique nitrogen source. On the other hand, Dhakar and Pandey [33] reported an optimum production of Laccase (0.390 U/ml) when $(NH_4)_2SO_4$ concentration was incorporated into Kirk and Farrell medium.

3. 4. 5. Effect of metallic ions on production of Laccase

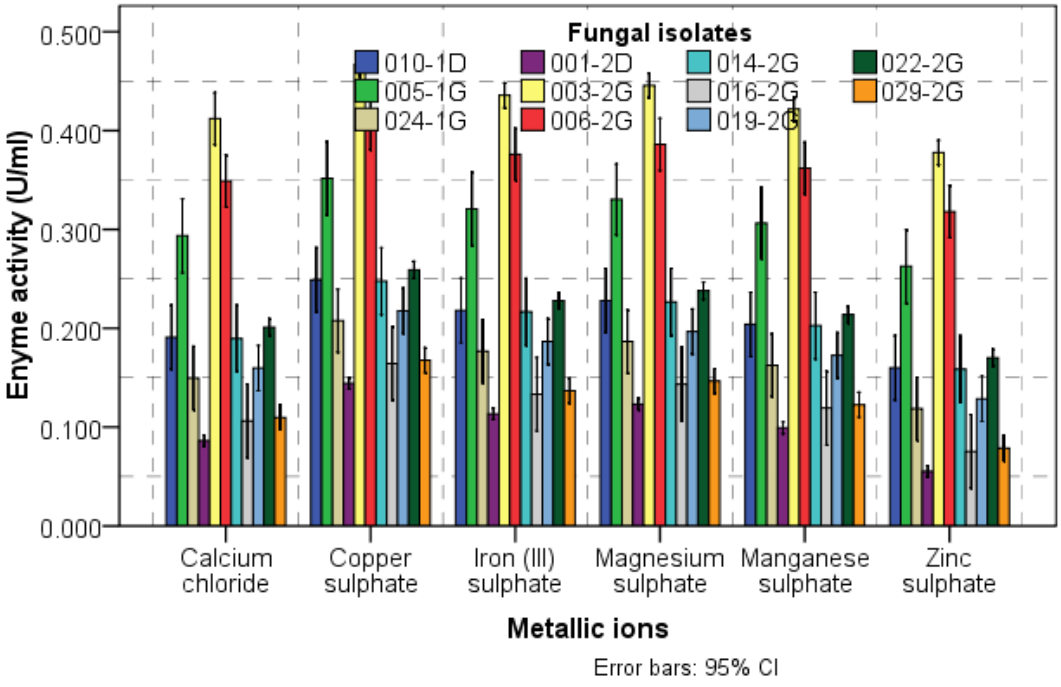

Fig. 17. Effect of metallic ions on the production of Laccase of the ligninolytic fungal isolates

Some heavy metals are essential for growth of fungi but can inhibit fungal activities when present in excess [37]. Of the metallic ions supplemented, magnesium sulphate had the highest positive effect on the production of Laccase which was followed by the effect of copper sulphate (Fig. 17). Isolates 003-2G (0.467 U/ml), 006-2G (0.407 U/ml) and 005-1G (0.330 U/ml) were found to be the top Laccase producers. Copper sulphate also increased the Laccase production of the fungal isolates. Different authors have reported the positive effect of addition of metal ions on the production of fungal ligninolytic enzymes [38-40]. Copper has been reported to be a strong Laccase inducer [41]. Dhakar and Pandey [33] reported an

optimum production of Laccase (0.580 U/ml) at 0.4 mM concentration of $CuSO_4$ incorporated into Kirk and Farrell medium.

4. CONCLUSION

By screening of wood rot fungi from their natural environment like forests, there is a high chance of getting high Laccase producing fungal isolates. The study sites got both natural and plantation forests which helped obtaining better isolates producing higher Laccases. The results indicated that the production of Laccase by WRF in both SmF and SSF significantly depends on the culture conditions. Supplementation of different sources into the growth media and adjusting growth conditions of the WRF resulted in production of higher Laccase. Higher Laccase activity found with sawdust of *E. grandis* also suggested the application of this substrate to large-scale processes so that high amount of Laccase is produced. Other ligninolytic enzyme having numerous applications would also be produced using this cheap lignocellulosic-based waste biomass in the country as well.

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