Color Variants of *Aureobasidium pullulans* Overproduce Xylanase with Extremely High Specific Activity

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Xylanase activity from naturally occurring color variants of *Aureobasidium pullulans* was associated with extracellular monomeric proteins of 20 to 21 kilodaltons. Xylanase represented nearly half the total extracellular protein, with a yield of up to 0.3 g of xylanase per liter. The specific activity of partially purified xylanase exceeded 2,000 IU/mg. Xylanase from typically pigmented strains appeared similar to that from color variants with respect to molecular weight, pH and temperature optima, and specific activity of purified (but not crude) enzyme. However, xylanase from typical strains made up only about 1.0% of total extracellular protein. Xylanase from strains of *Cryptococcus albidus* was associated with abundant proteins of about 43 kilodaltons and showed much lower specific activity.

Xylan is a heteropolymer characterized by a backbone of (β 1-4)-linked xylose (19). As the major component of hemicellulose, xylan accounts for 20 to 30% of the dry weight of agricultural residues (8). Since it has been estimated that 500 million tons of such material could be annually available from the residues of major crops (8), xylan is an abundant, underutilized resource.

While several species of fungi have been reported to produce extracellular xylanase (6), only three of these may have a yeastlike morphology. These are *Aureobasidium pullulans*, *Cryptococcus albidus*, and *Trichosporon beigeli* (1, 7, 9, 15). Xylanase has been purified from *T. beigeli* (16), and the enzyme from *C. albidus* has been extensively characterized by Biely and co-workers (see, e.g., 2-5). These yeastlike species offer an advantage in the study of xylanases in that they produce no cellulases (5, 11, 16). (Cellulases may have broad substrate specificities that can include xylan [17].) For this reason, as well as the manipulative advantages offered by yeastlike morphology, we sought a model strain from among these species to serve as a source of true xylanase with high specific activity.

In a survey of 58 yeastlike strains from the Agricultural Research Service Culture Collection, Peoria, Ill., certain isolates of *A. pullulans* were found to be extraordinary producers of xylanase (11). These high-level producers had previously been described as color variants of the species (19). In place of the off-white to black appearance of typically pigmented strains, color variant strains exhibited brilliant pigments of red, yellow, orange, or purple. Although *A. pullulans* is a ubiquitous species, color variant strains have been isolated only from tropical regions, including sites in Florida, Java, and Puerto Rico (19). Strains of *C. albidus* produced extracellular xylanase at levels and with crude specific activities similar to those of xylanases produced by typical strains of *A. pullulans* (11). The present study was undertaken to compare protein characteristics of xylanases from these sources of yeastlike fungi.

MATERIALS AND METHODS

Isolation of color variant strains of *A. pullulans*. Color variant strains of *A. pullulans* are commonly found in vegetation in tropical regions (19). Seagrass from Mangrove Cay, Fla., was the kind gift of J. Fell, University of Miami.

The material was vortexed vigorously in sterile water, and the resulting suspension was spread onto rich-medium plates (YM [11]) that contained 10 μ g of chloramphenicol per ml. Pigmented yeastlike colonies were chosen for purification. Strains Y-12,971 through Y-12,974 were classified as color variants of *A. pullulans* on the basis of morphological characteristics and assimilation tests. Other strains of *A. pullulans* and *C. albidus* were obtained from the Agricultural Research Service Culture Collection.

Xylanase preparation. Organisms were cultivated in a basal synthetic medium containing xylan from oat spelts (Sigma Chemical Co., St. Louis, Mo.) at 1.0% (wt/vol), as previously described (11). Cleared (cell-free) culture supernatants were dialyzed against 12.5 mM Tris (pH 7.3) containing 0.02% sodium azide, as previously described (11).

Enzyme assays. Xylanase was assayed by a modification of the dinitrosalicylic acid method (13) previously described (11), except that assays were further modified to accommodate an automated microtiter dish reader (Dynatech Laboratories, Inc., Alexandria, Va.). Samples were assayed to within 5% standard deviation. Standard xylanase assay conditions were 30°C and pH 5.0. One unit of xylanase activity is defined as that releasing 1 μ mol of xylose equivalents per min (international units). Protein assays were performed by the method of Lowry et al. (12), with bovine serum albumin as standard.

Electrophoresis. Proteins were resolved by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 3.0% stacking, 12.35% resolving gels) by the method of Laemmli (10). Gels were silver stained by the method of Wray et al. (20). Molecular weight standards were the high-molecular-weight-range set from Bethesda Research Laboratories, Gaithersburg, Md. Unknowns were estimated by the method of Shapiro et al. (14).

Gel filtration. Extracellular proteins were separated by gel filtration on an Econo-column (2.5 by 75 cm; Bio-Rad Laboratories, Richmond, Calif.) packed with Sephadex G-75 (Pharmacia, Inc., Piscataway, N.J.). Dialyzed 1.5-ml culture supernatant samples (unconcentrated, in 10% glycerol containing 150 to 1,050 μ g of protein) were applied to the column and eluted with dialysis buffer (described above) at 0.6 ml/min; 4-ml fractions were collected. Fractions were assayed for protein and xylanase activity as described above.

TABLE 1. Extracellular xylanase from *C. albidus* and *A. pullulans* strains

Strain (pigment) ^a	Extracellular protein (mg/ml)	Xylanase ^b	
		Activity (U/ml)	Sp act (U/mg)
<i>A. pullulans</i> YB-4588 (CV)	0.381	47.2	124.0
<i>A. pullulans</i> YB-4026 (CV)	0.258	12.0	50.0
<i>A. pullulans</i> Y-6754a (CV)	0.381	62.9	165.0
<i>A. pullulans</i> Y-2311-1 (CV)	0.691	373.0	540.0
<i>A. pullulans</i> Y-12,971 (CV)	0.209	81.3	389.0
<i>A. pullulans</i> Y-12,972 (CV)	0.201	59.1	294.0
<i>A. pullulans</i> Y-12,973 (CV)	0.167	58.7	352.0
<i>A. pullulans</i> Y-12,974 (CV)	0.148	59.5	402.0
<i>A. pullulans</i> Y-2312 (TP)	0.245	2.67	10.9
<i>A. pullulans</i> Y-2567 (TP)	0.278	5.33	19.2
<i>A. pullulans</i> YB-4029 (TP)	0.271	7.29	26.9
<i>A. pullulans</i> YB-4587 (TP)	0.236	4.58	19.4
<i>A. pullulans</i> Y-6992 (TP)	0.266	2.59	9.7
<i>C. albidus</i> Y-10,953	0.158	2.41	15.3
<i>C. albidus</i> Y-1516	0.196	4.13	21.2
<i>C. albidus</i> Y-1400	0.170	3.61	21.2

^a Abbreviations: CV, color variant; TP, typically pigmented.^b Assayed under standard conditions.

RESULTS

Xylanase production by yeastlike strains. Table 1 summarizes crude xylanase activities from newly isolated as well as previously examined (11) strains under standard assay conditions (see Materials and Methods). All cultures were grown with xylan as the sole carbon source. The results in Table 1 represent the culture preparations that were the source of protein for the subsequent SDS-PAGE survey and are characteristic. *A. pullulans* color variants YB-4588, YB-4026, Y-6754a, and Y-2311-1 were isolated more than three decades ago (19). For this comparative study, four additional color variants (strains Y-12,971 through Y-12,974) were newly isolated as described in Materials and Methods. Typically pigmented strains of *A. pullulans* (Y-2312, Y-2567, YB-4029, YB-4587, and Y-6992) were chosen as representative with respect to xylanase production and regulation. Two of these (YB-4029 and YB-4587) were also coisolates of color variants (those with consecutive strain numbers). *C. albidus* strains were those found to produce at least 1.0 IU of xylanase per ml.

Under standard assay conditions (pH 5.0, 30°C), strains of *C. albidus* and typically pigmented strains of *A. pullulans* produced 2.4 to 7.3 IU of xylanase per ml, with similar specific activities of 10.0 to 27.0 IU/mg of protein (Table 1). Color variants of *A. pullulans*, with the exception of strain YB-4026, a relatively low producer, yielded between 47.0 and 373.0 IU/ml, with specific activities up to 540.0 IU/mg (Table 1).

Survey of extracellular proteins. Figure 1 shows extracellular proteins from the strains shown in Table 1 as resolved by SDS-PAGE and silver stained. Samples were normalized with respect to protein amount (10 µg per lane). Silver stains were exhaustively developed for maximal sensitivity; less abundant protein species are thus probably overrepresented.

Lanes A through H represent color variant strains of *A. pullulans*, as described in the legend. Although several other similarities are apparent, all color variant strains secreted an abundant protein, estimated to be 20 kilodaltons (kDa) in molecular mass. An abundant 21-kDa species was also produced by five of eight strains. When gels were stained with Coomassie brilliant blue R instead of silver, these two

were the only proteins smaller than 200 kDa observed. These proteins were produced when strains were grown on xylan or xylose but not on glucose; their appearance was thus correlated with the expression of xylanase activity. While very high-molecular-weight species were also common to all color variants, extracellular xylanases characteristically have low molecular weight (5, 16, 18). Strain Y-2311-1 (lane D) was chosen as a representative color variant for partial purification of xylanase, since it was the best producer of enzyme activity (Table 1), was typical with respect to xylanase regulation (11), showed a typical protein pattern, and produced both a 20- and a 21-kDa species (Fig. 1).

Lanes I through M show proteins secreted by typically pigmented strains of *A. pullulans*. In contrast to the patterns of color variants, no abundant proteins were found common to all five strains. However, all typical strains produced one or two proteins of low abundance in the 20- to 21-kDa range. These small species were also correlated with xylanase production, in that they were found in xylan- and xylose- but not glucose-grown culture supernatants. Strain Y-2567 (lane J) was chosen as representative for further study.

Strains of *C. albidus* (lanes N through P) showed a less complex protein pattern that featured a major 43-kDa species. These abundant 43-kDa proteins were also specifically correlated with the production of xylanase. Two additional *C. albidus* strains, Y-2540 and Y-1501, also produced 43-kDa proteins when grown on xylan, although protein and activity levels were low (data not shown).

Gel filtration of proteins from representative strains. Figure 2A illustrates the separation of proteins from color variant *A. pullulans* Y-2311-1 (Fig. 1, lane D) on Sephadex G75. Blue dextran 2000 eluted in fractions 15 to 22, which thus marked the void volume of the column. One narrow and one broad peak of protein, centered at fractions 28 and 37, respectively, were resolved. Xylanase activity was exclusively associated with the latter, broad peak. This peak made up 45% of total protein eluted. SDS-PAGE of the G75 column fractions is shown in Fig. 3. Fraction 28 was found to be composed exclusively of the 34-kDa protein secreted by strain Y-2311-1 (Fig. 3, lane B). Fraction 37 contained a mixture of 20- and 21-kDa proteins (Fig. 3, lane C). When assayed under optimal conditions of temperature and pH (as determined below), this fraction exhibited a specific activity of 2,110 IU/mg. The 20- and 21-kDa species may both

FIG. 1. SDS-PAGE of extracellular proteins produced by color variant strains of *A. pullulans* and strains of *C. albidus* grown on xylan. Protein samples (10 µg per lane) were resolved on 12.5% SDS-polyacrylamide gels and silver stained. Lanes A through H show color variant strains of *A. pullulans*: A, YB-4588; B, YB-4026; C, Y-6754a; D, Y-2311-1; E, Y-12,971; F, Y-12,972; G, Y-12,973; H, Y-12,974. Lanes I through M show typically pigmented strains of *A. pullulans*: I, Y-2312; J, Y-2567; K, YB-4029; L, YB-4587; M, Y-6992. Lanes N through P show strains of *C. albidus*: N, Y-10,953; O, Y-1516; P, Y-1400.

represent xylanase isozymes, as suggested by the symmetry of the activity and protein peaks in Fig. 2A. As addressed below, the native subunit size of xylanase appears to be monomeric. Further, inactive aggregates of these species must not exist, since the 20- and 21-kDa xylanase species were not released from high-molecular-weight material of the void volume (Fig. 3, lane A).

A very different gel filtration elution pattern was observed for extracellular proteins from the typically pigmented strain Y-2567 (Fig. 2B). Nearly all protein eluted in the void volume, before fraction 23. Xylanase activity, however, was found at the same position as xylanase from strain Y-2311-1. Figure 3, lanes D and E, show protein patterns of fractions 17 and 37. Fraction 37 was enriched for the two low-abundance proteins of about 20 kDa. Because of the baseline protein levels in the xylanase peak, it was necessary to concentrate pooled xylanase fractions 32 through 41 by ultrafiltration to obtain an estimate of specific activity. The process of ultrafiltration resulted in an 80% loss of enzyme activity without loss of protein. On the basis of the initial, preconcentrated activity of fractions 32 through 41, the specific activity of this partially purified xylanase was estimated to be 1,690 IU/mg. Similar inactivation of xylanase from color variant strains was observed upon concentration, suggesting the validity of the higher estimate.

Protein from *C. albidus* Y-1516 (illustrated in Fig. 1, lane P) produced, upon gel filtration, a single sharp peak of xylanase activity centered at fraction 26. We conclude that the abundant 43-kDa protein species common to strains of *C. albidus* is xylanase.

Determination of native subunit composition of xylanase.

FIG. 2. G75 Sephadex column filtration of extracellular proteins from representative strains of *A. pullulans*. (A) Color variant strain Y-2311-1; (B) typically pigmented strain Y-2567. Symbols: ■, protein (in micrograms per milliliter); ▲, xylanase activity (in international units per milliliter).

FIG. 3. SDS-PAGE of proteins from G75 Sephadex column gel filtration fractions. Samples are from the fractionations illustrated in Fig. 2 for representative color variant and typically pigmented strains of *A. pullulans*. Fraction samples containing 10 μ g of protein, concentrated by ultrafiltration if necessary, were resolved on 12.5% SDS-polyacrylamide gels and silver stained. Lanes A through C show samples from fractionation of color variant strain Y-2311-1 (illustrated in Fig. 2A): A, fraction 17; B, fraction 28; C, fraction 37. Lanes E and F show samples from fractionation of typically pigmented strain Y-2567 (illustrated in Fig. 2B): E, fraction 18; F, fraction 37.

Appropriate molecular weight standards were separated under our conditions of gel filtration. On the basis of this calibration curve, the native molecular masses of proteins resolved by gel filtration were compared with their subunit molecular masses as estimated under the denaturing conditions of SDS-PAGE. The native molecular mass of the 20- to 21-kDa subunit xylanases from *A. pullulans* Y-2311-1 and Y-2567 was estimated to be 14 kDa. Xylanase from *C. albidus* Y-1516 was estimated to have a native molecular mass of 39 kDa, which compares with its apparent subunit size of 43 kDa. The 34-kDa protein of unknown identity from strain Y-2311-1 was estimated to have a native size of 32 kDa. Thus, xylanases from all three strains were found to be monomeric. Since the column retention times of these native proteins were in fact slightly longer than predicted by their subunit size, it is possible that they are particularly spherical proteins as compared with the molecular weight standards used. It may be noted that the molecular weight standards are all proteins of intracellular origin.

pH and temperature optima of xylanase from representative strains. pH and temperature optima were determined for crude xylanase from color variant *A. pullulans* Y-2311-1, typically pigmented *A. pullulans* Y-2567, and *C. albidus* Y-1516. Xylanase from both strains of *A. pullulans* shared a broad pH optimum (Fig. 4A). Maximal activity was at pH 4.0 to 4.5, but at least 80% of maximal activity remained between pH 3.0 and 5.5. In contrast, xylanase from *C. albidus* Y-1516 exhibited maximal activity at pH 5.0 to 5.5.

Figure 4B compares temperature optima of xylanase from the three strains. Xylanase from color variant and typical strains of *A. pullulans* again shared similar broad optima, with maximal activity between 35 and 50°C. Xylanase from *C. albidus* Y-1516 exhibited a quite different temperature curve, with a sharp peak of maximal activity at 60°C.

Time course of growth and xylanase production by *A. pullulans* strains with xylanase as the sole carbon source. Parallel time course cultures of strains Y-2311-1 (*A. pullulans* color variant strain) and Y-2567 (*A. pullulans* typically pigmented strain) were monitored for growth and xylanase production (Fig. 5). During exponential growth, color variant and typi-

FIG. 4. pH and temperature optima of xylanase from representative strains. Assays were performed under standard conditions of 30°C and pH 5.0, with the exception of the tested variable. For studies of pH optima, substrate buffer was titrated to the desired test pH with acetic acid or sodium hydroxide and returned to pH 5.0 before assays were developed, since the dinitrosalicylic acid assay was found to be pH sensitive. (A) pH optima; (B) temperature optima. Symbols: ■, color variant *A. pullulans* Y-2311-1; ▲, typically pigmented *A. pullulans* Y-2567; X, *C. albidus* Y-1516.

cal strains of *A. pullulans* showed identical doubling times of 4.1 h. While the color variant strain showed a slightly higher yield of cells by direct count, this difference was not seen in dry-weight measurements of larger cultures and probably reflected the greater tendency of strain Y-2567 to become hyphal in older cultures. Interestingly, typical strain Y-2567 secreted xylanase only during exponential growth, while color variant strain Y-2311-1 produced xylanase at a constant rate up to 1 day into stationary phase (and continued to slowly accumulate enzyme through 1 week [data not shown]). This difference, while of practical importance for enzyme production, does not, however, account for the greater enzyme yield of the color variant.

DISCUSSION

Color variants of *A. pullulans* produced a remarkable xylanase that has a specific activity 1 order of magnitude higher than those of previously characterized xylanases (5, 6, 16). Xylanase was abundantly produced, with a yield of up to 1/3 g/liter and made up about half the total extracellular protein.

Color variants further appeared to be overproducers of xylanase, since typically pigmented strains secreted a similar enzyme, but in far lower amounts. Xylanase from a representative typical strain was found to be similar with respect to molecular weight, pH and temperature optima, and spe-

cific activity of the partially purified enzyme (Table 2). Regulation of xylanase was also similar among color variant and typical strains of *A. pullulans* and distinct from *C. albidus* strains in that xylose serves to induce xylanase in the former species. Overproduction is also an apt term in that the high xylanase levels in color variants serve no obvious purpose. Under laboratory conditions of growth on xylan, typical strains achieved the same doubling times and similar growth yields as color variants. *C. albidus* Y-1516 showed a similar growth rate (Table 2), further suggesting that the moderate levels of xylanase activity are not limiting for growth. Color variant and typical isolates of *A. pullulans* have been collected side-by-side in tropical regions (19). Nevertheless, overproduction might offer a competitive advantage under natural conditions. Overproduction might also serve to create a distinctive ecological niche within a single environment. As noted, *A. pullulans* strains produce no cellulases. Excess xylanase could serve to promote mutualistic relationships by benefitting cellulase producers.

We do not suspect that pigment production itself is related to xylanase overproduction. Pigments are brightest during growth on simple sugars, including glucose, and faint during growth on xylan. Furthermore, our best xylanase producer, strain Y-2311-1, is a faintly pigmented derivative of red strain Y-2311, and no differences in xylanase production were found between these strains. Rather, we view color variation as a taxonomic marker. DNA-DNA hybridization experiments suggest that color variants may constitute a species that is closely related to but distinct from *A. pullulans* (C. P. Kurtzman, unpublished data).

Although the activity concentration and crude specific activity of xylanase from *C. albidus* strains resembled those of xylanase from typical strains of *A. pullulans* (Table 1), the enzyme from *C. albidus* was far different. While typical strains of *A. pullulans* produced low levels of highly active

FIG. 5. Time course of growth and xylanase production by *A. pullulans* strains with xylan as sole carbon source. (A) color variant *A. pullulans* Y-2311-1; (B) typically pigmented *A. pullulans* Y-2567. Symbols: ■, log cells per ml; ▲, percent maximal xylanase activity.

TABLE 2. Summary of xylanase characteristics

Strain (pigment) ^a	Xylanase characteristic							
	Induced during growth on xylose	Doubling time on xylan (h)	Mol mass (kDa)	Sp act (U/mg ^b)	pH optimum	Temp optimum (°C)	Yield (μg/ml)	% Total
<i>A. pullulans</i> Y-2311-1 (CV)	Yes	4.1	20–21	2,110	4.0–4.5	35–50	300	45
<i>A. pullulans</i> Y-2567 (TP)	Yes	4.1	20–21	1,690	4.0–4.5	35–50	2–3	1
<i>C. albidus</i> Y-1516	No	4.4	43	50	5.0–5.8	60	200	>90

^a Abbreviations: CV, color variant; TP, typically pigmented.

^b Partially purified, assayed under optimal conditions.

enzyme, strains of *C. albidus* produced an abundant xylanase, of 43 kDa, with very different enzyme properties that include much lower specific activity.

The well-characterized xylanase from *C. albidus* CCY 17-4-1, studied by Biely et al. (2–4) has been described as a 26-kDa enzyme with a specific activity of 88 IU/mg when pure (5). Strain CCY 17-4-1 was chosen by those workers from a screen of yeastlike strains because of its xylanase yield (1), but nevertheless it is considered to be the type strains of the species (Centraalbureau voor Schimmelcultures strain 142) (1). Our results suggest that it may be atypical with respect to xylanase production.

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