Laccase production by diverse phylogenetic clades of *Aureobasidium pullulans*

Joseph O. Rich[1], Pennapa Manitchotpisit[2], Stephen W. Peterson[3], and Timothy D. Leathers[1]*

[1]Renewable Product Technology Research Unit, National Center for Agricultural Utilization Research, ARS, USDA, Peoria, IL 61604, USA
E-mail: Joseph.Rich@ars.usda.gov; Tim.Leathers@ars.usda.gov
Phone: +1 309-681-6377; Fax: +1 309-681-6040
[2]Biochemistry Unit, Department of Medical Sciences, Faculty of Science, Rangsit University, Patumthani 12000, Thailand
E-mail: pennapam@hotmail.com
[3]Baterial Foodborne Pathogens and Mycology Research Unit, National Center for Agricultural Utilization Research, ARS, USDA, Peoria, IL 61604, USA
E-mail: Stephen.Peterson@ars.usda.gov

*Corresponding author

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Abstract

Laccases (EC 1.10.3.2) have numerous potential industrial applications including the degradation of dyes and toxic materials. Novel sources of this enzyme would be desirable to improve activity yields and substrate specificities. In this study we tested 51 strains of *Aureobasidium pullulans* representing 13 diverse phylogenetic clades for laccase production. Most strains grew on three different lignin-related substrates as sole carbon sources. Thirteen strains that grew well on these substrates, representing five clades, were chosen for a test of laccase production in an induction assay. Four representative strains of clade 5 produced laccase, indicating that this genetic group may be a promising source of novel activities.

***Keywords*:** *Aureobasidium pullulans, laccase, p-hydroxybenzoic acid, veratric acid, ferulic acid*

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 (Messerschmidt & Huber, 1990). 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 (Thurston, 1994; Gianfreda, Xu, & Bollag, 1999). Laccases have broad substrate specificities, which can be further extended through the use of mediator systems (Riva, 2006). Potential industrial applications include the degradation of textile dyes and toxic materials (Novotny et al., 2000; Shah & Nervd, 2002). Laccases have been well studied in white-rot fungi (Thurston, 1994). However, these enzymes are widely distributed in nature, and recent studies have sought to identify new microbial sources of laccase with novel properties (Kiiskinen, Ratto, & Kruus, 2004; Viswanath et al., 2008).

Aureobasidium pullulans is a polymorphic fungus, considered to be a filamentous ascomycete in class Dothideomycetes, subclass Dothideomycetidae (Schoch et al., 2006; Hibbett et al., 2007). *A. pullulans* is well known as the source of the commercial polysaccharide, pullulan (Leathers, 2002; Singh, Saini, & Kennedy, 2008). Strains of *A. pullulans* also produce numerous degradative enzymes, including fructofuranosidase, glucoamylase, and xylanase (Leathers, 1989; Deshpande, Rale, & Lynch, 1992). *A. pullulans* is sometimes associated with the deterioration of painted wood (Cooke, 1959; Eveleigh, 1961), and certain strains can grow on lignin-related aromatic compounds (Henderson, 1961; Horvath, Brent, & Cropper, 1976; Bourbon nais & Paice 1987; Cernakova et al., 1980; Schoeman & Dickinson, 1996). However, very little research has been reported on laccase production by *A. pullulans* (Rösch, Liese & Berndt, 1969; Deshpande, Rale & Lynch, 1992). We recently completed a multilocus molecular phylogeny of *A. pullulans* (Manitchotpisit et al., 2009). Interestingly, certain phylogenetically defined clades produced high levels of specific bioproducts, including pullulan, xylanase, and a heavy oil (Manitchotpisit et al., 2009; Manitchotpisit, Peterson & Leathers, 2011; Manitchotpisit et al., 2011).

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2. Objectives

The objectives of the study are to examine laccase production from *A. pullulans* and determine whether laccase activity is correlated with specific phylogenetic clades.

3. Materials and methods

3.1 Strains and phylogenetic analysis

Thirty of the strains used in this study were isolated between October 2009 and February 2010, designated RSU strains. RSU strains 1-8 were collected from Iceland, with the remaining RSU strains collected from Thailand. These RSU strains were identified as *A. pullulans* using internal transcribed spacer (ITS) and classified into phylogenetic clades using beta-tubulin (*BT2)* locus sequence analysis and a phylogram was generated as previously described (Manitchotpisit et al., 2009; Manitchotpisit, Peterson, & Leathers, 2011). Isolates were grown in yeast malt broth and cells were disrupted using a FastPrep instrument (Qbiogene, Carlsbad, CA, USA). Total DNA was isolated and purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA). The ITS and *BT2* loci were amplified as previously described (Manitchotpisit et al., 2009), using different primers and standard conditions as shown in Table 1. The amplified fragments were purified using the Millipore MultiScreen PCR system (Millipore, Billerica, MA, USA) and sequenced using the amplification primers and BigDye v3.1 Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and an Hitachi ABI 3730 (capillary) DNA Analyzer (Applied Biosystems). DNA sequences determined in this study were deposited in GenBank under accession numbers (JF419588-JF419610, JF419612-JF419616, JF419619, JF419622, JF419624-JF419633, JF419635-JF419638, JF419641, JF419644, JF419646-JF419659, JF682346, and HQ702497). The remaining 21 strains used in this study were previously described (Manitchotpisit et al., 2009). All sequences were determined from bidirectional sequencing and aligned with CLUSTALW (Thompson, Higgins, & Gibson, 1994) and trimmed to equal length. A maximum parsimony tree of *A. pullulans* strains was calculated with PAUP* version 4.0b10 (Swofford, 2003). Strain CU 30 was chosen as an outgroup isolate based on Manitchotpisit et al. (2009). The ITS data were partitioned into three regions, ITS1, 5.8s rRNA, and ITS2. The *BT2* locus included protein coding and intron regions and the data were partitioned accordingly into intron and exon data.

3.2 Cell growth on lignin-related substrates

As a preliminary screen for laccase activity, all strains were cultured on lignin-related substrates as sole carbon sources. Each strain was cultured on yeast malt agar at 28°C for 3 days. Preinocula were grown in 10 mL of medium containing 1.0% (w/v) glucose in a basal medium consisting of 0.06% (w/v) peptone, 0.04% (w/v) yeast extract, 0.5% (w/v) K_2HPO_4, 0.04% (w/v) $MgSO_4.7H_2O$, 0.1% (w/v) NaCl, pH 6.5, in 50-mL flasks incubated at 200 rpm, 25°C for 3 days. Preinocula were used to inoculate test cultures at 1% (v/v). Test cultures were 10 mL of basal medium containing 0.05% (w/v) of different lignin-related substrates (filtered sterilized *p*-hydroxybenzoic acid, veratric acid, or ferulic acid) as sole carbon sources. Growth on these substrates has been used as a preliminary screen for potential lignin degrading strains of *A. pullulans* (Bourbonnais & Paice, 1987). Test cultures were in 50-mL flasks, incubated at 25°C and 200 rpm for 10 days. Growth was measured at OD_{600}. Cultures containing no carbon source served as a negative control. Cultures with 0.05% (w/v) glucose served as a positive control.

3.3 Laccase induction cultures

Selected strains were cultured on potato dextrose agar plates at 28ºC for 3 days. Preinocula were grown in 50 mL of malt extract broth in 300 mL flasks, incubated at 28ºC, 130 rpm for 3 days. Preinocula were used to inoculate laccase induction cultures at 5% (v/v). Induction cultures were in duplicate flasks of 50 mL of basal liquid medium containing phenylalanine, adenine, and $CuSO_4$ (Fahraeus & Reinhammar, 1967), incubated at 28ºC and 130 rpm. After two days, cultures were induced by the addition of 2,5-xylidine to a final concentration of 200 µM (Fahraeus & Reinhammar, 1967). After an additional two days, cultures were assayed for laccase activity.

3.4 Laccase activity assay

One mL samples taken from induced cultures were clarified by centrifugation for 5 min at 13,000 rpm in 1.5 mL microcentrifuge tubes. Culture supernatants were transferred to new tubes to measure laccase activity. To confirm that laccase activity was enzymatic, duplicate samples were boiled for 15 min to serve as negative controls. Laccase activity assays were performed by measurement of enzymatic oxidation of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, Fluka,

Table 1 Primers used for PCR and sequencing reactions

Target DNA region	Primer[a]	Sequence 5'-3'	Cycling reaction (35 cycles)	Approximately PCR product (bp)
ITS[b]	ITS5 (F)	GGAAGTAAAAGTCGTAACAAGG	95°C, 20 Sec	550
	ITS4 (R)	TCCTCCGCTTATTGATATGC	56°C, 30 Sec	
			72°C, 1 min	
BT2[c]	BT-2A (F)	GGTAACCAAATCGGTGCTGCTTTC	95°C, 30 Sec	450
	BT-2B (R)	ACCCTCAGTGTAGTGACCCTTGGC	58°C, 1 min	
			72°C, 1 min	

[a] F and R in the parentheses mean forward and reverse primers, respectively.
[b] White et al., 1990
[c] Glass & Donaldson, 1995

Switzerland) at 420 nm kinetically for 30 min using a Molecular Devices SpectraMax M5 plate reader. The ABTS assay is widely used for determination of laccase activity and remains one of the best tests for determining the activity of oxidative enzymes (Johannes & Majcherczyk, 2000). Duplicate reactions were prepared for each supernatant and contained 60 µl McIlvaine buffer (pH 5.0), 30 µl culture supernatant, and 10 µl ABTS (13 mM) in McIlvaine buffer (pH 5.0) at 30°C. At pH 5.0, 100 mL of McIlvaine buffer contains 51.50 mL of 0.2 M Na_2HPO4 and 48.50 ml of 0.1 M citric acid (McIlvaine, 1921). The enzyme activity was expressed in units/mL (U = 1 µmol/min).

4. Results

4.1 Phylogenetic classification of recently isolated strains used in this study

The thirty recently isolated strains used in this study were classified into phylogenetic clades as previously described (Manitchotpisit et al., 2009). A tree generated from the ITS locus was not sufficiently informative to differentiate individual strains (data not shown). However, it was useful to identify this species. *BT2* sequences had sufficient informative characters to classify all *A. pullulans* strains into distinct phylogenetic clades with the exception of strains RSU 10 and RSU 35, which were classified as clade 1 or 2. Fig.1 shows a maximum parsimony tree of *BT2* sequences of *A. pullulans* strains, including the recent isolates. Eight strains isolated from Iceland (RSU 1-8) all belong to recently described clade 13, which contains only strains from temperate climates (Manitchotpisit, Peterson, & Leathers, 2011). Two isolates from Thailand belong to a novel clade 14 (Table 2). The remaining 21 isolates from Thailand were distributed among 11 previously described phylogenetic clades. These results are consistent with our previous observation that Thailand is a source of genetically diverse strains of *A. pullulans* (Manitchotpisit et al., 2009). In addition to the 30 recently isolated strains described above, an additional 21 strains were chosen for screening on lignin-related substrates. Thus, the 51 strains employed in this study represent a range of genetic diversity.

4.2 Growth of *A. pullulans* on lignin-related substrates

All 51 isolates of *A. pullulans*, representing 13 phylogenetic clades, were tested for utilization of different lignin-related substrates as sole carbon sources (Table 2). Growth was estimated by an increase in the culture OD_{600}. Strains RSU 35 and RSU 2 grown in basal medium with 0.05 mg/mL glucose served as a positive control for growth. These cultures showed an OD_{600} of 0.274 and 0.233, respectively. Strain RSU 35 cultured in basal medium without a carbon source served as a negative control. This culture showed an OD_{600} of 0.084. By using this measure, almost all strains grew well on all three tested substrates, with three exceptions (RSU 22, RSU 23, and RSU 27). Veratric acid was sometimes more difficult to metabolize than other substrates, particularly by members of clade 3 (Table 2). Generally, growth appeared to be greatest among members of clades 4, 5, 6, 11 and 14. Growth on these lignin-related substrates has been taken as evidence for the potential to degrade lignin in *A. pullulans* (Bourbonnais & Paice, 1987).

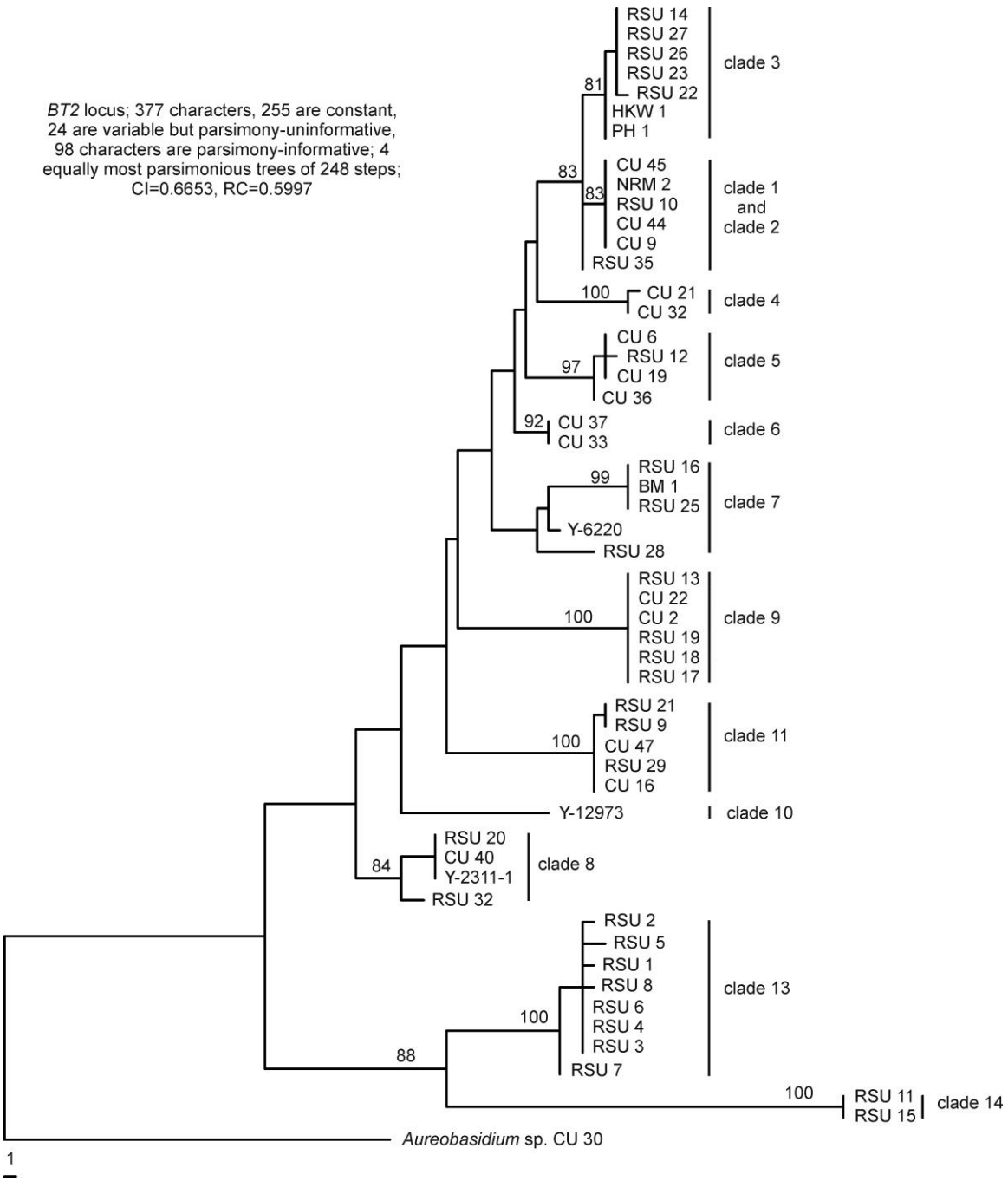

Figure 1 Maximum parsimony tree of *Aureobasidium pullulans* isolates calculated with PAUP*. The bootstrap proportion from 1000 repetitions is placed above a node when the value exceeded 70%. The clade numbering system and outgroup choice is from the *A. pullulans* multilocus phylogenetic study (Manitchotpisit et al., 2009).

Table 2 Growth of *Aureobasidium pullulans* on basal medium containing lignin-related substrates as sole carbon sources

Strains	Clade	Average OD_{600}		
		***p*-Hydroxy-benzoic acid**	**Veratric acid**	**Ferulic acid**
CU 44	1	0.295	0.343	0.250
CU 45		0.311	0.346	0.397
CU 9	2	0.311	0.352	0.332
NRM2		0.323	0.262	0.267
RSU 10	1 or 2	0.264	0.102	0.243
RSU 35	1 or 2	0.207	0.216	0.296
PH1	3	0.333	0.265	0.476
HKW1		0.365	0.227	0.405
RSU 14		0.208	0.129	0.303
RSU 22		0.262	0.068	0.307
RSU 23		0.176	0.060	0.297
RSU 26		0.203	0.101	0.281
RSU 27		0.161	-0.026	0.254
CU 21	4	0.345	0.387	0.376
CU 32		0.565	0.403	0.317
CU 6	5	0.377	0.506	0.432
CU 36		0.393	0.478	0.432
RSU 12		0.337	0.332	0.334
CU 33	6	0.430	0.418	0.404
CU 37		0.329	0.415	0.392
Y-6220	7	0.257	0.283	0.260
BM1		0.317	0.144	0.135
RSU 16		0.295	0.282	0.335
RSU 25		0.260	0.177	0.301
RSU 28		0.150	0.134	0.278
Y-2311-1	8	0.289	0.363	0.322
CU 40		0.281	0.297	0.375
RSU 20		0.264	0.206	0.400
RSU 32		0.311	0.164	0.374
CU 2	9	0.315	0.304	0.401
CU 22		0.203	0.113	0.413
RSU 13		0.200	0.135	0.202
RSU 17		0.250	0.213	0.313
RSU 18		0.132	0.184	0.327
RSU 19		0.398	0.151	0.253
Y-12973	10	0.392	0.331	0.404
CU 16	11	0.280	0.313	0.300
CU 47		0.281	0.375	0.215
RSU 9		0.399	0.293	0.355
RSU 21		0.279	0.321	0.349
RSU 29		0.165	0.287	0.350
RSU 1	13	0.247	0.269	0.285
RSU 2		0.259	0.204	0.309
RSU 3		0.247	0.156	0.339
RSU 4		0.268	0.140	0.214
RSU 5		0.269	0.110	0.186
RSU 6		0.286	0.154	0.270
RSU 7		0.238	0.147	0.197
RSU 8		0.385	0.164	0.295
RSU 11	14	0.403	0.400	0.307
RSU 15		0.403	0.323	0.374

4.3 Laccase induction assay

Thirteen strains that grew well on lignin-related substrates were chosen for further screening in a laccase induction assay (Table 3). Despite the fact that both strains from novel clade 14 showed good growth on lignin-related substrates, only one of these (RSU 11) exhibited laccase activity. Two strains from clade 4 showed no laccase activity, and only one strain from each of clades 6 and 11 had relatively low enzyme activity. On the other hand, every strain from clade 5 expressed laccase, with strains CU 6 and RSU 12 having the greatest activity. Strain CU 19, which was not included in the survey of strains grown on lignin-related substrates, was added to the laccase induction assay study as an additional representative of clade 5. This strain also produced laccase (Table 3).

Table 3 Laccase activity produced by strains of *Aureobasidium pullulans*

Strains	Clade	Laccase (mU/mL)
CU 21	4	< 0.01*
CU 32		< 0.01
CU 6	5	15 ± 0.4
CU19		7.3 ± 0.4
CU 36		1.4 ± 0.4
RSU 12		11 ± 1.7
CU 33	6	0.04 ± 0.1
CU 37		< 0.01
RSU 9	11	< 0.01
RSU 21		< 0.01
RSU 29		0.7 ± 0.3
RSU 11	14	1.9 ± 0.02
RSU 15		< 0.01

* Below limit of detection

5. Discussion

Novel sources of the enzyme laccase are needed to improve activity yields and expand substrate specificities. The metabolically versatile fungus *A. pullulans* is commonly associated with the deterioration of plant materials and produces numerous degradative enzymes, including hemicellulases. Although strains of *A. pullulans* are capable of utilizing lignin-related aromatic compounds, little has been reported concerning laccase production by this organism. In this study, we examined 13 diverse phylogenetic clades of *A. pullulans* for laccase production. Initially, strains were tested for utilization of three lignin-related substrates as sole carbon sources. Most strains grew well on all three substrates, consistent with previous reports on the utilization of aromatic compounds by *A. pullulans*. Thirteen strains that

grew best on these substrates, representing five clades, subsequently were tested for laccase production in an induction assay. Six of 13 strains failed to produce laccase, indicating that growth on lignin-related compounds may not be a useful indicator of laccase production. Interestingly, all representatives of clade 5 produced laccase, indicating that this genetic group may be a promising source of novel laccase activities. Under similar assay conditions in our laboratory, laccase activities from these strains were comparable with those of other fungal laccases, including *Phlebia radiata* and *Pycnoporus cinnabarinus* (Kantelinen, Hatakka, & Viikari, 1989; Eggert, Temp, & Eriksson, 1996). *A. pullulans* clade 5 is distinctive in that it produces a dark purple or vinaceous pigment (Manitchotpisit et al., 2009). Thurston (1994) points out that in a number of fungi, laccase activity is associated with pigment formation, and it is certainly possible that this is its natural role in *A. pullulans*. This also suggests that it might be possible to rapidly screen potential laccase producers in *A. pullulans* by their colonial morphology, based on pigment. However, this suggestion should be studied further, since 7 of 13 strains tested for laccase activity exhibited enzyme production.

6. Conclusion

A. pullulans is the source of a number of valuable bioproducts, including degradative enzymes. In this study, 51 strains from 13 diverse phylogenetic clades were examined for growth on lignin-related substrates. Although most strains grew well on these substrates, laccase activity was consistently found only in four representative strains belonging to phylogenetic clade 5, a distinctive group that produces a dark purple or vinaceous pigment. Thus, *A. pullulans* may serve as a source of novel laccase activities.

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