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Laccases from *Aureobasidium pullulans*☆

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ABSTRACT

Laccases are polyphenol oxidases (EC 1.10.3.2) that have numerous industrial and bioremediation applications. Laccases are well known as lignin-degrading enzymes, but these enzymes can play numerous other roles in fungi. In this study, 41 strains of the fungus *Aureobasidium pullulans* were examined for laccase production. Enzymes from *A. pullulans* were distinct from those from lignin-degrading fungi and associated with pigment production. Laccases from strains in phylogenetic clade 5, which produced a dark vinaceous pigment, exhibited a temperature optimum of 50–60 °C and were stable for an hour at 50 °C, unlike enzymes from the lignin-degrading fungi *Trametes versicolor* and *Pycnoporus cinnabarinus*. Laccase purified from *A. pullulans* strain NRRL 50381, a representative of clade 5, was glycosylated but had a molecular weight of 60–70 kDa after Endo H treatment. Laccase purified from strain NRRL Y-2568, which produced a dark olivaceous pigment, was also glycosylated, but had a molecular weight of greater than 100 kDa after Endo H treatment.

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1. Introduction

Laccases are polyphenol oxidases (EC 1.10.3.2) containing four copper atoms in their active sites, and are the largest subclass of blue multicopper oxidases [1]. Laccases are well known as a component of fungal enzyme systems for lignin degradation. However, these enzymes can play numerous other roles in fungi, such as in host-pathogen interactions, sporulation, and morphogenesis [2]. Laccases have broad substrate specificities, which can be further extended through the use of mediator systems [3,4]. Potential industrial applications include the degradation of textile dyes and toxic materials [5–9]. Laccases have been well studied in white-rot fungi. However, these enzymes are widely distributed in nature, and studies have sought to identify new microbial sources of laccase with novel properties [10,11].

Aureobasidium pullulans is considered to be a filamentous ascomycete in class Dothideomycetes, subclass Dothideomycetidae [12,13]. *A. pullulans* is well known as the source of the commercial polysaccharide, pullulan [14]. It can also produce degradative enzymes, including xylanase [15,16]. *A. pullulans* is sometimes associated with the deterioration of painted wood [17,18], and certain strains can grow on lignin-related aromatic compounds [19–21]. However, little has been reported on laccase production by *A. pullulans* [22,23]. In the current study, 41 strains of *A. pullulans* were examined for their capacity to produce laccase.

2. Methods

2.1. Organisms and growth conditions

A. pullulans strains used in this study were obtained from the ARS Culture Collection, Peoria, IL (NRRL strains). Control strains of the lignin-degrading fungi *Trametes versicolor* (strain ATCC 11235) and *Pycnoporus cinnabarinus* (ATCC 200478) were purchased from the American Type Culture Collection. Strains were cultured on potato dextrose agar plates at 28 °C for 2 days. Preinocula were grown in 30 mL of malt extract broth in 300 mL flasks, incubated at 28 °C, 130 rpm (2 in displacement) for 3 days. Preinocula of *T. versicolor* and *P. cinnabarinus* were composed of mycelia clumps and required homogenization for 30 s with a PowerGen 700 homogenizer (Fisher Scientific). Preinocula were used to inoculate laccase induction cultures at 5% (v/v). Laccase induction medium contained the following per liter: 20 g glucose, 2.5 g L-asparagine, 0.05 mg thiamine–HCl, 1 g KH_2PO_4, 0.1 g $Na_2HPO_4{\cdot}2H_2O$, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.01 g $CaCl_2$, 0.01 g $FeSO_4{\cdot}7H_2O$, 0.001 g $MnSO_4{\cdot}4H_2O$, 0.001 g $ZnSO_4{\cdot}7H_2O$, and 0.002 g $CuSO_4{\cdot}5H_2O$ [24]. Laccase induction cultures were 25 mL, grown in triplicate 125 mL flasks at 30 °C, 130 rpm. After two days, cultures were induced by the addition of 2,5-xylidine to a final concentration of 200 μM [24]. After an additional day, cultures were assayed for laccase activity. Time course studies showed that one day after induction produced maximal activities (data not shown).

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2.2. Laccase activity assay

One mL samples taken from induced cultures were clarified by centrifugation for 5 min at 15,871 × *g* and assayed for laccase activity by measurement of the enzymatic oxidation of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, Fluka, Switzerland). Reaction kinetics were followed at 420 nm for 5 min using a Molecular Devices SpectraMax M5 plate reader. Reactions contained 30 μL culture supernatant, 60 μL McIlvaine buffer (pH 4.4), and 10 μL ABTS (13 mM) in McIlvaine buffer (pH 4.4) at 30 °C. At pH 4.4, 100 mL of McIlvaine buffer contains 44.1 mL of 0.2 M Na_2HPO4 and 55.9 mL of 0.1 M citric acid [25]. Enzyme activity was expressed in units/mL (1 U = 1 μmol product formed/min) and as specific activity (U/mg protein). Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA), based on the Bradford dye-binding method [26]. To confirm that laccase activity was enzymatic, duplicate samples were boiled for 15 min to serve as negative controls.

2.3. Polyacrylamide gel electrophoresis and zymogram analysis

Samples were denatured by boiling for 5 min at 95 °C in 2× SDS sample buffer (4.0% (w/v) SDS, 20% (v/v) glycerol, 0.005% (w/v) bromophenol blue, 0.126 M Tris–HCl pH 6.8 and 5.0% (v/v) β-mercaptoethanol) and applied to an SDS-PAGE gel (5.0% stacking, 10% resolving). After electrophoresis at 100 V for approximately 1 h, the gel was stained with Coomassie Brilliant Blue. Molecular masses of bands were estimated using the Bio-Rad Precision Plus All Blue Protein Standards (Bio-Rad, #161-0373) and Kodak 1D analysis software. SDS-PAGE gels of purified laccases were stained with SYPRO Ruby protein gel stain (Invitrogen, Grand Island, NY) and used Bio-Rad Precision Plus Unstained Protein Standards (Bio-Rad, #161-0363).

Non-denaturing polyacrylamide gel electrophoresis was performed under similar conditions, except that SDS was omitted, β-mercaptoethanol was excluded from the sample buffer, and samples were not boiled. Zymograms were prepared from duplicate lanes of non-denaturing gels by staining with 2 mM ABTS in McIlvaine buffer (pH 4.4) for 30 min on a rocker shaker at room temperature [27].

2.4. Enzyme purification by medium pressure liquid chromatography

Enzyme samples were purified using a medium pressure liquid chromatography system (Biologic Duoflow, Bio-Rad, Hercules, CA). Cell-free culture supernatants were applied to a Superose 6 10/300 GL gel filtration column (GE Healthcare, Piscataway, NJ) equilibrated with McIlvaine buffer, pH 5.0. Alternatively, samples were applied to a 5.0 mL High Q anion exchange column (Bio-Rad) equilibrated in 20 mM sodium phosphate buffer, pH 6.5, and bound protein was eluted with an increasing gradient of 0.0–1.0 M NaCl in the same buffer.

Purified laccases were treated with endoglycosidase H (Endo H, New England Biolabs, Ipswich, MA) according to manufacturer's instructions.

3. Results and discussion

3.1. Strains of *A. pullulans* used in this study

In a preliminary study, laccase production was observed in certain *A. pullulans* strains that also produce a dark vinaceous pigment [23]. In some fungi, laccase production appears to be associated with pigment formation rather than lignin degradation [2]. For the current study, 41 strains of *A. pullulans* were chosen based on their capacity to form pigment (Table 1). We recently completed a multilocus molecular phylogeny of *A. pullulans* [28]. Interestingly, certain phenotypic traits, including pigment production, were

Table 1
Laccase production by strains of *Aureobasidium pullulans*.

Clade[a]	Strain number	Equivalent number	Maximal laccase (mU/mL)	Specific activity (U/mg protein)	Color of culture[b]
1	NRRL 58530	CU 17	<0.01	n/a	Cream
	NRRL 58533	CU 20	<0.01	n/a	Cream
	NRRL 58537	CU 24	0.5 ± 0.3	0.04 ± 0.03	Cream
	NRRL 58555	CU 44	<0.01	n/a	Cream
	NRRL 58556	CU 45	<0.01	n/a	Cream
5	NRRL 58519	CU 6	18 ± 8.6	1.2 ± 0.2	Vinaceous
	NRRL 58532	CU 19	20 ± 3.4	1.6 ± 0.5	Vinaceous
	NRRL 58548	CU 36	39 ± 1.5	3.6 ± 0.2	Vinaceous
	NRRL 50381	RSU 12	43 ± 1.3	3.5 ± 0.2	Vinaceous
6	NRRL 58546	CU 33	<0.01	n/a	Cream
	NRRL 58549	CU 37	<0.01	n/a	Cream
8	NRRL 58523	CU 10	6.5 ± 1.7	0.6 ± 0.1	Gray
	NRRL 58524	CU 11	4.6 ± 0.1	0.4 ± 0.1	Gray
	NRRL 58526	CU 13	<0.01	n/a	Cream
	NRRL 58536	CU 23	<0.01	n/a	Cream
	NRRL 58550	CU 38	<0.01	n/a	Cream
	NRRL 58552	CU 40	<0.01	n/a	Cream
	NRRL Y-2311		<0.01	n/a	Cream
	NRRL Y-2311-1	ATCC 62921	<0.01	n/a	Cream
	NRRL Y-6754a		<0.01	n/a	Cream
	NRRL Y-12971	ATCC 62922	<0.01	n/a	Cream
	NRRL Y-12972		<0.01	n/a	Cream
	NRRL YB-4026		<0.01	n/a	Cream
	NRRL YB-4588		<0.01	n/a	Cream
9	NRRL 58515	CU 2	0.1 ± 0.2	0.0 ± 0.01	Cream
	NRRL 58517	CU 4	1.0 ± 0.2	0.07 ± 0.01	Cream
	NRRL 58518	CU 5	<0.01	n/a	Cream
	NRRL 58520	CU 7	0.3 ± 0.0	0.03 ± 0.0	Cream
	NRRL 58535	CU 22	<0.01	n/a	Cream
	NRRL 58553	CU 41	0.1 ± 0.2	0.01 ± 0.01	Cream
10	NRRL Y-12973		<0.01	n/a	Cream
	NRRL Y-12974		2.0 ± 0.5	0.2 ± 0.1	Cream
11	NRRL 58525	CU 12	<0.01	n/a	Cream
	NRRL 58527	CU 14	<0.01	n/a	Cream
	NRRL 58528	CU 15	<0.01	n/a	Cream
	NRRL 58529	CU 16	<0.01	n/a	Cream
	NRRL 58538	CU 25	<0.01	n/a	Cream
13	NRRL 58012	CBS 584.75	<0.01	n/a	Cream
14	NRRL 62033	RSU 11	1.5 ± 0.2	0.1 ± 0.02	Cream
	NRRL 62036	RSU 15	<0.01	n/a	Cream
ND[c]	NRRL Y-2568		110 ± 3.2	4.4 ± 0.1	Dark olivaceous

[a] Phylogenetic clade according to the system of Manitchotpisit et al. [28].
[b] Grown on laccase induction medium.
[c] Not determined.