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ORIGINAL RESEARCH PAPER

Lipase production by diverse phylogenetic clades of *Aureobasidium pullulans*

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Abstract Thirty-nine strains representing 12 diverse phylogenetic clades of *Aureobasidium pullulans* were surveyed for lipase production using a quantitative assay. Strains in clades 4 and 10 produced 0.2–0.3 U lipase/ml, while color variant strain NRRL Y-2311-1 in clade 8 produced 0.54 U lipase/ml. Strains in clade 9, which exhibit a dark olivaceous pigment, produced the highest levels of lipase, with strain NRRL 62034 yielding 0.57 U lipase/ml. By comparison, *Candida cylindracea* strain NRRL Y-17506 produced 0.05 U lipase/ml under identical conditions. *A. pullulans* strain NRRL 62034 reached maximal lipase levels in 5 days on lipase induction medium, while *A. pullulans* strain NRRL Y-2311-1 and strains in clades 4 and 10 were highest after 6 days. *A. pullulans* strain NRRL Y-2311-1 and strains in clade 9 produced two extracellular proteins in common, at >50 and <37 kDa.

Keywords *Aureobasidium pullulans* · *Candida cylindracea* · Lipase · Phylogenetic clades

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Introduction

Aureobasidium pullulans is a polymorphic fungus, considered to be a filamentous ascomycete in class Dothideomycetes, subclass Dothideomycetidae (Hibbett et al. 2007; Schoch et al. 2006). It is well known as the source of the commercial polysaccharide, pullulan (Leathers 2002; Singh et al. 2008). Strains of *A. pullulans* also produce numerous degradative enzymes, including fructofuranosidase, glucoamylase, laccase, and xylanase (Leathers 1989; Deshpande et al. 1992; Rich et al. 2013). We recently developed a multilocus molecular phylogeny of *A. pullulans* based on sequences of five genetic loci (Manitchotpisit et al. 2009). Interestingly, certain phylogenetically defined clades produced high levels of specific valuable bioproducts, including pullulan, xylanase, poly(β-L-malic acid) (PMA), laccase, and liamocins (heavy oil) (Manitchotpisit et al. 2009, 2011, 2012; Rich et al. 2013). However, relatively little information has been available concerning lipase production by *A. pullulans.*

Microbial lipases have numerous biotechnological applications in the detergent, food, and pharmaceutical industries (Hasan et al. 2006; Salihu and Alam 2012; Singh and Mukhopadhyay 2012). Lipases also can be used in the production of biodiesel fuels (Tan et al. 2010; Ghaly et al. 2010). Numerous bacteria and fungi produce lipases, including species of the yeast-like fungus *Candida* (Sharma et al. 2011; Patil et al. 2011). Federici (1982) reported that all of 198 strains of *A. pullulans* produced lipase activity in semi-quantitative plate assays. Buzzini and Martini (2002) found that 20 of 46 tropical isolates from the Brazilian rain forest produced lipase activity in a qualitative plate assay. Similarly, Kudanga et al. (2007) reported that 20 of 42 tropical isolates from Zimbabwe produced lipase. Wang et al. (2007) isolated a lipase-producing marine fungus which they described as *A. pullulans*, and only lipase from this single strain has been studied in greater detail (Liu et al. 2008a, b). In this study we survey 39 strains representing 12 diverse phylogenetic clades of *A. pullulans* for lipase production using quantitative assays and identify specific clades that produce lipase.

Materials and methods

Organisms and growth conditions

*S*trains used in this study were obtained from the ARS Culture Collection, Peoria, IL (Table 1). Strains were maintained at 28 °C on potato/dextrose/agar (PDA). Single colonies from 24 h plates were used to inoculate liquid preinocula, which were grown overnight in 10 ml yeast peptone/dextrose broth (YPD) in 50 ml flasks at 28 °C, 200 rpm. Preinocula were used to inoculate triplicate 250 ml flasks to a final OD_{600} of 0.04 in 50 ml lipase induction medium (Liu et al. 2008a) containing 0.4 % (w/v) glucose, 0.6 % (w/v) $(NH_4)_2SO_4$, 0.1 % (w/v) K_2HPO_4, and 0.05 % (w/v) $MgSO_4{\cdot}7H_2O$, pH 7.0. After 6 h at 25 °C, 130 rpm, 3 % (v/v) sterile olive oil was added and cultures were further incubated for up to 6 days (Liu et al. 2008a).

Lipase activity assay

One ml samples of lipase induction cultures were centrifuged for 20 min at $\sim 16000 \times g$ and supernatants were assayed for lipase activity by measurement of *p*-nitrophenol released from *p*-nitrophenyl laurate. Substrate emulsions were prepared as described by Liu et al. (2008a). Six microliters of sample were added to 194 µl of freshly prepared substrate emulsion and incubated in a Molecular Devices SpectraMax M5 plate reader at 35 °C for 25 min. Product was detected at 410 nm. Lipase from porcine pancreas served as a standard and positive control. Boiled samples served as negative controls. Enzyme activity was expressed in units/ml (1 U = 1 µmol product formed/min) and as specific activity (U/mg protein). Standard errors are reported. Protein concentrations were determined using the Bio-Rad protein assay, based on the Bradford dye-binding method with bovine serum albumin as standard.

Polyacrylamide gel electrophoresis

Samples were concentrated 50-fold by ultrafiltration (Nanosep 3 K Omega low protein binding spin cells) and denatured for 2 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). Samples and Bio-Rad Precision Plus Unstained Protein Standards (Bio-Rad) were applied to an SDS-PAGE gel (5 % v/v stacking, 10 % v/v resolving). After electrophoresis at 100 V for ~1 h, the gel was stained with SYPRO Ruby protein gel stain (Invitrogen, Grand Island, NY).

Results and discussion

Production of lipase by diverse phylogenetic clades of *A. pullulans*

Based on a multilocus molecular phylogeny of *A. pullulans* (Manitchotpisit et al. 2009), we surveyed 39 strains representing 12 diverse phylogenetic clades for production of lipase (Table 1). Seven reference strains previously described as lipase producers also were tested as controls. *A. pullulans* strains in phylogenetic clade 7 produced no detectable lipase (<0.01 U/ml) after 6 days of induction under conditions tested (Table 1). Several clades (1, 2, 3, 5, 6, 11, and 13) produced lipase at levels generally less than 0.1 U lipase/ml. Some of these clades produce other valuable bioproducts. For example, strains in clade 1

Table 1 Lipase production by strains of *Aureobasidium pullulans* and reference strains

Aureobasidium pullulans strains				
Clade[a]	Strain number	Equivalent number	Lipase activity[b]	
			Protein (U/mg)	U/ml
1	NRRL 58555	CU 44	4.3 ± <0.1	0.12 ± 0.02
	NRRL 58556	CU 45	8.6 ± 0.2	0.05 ± <0.01
1 or 2	NRRL 62032	RSU 10	28 ± 1.5	0.24 ± <0.01
	NRRL 62054	RSU 35	1.4 ± 0.2	0.05 ± <0.01
2	NRRL 58522	CU 9	12 ± 1.9	0.09 ± 0.01
	NRRL 58560	NRM2	4.3 ± 0.9	0.03 ± <0.01
3	NRRL 58562	HKW 1	4.8 ± 0.3	0.03 ± <0.01
	NRRL 58563	PH 1	2.6 ± 0.1	0.02 ± <0.01
	NRRL 62035	RSU 14	11 ± 1.1	0.08 ± <0.01
	NRRL 62043	RSU 22	9.1 ± 2.2	0.10 ± 0.01
	NRRL 62044	RSU 23	8.1 ± 0.4	0.05 ± <0.01
	NRRL 62047	RSU 26	5.0 ± 0.4	0.07 ± <0.01
	NRRL 62048	RSU 27	5.3 ± 1.0	0.04 ± 0.01
4	NRRL 58534	CU 21	5.9 ± 0.8	0.29 ± 0.08
	NRRL 58545	CU 32	4.1 ± 0.1	0.24 ± 0.03
5	NRRL 50381	RSU 12	1.9 ± 0.3	0.04 ± <0.01
	NRRL 58519	CU 6	12 ± 1.7	0.09 ± <0.01
	NRRL 58548	CU 36	1.5 ± 0.1	0.07 ± 0.02
6	NRRL 58546	CU 33	2.3 ± 0.8	0.03 ± 0.01
	NRRL 58549	CU 37	4.3 ± 0.5	0.05 ± <0.01
7	NRRL 62037	RSU 16	<0.1	<0.01
	NRRL Y-6220		<0.1	<0.01
8	NRRL 58552	CU 40	5.9 ± 2.8	0.11 ± 0.03
	NRRL 62041	RSU 20	1.8 ± 0.1	0.16 ± <0.01
	NRRL Y-2311-1	ATCC 62921	9.7 ± 0.5	0.54 ± 0.03
9	NRRL 58515	CU 2	14 ± 1.9	0.51 ± 0.02
	NRRL 58535	CU 22	8.0 ± 0.8	0.43 ± 0.04
	NRRL 62034	RSU 13	19 ± 3.6	0.57 ± 0.03
	NRRL 62038	RSU 17	24 ± 2.3	0.35 ± 0.09
	NRRL 62039	RSU 18	9.7 ± 0.6	0.42 ± <0.01
	NRRL 62040	RSU 19	18 ± 0.3	0.32 ± <0.01
10	NRRL Y-12973		14 ± 1.1	0.20 ± 0.01
	NRRL Y-12974		6.5 ± 0.3	0.21 ± 0.01
11	NRRL 58529	CU 16	1.5 ± 0.3	0.03 ± <0.01
	NRRL 62031	RSU 9	6.2 ± 0.1	0.15 ± <0.01
	NRRL 62042	RSU 21	3.9 ± 0.3	0.12 ± <0.01
13	NRRL 58012	CBS 584.75	3.4 ± 0.1	0.05 ± <0.01
	NRRL 58013	CBS 100524	2.9 ± < 0.1	0.07 ± <0.01
	NRRL 62025	RSU 2	1.4 ± 0.1	0.02 ± <0.01

Table 1 continued

Aureobasidium pullulans strains				
Clade[a]	Strain number	Equivalent number	Lipase activity[b]	
			Protein (U/mg)	U/ml
Reference strains				
Candida cylindracea	NRRL Y-17506[c]	ATCC 14830	7.3 ± 1.9	0.05 ± <0.01
Candida sp.	NRRL YB-2064[d]		<0.1	<0.01
Penicillium citrinum	NRRL 3754[d]		2.7 ± 0.5	0.02 ± <0.01
Penicillium citrinum	NRRL 5907[d]		0.5 ± 0.1	0.03 ± <0.01
Penicillium funiculosum	NRRL 6014[d]		<0.1	<0.01
Pseudomonas fluorescens	NRRL B-14678[e]	JCM 5963	<0.1	<0.01
Pseudomonas fluorescens	NRRL B-2641		<0.1	<0.01

[a] Phylogenetic clade according to the system of Manitchotpisit et al. (2009)

[b] Grown for 6 days on lipase induction medium

[c] Salihu et al. (2011)

[d] Hou and Johnston (1992)

[e] Zhang et al. (2009)

produce high levels of pullulan without contaminating pigment (Manitchotpisit et al. 2009). Strains in clade 5 produce relatively high levels of laccase (Rich et al. 2013), while strains in clade 13 produce high levels of PMA (Manitchotpisit et al. 2012). Strains in clades 4 and 10 produced 0.2–0.3 U lipase/ml. *A. pullulans* strain NRRL Y-2311-1 in clade 8 produced 0.54 U lipase/ml. Clade 8 includes the so-called "color variant" strains that produce brilliant pigments of red, yellow, and orange (Wickerham and Kurtzman 1975). These strains produce high levels of xylanase (Leathers 1986; Manitchotpisit et al. 2009). Strains in clade 9 consistently produced the highest levels of lipase among those tested (Table 1). In particular *A. pullulans* strain NRRL 62034 produced 0.57 U lipase/ml under these conditions. By comparison, the best of seven reference strains, *Candida cylindracea* strain NRRL Y-17506, produced 0.05 U lipase/ml (Table 1). Under optimized conditions, strain NRRL Y-17506 has been reported to produce 20 U lipase/ml (Salihu et al. 2011). Strain HN2.3 of a marine fungus described as *A. pullulans*, of unknown phylogenetic affiliation, reportedly produced 8.02 U lipase/ml (Liu et al. 2008a). Lipase activities from other fungi vary considerably, but are often in this range (Singh and Mukhopadhyay 2012). *A. pullulans* phylogenetic clade 9 was not previously recognized for production of valuable bioproducts. However, this clade is distinguished by production of dark olivaceous pigment (Manitchotpisit et al. 2009).

We have observed a general relationship in *A. pullulans* between the capacity to produce pigment and the production of certain hydrolytic enzymes. Color variant strains in clade 8 are high-level producers of xylanase, while strains in clade 5, which exhibit a dark vinaceous pigment, produce relatively high levels of laccase. In this study, clade 9, strains of which exhibit a dark olivaceous pigment, produced the highest levels of lipase. On the contrary, clades that produce high levels of pullulan, PMA, and liamocins are not associated with pigment production (Manitchotpisit et al. 2009, 2011, 2012). The reason for the association of enzyme production with pigment is unclear. Most of the genetic diversity of *A. pullulans* occurs in tropical environments, and in fact most isolates from temperate climates have thus far been found in clade 13. Although pigment production might be assumed to be protective against environmental exposure to sunlight, strains from diverse phylogenetic clades have been isolated side-by-side from similar collection sites (Manitchotpisit et al. 2009). In the case of laccase production, the enzyme may be directly involved in pigment biosynthesis (Rich et al. 2013).

Extracellular proteins from lipase-producing strains of *A. pullulans*

Total extracellular proteins from strains representing diverse phylogenetic clades of *A. pullulans* were compared by SDS-PAGE (Fig. 1). Strains from clade 9 showed two extracellular proteins in common, at >50 and <37 kDa (Fig. 1). Interestingly, clade 8 strain NRRL Y-2311-1, which made similar levels of

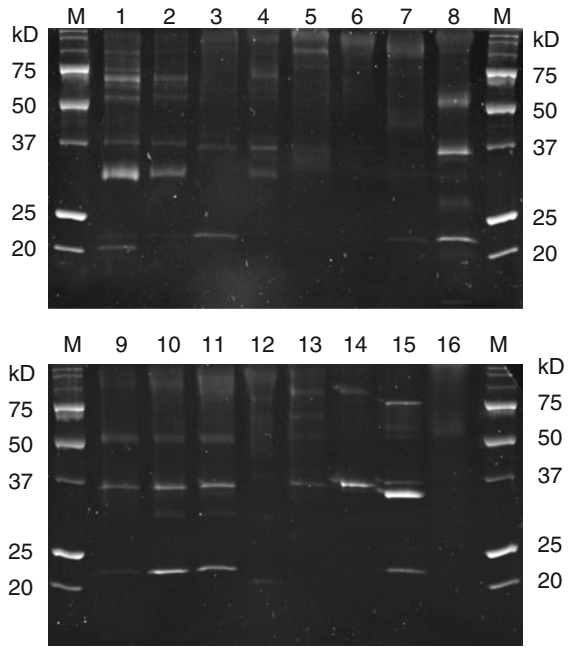

Fig. 1 SDS-PAGE of extracellular proteins produced by *Aureobasidium pullulans* strains from diverse phylogenetic clades and reference strains after 6 days on lipase induction medium. *Lane M* Bio-Rad Precision Plus protein standards; *lane 1 A. pullulans* strain NRRL 58555 (clade 1); *lane 2 A. pullulans* strain NRRL 62032 (clade 1 or 2); *lane 3 A. pullulans* strain NRRL 58522 (clade 2); *lane 4 A. pullulans* strain NRRL 62043 (clade 3); *lane 5 A. pullulans* strain NRRL 58534 (clade 4); *lane 6 A. pullulans* strain NRRL 58519 (clade 5); *lane 7 A. pullulans* strain NRRL 58546 (clade 6); *lane 8 A. pullulans* strain NRRL Y-2311-1 (clade 8); *lane 9 A. pullulans* strain NRRL 62034 (clade 9); *lane 10 A. pullulans* strain NRRL 62039 (clade 9); *lane 11 A. pullulans* strain NRRL 62040 (clade 9); *lane 12 A. pullulans* strain NRRL Y-12974 (clade 10); *lane 13 A. pullulans* strain NRRL 62031 (clade 11); *lane 14 A. pullulans* strain NRRL 58012 (clade 13); *lane 15 Penicillium citrinum* strain NRRL 3754 (reference strain); *lane 16 Candida cylindracea* strain NRRL Y-17506 (reference strain)

lipase, produced similar protein species (Fig. 1). Strains in clades 1, 2, 3, 4, 11, and 13 also produced one or more of these species, as did *Penicillium citrinum* reference strain NRRL 3754. Fungal lipases vary considerably in molecular weight but have often been reported in this range (Sharma et al. 2011; Patil et al. 2011). Lipase isoforms of approximately 60 kDa have been purified from *C. cylindracea* strain NRRL Y-17506 (Rua et al. 1993; Benjamin and Pandey 2001). Similarly, a lipase purified from *A. pullulans* strain HN2.3 was estimated to be 63.5 kDa (Liu et al. 2008a). Strains in clades 5, 6, and 10 produced diffuse,

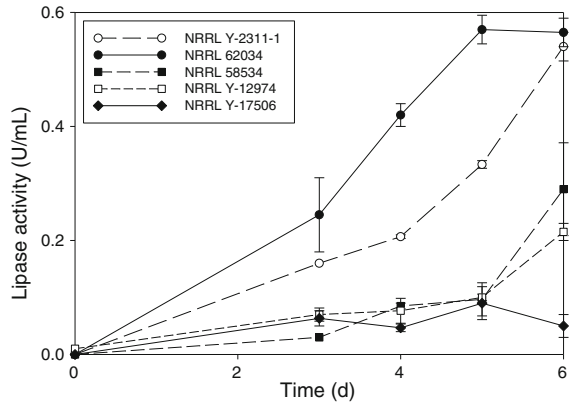

Fig. 2 Time course of lipase production by *Aureobasidium pullulans* strains and *Candida cylindracea* strain NRRL Y-17506 grown for 6 days on lipase induction medium

high molecular weight protein species, which may be glycosylated forms (Fig. 1).

Time course of lipase production by *A. pullulans* strains

Aureobasidium pullulans strains representing the highest lipase-producing clades (4, 8, 9, and 10) were cultured for 6 days in lipase induction medium. Clade 9 strain NRRL 62034 reached maximal lipase production levels (~0.6 U lipase/ml) within 5 days, while clade 8 strain NRRL Y-2311-1 required an additional day to reach equivalent levels (Fig. 2). Strains in clades 4 and 10 produced about 0.2 U lipase/ml in 6 days. By comparison *C. cylindracea* strain NRRL Y-17506 produced a maximum of about 0.1 U lipase/ml in 5 days. Lipase from *C. cylindracea* strain NRRL Y-17506 is widely used in research due to its high activities in hydrolysis and synthesis (Rua et al. 1993), and the enzyme is commercially produced (Singh and Mukhopadhyay 2012).

Conclusion

we report here for the first time lipase production by diverse phylogenetic clades of *A. pullulans*. Strains in phylogenetic clade 9 are most promising for lipase production, the best of which is strain NRRL 62034. Strains in this clade are distinguished by production of dark olivaceous pigment. This may provide a simple method to rapidly screen new isolates for lipase

production. Under identical conditions, strain NRRL 62034 produced approx. six times as much lipase as commercial strain *C. cylindracea* NRRL Y-17506.

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