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Effect of Copper and Manganese Ions on Activities of Laccase and Peroxidases in Three *Pleurotus* Species Grown on Agricultural Wastes

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Abstract

Copper (Cu^{2+}) and manganese (Mn^{2+}) ions influenced laccase (Lac) and peroxidase production in *Pleurotus eryngii*, *Pleurotus ostreatus*, and *Pleurotus pulmonarius*. In *P. eryngii*, the optimum Cu^{2+} concentration for Lac production was 1 m*M* and for peroxidases 10 m*M*, and Mn^{2+} concentration of 5 m*M* led to peaks of Lac and peroxidase activity. In *P. ostreatus* HAI 493, the highest level of Lac activity was at Cu^{2+} concentrations of 1 and 10 m*M* and Mn^{2+} concentration of 1 m*M*, respectively. The absence of Cu^{2+} and Mn^{2+} caused the highest levels of peroxidase production. In *P. ostreatus* HAI 494, the highest level of Lac activity was at a Cu^{2+} concentration of 5 m*M* and at Mn^{2+} concentration of 1 m*M*, respectively. High levels of peroxidase activity were found in the medium without and with 1 m*M* Cu^{2+}, and at 1 and 5 m*M* Mn^{2+}, respectively. In *P. pulmonarius*, the highest Lac activity was found in the presence of 5 m*M* Cu^{2+} and 5 m*M* Mn^{2+}, respectively. The absence of Cu^{2+} and Mn^{2+} as well as their presence at a concentration of 1 m*M* led to the peaks of peroxidase activities.

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Index Entries: *Pleurotus eryngii*; *Pleurotus ostreatus*; *Pleurotus pulmonarius*; laccase; peroxidases; copper; manganese.

Introduction

The *Pleurotus* species belong to the group of white-rot fungi and their ligninolytic system is composed of laccase (Lac), Mn-dependent peroxidase, versatile peroxidase, and aryl-alcohol oxidase *(1)*.

Metal ions comprise an important group of ligninolytic enzyme activity modulators and are present in the environment either naturally (Cu, Mn) or as a result of human activities (Cd, Hg, Pb) *(2,3)*. Cu^{2+} regulates the transcription of Lac gene *(4,5)* and also positively affects the activity and stability of this enzyme *(3)*. Mn^{2+} is both an active mediator for manganese peroxidase (MnP) and a regulator of MnP and Lac production *(6)*. It was shown that MnP production by different *Pleurotus* species, which were grown under conditions of submerged fermentation *(7)* as well as solid-state fermentation *(8)*, depended on the concentration of Mn^{2+}.

Grapevine sawdust and mandarine peels represent very common agricultural wastes in some regions, and they are prospective substrates for bioconversion into fungal biomass and lignocellulytic enzymes. Grapevine sawdust contains significant concentrations of metal ions (13 ppm Cu, 107 ppm Mn, 30 ppm Fe, and 73 ppm Zn) *(9)*, and their proportion is lower in mandarine fresh fruit (0.6 g/t of Cu, 0.4 g/t of Mn, 2.6 g/t of Fe, and 0.8 g/t of Zn) *(10)*.

The purpose of the present investigation was to study the effect of Cu^{2+} and Mn^{2+} ions on the production of Lac and peroxidases in *Pleurotus eryngii*, *Pleurotus ostreatus*, and *Pleurotus pulmonarius* grown under optimum conditions (submerged fermentation and solid-state fermentation, respectively, and the best carbon and nitrogen sources and concentrations).

Materials and Methods

Organisms and Growth Conditions

Table 1 provides the investigated *Pleurotus* species and strains and their origin. The four selected *Pleurotus* strains were the best producers of Lac and peroxidases among 32 previously investigated strains of 11 *Pleurotus* species *(11)*. The cultures are preserved on wort agar medium *(11)*, in the culture collection of the Institute of Evolution, University of Haifa, Israel (HAI), and documented in the *Catalogue of Cultures (12)*.

The inocula were prepared by growing fungi at room temperature (22 ± 2°C) on a rotary shaker at 180 rpm in 250-mL flasks containing 100 mL of synthetic medium *(11)*. After 7 d of cultivation, mycelial pellets were harvested and homogenized using a laboratory homogenizer at 10,000 rpm.

Table 1
Investigated Species and Strains of Genus *Pleurotus*

Species	HAI no. of strain	Origin of strain
P. eryngii (DC.:Fr.) Quél. var. *eryngii*	616	Israel, Tabor, on *Ferula* sp.
P. ostreatus (Jacq.:Fr.) Kumm.	493	United States, Hawaii, Nextlab
	494	United States, Hawaii, Nextlab
P. pulmonarius (Fr.) Quél.	572	Czech Republic, KW, A. S. Buchalo (194), -CCBAS, M. Semerdzieva (478)

Submerged Fermentation of Dry Ground Mandarine Peels

P. eryngii HAI 616 was a good Lac producer under conditions of submerged cultivation in the presence of dry ground mandarine peels as the carbon source and $(NH_4)_2SO_4$ as the nitrogen source at a nitrogen concentration of 20 m*M (13)*. Submerged fermentation was carried out at room temperature (22 ± 2°C) on a rotary shaker at 180 rpm in 250-mL flasks containing 2.0 g of dry ground mandarine peels and 50 mL of the modified synthetic medium (with $[NH_4]_2SO_4$ in a nitrogen concentration of 20 m*M*) enriched with 0.06 g/L of $CaCl_2 \cdot 2H_2O$, 0.002 g/L of $ZnSO_4 \cdot 7H_2O$, 0.005 g/L of $FeSO_4 \cdot 7H_2O$ and the following concentrations of Cu^{2+} and Mn^{2+}:

1. 0.05 g/L of $MnSO_4 \cdot H_2O$, $CuSO_4 \cdot 7H_2O$ in Cu^{2+} concentrations of 0, 1, 5, and 10 m*M*.
2. 0.02 g/L of $CuSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$ in Mn^{2+} concentrations of 0, 1, 3, and 5 m*M*.

The initial pH of the medium was adjusted to 9.4 prior to sterilization by adding 10% NaOH, so that the pH would be 6.0 after sterilization. Three replications for each Cu^{2+} and Mn^{2+} concentration were performed. Homogenized inocula of 5 mL were used per flask. The biomasses were separated by centrifugation (4°C, 5000 rpm, 20 min) after 5 and 7 d of cultivation, which is known as the optimum period of cultivation for enzyme production *(14)*. Clean supernatants were used to estimate enzyme activity.

Solid-State Fermentation of Grapevine Sawdust

High levels of Lac and peroxidase production in the three other investigated *Pleurotus* strains (Table 1) occurred under conditions of solid-state cultivation in the presence of grapevine sawdust as the carbon source and $(NH_4)_2SO_4$ in a nitrogen concentration of 40 m*M* (in HAI 493), peptone in a concentration of 0.5% (in HAI 494), and NH_4NO_3 in a nitrogen concentration of 30 m*M* (in HAI 572) as the optimum nitrogen sources and concentrations *(13)*. Solid-state fermentation was carried out at 25°C in 100-mL flasks containing 4.0 g of grapevine sawdust and 12 mL of the modified

synthetic medium (with the best nitrogen source and concentration) enriched with the same concentrations of metal ions as in submerged fermentation.

The initial pH of the synthetic medium was adjusted to 6.0 prior to sterilization, and three replications for each Cu^{2+} and Mn^{2+} concentration were used. Homogenized inocula of 3 mL were used per flask. The extracellular enzymes were extracted on d 7 and 10 of cultivation *(15)* by samples grinding in a mortar with 20 mL of distilled water for 5 min on ice. This procedure was repeated three times and obtained extracts were mixed (total volume of 60 mL). Solids were separated by centrifugation (4°C, 5000 rpm, 10 min), and supernatants were used for measurements of Lac and peroxidase activities.

Enzyme Activity Assays

Lac and peroxidase activities were determined spectrophotometrically. Lac activity was assayed using syringaldazine as a substrate (ε_{525} = 65,000 $M^{-1}cm^{-1}$) *(11)*. Peroxidase activities were determined with 3 m*M* phenol red as the substrate (ε_{610} = 22,000 $M^{-1}cm^{-1}$) *(13)*.

One unit of enzyme activity was defined as the amount of enzyme that transformed 1 µmol of substrate/min. A UV-160A Spectrophotometer (Shimaden) was used for these assays.

Results

Effect of Cu^{2+} on Laccase and Peroxidase Production

P. eryngii, *P. ostreatus*, and *P. pulmonarius* produced Lac and peroxidases in media with all of the investigated Cu^{2+} concentrations (*see* Tables 2 and 3). In *P. eryngii* the highest level of Lac activity was at 1 m*M* Cu^{2+}, where the activity was approximately four times higher than in the control (300.12 and 77.73 U/L, respectively, after 7 d of cultivation) (Table 2). However, with a further increase in Cu^{2+} concentration (5 and 10 m*M*), a significant decrease in Lac activity (0.78 and 1.66 U/L, respectively) was noticed. The addition of Cu^{2+} led to an increase in the level of phenol red oxidation in both the presence and absence of external Mn^{2+}, and peaks were recorded at a Cu^{2+} concentration of 10 m*M* (28.15 and 29.92 U/L, respectively, after 5 d of cultivation) (Table 2).

In *P. ostreatus* HAI 493, a Cu^{2+} concentration of 10 m*M* caused a peak in Lac activity (494.00 U/L, on d 7 of cultivation). High levels of activity against phenol red in both the presence and absence of external Mn^{2+} were found in the control medium (35.14 and 14.04 U/L, respectively, after 10 d of cultivation), whereas these activities were significantly lower at other investigated Cu^{2+} concentrations (Table 3).

In *P. ostreatus* HAI 494, Lac activity showed the highest level at a Cu^{2+} concentration of 5 m*M* (432.20 U/L, on d 10 of cultivation), while the highest levels of phenol red oxidation in the presence and absence of external

Table 2
Lac Activity and Activity Against Phenol Red in Presence and Absence of External Mn^{2+} in *P. eryngii* Under Submerged Fermentation Conditions of Dry Ground Mandarine Peels Depending on Concentration of Added Cu^{2+} and Mn^{2+} in Medium

Species	Strain (HAI)	Concentration of added Cu^{2+} and Mn^{2+} in medium	Period of cultivation (d)	Lac activity (U/L)	Activity against phenol red (U/L)	
					$+Mn^{2+}$	$-Mn^{2+}$
P. eryngii var. *eryngii*	616	Control (–Cu)	5	6.27 ± 1.70	11.02 ± 0.30	12.61 ± 0.31
			7	77.73 ± 2.54	6.17 ± 0.24	7.91 ± 0.26
		1 m*M* Cu	5	0.26 ± 0.08	16.77 ± 0.61	18.39 ± 0.66
			7	300.12 ± 12.08	8.35 ± 0.22	10.26 ± 0.14
		5 m*M* Cu	5	1.03 ± 0.45	24.55 ± 0.37	26.17 ± 0.05
			7	0.78 ± 0.01	22.11 ± 0.97	23.85 ± 0.91
		10 m*M* Cu	5	0.43 ± 0.04	28.15 ± 0.12	29.92 ± 0.38
			7	1.66 ± 0.19	26.61 ± 0.45	28.50 ± 0.21
		Control (–Mn)	5	65.70 ± 3.47	7.85 ± 0.30	10.03 ± 0.42
			7	144.72 ± 3.13	11.21 ± 1.75	10.09 ± 0.70
		1 m*M* Mn	5	77.45 ± 2.34	7.36 ± 0.39	9.77 ± 0.23
			7	100.52 ± 18.76	4.88 ± 0.16	6.70 ± 0.19
		3 m*M* Mn	5	124.71 ± 10.71	7.20 ± 0.16	10.58 ± 0.60
			7	154.43 ± 12.28	4.91 ± 0.14	7.03 ± 0.24
		5 m*M* Mn	5	261.22 ± 0.34	14.50 ± 1.15	20.16 ± 1.09
			7	322.62 ± 5.40	4.85 ± 0.18	7.10 ± 0.34

Table 3
Lac Activity and Activity Against Phenol Red in Presence and Absence of External Mn^{2+} in *P. ostreatus* and *P. pulmonarius* Under Solid-State Fermentation Conditions of Grapevine Sawdust Depending on Concentration of Added Cu^{2+} and Mn^{2+} in Medium

Species	Strain (HAI)	Concentration of added Cu^{2+} and Mn^{2+} in medium	Period of cultivation (d)	Lac activity (U/L)	Activity against phenol red (U/L)	
					$+Mn^{2+}$	$-Mn^{2+}$
P. ostreatus	493	Control (–Cu)	7	255.46 ± 31.83	2.23 ± 0.05	1.43 ± 0.05
			10	362.79 ± 1.72	35.14 ± 4.85	14.04 ± 2.06
		1 m*M* Cu	7	411.57 ± 2.31	1.63 ± 0.04	1.45 ± 0.07
			10	460.46 ± 137.35	8.26 ± 0.45	7.37 ± 1.04
		5 m*M* Cu	7	255.03 ± 5.07	1.94 ± 0.11	2.64 ± 0.15
			10	346.42 ± 20.00	8.52 ± 0.28	8.42 ± 0.56
		10 m*M* Cu	7	494.00 ± 56.09	1.47 ± 0.04	2.16 ± 0.04
			10	261.19 ± 13.08	7.99 ± 0.71	6.86 ± 0.33
		Control (–Mn)	7	283.70 ± 34.14	2.93 ± 0.21	1.50 ± 0.13
			10	213.41 ± 127.93	38.28 ± 2.30	13.30 ± 1.02
		1 m*M* Mn	7	324.41 ± 5.91	2.43 ± 0.21	1.32 ± 0.04
			10	113.40 ± 5.64	15.83 ± 1.02	9.11 ± 1.10
		3 m*M* Mn	7	125.71 ± 3.83	1.89 ± 0.14	1.48 ± 0.07
			10	261.39 ± 31.86	8.68 ± 1.13	6.70 ± 0.54
		5 m*M* Mn	7	253.77 ± 4.16	1.94 ± 0.06	1.48 ± 0.06
			10	59.90 ± 16.09	8.07 ± 0.16	5.93 ± 0.54
P. ostreatus	494	Control (–Cu)	7	265.19 ± 9.06	74.12 ± 4.42	61.87 ± 7.49
			10	191.65 ± 4.58	48.98 ± 3.16	58.64 ± 6.31
		1 m*M* Cu	7	201.08 ± 6.28	67.55 ± 4.62	29.63 ± 0.58
			10	134.11 ± 7.48	47.56 ± 6.08	80.00 ± 2.17
		5 m*M* Cu	7	230.54 ± 24.30	50.94 ± 6.28	43.54 ± 4.81
			10	432.20 ± 1.51	26.32 ± 2.87	51.55 ± 2.06

		10 m*M* Cu	7	139.77 ± 11.99	22.42 ± 2.39	26.29 ± 2.78
			10	232.82 ± 37.97	47.73 ± 5.75	40.74 ± 1.81
		Control (–Mn)	7	211.10 ± 30.13	79.32 ± 13.51	39.42 ± 7.58
			10	214.37 ± 39.57	46.42 ± 2.46	64.20 ± 5.85
		1 m*M* Mn	7	325.00 ± 2.51	58.30 ± 1.70	73.64 ± 7.67
			10	303.59 ± 6.26	87.77 ± 3.31	98.98 ± 4.83
		3 m*M* Mn	7	237.68 ± 3.93	79.72 ± 3.54	70.68 ± 1.61
			10	240.48 ± 62.14	76.08 ± 2.27	92.56 ± 3.76
		5 m*M* Mn	7	273.25 ± 17.00	74.85 ± 2.04	81.48 ± 1.39
			10	208.59 ± 13.94	67.28 ± 4.92	120.74 ± 1.81
P. pulmonarius	572	Control (–Cu)	7	308.37 ± 9.36	14.09 ± 0.31	58.22 ± 3.06
			10	216.49 ± 11.66	46.73 ± 0.07	58.91 ± 0.96
		1 m*M* Cu	7	355.21 ± 34.56	13.79 ± 1.12	65.25 ± 2.22
			10	209.13 ± 1.30	45.00 ± 1.11	25.46 ± 4.95
		5 m*M* Cu	7	413.19 ± 6.72	9.83 ± 0.79	13.35 ± 1.34
			10	147.92 ± 28.54	15.64 ± 0.38	23.69 ± 1.86
		10 m*M* Cu	7	358.54 ± 6.00	3.71 ± 0.40	8.66 ± 0.40
			10	287.32 ± 61.03	27.33 ± 6.64	14.39 ± 1.03
		Control (–Mn)	7	308.90 ± 8.97	17.41 ± 1.66	37.28 ± 1.25
			10	199.57 ± 13.63	62.87 ± 9.54	9.25 ± 1.00
		1 m*M* Mn	7	295.62 ± 8.90	17.14 ± 0.62	67.33 ± 1.01
			10	87.10 ± 14.47	38.14 ± 2.86	59.12 ± 6.67
		3 m*M* Mn	7	309.60 ± 20.04	17.10 ± 1.19	58.49 ± 5.77
			10	185.18 ± 5.45	17.37 ± 1.93	45.15 ± 5.14
		5 m*M* Mn	7	345.59 ± 19.00	20.06 ± 0.91	61.91 ± 0.53
			10	229.95 ± 18.71	58.45 ± 1.63	54.62 ± 4.11

Mn^{2+} were obtained in the medium without and with 1 m*M* Cu^{2+}, respectively (Table 3). The same Cu^{2+} effect on Lac and peroxidase production was obtained in *P. pulmonarius*.

Effect of Mn^{2+} on Laccase and Peroxidase Production

In *P. eryngii*, the addition of Mn^{2+} to the medium led to an increase in Lac activity in comparison to the control, and the highest level of Lac was at Mn^{2+} concentration of 5 m*M* (322.62 U/L, after 7 d of cultivation) (Table 2). The same Mn^{2+} concentration caused the highest Lac activity in *P. pulmonarius* (345.59 U/L, after 7 d of cultivation), whereas in both strains of *P. ostreatus*, the maximum increase in Lac activity was at Mn^{2+} concentration of 1 m*M* (324.41 U/L in HAI 493 and 325.00 U/L in HAI 494, after 7 d of cultivation) (Table 3).

In *P. eryngii*, levels of phenol red oxidation in the presence as well as in the absence of external Mn^{2+} were the highest at an Mn^{2+} concentration of 5 m*M* (14.50 and 20.16 U/L, respectively), whereas at Mn^{2+} concentrations of 1 and 3 m*M*, they were significantly lower than in the control on d 7 of cultivation (Table 2). *P. ostreatus* HAI 493 had peaks of these activities in the medium without Mn^{2+}, while they decreased with an increase of Mn^{2+} concentration (Table 3). However, in *P. ostreatus* HAI 494, Mn^{2+} concentrations of 1 and 5 m*M*, respectively, caused the maximum level of phenol red oxidation in the presence and absence of external Mn^{2+} (87.77 and 120.74 U/L, respectively, after 10 d of cultivation). In *P. pulmonarius*, peaks of these activities were obtained in the medium without and with 1 m*M* Mn^{2+}, respectively (Table 3).

Discussion

Copper is a metal present in the environment that has an important role in enzyme synthesis and stability. Palmieri et al. *(4)* demonstrated in *P. ostreatus* grown under submerged fermentation conditions that the increase in Lac activity is proportional to the added Cu^{2+} concentration and that the maximal effect is obtained at a $CuSO_4$ concentration of 150 µ*M*. In *P. eryngii* cultivated under submerged fermentation conditions of dry ground mandarine peels, the highest level of Lac activity was at a Cu^{2+} concentration of 0.07 m*M (11)*. Likewise, in one strain of *P. ostreatus* grown under solid-state fermentation conditions of wheat straw, the addition of Cu^{2+} at a concentration of 1 m*M* led to the highest increase in Lac activity (eight times higher than in the control flasks), whereas at Cu^{2+} concentrations that were lower or higher than this value, the increase was not as high *(3)*. The positive effect of Cu^{2+} on Lac synthesis can be explained by the results of Palmieri et al. *(16)*, who showed that Cu^{2+} at a concentration of 1 m*M* decreased the activity of extracellular protease (to 77%) that is responsible for Lac degradation.

In the present study, obtained values for Lac activity in selected *P. ostreatus* and *P. pulmonarius* strains under solid-state fermentation con-

ditions were significant in the presence of high Cu^{2+} concentrations, which is not in accordance with the aforementioned results. However, in *P. eryngii*, an increase in Cu^{2+} concentration in the medium led to a decrease in Lac production, especially at concentrations of 5 and 10 m*M*, when it was in trace (Table 2).

According to Wariishi et al. *(2)*, MnP requires the presence of Mn^{2+} in order to complete its catalytic cycle efficiently, and this metal is naturally present in wood. Camarero et al. *(8)* showed that in *P. pulmonarius* grown under solid-state fermentation conditions of wheat straw, the addition of Mn^{2+} significantly stimulated lignin mineralization. They emphasized that nonenzymatic oxidation of Mn^{2+} cannot be completely excluded from wheat lignin degradation, but MnP had the main role in that process.

Cohen et al. *(17)* found in *P. ostreatus* five peaks of versatile peroxidase and only one peak of MnP in Mn^{2+} unamended peptone medium under solid-state fermentation conditions, whereas in an amended peptone medium with 500 µ*M* Mn^{2+}, MnP activity significantly increased, the activity of five versatile peroxidase peaks significantly decreased, and one new versatile peroxidase peak with very low activity appeared.

The results obtained in the present study differ from the aforementioned results. Considering the significant Mn^{2+} concentration in grapevine sawdust *(9)*, and the fact that only trace Mn^{2+} concentrations could be enough for MnP action, without any external Mn^{2+} addition to the mixture for estimation of peroxidases activities, it cannot be spoken about MnP and versatile peroxidase activities, but only about phenol red oxidation levels in the presence and absence of external Mn^{2+}, respectively. However, high peaks of activity against phenol red in the absence of external Mn^{2+} in *P. ostreatus* and *P. pulmonarius* after purification of the crude enzyme mixture *(13)* showed that grapevine sawdust Mn^{2+} content inhibited MnP activity.

In a study by Martínez et al. *(7)*, in *P. eryngii* grown in a glucose/peptone/yeast extract medium under submerged fermentation conditions, the highest level of MnP activity was obtained in the medium without Mn^{2+}, whereas Mn^{2+} concentration of 5 µ*M* produced a significant decrease (approx 90%) in MnP activity and no activity was found at Mn^{2+} concentration of 25 µ*M*. After purification, two peaks of peroxidase activity (MP-1 and MP-2) that possess high Mn-independent activity were found. In the present study, for a selected *P. eryngii* strain cultivated under submerged fermentation conditions of dry ground mandarine peels, peroxidase activities were the highest at Mn^{2+} concentration of 5 m*M*. Stajić et al. *(13)* obtained only one peak of versatile peroxidase activity by purification of a crude enzyme mixture of this strain.

Selected *Pleurotus* species and strains showed the best Lac production under both submerged fermentation and solid-state fermentation conditions at high Mn^{2+} concentrations, which is not in accordance with the results of a previous study of *Trametes versicolor (5)*, in which an Mn^{2+} concentration of 1 m*M* had a positive effect on Lac production.

Our results show that Cu^{2+} and Mn^{2+} ions have different effects on Lac and peroxidase production in selected *Pleurotus* species. The effect depends on the *Pleurotus* species and strain, cultivation conditions (submerged or solid-state fermentation), as well as concentration of the metal ions.

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