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ORIGINAL PAPER

α-Amylase activity during pullulan production and α-amylase gene analyses of *Aureobasidium pullulans*

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Abstract *Aureobasidium pullulans* is the source of commercially produced pullulan, a high molecular weight polysaccharide that is used in the manufacture of edible films. It has been proposed that α-amylase decreases the molecular weight of pullulan in late cultures. Based on a recent phylogenetic analysis, five representative strains were chosen to study the relationship between α-amylase and pullulan production. In sucrose-grown cultures, pullulan yields increased over time while the molecular weight of pullulan generally decreased. However, no α-amylase activity was detected in these cultures. Low levels of α-amylase were present in starch-grown culture, but pullulan analysis was complicated by residual starch. To facilitate further studies on the role of α-amylase in the reduction of pullulan molecular weight, the α-amylase gene from *A. pullulans* NRRL Y-12974 was cloned and characterized. The coding region of the complete α-amylase gene contains 2,247 bp, including 7 introns and 8 exons. The putative mRNA was 1,878 bp long, encoding an α-amylase of 625 amino acid residues. Southern blot analysis indicated that there was only one copy of this gene in the genome. Reverse transcription–polymerase chain reaction (RT–PCR) analysis indicated that the gene was transcribed in both sucrose- and starch-grown cultures. It is possible that very low levels of α-amylase attack the minor maltotetraose subunits of pullulan and cause the reduction of molecular weight.

Keywords *Aureobasidium pullulans* · α-Amylase · Molecular weight · Pullulan

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Introduction

Aureobasidium pullulans is well known for producing the exopolysaccharide (EPS) pullulan. In nature, pullulan helps the fungus adhere to environmental habitats and also protects the cells from desiccation [2]. Commercially, pullulan has been used for making biofilms and adhesives utilized in food, drug, and cosmetic industries [12]. The major pullulan structure is a polymer of α-1,6-linked maltotriose subunits, or a linear glucan containing α-1,4 and α-1,6 linkages in a ratio of 2:1 [22]. Pullulan also contains as a minor structural feature a low percentage of α-1,6-linked maltotetraose subunits [4, 5]. These maltotetraose subunits are distributed randomly, about 1–7% of total residues, throughout the pullulan molecule [6, 23].

It has been reported that pullulan is susceptible to degradation by pullulanase (EC 3.2.1.41), α-amylase (EC 3.2.1.1), and glucoamylase (EC 3.2.1.3). The maltotetraose subunits in pullulan are substrates for α-amylase [4]. The addition of amylase inhibitor to the culture medium had a slightly positive effect in maintaining the high molecular weight of pullulan [18]. However, levels of α-amylase have been reported to be low in pullulan-producing cultures, and not clearly correlated with the decrease in pullulan molecular weight [10, 11, 18, 20]. *A. pullulans* produces extracellular α-amylase at a low level in cultures grown on various non-starch media and at a slightly higher level on starch media [20]. Furthermore, *A. pullulans* has been reported to produce other enzymes that might attack pullulan, including glucoamylase [20] and pullulanase [18]. Therefore, further study of the relationship between α-amylase activity and the molecular weight of pullulan is needed, including an analysis of α-amylase gene.

Tropical strains of *A. pullulans* were isolated from various habitats in Thailand and were classified based on multilocus phylogenetic analyses using concordance analysis of DNA sequences from the rRNA ITS region, the rRNA IGS1 region, *EF-1α*, *BT2*, and *RPB2* [14]. Representative isolates were selected to study the relationship between α-amylase and pullulan in cultures grown on different media. Pullulan profiles, including NMR spectra, α-amylase sensitivity, pullulanase sensitivity, molecular weight, and viscosity were determined and compared with the production of α-amylase activity. Finally, the putative α-amylase gene was characterized and the transcription of α-amylase mRNA during cultivation was detected by using reverse transcription–polymerase chain reaction (RT–PCR).

Materials and methods

Aureobasidium pullulans isolates used in this study

To investigate the potential relationship between α-amylase and the pullulan molecular weight, five representative *A. pullulans* isolates were chosen according to the first multilocus phylogenetic analysis of *A. pullulans* [14] (Table 1). Strain CU 3 is representative of *A. pullulans* clade 7. It produces olivaceous (dark green) pigment in culture and produced pullulan contaminated with pigment. Strain CU 20 is representative of clade 1, which produces high levels of pullulan without pigmentation. Strain CU 36 is in clade 5, and produces a vinaceous (purple) pigment in production medium (PM), although the pigment does not contaminate pullulan precipitated from cultures. Strain NRM2 is a reference strain from tropical isolates [18, 19]. It is classified in clade 2 with a moderate pullulan yield. Reference strain NRRL Y-12974 [13] is in clade 10 and produces a high yield of pullulan.

Table 1 *Aureobasidium pullulans* isolates used in this study

Strain number	Synonyms	Host/source	Clade[a]
CU 3	NRRL 58516	Painted wall	7
CU 20	NRRL 58533	Mango leaf	1
CU 36	NRRL 58548	Mango leaf	5
NRM2	NRRL 58560	Unknown leaf	2
NRRL Y-12974		Seagrass	10

[a] Phylogenetic group based on Manitchotpisit et al. [14]

Growth and production of EPSs

Cultures were maintained on yeast malt extract agar (YMA). Representative isolates were cultured in standard pullulan production medium (PM) containing 5.0% sucrose and 0.1% N-sources [14, 18]. Standard PM is composed of 5% (w/v) sucrose, 0.06% (w/v) peptone, 0.04% (w/v) yeast extract, 0.5% (w/v) K_2HPO_4, 0.04% (w/v) $MgSO_4 \cdot 7H_2O$, and 0.1% (w/v) NaCl.

Strain NRRL Y-12974 also was grown in modified PM containing 5.0% (w/v) sucrose and 0.3% (w/v) N-sources; 5.0% (w/v) soluble starch and 0.1% (w/v) N-sources; or 5.0% (w/v) soluble starch and 0.3% (w/v) N. Media with 0.3% (w/v) N-sources contained 0.18% (w/v) peptone and 0.12% (w/v) yeast extract.

In all cases, a fresh colony growing on YMA (2–3 days cultivation) was used to inoculate a 25-ml flask containing 10 ml of YM, which was incubated overnight at 28°C with shaking at 150 rpm. The concentration of cells in this preinoculum was determined by using a hemocytometer, and 4×10^7 cells were used to inoculate a 250-ml flask containing 80 ml of PM or modified PM. Cultures were incubated at 28°C with 150 rpm shaking, and sampled at days 2, 4, 6, and 8 of cultivation. Samples were evaluated for OD_{600} (cell growth), pH of culture, EPS or pullulan yield, molecular weight and viscosity of EPS, and also α-amylase and pullulanase activities.

EPS analyses

Determination of the molecular weight of EPS using high-performance size-exclusion chromatography (HPSEC) was modified from Prasongsuk et al. [18]. Freeze-dried EPS was dissolved in distilled water to a concentration of 1% (w/v) and filtered by using Nanosep MF 0.45 μm microcentrifuge filter tubes (Pall Corporation, East Hills, NY). Ten microliters of filtrate was applied to a Shodex SB 806 M HQ HPSEC column (300 × 8 mm, Showa Denko, Tokyo, Japan) equilibrated with 50 mM $NaNO_3$ using a flow rate of 0.5 ml/min. Eluate from the column was analyzed by optical rotation using a Shodex OR-1 detector. Molecular weight estimates based on elution positions

were made using pullulan molecular weight standards (Shodex 5,800–1.66 million).

Viscosity determinations also were performed on freeze-dried EPS dissolved in distilled water at 1% (w/v), using a Brookfield Digital Rheometer model DV-III + (Brookfield, Middleboro, MA) at 25°C with a rotation of 30 rpm (shear rate of 39.6 1/s) using spindle SC4-18 and chamber 13R.

EPS or pullulan structure was analyzed by ^{1}H-NMR (nuclear magnetic resonance spectroscopy) as described by Manitchotpisit et al. [14].

Assay for α-amylase and pullulanase activities

Activities of α-amylase and pullulanase were determined by using Blue-starch (Starch Azure, Sigma, St. Louis, MO) and Red-pullulan (Procion Red MX-5B, Megazyme, Co. Wicklow, Ireland) as substrates, respectively. High molecular weight material was removed by centrifugation, and the color of the reaction mixture was measured at 595 nm (for α-amylase assay) or 510 nm (for pullulanase assay). The reaction mixture containing 200 µl of 1% (w/v) substrate in 50 mM sodium acetate buffer (pH 5.0), 50 µl of supernatant from each culture broth, and 2.5 µl of 2% (w/v) sodium azide was incubated at 28°C for 20 h. Five hundred microliters of precipitation buffer (4% (w/v) sodium acetate trihydrate, 0.4% (w/v) zinc acetate, 800 ml of 100% ethyl alcohol, and 200 ml of deionized water per liter) was added and mixed by briefly vortexing. The reaction mixture was incubated at room temperature for 10 min and then centrifuged at 10,000 rpm for 10 min. Two hundred microliters of reaction mixture was applied to a microtiter plate and the absorbance measured as described above. The assays were calibrated by using commercial enzymes of known activities. One unit of enzyme activity is defined as the amount necessary to release 1 µmol of maltose (for α-amylase) or glucose (for pullulanase) equivalents per minute, determined by the dinitrosalicylic acid method [15].

α-Amylase and pullulanase sensitivity tests

Sensitivity tests were based on the pullulanase sensitivity assay described by Leathers et al. [13]. Lyophilized EPS from each sample at day 2 was resuspended at 0.1% (w/v) in 50 mM sodium acetate buffer, pH 5.0, with 0.01% sodium azide added. α-Amylase (type II-A: *Bacillus* sp., Sigma, St. Louis, MO) or pullulanase (*Klebsiella pneumoniae,* Sigma, St. Louis, MO) was added to 0.1 U/ml and samples were incubated at 28°C for 20 h. Reducing sugar equivalents were determined by the dinitrosalicylic acid method [15].

α-Amylase gene analyses

Genomic DNA sequence analysis

Aureobasidium pullulans strain NRRL Y-12974 was employed to determine the genomic DNA sequence of the α-amylase gene. Collected mycelia were immediately frozen in liquid nitrogen and disrupted by using the Bead Mill MM 301 (Retsch, Newtown, PA) followed by DNA purification with the DNeasy Plant Mini kit (QIAGEN, Valencia, CA). Degenerate primers (APamylF1 and APamylR2.2, Table 2) were designed to anneal to conserved regions GFTAIWI and DGIPIIY identified with amino acid sequence alignment of fungal α-amylase. After confirming that the amplified 1,072-bp product was likely a partial α-amylase fragment, flanking regions (both 3′ and 5′ ends) extending beyond the predicted coding region of the gene were further determined by using the GenomeWalker Universal kit (Clontech, Mountain View, CA). The mRNA sequence, amino acid sequence, initial start codon, stop codon, and number of exons were predicted by the hidden Markov model plus protein-based gene prediction FGENESH + (Softberry, Inc.). Calculations utilized the *Aspergillus* splice junction consensus and *Aspergillus terreus* α-amylase (XP_001209405) as a homologous protein sequence. The α-amylase gene sequence from *A. pullulans* strain NRRL Y-12974 is assigned GenBank accession number HM590632. The α-amylase amino acid sequences of *A. pullulans* and other species were analyzed by using DNA Star program (DNASTAR, Inc, Madison, WI). DNA sequencing was performed by using the amplification primers and BigDye v3.1 Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and a Hitachi ABI 3730 (capillary) DNA Analyzer (Applied Biosystems, Foster City, CA).

Table 2 Primers used for amplification of α-amylase gDNA and mRNA of *A. pullulans*

Primer	Sequence 5′–3′
APamylF1 (F)[a]	GGNTTYACNGCNATHTGGATH[b]
APamylR2.2 (R)	RTADATDATYGGDATYCCRTC[b]
AP-GSP3f (F)	CTACTGGATCACACAAGCCTTCCAGTC
AP-GSP5r (R)	GTAATACGACTCACTATAGGGCACGC
AP-LS1f (F)	GAAACCAACAGGGATTGCCCTAG
AP-LS3r (R)	GACGGGTCGCTTACAACCATTAC
APamylf3 (F)	GATGGTTGACATTGTCACAAACC
APamylr3 (R)	CCAGTAGTATGTTGGGTAGTTG

[a] F and R in the parentheses mean forward and reverse primers, respectively

[b] Degeneracy codes: R = A or G, Y = C or T, H = A or T or C, D = G or A or T, N = A or C or G or T

Southern blot hybridization

Southern hybridization analysis was performed with genomic DNA of strain NRRL Y-12974 cut with 5 different restriction enzymes (*Xba*I, *Kpn*I, *Pst*I, *Pvu*II, and *Eco*RI). AP-GSP3f and AP-GSP5r (Table 1) were used as primers for amplifying a 575-bp DIG (digoxygenin)-labeled fragment (Roche, Indianapolis, IN) of the α-amylase gene to serve as the hybridization probe. Transfer, hybridization, washing, and detection were performed according to manufacturer's recommendations for DIG analysis. The Kodak Image Station 1000 was used for chemiluminescent detection.

Detection of α-amylase mRNA

Total RNA was isolated by using the RNeasy Plant Mini kit (QIAGEN, Valencia, CA). Contaminating genomic DNA was removed from total RNA using the DNA-free kit (Applied Biosystems/Ambion, Austin, TX). The first-strand cDNA was synthesized by using the SuperScript III First-Strand Synthesis System for RT–PCR (Invitrogen, Carlsbad, CA). RNA samples not treated with reverse transcriptase (no-RT) served as controls to confirm that subsequent amplification was specific to cDNA. Both cDNA and no-RT RNA samples were used as the templates for amplification of the α-amylase using primers APamylf3 and AP amylr3 and REDTaq DNA Polymerase (Sigma, St. Louis, MO). Denaturation was at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 58°C for 1 min, and 72°C for 1 min. The large subunit (26S) of rDNA was used as an internal control by confirming the ability to amplify product from all samples with primers AP-LS1f and AP-LS3r.

Results

Extracellular polysaccharide (EPS) and amylase production by strains of *A. pullulans*

Growth and EPS production in standard PM

Strains listed in Table 1 were grown in a standard pullulan production medium, which has a high level of sucrose (5%) and a low level of total nitrogen sources (0.1%), shown to favor pullulan production [18]. Cell growth gradually increased over 8 days in standard PM for all isolates except NRRL Y-12974, which showed a drop in OD_{600} after day 4 (Fig. 1a). Cell growth was accompanied by a drop in initial pH of 6.5 to a final pH of 3.5, except for cultures of strain NRRL Y-12974, which dropped to about pH 4.2 (data not shown). *A. pullulans* has been reported to produce such acids as gluconic acid and uronic acid [1, 17].

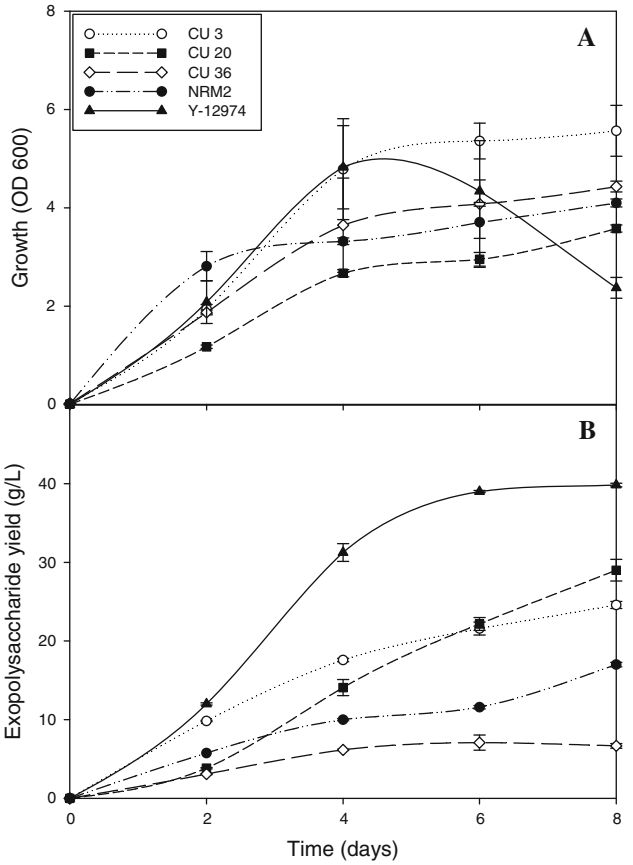

Fig. 1 Growth and exopolysaccharide production by strains of *Aureobasidium pullulans* cultured in standard PM (5.0% sucrose and 0.1% nitrogen sources). **a** Growth (OD_{600}). **b** Exopolysaccharide production. *Bars* represent standard error

Extracellular polysaccharide yields of the five representative isolates increased during cultivation on standard PM (Fig. 1b). Highest yields were from strain NRRL Y-12974 (approx. 40 g/l), followed in order by isolates CU 20, CU 3, NRM2, and CU 36. As previously described, these yields are characteristic of the phylogenetic clades of these isolates [14].

EPS subsequently was analyzed for molecular weight and solution viscosity. With the exception of isolate CU 3, the molecular weight of EPS from each isolate gradually decreased after day 2 (Fig. 2a). This is consistent with previous reports of loss of pullulan molecular weight during fermentation [12]. The molecular weight of EPS from isolate CU 3 fell from day 2 to 4 and then appeared to recovery partially at day 6 (Fig. 2a). Generally, the viscosity of EPS decreased as a function of molecular weight (Fig. 2b). Isolate CU 36 was clearly exceptional in this regard, as EPS gained relatively high viscosity despite apparent loss of molecular weight. Less apparent is the gradual increase in viscosity of isolate CU 3 (Fig. 2b). It is possible that these isolates produce polysaccharides other than pullulan, particularly in late cultures. Although

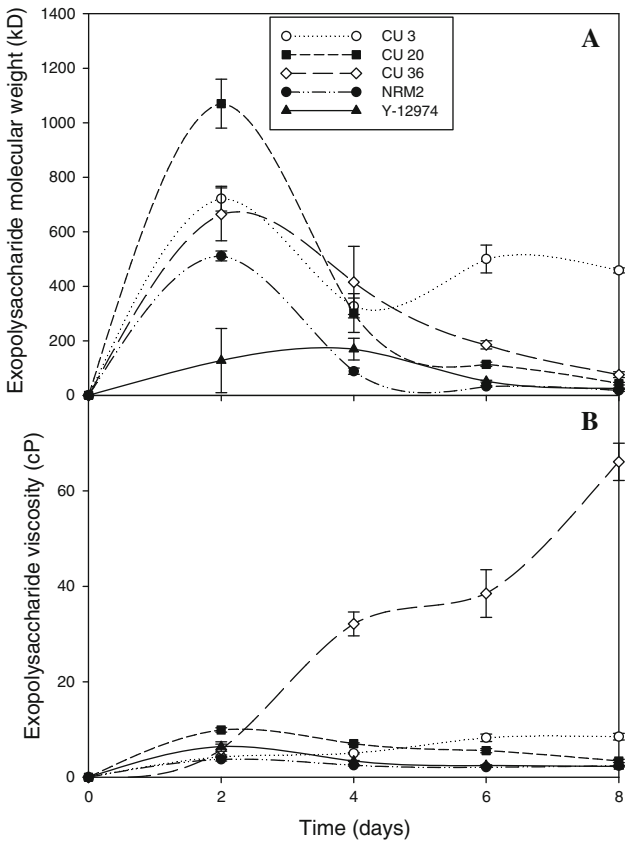

Fig. 2 Molecular weight and viscosity of exopolysaccharides produced by strains of *Aureobasidium pullulans* cultured in standard PM (5.0% sucrose and 0.1% N-sources). **a** Exopolysaccharide molecular weight. **b** Exopolysaccharide viscosity. *Bars* represent standard error

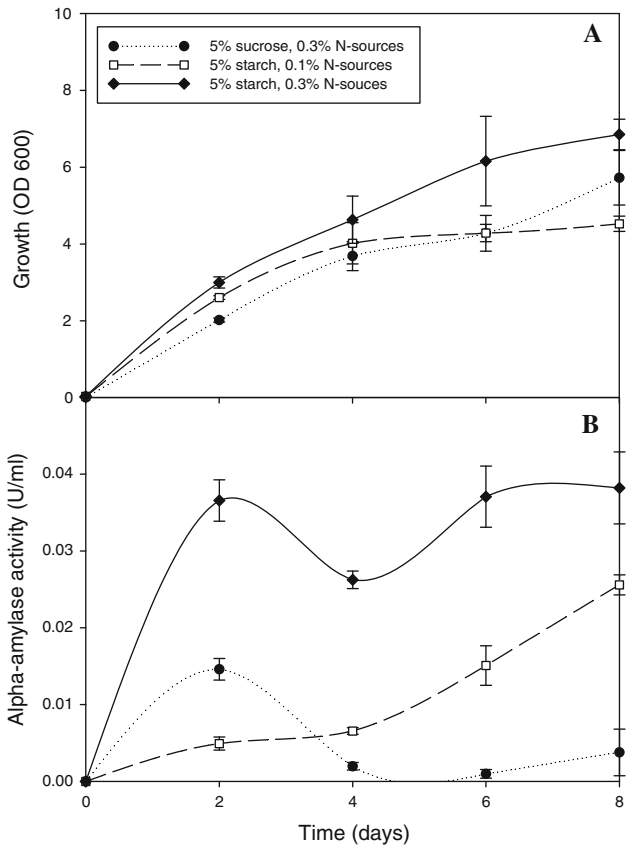

Fig. 3 Growth and α-amylase production by *Aureobasidium pullulans* strain NRRL Y-12974 grown in modified PM (5.0% sucrose and 0.3% nitrogen sources; 5.0% starch and 0.1% nitrogen sources; or 5.0% starch and 0.3% nitrogen sources). **a** Growth (OD_{600}). **b** α-Amylase production. *Bars* represent standard error

non-pullulan polysaccharides are not evident from NMR spectra, EPS from isolate CU 36 was only 41% sensitive to pullulanase. Strain CU 36 is a member of clade 5, and it should be noted that other isolates in this clade also make highly viscous EPS [14]. *A. pullulans* has been reported to produce a variety of polysaccharides other than pullulan [12].

Despite the fact that EPS molecular weight and viscosity generally fell during culture in standard PM, all samples contained less than 0.005 U/ml amylase and less than 0.002 U/ml pullulanase, considered the limits of detection (data not shown).

Growth and amylase production by strain NRRL Y-12974 in modified PM

Since *A. pullulans* strain NRRL Y-12974 produced the greatest yields of pullulan in standard PM, it was chosen for further studies in modified PM (Fig. 3). Cultures grown in medium containing 5.0% sucrose and 0.3% N-sources grew through the 8 days time course without the decrease in OD_{600} observed in standard PM (Fig. 3a). At the same time, EPS yields from this culture were reduced by about one third (data not shown). This supports the common observation that conditions of limiting N, as in standard PM, favor pullulan production at the expense of cell growth [12]. Strain NRRL Y-12974 also grew well in medium containing 5.0% starch and 0.1% N-sources, and even better in medium containing 5.0% starch and 0.3% N-sources (Fig. 3a). Despite good growth, the pH of all modified PM fell to only approx. pH 5.7, suggesting that different carbon and nitrogen sources may affect the amounts or types of acids produced.

NMR spectra of EPS from all five strains grown in standard PM were similar to the spectrum of pullulan standards (data not shown). However, the NMR spectra of EPS from 8 day starch-grown cultures of strain NRRL Y-12974 suggested a mixture of pullulan and residual starch, which was confirmed by pullulanase and amylase sensitivity assays (data not shown).

Even though cultures grew well in modified PM containing starch, α-amylase activities were relatively low (<0.04 U/ml, Fig. 3b). Activities were slightly higher in

cultures containing 5.0% starch and 0.3% N-sources than in cultures containing 5.0% starch and 0.1% N-sources. None of the cultures produced detectable pullulanase activity. The apparent molecular weight and viscosity of EPS from starch-grown cultures was low; however, this result is difficult to interpret since EPS included residual starch (data not shown).

Analysis of the α-amylase gene from *A. pullulans*

Cloning and sequence analysis

To further investigate the possible role of α-amylase in the degradation of pullulan, the gene was cloned and sequenced from *A. pullulans* strain NRRL Y-12974. The cloned gene then was used to estimate gene expression in cultures producing pullulan and amylase. Sequence analysis revealed a putative mRNA of 1,878 bp encoding an α-amylase of 625 amino acid residues. The complete genomic DNA sequence contains 2,247 bp, including 7 introns and 8 exons. The putative protein sequence is predicted (WoLF PSORT, [9]) to be secreted to the extracellular environment and likely contains a 22 amino acid signal peptide (SignalP, [3]). BlastP sequence analysis of the putative protein sequence reveals an α-amylase superfamily domain and C-terminal carbohydrate-binding module, family 20 domain. Figure 4 shows a phylogenetic tree of α-amylases, based on Clustal-W analysis of the entire coding region minus signal peptide of the most similar α-amylases in GenBank. The *A. pullulans* α-amylase was found to be unique with only 51–59% identity (Lipmann Pearson) to other amylases from fungi in the phylum Ascomycota, class Eurotiomycetes, Sordiomycetes, and Tremellomycetes [8]. Results show that the α-amylase from *A. pullulans* is the first described from a Dothideomycete fungus. Southern hybridization analysis showed only a single band of α-amylase fragment in genomic DNA digested with five restriction enzymes (Fig. 5), suggesting that *A. pullulans* strain NRRL Y-12974 carries only a single copy of this gene.

Expression of the α-amylase gene

RT–PCR was used to detect α-amylase gene expression in cultures of *A. pullulans* strain NRRL Y-12974 grown on either starch or sucrose. Amplification of an internal α-amylase fragment occurred with cDNA made from cells grown in PM containing both carbon sources. In both cases, PCR products were obtained only from cultures grown for 4, 6, and 8 days and not from 2-day-old cultures. More intense bands were produced from cultures grown on starch (Fig. 6a, lanes 9–12), compared to sucrose (Fig. 6a, lanes 13–16). No products were amplified from RNA controls lacking RT step, indicating that samples were free of DNA contamination. In order to determine if the lack of amplification from cDNA associated with cultures at day 2 was due to PCR inhibitors or incomplete RT step, we tested the ability to amplify part of the 26S rDNA cDNA in all samples. Product was amplified for all RNA (Fig. 6b); however, there was also some weak amplification in the no-RT samples, suggesting residual DNA contamination. Results indicate that the α-amylase transcript is present

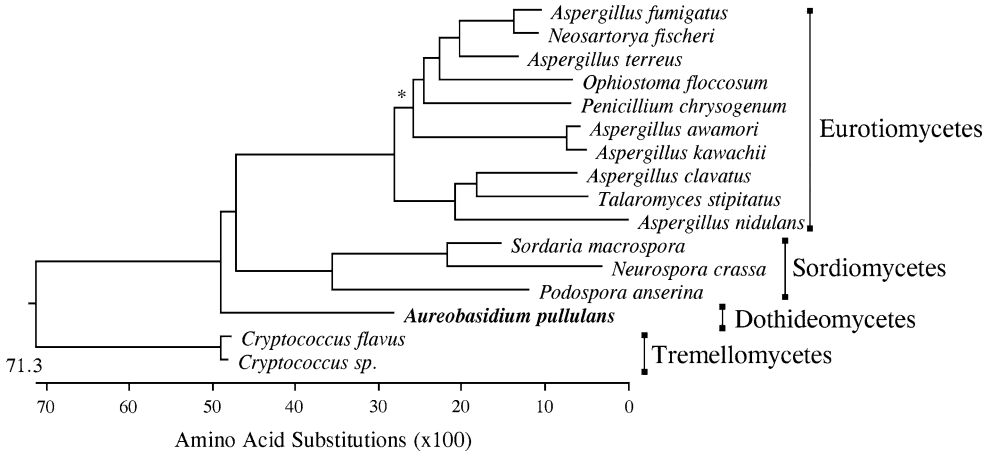

Fig. 4 Phylogenetic tree of α-amylases. Protein sequences minus signal peptide of the most similar α-amylases in GenBank were aligned by using Lasergene MegAlign (DNAStar, Madison, WI) with ClustalW parameters set to default. All nodes had bootstrap values of 90–100 (Bootstrap analysis included 1,000 trials with random seed of 111) with the exception of nodes marked **a** bootstrap values = 55–90, and **b** unable to calculate bootstrap due to Megalign rooting tree at this node. Amino acid substitutions shown at *bottom* of figure and fungal classes at the *right*. The GenBank accession numbers for the protein sequences are as follows: *A. fumigatus* (XP_749208), *N. fischeri* (XP_001265628), *A. terreus* (XP_001209405), *O. floccosum* (ABF72529), *P. chrysogenum* (XP_002560482), *A. awamori* (BAD06003), *A. kawachii* (BAA22993), *A. clavatus* (XP_001272245), *T. stipitatus* (XP_002478703), *A. nidulans* (XP_661006), *S. macrospora* (CBI56782), *N. crassa* (XP_964065), *P. anserina* (XP_001908940), *A. pullulans* (HM590632), *C. flavus* (ABS76467), and *Cryptococcus* sp. (BAA12010)

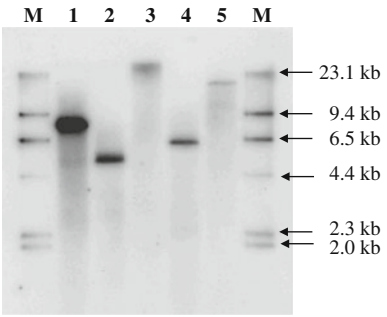

Fig. 5 Southern hybridization analysis of the α-amylase gene in *Aureobasidium pullulans* strain NRRL Y-12974. *Lanes 1–5* represent total DNA digested with *Xba*I, *Kpn*I, *Pst*I, *Pvu*II, and *Eco*RI, respectively. *Lane M* is a *Hin*dIII digested Lambda ladder [mixture of DIG labeled *Hin*dIII digested Lambda (20 ng) and unlabeled *Hin*dIII digested Lambda (150 ng)]

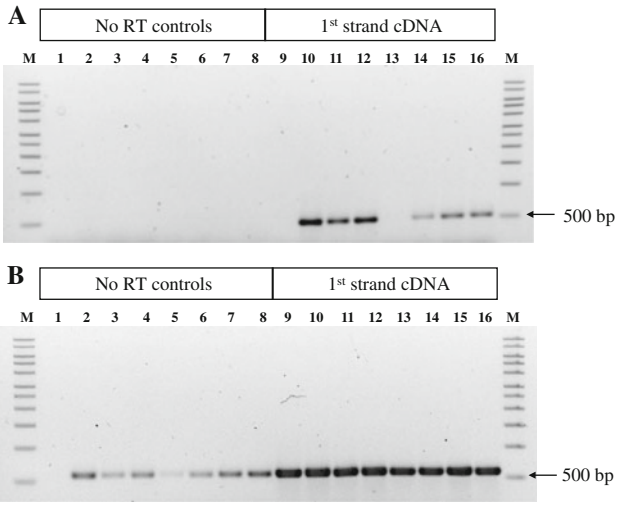

Fig. 6 RT–PCR amplification of **a** the α-amylase gene and **b** 26S rDNA from *A. pullulans* strain NRRL Y-12974. *Lanes 1–4* are RNA (no RT control) from NRRL Y-12974 cultured in PM (5.0% starch, 0.3% N-sources) for 2, 4, 6, and 8 days, respectively. *Lanes 5–8* are RNA (no RT control) from NRRL Y-12974 cultured in PM (5.0% sucrose, 0.3% N-sources) for 2, 4, 6, and 8 days, respectively. *Lanes 9–12* are the 1st strand cDNA from NRRL Y-12974 cultured in PM (5.0% starch, 0.3% N-sources) for 2, 4, 6, and 8 days, respectively. *Lanes 13–16* are the 1st strand cDNA from NRRL Y-12974 cultured in PM (5.0% sucrose, 0.3% N-sources) for 2, 4, 6, and 8 days, respectively. *Lane M* is a DNA ladder, Directload, 1kB (Sigma), 250 μg

from days 4 to 8 in cells grown in PM containing either sucrose or starch.

Discussion

Pullulan is an extracellular polysaccharide produced by strains of the fungus *A. pullulans*. The molecular weight of pullulan depends on the specific strain [16] and culture conditions used [7, 21, 24]. Furthermore, molecular weight generally decreases with culture age and this is generally believed to be the result of hydrolytic enzymes in the culture supernatant [12]. Among these enzymes, α-amylase is of particular interest. Early studies demonstrated that the minor maltotetraose subunits in pullulan are substrates for α-amylase [4]. Hydrolysis of these sites would result in a rapid reduction of pullulan molecular weight and viscosity, conceivably regulated in response to environmental conditions. Consistent with these observations, the five representative strains tested here produced EPS on standard PM that varied in initial molecular weight and generally fell to lower molecular weight forms during cultivation. However, α-amylase activities in these cultures were below the limits of detection. Some previous studies found that levels of α-amylase were low in pullulan-producing cultures [10, 18, 20]. Starch-grown cultures had low levels of α-amylase and low molecular weight EPS; however, this EPS included residual starch, which made analysis of pullulan difficult. It is conceivable that low levels of amylase in standard PM were responsible for the reduction in pullulan molecular weight. The reduction in pullulan molecular weight did appear to be enzymatic. In control experiments, purified EPS of the isolate CU 20 obtained at day 2 was resuspended in 50 mM sodium acetate buffer at pH 3.5 and agitated for 8 days. This sample was chosen as a control because it contained by far the highest molecular weight pullulan (Fig. 2). The sample suffered no loss of molecular weight or viscosity in the absence of enzyme (data not shown). When either bacterial α-amylase or glucoamylase was added to these controls, the EPS showed a slight reduction in molecular weight and viscosity over 8 days (data not shown). This confirms that pullulan is susceptible to these enzymes; however, the reduction in molecular weight was not nearly as great as that observed in vivo. It is possible that the corresponding enzymes from *A. pullulans* have a higher specificity for pullulan. Alternatively, other uncharacterized enzymes may be involved in the degradation of pullulan in late cultures.

RT–PCR was used to detect expression of α-amylase from *A. pullulans*. Results showed that the gene was transcribed during cultivation in both starch and sucrose media. However, there were no PCR products from either sucrose or starch cultures at day 2, suggesting that gene expression began after this time. In contrast, amylase assays showed low activities at days 2–8 in starch-grown cultures, and no detectable activity in sucrose-grown cultures. At this time it is unclear why there is an imperfect correlation between transcription and the appearance of enzyme activity.

The α-amylase gene sequence of *A. pullulans* will be useful for future studies on expression of this gene or to study the structure and properties of the α-amylase enzyme

and determine its activity towards pullulan. The cloned gene could also be used in genetic engineering studies to eliminate or modify α-amylase expression in *A. pullulans* in order to more conclusively determine if this enzyme impacts the molecular weight of the pullulan. In conclusion, the results obtained from this study contribute to a better understanding of pullulan production which can be applied to strain improvements in the future.

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