Screening Diverse Fungi for Laccases of Varying Properties

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Abstract Qualitative screening of 295 fungi for laccases yielded 125 laccase positive ones, mostly basidiomycetes. Fifty of these were tested for laccase activity at pH 3.0, 4.5 and 6.0. Most showed maximum activity at pH 4.5, a few showed a broad activity range, two were optimal at pH 3.0 and only the mitosporic fungus *Beltraniella* sp. was best at pH 6. Most of the 25 fungi assayed at three different temperatures had an optimum at 45°C. The basidiomycete *Auricularia* sp. acted best at 30°C, while three others showed best activity at 60°C. This study shows the potential of screening diverse fungi for laccase with varying pH and temperature preferences for different applications.

Keywords Fungi · Laccase · pH · Temperature · Optima

Laccases are multicopper enzymes found in bacteria, fungi and plants. They catalyze the oxidation of phenols and polyphenols, besides many nonphenolic compounds, resulting in reduction of molecular oxygen to water [1]. Fungal laccases occur in Ascomycota, Basidiomycota and mitosporic fungi [2]. Basidiomycetous white rot fungi such as *Trametes versicolor*, *Pleurotus ostreatus* and *Ganoderma lucidum* are excellent laccase producers [3]. Laccases are used in paper, textile, bioremediation and other industries [1, 2]. Such applications would benefit by laccases

with different pH and temperature optima. We screened a large number of fungi for laccases for these properties. Cultures from in-house collection of the organization were used. These were identified based on morphology, using standard monographs. Initial qualitative screening was by inoculating a 5 mm. diam mycelial disc of 5 day old culture onto PDA plates containing 4 mM guaiacol. Intense brown coloration around the fungal colony was considered positive for laccase production [4].

A 5 mm mycelial disc of 7 day old culture was inoculated into 150 ml Erlenmeyer flask containing 30 ml Malt Extract broth (MEB) and grown stationarily. After 7 days, sterile glass beads were added and the mycelium was homogenized by shaking. A 5% inoculum (v/v) was transferred to 20 ml of MEB in a 100 ml Erlenmeyer flask. Three replicates were incubated for 20 days at 25°C in stationary condition. Copper sulphate (2 mM end concentration) was added in some experiments after 7 days. Enzyme was harvested by filtration using 0.45 µm Millipore filter. Spectrophotometric assays were carried out at 405 nm using 1 mM 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) [5] at pH, 3.0, 4.5 and 6.0, using appropriate buffers and further at 30, 45 and 60°C temperatures. One laccase unit is defined as µmoles of product formed per minute per ml of culture supernatant.

Of the total 295 fungi screened qualitatively, 125 were positive (Fig. 1). Forty-seven of fifty-four basidiomycetes (87%) and 9 of 14 ascomycetes (64%), but only 69 of 227 mitosporic and non-sporulating fungi (30%) indicated laccase activity. White-rot basidiomycetes commonly produce laccase [3]. Guaiacol plate assay helps in identifying laccase-positive fungi, but is not specific, several other oxidases also producing a colour on guaiacol plates [6]. Therefore, fifty cultures producing the broadest bands of guaiacol oxidation were selected for further quantitative estimation.

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Fig. 1 Qualitative screening of fungi for laccase of cultures

Fungi differ in their optimal media for laccase. MEB, which is a useful medium for laccase production [7] was chosen as the medium for comparative purposes. Among the 50 cultures comprising 34 basidiomycetes, 1 ascomycete, 13 mitosporic and 2 unidentified fungi assayed, several isolates of *Lentinus*, as well as an isolate of *Ganoderma* and *Fomitopsis* produced >1500 Units l^{-1} laccase even under unoptimized MEB media conditions, emphasizing the importance of basidiomycetes in laccase production (Table 1). The first two genera are known to produce high amounts of laccase [8, 9]. Copper sulphate induced laccase in three of four fungi (Table 1). Although basidiomycetes are generally the candidates for laccase, a few mitosporic fungi are also good laccase producers [1]. Interestingly, the mitosporic fungus, *Geotrichum* sp., isolated from mushrooms produced high amounts of 1723 Units l^{-1} of laccase. *Geotrichum* is known to transform several azo- and anthraquinone dyes, presumably owing to laccase [10]. Although a few ascomycetes produce laccase, we found only a single ascomycete with moderate amounts of laccase (319 Units l^{-1} ; Table 2).

Twenty four of 50 fungi showed best laccase activity at pH 4.5 (Table 1). Of these, 19 were basidiomycetes and 5 were mitosporic fungi. Laccases of white-rot fungi are mostly optimal at pH 3.0 to 5.5 [11, 12]. Two mitosporic fungi *Dictyochaeta* sp., *Excipularia* sp. and an unidentified fungus showed an optimal pH solely at 3.0, as has also been reported in a marine isolate of the basidiomycete *Cerrena versicolor*, *Ganoderma lucidum* and *Phanerochaete flavidio-alba* [9, 13, 14]. Only the mitosporic species, *Beltraniella* sp. preferred pH 6.0. Four basidiomycetes, *Mycena* sp., *Hygrocybe* sp., *Ganoderma* sp. and *Fomitopsis* sp., besides an unidentified mitosporic fungus preferred pH 3.0 and 4.5. Three basidiomycetes, *Pleurotus* sp., *Russula* sp., and a polypore, an unidentified ascomycete, and the mitosporic fungus *Dictyochaeta* sp.

Table 1 Laccase activity at different pH

No.	Fungus	Substrate	Laccase Units l^{-1} at three different pH		
			3.0	4.5	6/8*
1	<i>Agaricus heterocystis</i>	Soil	72.34	122.4	20.6
2	<i>Agaricus</i> sp.	Soil	10.29	17.49	0*
3	<i>Agaricus</i> sp.	Soil	13.99	173.02	13.7
4	<i>Anthracophyllum nigratum</i>	Wood	6.34	ND	ND
5	<i>Auricularia</i> sp.	Wood	4.46	113.14	115.2
			4.9	143.31	90.5
6	<i>Chlorophyllum molybdites</i>	Soil	113.83	752.57	0*
7	<i>Clarkiella</i> sp.	Soil	25.44	179.31	80.2
8	<i>Coprinus</i> sp.	Soil	12.82	879.43	1.13*
9	<i>Fomitopsis</i> sp.	Wood	1464	1194	249
10	<i>Ganoderma</i> sp.	Wood	1848	1933	751
11	<i>Hygrocybe</i> sp.	Soil	63.09	62.57	12.1
12	<i>Lentinus bambussinus</i>	Soil	428.57	1498.3	325
13	<i>Lentinus</i> sp.	Soil	764.57	1968	2.06*
			574.8	989.14	269.66
14	<i>Lentinus</i> sp.	Wood	11.38	ND	ND
15	<i>Lentinus</i> sp.	Wood	90.77	438.86	144.7
			90.17	574.63	82.63
16	<i>Lentinus</i> sp.	Wood	318	1777.7	672
17	<i>Leucocoprinus</i> sp.	Wood	ND	ND	56.81
			36	94.87	37.37
18	<i>Leucocoprinus</i> sp.	Wood	18.86	80.06	18.7
19	<i>Macrolepiota</i> sp.	Soil	81.26	380.57	131.8
			54.34	ND	ND
20	<i>Marasmiellus</i> sp.	Wood	0	15.09	3.43*
21	<i>Marasmius</i> sp.	Wood	0	ND	ND
22	<i>Marasmius</i> sp.	Wood	3.09	33.6	17.1
23	<i>Microporus</i> sp.	Wood	0.34	0	0
24	<i>Mycena</i> sp.	Soil	24	18.86	0*
25	<i>Mycena</i> sp.	Wood	11.93	ND	ND
26	<i>Pleurotus</i> sp.	Wood	121.37	134.74	136.11
27	<i>Pleurotus</i> sp.	Wood	60	202.29	97.4
28	Polypore	Wood	4.17	0	5.04
29	Polypore	Wood	0	0	0*
30	Polypore	Wood	37.71	40.8	34.3
31	<i>Russula</i> sp.	Soil	8.38	9.09	9.6
32	Unidentified basidiomycete	Soil	6.5	ND	ND
33	Unidentified basidiomycete	Wood	0	0	0*
34	Unidentified basidiomycete	Soil	28.8	96	44.4
			33.94	157	56.9
35	<i>Beltraniella</i> sp.	Endophyte	26.79	28.11	51.4
36	<i>Bisporomyces</i> sp.	Litter	0.82	2.5	0

Table 1 continued

No.	Fungus	Substrate	Laccase Units l ⁻¹ at three different pH		
			3.0	4.5	6/8*
37	<i>Dictyochaeta</i> sp.	Litter	2.54	0.39	0
38	<i>Dictyochaeta</i> sp.	Litter	5.31	6.34	4.29
39	<i>Excipularia</i> sp.	Litter	9.27	0	0
40	<i>Geotrichum</i> sp.	Mushroom	1040.6	1722.9	1126
41	<i>Gyrophthrix</i> sp.	Litter	1.18	9.43	3.31
42	<i>Monodictys</i> sp.	Litter	11.67	61.87	1.87
43	<i>Robillarda</i> sp.	Endophyte	0	0	0*
44	<i>Selenodriella</i> sp.	Litter	4.71	14.86	4.3
45	Unidentified fungus	Litter	0	0	0
46	Unidentified fungus	Litter	0.75	0.27	0
47	Unidentified mitosporic fungus	Litter	30.39	27.77	17.5
48	Unidentified mitosporic fungus	Litter	0	0	0
49	Unidentified mitosporic fungus	Litter	137.83	192.34	164
50	Unidentified ascomycete	Litter	99.26	77.34	107

Highest activity for each fungus and values that fell within 75% of that are shown in bold and considered optimal pH for that fungus

Italicized rows cultures grown in MEB medium; 2 mM CuSO₄ added to culture on 7th day

Non italicized rows cultures grown in MEB medium in absence of CuSO₄

ND not determined

*Fungi assayed at pH 8.0

showed a broad pH range for laccase activity at all three pH. None of the nine fungi tested at pH 8.0 instead of 6.0 showed good activity at this pH (Table 1).

Twenty fungi from the 50 in Table 1, and also 5 others were selected based on high laccase activity and different pH preferences for further assays on temperature optima at 30, 45 and 60°C (Table 2). Six showed optimum activity at 45°C. Only the basidiomycete *Auricularia* sp. showed best activity at 30°C. Enzymes with room temperature optima are advantageous. A temperature optimum of 20°C has been reported for *Ganoderma lucidum* [9]. A preference for 60°C was rare, only *Pleurotus* sp., *Termitomyces* sp. and *Excipularia* sp. showing this (Table 2). D'Souza et al. [13] found such a high temperature optimum in a marine isolate of *Cerrena unicolor*. *Lentinus bambussimus* and a polypore showed high laccase activity at all three temperatures.

Wood-degrading basidiomycetes are generally the best laccase sources. This study suggests that fungi from other sources are equally useful, often yielding higher amounts of laccase than the former (Fig. 2; Tables 1, 2). Thus, the best laccase producers were two non-wood isolates,

Table 2 Laccase activity at different temperatures

No.	Isolates	30°C (U/l)	45°C (U/l)	60°C (U/l)	Substrate
1	<i>Agaricus</i> sp.	393	385	5	Soil
2	<i>Agaricus heterocystis</i>	1611	2400	2194	Soil
3	<i>Auricularia</i> sp.	80.57	22	37.7	Wood
4	<i>Chlorophyllum molybdites</i>	85.71	624	178	Wood
5	<i>Clarkiella</i> sp.	160	277	341	Soil
6	<i>Excipularia</i> sp.	9.89	15.89	27	Litter
7	<i>Ganoderma</i> sp.	494	778	668	Wood
8	<i>Geotrichum</i> sp.	3925	4892	2965	Mushroom
19	<i>Hygrocybe</i> sp.	98	226	217	Soil
10	<i>Lentinus bambussimus</i>	3918	5434	2909	Soil
11	<i>Lentinus</i> sp.	1275	1474	1251	Wood
12	<i>Lentinus</i> sp.	306	579	126	Wood
13	<i>Lentinus</i> sp.	685.7	377	771	Soil
14	<i>Lentinus</i> sp.	150	996	1119	Wood
15	<i>Macrolepiota</i> sp.	341	720	866	Soil
16	<i>Monodictys</i> sp.	52.46	65.31	80.9	Litter
17	<i>Pleurotus</i> sp.	113	156	93	Wood
18	<i>Pleurotus</i> sp.	0.12	0.29	0.49	Wood
19	<i>Schizophyllum</i> sp.	3.21	4.29	5.26	Wood
20	<i>Selenodriella</i> sp.	38.22	39.25	17.6	Litter
21	<i>Termitomyces</i> sp.	428	514	798.8	Wood
22	Polypore	3497	4261.7	4479	Wood
23	Unidentified ascomycete	123.9	318.86	43.37	Litter
24	Unidentified	12	21.6	10	Litter
25	Unidentified	18.69	28.6	15.43	Litter

Highest activity for each fungus and values that fell within 75% of that are shown in bold and considered optimal temperature for that fungus

Lentinus bambussimus and *Geotrichum* sp., which produced 5434 and 4892 Units l⁻¹ respectively (Table 2).

This study shows the usefulness of screening diverse fungi to obtain laccases of varying pH and temperature

Fig. 2 Laccase activity of fungi isolated from soil and wood

optima. It yielded a high laccase-producing mitosporic fungus, *Geotrichum* sp., with a pH optimum of 4.5 and temperature optimum of 30 and 45°C (Table 2). Fungi with laccases of different pH and temperature optima could be the basis of further studies, leading to specific applications.

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