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CHARACTERIZATION AND PRODUCTION OF MnP AND LiP FROM WHITE ROT FUNGI OF PLANTATION AND NATURAL FORESTS OF ARSI FOREST ENTERPRISE, ETHIOPIA

Shasho Megersa [*]
Abdella Gure [*]
Sisay Feleke [**]
Melaku Alemu [***]

Abstract

A study was carried out to screen the wood rot fungal isolates and quantify their ligninolytic enzymes. Fifty six fungal isolates obtained from natural and plantation forests of Arsi forest enterprise were screened for the presence of ligninolytic enzymes and then MnP and LiP enzymes of eleven isolates were quantified. Results obtained from qualitative ligninolytic screening and quantitative MnP and LiP determinations showed that the fungal isolates coded as 003-2G, 006-2G and 005-1G measured the highest colored zone diameter and colony diameter in agar plate screening test with tannic acid supplemented and the highest MnP and LiP activities in *E. grandis* sawdust in both submerged fermentation (SmF) and solid state fermentation (SSF). The isolates displayed their highest MnP and LiP activities on 8^{th} day in SmF and on 12^{th} day in SSF. Different supplementations into the standard media also resulted in higher MnP and LiP productions. The three WRF isolates were efficient delignifying fungi and production of their enzymes was effective on 12^{th} day in SSF. This isolates could be used in biotreatement of sawdust for ethanol production.

Keywords:

Arsi;
WRF;
ligninolytic enzymes;
MnP;
LiP.

[*] **Wondo Genet College of Forestry and Natural Resources, Ethiopia**
[**] **Wood Technology Research Center, Addis Ababa, Ethiopia**
[***] **National Agricultural Biotechnology Research Center, Holeta, Ethiopia**

1. Introduction

Lignocellulosic substrates are mainly composed of cellulose, hemicellulose, and lignin (Dashtban et al., 2010). Lignin is the second most abundant polymer (Mabrouk et al., 2010). It is a non-carbohydrate aromatic polymer and gives rigidity to plants and resistance to attack by microorganisms. Research on lignin is important for conversion of solid organic wastes into production of valuable compounds like biofuels. This recalcitrant organic compound has been effectively degraded by microbes, particularly by white rot fungi (WRF). WRF are the most efficient lignin degrading basidiomycete fungi through ligninolytic enzymes secretion (Elisashvili and Kachlishvili, 2009). Recent studies have shown that individual groups of ligninolytic enzymes can be produced in SmF and SSF using plant raw materials as substrates (Couto and Sanroman, 2005; Silva et al., 2014). In the current study, the ligninolytic potential of the WRF isolated from the plantation and natural forests of Arsi forest enterprise were qualitatively screened. Manganes peroxidase (MnP) and Lignin peroxidase (LiP) of the potential ligninolytic fungal isolates were also quantified and characterized.

2. Materials and methods

Culture and substrate sources

The cultures were obtained from previous fungal diversity study of the natural and plantation forests of Arsi forest enterprise, Oromia, Ethiopia (Megersa et al., 2017) (Fig. 1). Fresh sawdust of *E. grandis* was obtained from sawmill of Arsi forest enterprise.

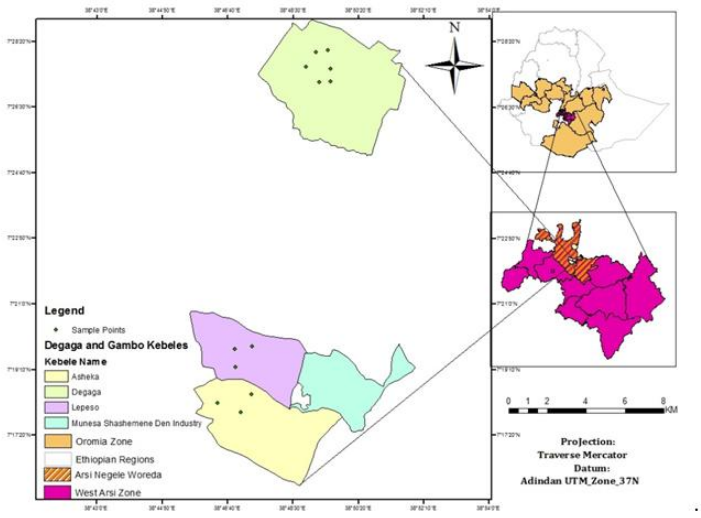

Fig. 1 Site map of the culture collection area

Qualitative screening of the WRF

Ligninolytic activities of the WRF isolates were screened using the lignin modifying fungal enzymes basal medium (LBM) supplemented with tannic acid (Atri and Sharma, 2012). Aqueous tannic acid solution (1% w/v) were separately sterilized and aseptically added to LBM (1% v/v). Each test fungus was separately inoculated and incubated at 25^{o}C in darkness. The brown oxidation zone and culture diameter were measured and enzymatic index (EI) was calculated for each isolate.

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

Fungal isolates with EI higher than 1.50 were considered as potential producers of ligninolytic enzymes (Damaso et al., 2012).

Quantitative determination of MnP and LiP

Inoculum preparation

The screened efficient ligninolytic fungal isolates (Table 1) were prepared using the standard medium of Altaf *et al*. (2012). The pH of the medium was adjusted to 6.0 with 2M NaOH. Four disks (Ø 0.5 mm) of each isolate were inoculated and grown on a rotary shaker at 150 rpm and room temperature in 250 ml flasks containing 100 ml of the medium. After six days of fungal cultivation mycelial pellets were homogenized and used as inocula for both SmF and SSF experiments in quantitative estimation of MnP and LiP activities.

Submerged fermentation (SmF)

The sawdust was ground and sieved with 4.0 mm sieve before use and SmF of the fungal isolates was carried out replacing glucose of the standard medium with 10.0 g of *E. grandis* sawdust. 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 2 M NaOH. Each flask was inoculated with 3 ml of mycelial homogenate and incubated on a rotary shaker at 150 rpm and room temperature. After 5, 8 and 12 days, solids were separated by filtration through nylon cloth followed by centrifugation at 4000 rpm for 15 minutes.

Solid state fermentation (SSF)

10.0 g of sawdust was moistened with 12 ml of the standard medium in 250 ml flasks. The flasks were autoclaved and inoculated with 3 ml of mycelial homogenate and incubated at room temperature. After 7, 12 and 15 days, 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.

Determination of MnP and LiP activities

Determination of MnP activity

MnP activity of the enzyme extract was measured using guaiacol as a substrate (Mabrouk et al., 2010). The increase in absorbance at 465 nm due to oxidation of guaiacol (2-methoxyphenol) (ε_{465} = 12100 $LM^{-1}cm^{-1}$) was measured using JENWAY6305 UVis-Spectrophotometer. One unit (U) of MnP activity was defined as activity of an enzyme that catalyzes the conversion of 1 μmole of guiacol per minute.

Determination of LiP activity

LiP activity of the enzyme extract was determined spectrophotometrically at 310 nm through the oxidation of veratryl alcohol (Dimethoxybenzyl alcohol) to veratryl aldehyde (ε_{310} = 9300 LM^{-1} cm^{-1}) (Irshad and Asgher, 2011). One unit (U) of LiP activity was defined as activity of an enzyme that catalyzes the conversion of one μmole of veratryl alcohol per minute.

Finally MnP and LiP activities were calculated using Beer-Lambert equation $A = \varepsilon dC \Rightarrow C = \frac{A}{\varepsilon d}$, 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}) and enzyme unit was reported as U/ml.

Partial characterization of MnP and LiP

Effect of temperature on the activity and stability of MnP and LiP

The optimum temperatures for the crude MnP and LiP activities were determined by incubating the mixture at 5°C intervals from 30-70°C for 30 minutes in a digital incubator (Snajdr and Baldrian, 2007). After 30 minutes of incubation, the mixtures were removed and measured for absorbance using UV-Vis spectrophotometer.

Heat stability studies of the crude ligninolytic enzyme were performed by pre-incubating the crude enzyme in 0.05 M acetate buffer (pH 4.5) at 5^{o}C intervals from 30-70^{o}C for 120 minutes. After 120 minutes of pre-incubation, enzyme activities were determined.

Effect of pH on the activity and stability of MnP and LiP

The optimum pH values for the crude MnP and LiP activities were determined by incubating the mixture of crude enzyme in the presence of appropriate pH buffer solutions 3.0-9.0 and incubated 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. After 30 minutes of incubation, the enzyme activities were measured.

For the pH stability experiments, the crude extracts were pre-incubated under initial conditions (pH values 3.0-8.0) for 120 minutes at 30^{o}C. After 120 minutes of crude enzyme incubation, the enzyme activities were measured.

Optimization of culture conditions for MnP and LiP productions

Three fungal isolates were incubated under the following test conditions for eight days and the cell free extracts from each flask were analyzed for MnP and LiP activities.

Effect of temperature on MnP and LiP

SmF were carried out in an incubator at 5^{o}C intervals in the range of 20-45°C using the standard medium.

Effect of pH on production of MnP and LiP

SmF were carried out at different pH ranges of 3.0 to 9.0 using the standard medium. The pH of the medium was adjusted by using 1N HCl or 1N NaOH.

Effect of carbon sources on production of MnP and LiP

SmF were carried out by replacing glucose of the standard medium with 0.6% dextrose, maltose, lactose, sucrose, and starch as carbon sources.

Effect of nitrogen sources on production of MnP and LiP

SmF of the fungal isolates was conducted by supplementing the nitrogen source of the standard medium with yeast extract, peptone, $NaNO_3$, NH_4NO_3, $(NH4)_2SO_4$, NH_4Cl at 0.3% concentration.

Effect of divalent metallic ions on production of MnP and LiP

SmF of the fungal isolates were conducted by replacing the divalent 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%.

Statistical Analysis

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

3. Results

Qualitative assays of WRF

Of the 56 fungal cultures screened many displayed oxidation zones under and round their cultures which indicated the secretions of ligninolytic enzymes on LBM supplemented with tannic acid. Those cultures which showed significant colored zone round the growing mycelia and hence showed higher EI were particularly considered as potential WRF and were selected for quantitative determination of MnP and LiP (Table 1). Wider oxidation zone round the fungal culture means the more the fungal isolate could secrete ligninolytic enzymes into the solid media and degrade tannic acid. Isolate 005-1G, 003-2G and 006-2G in particular showed wider color zones round colony and selected for quantitative determination experiments (Table 1).

Table 1 WRF isolates selected for MnP and LiP 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

Quantitative determination of MnP and LiP

Determination of MnP and LiP activities using SmF and SSF

All fungal isolates secreted their highest MnP and LiP on the 8th day of fermentation. The enzyme activities were significantly differed. The highest MnP activity was shown by isolate 003-2G (0.180 U/ml) which was followed by MnP activities of 006-2G (0.173 U/ml) and 005-1G (0.120 U/ml) (Fig. 2). On the other hand the highest LiP activity was displayed by isolate 006-2G (0.743 U/ml) which was not significantly different from LiP activity of 003-2G (0.700 U/ml). Considerable amount of LiP was also secreted by isolate 005-1G (0.687 U/ml).

On the other hand, the fungal isolates secreted their highest respective MnP and LiP enzymes 12th day of sawdust' SSF (Fig. 3). MnP activities of isolates 003-2G (0.184 U/ml) and 006-2G (0.179 U/ml) were significantly higher than the MnP activities of the other isolates. LiP activity obtained from isolate 006-2G (0.771 U/ml) was significantly higher and followed by the LiP activities of isolate 003-2G (0.723 U/ml) and isolate 005-1G (0.709 U/ml).

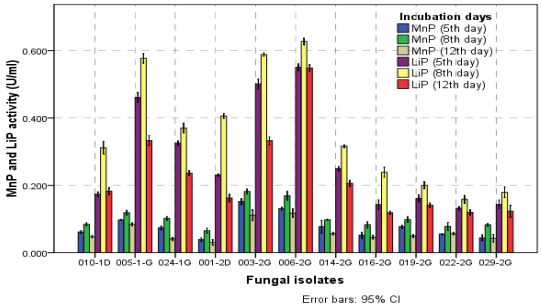

Fig. 2 MnP and LiP activities of the fungal isolates grown in SmF sawdust

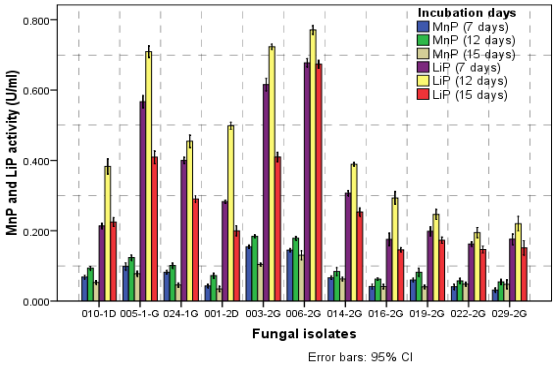

Fig. 3 MnP and LiP activities of the fungal isolates grown in SSF of sawdust

Partial characterization of MnP and LiP

For the partial characterization and production optimization experiments, three most efficient WRF isolates (005-1G, 003-2G and 006-2G) were selected based on the qualitative and quantitative ligninolytic assays (Table 1, Fig. 2 and Fig. 3). Since the enzymes secreted by the fungal isolates in SSF growth conditions is higher than the amount secreted in SmF growth conditions, enzyme extracts of the fungal isolates grown in SSF sawdust for 12 days were used for further partial characterization of the enzymes.

Temperature optima and stabilities of MnP and LiP

MnP and LiP activities of the fungal isolates have been affected by incubation temperature. Isolate 006-2G showed the highest activities of MnP (0.311 U/ml) and LiP (0.801 U/ml) at 45^{o}C (Fig. 4) indicating that this temperature is most optimum for their activities. On the other hand, isolate 003-2G showed the highest MnP (0.313 U/ml) activity at 60^{o}C and LiP (0.707 U/ml) 40^{o}C.

MnP and LiP of isolate 006-2G were most stable at 45°C, displaying enzyme activities of 0.298 U/ml 0.728 U/ml, respectively. Declined enzyme activities were obtained with the further rising incubation temperatures (Fig. 5). Isolate 003-2G showed most stable MnP (0.297 U/ml) and LiP (0.678 U/ml) at 60°C and 40°C, respectively. Isolate 005-1G showed most stable activity of MnP (0.238 U/ml) at 65°C, and stable activities of LiP (0.634 U/ml) at 45°C.

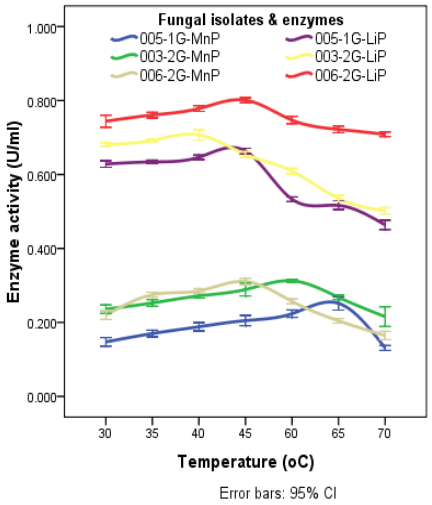

Fig. 4 Temperature optima of the MnP and LiP of the fungal isolates

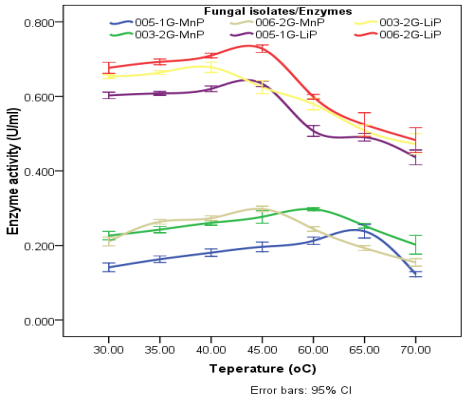

Fig. 5 Temperature stabilities of MnP and LiP of the fungal isolates

pH optima and stabilities of MnP and LiP

pH 6.0 was found most optimum for the activities of MnP (0.310 U/ml) and LiP (0.743 U/ml) of isolate 006-2G (Fig. 6). But isolate 003-2G displayed the highest activities of MnP (0.293 U/ml) at pH 4.0 and LiP (0.744 U/ml) at pH 5.0. Similarly, isolate 005-1G displayed its highest MnP (0.241 U/ml) and LiP (0.709 U/ml) activities at pH 4.0 and 6.0, respectively, showing that these enzymes perform most at mentioned pH values.

Isolate 006-2G showed the most stable MnP (0.252 U/ml) activity at pH 6.0 and LiP (0.723 U/ml) activity at pH 5.0 (Fig. 7). Isolate 003-2G showed the highest MnP (0.249 U/ml) activity at pH 4.0 and LiP (0.632 U/ml) activity at pH 5.0 indicating that these enzymes are stable at these temperatures. Isolate 005-1G also showed the highest MnP (0.205 U/ml) and LiP (0.603 U/ml) activities at pH values of 4.0 and 6.0, respectively.

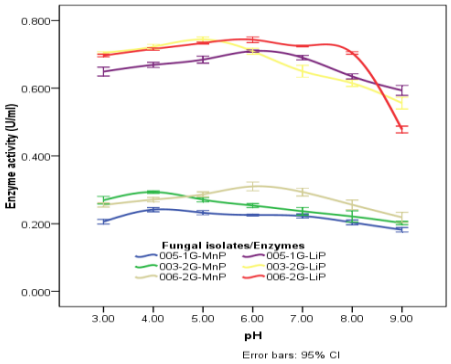

Fig. 6 pH optima of the MnP and LiP of the fungal isolates

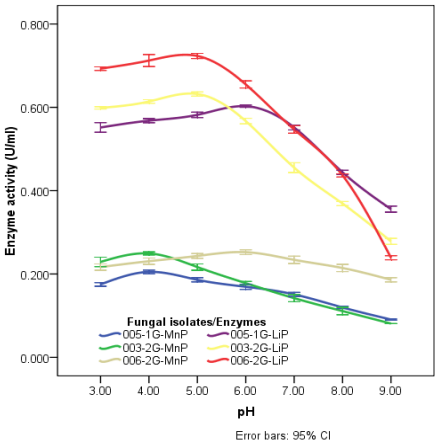

Fig. 7 pH stabilities of MnP and LiP of the fungal isolates

Optimization of culture conditions for MnP and LiP productions

Productions of MnP and LiP were optimized by supplementing different components into the standard culture media of Altaf et al. (2012).

Effect of temperature on production of MnP and LiP

Effect of incubation temperature on enzyme productions was presented in table 2. The optimum temperature for both MnP and LiP productions by all isolates was found to be 30°C. The amounts of enzymes were decreased before and beyond this temperature. Isolates 005-1G, 003-2G and 006-2G secreted their respective highest MnP of 0.135 U/ml, 0.194 U/ml and 0.186 U/ml, respectively, at 30°C. Similarly, the isolates secreted their highest LiP of 0.532, 0.564 and 0.617 U/ml, respectively, at the same incubation temperature.

Table 2 Effect of temperature on production of MnP and LiP

Fungal isolates	Temperature (oC)					
	20	25	30	35	40	45
	MnP activity (U/ml) (mean±s.d.)					
005-1G	.104±.003^{b}	.119±0.003^{c}	.135±.003^{c}	.097±.003^{c}	.074±.003^{c}	.060±.003^{c}
003-2G	.157±.002^{a}	.172±0.002^{a}	.194±.003^{a}	.160±.003^{a}	.116±.003^{a}	.088±.003^{a}
006-2G	.152±.003^{a}	.164±0.003^{b}	.186±.002^{b}	.147±.005^{b}	.108±.002^{b}	.075±.002^{b}
	LiP **activity (U/ml) (mean±s.d.)**					
005-1G	.398±.005^{c}	.479±.005^{c}	.532±.005^{c}	.440±.006^{b}	.230±.003^{b}	.104±.003^{b}
003-2G	.472±.002^{b}	.515±.002^{b}	.564±.002^{b}	.451±.002^{b}	.230±.006^{b}	.118±.005^{b}
006-2G	.541±.004^{a}	.592±.003^{a}	.617±.004^{a}	.565±.004^{a}	.347±.004^{a}	.219±.006^{a}

Mean followed by similar lower case superscript letters in each column do not differ significantly at 5% level by Tukey's multiple comparison

Effect of pH on production of MnP and LiP

The fungal isolates produced their respective highest MnP and LiP at pH 5.0 (Table 3). But when the amount of MnP secreted under initial pH media were compared, the highest MnP amount was secreted by isolate 003-2G (0.173 U/ml) at pH 5.0. This was followed by the MnP amounts from isolate 006-2G (0.167 U/ml) at pH 5.0 and isolate 003-2G (0.163 U/ml) at pH 6.0. But the highest LiP production was obtained from isolate 006-2G (0.757 U/ml) at pH 5.0 which was followed by LiP amounts the same isolate at pH 6.0 (0.702 U/ml) and at pH 4.0 (0.687 U/ml).

Table 3 Effect of pH on production of MnP and LiP

Fungal isolates	pH						
	3	4	5	6	7	8	9
	MnP activity (U/ml) (mean±s.d.)						
005-1G	.109±.003[b]	.116±.003[b]	.138±.003[b]	.123±.003[c]	.107±.003[b]	.078±.003[b]	.069±.003[b]
003-2G	.146±.005[a]	.151±.004[a]	.173±.003[a]	.163±.002[a]	.142±.003[a]	0.113±.003[a]	.104±.003[a]
006-2G	.141±.006[a]	.145±.01[a]	.167±.006[a]	.153±.004[b]	.136±.005[a]	.105±.006[a]	.098±.006[a]
	LiP activity (U/ml) (mean±s.d.)						
005-1G	.539±.006[b]	.562±.006[b]	.632±.006[b]	.577±.006[b]	.516±.006[b]	.416±.006[b]	.386±.006[b]
003-2G	.551±.002[b]	.573±.002[b]	.643±.002[b]	.588±.002[b]	.527±.002[b]	.427±.002[b]	.397±.002[b]
006-2G	.667±.004[a]	.687±.004[a]	.757±.004[a]	.702±.004[a]	.641±.004[a]	.541±.004[a]	.511±.004[a]

Mean followed by similar lower case superscript letters in each column do not differ significantly at 5% level by Tukey's multiple comparison

Effect of carbon sources on production of MnP and LiP

Different inexpensive carbon sources such as glucose, dextrose, maltose, sucrose and starch were used in SSF culture conditions to get maximum enzyme yields. Sucrose supplementation had superior positive effects on the enzymes productions of the fungal isolates (Table 4). The highest MnP production was obtained from 003-2G (0.194 U/ml) when sucrose was supplemented. The next two top MnP productions were from isolate 006-2G (0.179 U/ml) when sucrose was supplemented and from isolate 003-2G (0.146 U/ml) when starch was supplemented. On the other hand, three highest consecutive LiP productions of 0.664, 0.638 and 0.632 U/ml were obtained from isolate 006-2G when sucrose, glucose and dextrose were supplemented, respectively. It was observed that glucose and dextrose had similar effects on the amount of MnP and LiP productions.

Table 4 Effect of carbon sources on production of MnP and LiP

Fungal isolates	Carbon sources					
	Glucose	**Dextrose**	**Maltose**	**Lactose**	**Sucrose**	**Starch**
	MnP activity (U/ml) (mean±s.d.)					
005-1G	.107±.006[b]	.106±.005[b]	.078±.007[b]	.079±.006[b]	.134±.005[b]	.082±.007[b]
003-2G	.167±.008[a]	.166±.011[a]	.141±.007[a]	.137±.007[a]	.194±.005[a]	.146±.007[a]
006-2G	.155±.004[a]	.155±.009[a]	.124±.008[a]	.122±.004[a]	.179±.008[a]	.130±.013[a]
	LiP activity (U/ml) (mean±s.d.)					
005-1G	.545±0.015[b]	.544±.006[b]	.516±.010[b]	.510±.006[b]	.576±.006[b]	.525±.006[b]
003-2G	.561±0.002	.555±002[b]	.530±.002[b]	.521±.002[b]	.588±.002[b]	.536±.002[b]
006-2G	.638±0.004	.632±.004[a]	.607±.004[a]	.598±.004[a]	.664±.004[a]	.613±.004[a]

Mean followed by similar lower case superscript letters in each column do not differ significantly at 5% level by Tukey's multiple comparison

Effect of nitrogen sources on production of MnP and LiP

Nitrogen sources such as yeast extract, peptone, ammonium sulphate, ammonium nitrate and ammonium chloride were used to optimize MnP and LiP productions from the three fungal isolates. Different enzyme amounts were produced. It was observed that ammonium sulphate was most suitable for MnP and LiP productions by the three isolates (Table 5). Supplementation of peptone also had higher positive effects on the productions of the enzymes. When each supplementation considered, three top MnP amounts of 0.167 U/ml, 0.165 U/ml and 162 U/ml were obtained from the cultures which were supplemented with ammonium sulphate, ammonium chloride and peptone, respectively. On the other hand, three top LiP productions of 0.705 U/ml, 0.671 U/ml and 0.660 U/ml were secreted with supplementations of ammonium sulphate, peptone and ammonium nitrate, respectively.

Table 5 Effect of nitrogen sources on production of MnP and LiP

Fungal isolates	Nitrogen sources					
	Yeast extract	Peptone	Sodium nitrate	Ammonium sulphate	Ammonium nitrate	Ammonium chloride
	MnP activity (U/ml) (mean±s.d.)					
005-1G	.102±.003^{c}	.117±.003^{c}	.105±.003^{c}	.126±.003^{b}	.107±.003^{c}	.102±.003^{c}
003-2G	.145±.003^{a}	.162±.003^{a}	.147±.003^{a}	.167±.005^{a}	.149±.003^{a}	.165±.003^{a}
006-2G	.134±.006^{b}	.140±.013^{b}	.129±.004^{b}	.159±.006^{a}	.136±.006^{b}	.136±.003^{b}
	LiP activity (U/ml) (mean±s.d.)					
005-1G	.535±.006^{b}	.551±.006^{b}	.536±.006^{b}	.579±.006^{b}	.542±.006^{b}	.528±.006^{b}
003-2G	.546±.002^{b}	.561±.003^{b}	.548±.002^{b}	.589±.004^{b}	.552±.003^{b}	.539±.002^{b}
006-2G	.653±.004^{a}	.671±.004^{a}	.654±.004^{a}	.705±.009^{a}	.660±.004^{a}	.646±.004^{a}

Mean followed by similar lower case superscript letters in each column do not differ significantly at 5% level by Tukey's multiple comparison

Effect of divalent metallic ions production of MnP and LiP

The effects of metal ions such as calcium chloride, copper sulphate, iron sulphate, magnesium sulphate, manganese sulphate and zinc sulphate on MnP and LiP productions were also investigated. Of all metallic ions supplementations, Magnesium sulphate had the highest effect on the MnP and LiP productions by the fungal isolates and was followed by positive effects of copper sulphate (Table 6).

Table 6 Effect of divalent metallic ions production of MnP and LiP

Fungal isolates	Metallic ions					
	Calcium chloride	Copper sulphate	Iron (II) sulphate	Magnesium sulphate	Manganese sulphate	Zinc sulphate
	MnP activity (U/ml) (mean±s.d.)					
005-1G	.072±.008[b]	.082±.002[b]	.079±.008[b]	.091±.009[b]	.075±.006[b]	.068±.015[b]
003-2G	.119±.009[a]	.127±.006[a]	.123±.005[a]	.135±.005[a]	.120±.008[a]	.112±.010[a]
006-2G	.107±.004[a]	.117±.004[a]	.114±.004[a]	.126±.004[a]	.111±.004[a]	.103±.004[a]
	LiP activity (U/ml) (mean±s.d.)					
005-1G	.410±.005[b]	.428±.005[b]	.436±.004[b]	.442±.004[b]	.421±.004[b]	.404±.005[b]
003-2G	.419±.002[b]	.437±.002[b]	.444±.002[b]	.451±.002[b]	.430±.002[b]	.412±.001[b]
006-2G	.504±.003[a]	.523±.003[a]	.530±.003[a]	.537±.003[a]	.516±.003[a]	.498±.003[a]

Mean followed by similar lower case superscript letters in each column do not differ significantly at 5% level by Tukey's multiple comparison.

4. Discussions

Qualitative assays of WRF

According to Brijwani et al. (2010), enzyme production by WRF depends on different factors mainly on fungal species and fungal strains. Damaso et al. (2012) also explained that size of colored zone round culture colony is a good indication of secreting high amount of ligninolytic enzymes. Similarly, wider oxidation zones were observed round the cultures of the isolate 005-1G, 003-2G and 006-2G during qualitative screenings. Higher MnP and LiP were also quantified from these isolates.

Quantitative determination of MnP and LiP

Screening fungi from natural environment is one strategy to obtain efficient isolates for ligninolytic enzyme production. Three isolate (003-2G, 006-2G and 005-1G) were found to be efficient MnP and LiP producers both in SmF and SSF culture conditions. Enzyme quantification results of the current work were comparable to results reported by different scholars. Knezevic et al. (2010) reported the highest MnP (0.111 U/ml) and LiP (0.767 U/ml) activities on the 7th day using oak sawdust in SmF of WRF *Trametes suaveolens*. Similarly, Cilerdzic et al. (2016)

obtained the highest MnP activity from the WRF *G. lucidum* during the SmF of oak sawdust on the 14th day.

Sukarta and Sastrawidana (2014) reported the highest activities of MnP (0.210 U/ml) and LiP (0.260 U/ml) by cultivating *Polyporus* sp. on sawdust in SSF on the 7th day. On the other hand, Cilerdzic et al. (2016) reported the highest MnP activity during the SSF of oak sawdust on the 14th day with *G. lucidum*.

Partial characterization of MnP and LiP

Temperature optima and stabilities of MnP and LiP

Temperature optima and stability of the fungal isolates of the current work (Fig. 3 and Fig. 4) is in line with the report of Singh et al. (2012) who stated that temperature profile of extracellular ligninolytic enzymes usually lies between 25°C and 60°C. MnP activity of the mushroom *Stereum ostrea* was optimum at 35°C (Praveen et al., 2012). Aslam and Asgher (2011) conducted an experiment to evaluate the effect of different temperatures on ligninolytic activity of *P. ostreatus* and obtained the optimum temperature at 30°C. Praveen *et al*. (2012) reported stable MnP activity of the mushroom *Stereum ostrea* over the temperature ranges of 20-35°C. The works of Zeng et al. (2013) on thermal stability profiles revealed that LiP and MnP were highly stable at temperatures between 35°C and 40°C.

pH ptima and stabilities of MnP and LiP

pH optima of the fungal isolates (Fig. 5 and Fig. 6) were similar to what Praveen et al. (2012) reported explaining pH 4.5 as an optimum MnP activity for the mushroom *Stereum ostrea*. Similarly, the authors reported stable MnP activity of the mushroom was over the pH ranges of 4.5-6.0. On the other hand, Aslam and Asgher (2011) reported the maximum MnP and LiP activities at pH value of 4.0 for *P. ostreatus*. pH optima of 4.5 for MnP and 3.0 for LiP were reported for *Phanerochaete chrysosporium* (Zeng et al., 2013).

Optimization of culture conditions for MnP and LiP productions

Temperature required for maximum MnP and LiP productions by the fungal isolates was slightly less than reported by different authors (table 2). Vassilev et al. (2009) evaluated the influence of

initial temperature on the production of MnP by *Phanerochaete chrysosporium* on dry olive and sugar beet wastes under SSF and obtained optimum MnP secretion at 37^{o}C. The maximum activities of MnP and LiP were produced in the flasks incubated at 35°C when *Schizophyllum commune* grown in solid state medium banana stalks (Irshad and Asgher, 2011).

pH 5.0 was suitable for enzyme production of the fungal isolates (table 3) which was similar to other reports. Wen et al. (2010) reported the optimum pH of 4.8 for MnP production from *P. chrysosporium*. Vassilev et al. (2009) also evaluated the influence of initial pH on the production of MnP by *Phanerochaete chrysosporium* on olive and sugar beet wastes under SSF and reported the highest MnP secretion at pH 5.0.

Supplementation of sucrose was found to be superior to other carbon sources in the current research results. MnP activity was observed to be maximum in glucose followed by fructose and sucrose and the highest LiP activity was expressed in glucose, maltose and lactose when *Dictyoarthrinium Synnematicum* grown on different carbon sources (Prasher and Chauhan, 2015). On the other hand, Mikiashvili et al. (2006) reported the highest MnP (0.10 U/ml) and LiP (0.71 U/ml) productions in peptone supplemented media for *P. ostreatus* in SmF for 10 days.

Different authors also have reported the positive effect of the addition of metal ions on the production of fungal MnP and LiP in lower concentrations (Makela et al., 2013; Viswanath et al., 2014).

Conclusion

With the rising interest in generating bioethanol from lignocellulosic resources, screening efficient ligninolytic fungi from natural habitat and optimizing their enzyme production is mandatory. Such isolates were screened qualitatively, their enzymes quantified and optimized for maximum production. The results indicated that the production of MnP and LiP by WRF in SmF and SSF significantly depends on fungal isolate type. Supplementation of different sources into the growth media and adjusting growth conditions of the WRF resulted in production of higher MnP and LiP. Higher MnP and LiP activities obtained with sawdust of *E. grandis* in SSF also suggested the application of this substrate to large-scale processes so those high amounts of the enzymes are produced.

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