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Isolation of New *Aureobasidium* Strains That Produce High-Molecular-Weight Pullulan with Reduced Pigmentation

THOMAS J. POLLOCK,* LINDA THORNE, AND RICHARD W. ARMENTROUT

Shin-Etsu Bio, Inc., 6650 Lusk Boulevard, Suite B-106, San Diego, California 92121

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New isolates of *Aureobasidium pullulans* were obtained from plant leaf surfaces gathered in San Diego County. The new fungal isolates were identified as *A. pullulans* on the basis of the appearance of polymorphic colonies formed on agar plates, the electrophoretic profiles of repeated genomic DNA sequences, and the production of pullulan in shake flask cultures. The isolates showed different degrees of pigmentation. One of the natural isolates was nonpigmented under mock production conditions in liquid culture, but was still able to synthesize a reduced amount of pigment on agar plates at late times. A mutagenic treatment with ethidium bromide produced derivatives of normally pigmented natural isolates that exhibited an increased tendency toward yeastlike growth and reduced pigmentation. Additionally, some of the new isolates and mutant derivatives accumulated pullulan of relatively high molecular weight in the culture broths.

Pullulan is one of the few neutral water-soluble polysaccharides that can be produced in large quantities by fermentation of a microorganism, in this case the fungus *Aureobasidium pullulans*. This fungus is believed to play a central part in initiating the natural breakdown and recycling of plant material, but is also saprophytic on a wide variety of substrates in diverse habitats, from painted surfaces to lymph nodes (4, 10). The genus name is misleading since the fungus produces a black pigment and since no basidia are known. Although its omnivorous appetite is impressive, we were interested mainly in improving the process for producing pullulan. Applications for the polymer are as films, oxygen-impermeable coatings, adhesives, and fibers (26); and a dielectric material in the form of cyanoethylpullulan (18). For these special uses, polymer purity and molecular weight are important properties.

The biosynthesis of pullulan was studied in detail and reviewed by Catley (3), and a microbial fermentation and recovery process was developed for large-scale production (8). Two undesirable features of fermentation of *A. pullulans* are readily observed. The first problem is a decrease in culture viscosity late during submerged growth, owing to a decrease in the average molecular weight of the accumulated extracellular pullulan from 3×10^6–6×10^6 to 1×10^5–2×10^5 (2, 7). Although it is possible to adjust the culture conditions (initial pH, phosphate concentration, carbon source, inoculum size, and time of harvest) to alter the average molecular weight of the polymer, these adjustments generally reduce the yield (8). The second problem is the simultaneous synthesis of dark melaninlike pigment, which contaminates the pullulan (14). A multistep decolorization process with activated carbon followed by filtration is currently used to remove the pigment (8).

Color variants of *A. pullulans* that accumulate less melanin are known (25). Some of these were analyzed for pullulan productivity, purity, and molecular weight and compared with the previously studied isolates in terms of these parameters. One strain, Y-12974, shows less pigmentation in polysaccharides recovered from the medium, but the yield of polysaccharide from sucrose is relatively low (20 to 30%) (13, 21). As much as 25 to 30% of this polysaccharide is not pullulan, and the molecular weight is low relative to that of the polysaccharides accumulated by the preexisting strains tested in parallel (13, 21). Other nonpigmented pullulan-producing strains were recently isolated from cocoons of Canadian leaf-cutting insects and exhibit similar productivities, but have not been as extensively characterized (17). In this report we describe new isolates of *A. pullulans* and derived mutant strains that produce pullulan not only with less pigmentation, but also with uniquely high molecular weight and in yields comparable to those of previously known strains.

MATERIALS AND METHODS

Fungal strains and culture media. Our strain designations, synonyms, and sources for preexisting and new isolates of *A. pullulans* and *Saccharomyces cerevisiae* are listed in Table 1. Shake flask cultures were grown in either a complex nitrogen medium (P1) or a minimal salts medium (P2). Medium P1 included the following (per liter of deionized water): 2 g of yeast extract (Difco); 0.5 g of $(NH_4)_2SO_4$; 1 g of NaCl; 0.2 g of $MgSO_4$; 3 g of K_2HPO_4; 0.01 g each of $FeSO_4$, $MnSO_4$, and $ZnSO_4$; and HCl to pH 6.0. Medium P2 included the following (per liter of deionized water): 1 g of $(NH_4)_2HPO_4$; 0.5 g of NaCl; 0.05 g of $MgSO_4 \cdot 7H_2O$; 2 g of K_2HPO_4; 0.01 g each of $FeSO_4$, $MnSO_4$, and $ZnSO_4$; and HCl to pH 7.0. Either sucrose or corn syrup (Globe 1632 [Corn Products], which was 82.5% as the dry substance and 43.2 Baume and 63-66 dextrose equivalents) was added to 1 to 5% (grams [dry weight] per 100 ml). Agar was included at 15 g/liter for solid plates.

Isolation of *A. pullulans* by selective enrichment. One attached leaf was removed from each plant and soaked in sterile water for 3 days at 25°C, and then 0.1 ml was transferred to 10 ml of P2 minimal salts medium (pH 4) containing 1% (wt/vol) corn syrup and 10 μg of chloramphenicol per ml. After 2 days of shaking at 25°C the turbid culture was allowed to sit undisturbed for 20 min to allow filaments and aggregates to settle to the bottom. About 10 μl from the upper, partially clarified phase that was enriched for yeastlike cells was spread onto agar plates containing P2 medium (pH 5), 1% (wt/vol) corn syrup, and 10 μg of

* Corresponding author.

TABLE 1. Fungal strains used in this study

No.	Original name[a]	Source	Reference
F6	*S. cerevisiae* DL1α	S. Subramani, UCSD	
AP1	IFO4464	Fermentation Institute, Osaka, Japan	8
AP2	IFO6353	Fermentation Institute, Osaka, Japan	8
AP3	QM3092	ATCC 9348	3
AP4	NRRL Y6220	ATCC 34647	7
AP5	NRRL Y12996	ATCC 42023	27
AP7	S-1	NRRL Y17005	23
AP9	Y12974	NRRL, Peoria, Ill.	11
AP11		*Ficus rubiginosa* leaf, Sorrento Mesa, Calif.	
AP24		Ethidium-treated derivative of AP11	
AP41		UV-treated derivative of AP24	
AP26		*Ficus rubiginosa* leaf, Sorrento Mesa, Calif.	
AP27		*Ficus rubiginosa* leaf, Sorrento Mesa, Calif.	
AP30		*Acacia* leaf, La Jolla, Calif.	
AP42		Ethidium-treated derivative of AP30	
AP31		*Nerium oleander* leaf, La Jolla, Calif.	
AP32		*Liquidambar styraciflua* leaf, La Jolla, Calif.	
AP33		*Quercus kelloggi* leaf, Palomar Mountain, Calif.	
AP34		*Raphiolepis indica* leaf, University City, Calif.	
AP35		*Vitis vinifera* leaf, University City, Calif.	
AP36		*Magnolia grandiflora* leaf, University City, Calif.	

[a] All strains except DL1α are *A. pullulans*.

chloramphenicol per ml. After 4 days, independent colonies were purified by replating and tested in shake flasks for secretion of isopropyl alcohol (IPA)-precipitable polysaccharides and culture pigmentation. One isolate from each plant was selected for further study on the basis of polysaccharide yield and low pigmentation.

DNA isolation, restriction, and electrophoretic analysis. Cells were cultured overnight to mid-log phase in P2 medium with 1% (wt/vol) sucrose, concentrated by centrifugation (5,000 × *g* for 5 min), and resuspended at 10^9 cells per ml in 1 M sorbitol–25 mM EDTA–25 mM dithiothreitol (pH 7.0). Following a second centrifugation, protoplasts were prepared by resuspending 2.5×10^8 cells for 15 min at 37°C in 0.5 ml of a solution containing 1 M sorbitol, 25 mM EDTA, and 2.5 mg of lysing enzyme (from *Trichoderma harzianum* [Sigma]). Following a third centrifugation, protoplasts were lysed by resuspension in 0.75 ml of 10× TE (50 mM Tris-HCl, 10 mM EDTA [pH 8]) containing 1% (wt/vol) sodium dodecyl sulfate and then immediately mixed with 0.5 ml of phenol-chloroform (1:1 and saturated with water) to reduce nucleolytic degradation. After centrifugation of the emulsion at 10,000 × *g* for 2 min, the viscous upper phase was precipitated with 1 volume of ethanol at 4°C and centrifuged at 10,000 × *g* for 10 min at 25°C. The precipitate was resuspended for 15 min at 25°C in 0.5 ml of 1× TE containing 100 μg of RNase A (Sigma) and then extracted with phenol-chloroform, precipitated with ethanol, and resuspended in 0.11 ml of 1× TE. The DNA was high molecular weight as seen by gel electrophoresis. DNA samples were digested to completion with *Eco*RI (Sigma) in 50 mM Tris-HCl–10 mM $MgCl_2$–50 mM NaCl–0.1 mg of bovine serum albumin (Sigma) per ml (pH 8), electrophoresed through 1% (wt/vol) agarose gels with Tris-acetate buffer, and stained with ethidium bromide (16).

Treatment with ethidium bromide. The parental strain was grown in 10 ml of P1 medium with 5% (wt/vol) sucrose to a density of about 4×10^6 to 5×10^6. Ethidium bromide (30 μl from a 10-mg/ml solution in 25% [vol/vol] ethanol in water) was added, and shaking was continued for 3 to 4 h at about 25°C. During this period the cells tripled in number. The treated cells were centrifuged (5,000 × *g* for 5 min), washed with deionized water, and recentrifuged and the cell pellet was suspended in 30 ml of P1 medium with 15% (vol/vol) glycerol replacing the sugar and lacking yeast extract and $(NH_4)_2SO_4$. A sample of the treated cells was diluted by about 10^4-fold in the same solution and spread onto agar plates containing modified P1 medium with 0.2% (wt/vol) Pharmamedia (Traders' Protein), no yeast extract, 0.025% (wt/vol) $(NH_4)_2SO_4$, and 0.2% (wt/vol) glucose and buffered to pH 7. The inclusion of Pharmamedia enhanced pigment formation. Nonpigmented or "yeasty" colonies were purified. In certain cases, ethidium-treated cells were exposed to a low dose of UV irradiation (20 to 50% survival) before plating.

Measurement of polysaccharide yield and viscosity. The fungal cultures were prepared as follows. A small sample of cells of each strain was removed from the −70°C freezer, spread on P1 (1% sucrose) agar plates, and incubated at 28°C for 5 days. A loopful of cells was added to 2.5 ml of P2 (5% sucrose) liquid medium and shaken overnight to prepare a seed culture. A sample of cells, equal in number and about 0.2 ml in volume, was added to each replicate flask containing 15 ml of P2 medium (5% sucrose), and the cultures were shaken at 25°C for 66 h. Each sample was diluted with 1 volume of deionized water and centrifuged, and the polysaccharides were recovered from the clarified broth by precipitation with IPA. One volume of IPA was mixed into an aqueous sample to precipitate polysaccharides. The precipitate was removed with forceps and dried to constant weight in an oven at 80°C. If a cohesive clot failed to form, the material was recovered by centrifugation. The yield of polysaccharide was expressed as grams (dry weight) per liter (1,000 g) of culture.

The precipitated samples of polysaccharide were pressed and blotted to remove excess fluid, redissolved in 30 ml of deionized water, and then placed in an oven for 1 h at 100°C to speed dissolution. Viscosities were measured at 20°C with a Brookfield LVTDV-II viscometer. Intrinsic viscosity [η] was the *y*-intercept for the linear best fit to $(\ln \eta_R)/C$ as a function of C (with C as grams per deciliter). The weight-average molecular weight (M_w) was calculated from the equation $[\eta] = (0.000258)M_w^{0.646}$ (1).

Polysaccharide composition. Samples of dry polysaccharide were dissolved in deionized water at a final concentration of 1.0 to 5.0 g/100 ml with 0.01% (wt/vol) sodium azide as a preservative. Commercial pullulan, PF10 (lot 90201) and PF20 (lot 90517), was obtained from Hayashibara. The fraction of each sample as acidic polysaccharide was determined gravimetrically. Polysaccharide samples (30 mg in 3 ml of H_2O) were mixed with an equal volume of 1% (wt/vol) cetyltrimethylammonium bromide in 0.1 M sodium phosphate buffer (pH 7), incubated for 1 h at 25°C, and centri-

fuged at 5,000 × *g* for 5 min, and then the pellets were dried and weighed. The sensitivity to pullulanase (E.C. 3.2.1.41) from *Enterobacter aerogenes* (Sigma) was also determined gravimetrically. Samples of 30 mg of polysaccharide were completely dissolved in 3.0 ml of deionized water. Then 0.6 ml of deionized H_2O, 0.9 ml of 0.5 M sodium acetate buffer (pH 5.2), and 60 μl of 3.2 M $(NH_4)_2SO_4$ (pH 6.2) buffer with or without 1.6 U of pullulanase were added and the mixture was incubated for 3 h at 45°C. One unit liberates 1.0 μmol of maltotriose from pullulan per minute at pH 5.0 at 25°C. The fraction of pullulanase-resistant material was the weight of IPA-precipitable polysaccharide following enzyme treatment divided by the weight of IPA-precipitable polysaccharide with no enzyme treatment. The maltooligosaccharide products of digestion with pullulanase were separated by ascending thin-layer chromatography (5), with plates from E. Merck (Art.13145 Kieselgel 60CF245, 10 by 20 cm, prechanneled). The buffer (20 ml of IPA, 20 ml of acetone, 9.91 ml H_2O, 0.09 ml of 85% lactic acid) ascended for 4 to 6 h in a sealed glass container. The chromatogram was air dried, sprayed with stain (80 mg of naphthoresorcinol, 40 ml of ethanol, 0.8 ml of concentrated H_2SO_4), and incubated for 60 min at 80°C.

RESULTS

Colony appearance and growth properties of new isolates. On the basis of the growth properties of preexisting strains of *A. pullulans*, we devised selective culture conditions to enrich for new isolates of this fungus. The conditions included minimal salts medium plus sucrose, low pH, and 25°C. Following the chance isolation of an *A. pullulans* strain from a plant leaf taken near our laboratory, we obtained leaves from a variety of plants from different locations within San Diego County (Table 1). Surprisingly, almost every leaf yielded colonies whose general appearance resembled *A. pullulans*. We isolated the fungus from leaves collected from coastal suburban ornamental landscaping and natural mountain flora.

With the exception of strains AP2 and AP7, colonies of the preexisting strains (AP1 through AP9) and the new isolates described here (AP11 through AP36) were similar in general appearance when grown on P1 or P2 agar plates. However, there were also distinguishing features. The colony morphologies of preexisting isolates (AP1 and AP2) and our new strains (AP11, AP27, and AP30) are shown in Fig. 1. After incubation for 3 days at 28°C, colony colors ranged from off-white or light beige to pale pink or salmon. Branched septate filaments extended from the center of each colony to beyond the edge of a glistening, raised, semiround zone of yeast cells and also down into the agar. The densities and radial extents of the filaments were different among the strains. For example, new strain AP27 was the least pigmented of the natural isolates and formed the fewest filaments. The colonies continually expanded for about 10 days or until the plates became dry. After about 4 days of growth at 28°C, some of the filaments began to accumulate an olive pigment that appeared to blacken with age. If the plates were exposed to the light-dark cycle of the fluorescent room lights, concentric rings of pigmentation developed. Strains AP2 and AP7 became exceptionally dark. The filamentous zones of all strains eventually accumulated at least some of the dark pigment and sometimes appeared sectored. The new strains were most similar to AP9 (Y12974), which was isolated from Florida sea grass (11). Among the new natural isolates, AP27 was especially noteworthy since it appeared nonpigmented and grew almost exclusively in the yeast form. Although strain AP11 was more pigmented, it was isolated from the same plant as AP27.

Since *A. pullulans* is polymorphic on agar plates or in submerged liquid culture, it was not easy to positively identify the fungus with the unaided eye. Microscopic observations of the new isolates revealed the same three predominant types of cells seen for the preexisting strains: budding yeast-like cells, branched filaments, and chlamydospores, which are pigmented cells about twice the size of the yeast forms. In submerged liquid culture the new isolates appeared similar to strains AP1, AP3, AP4, AP5, and AP9 but unlike strains AP2 and AP7, which were more highly pigmented. For strains AP2 and AP7 the medium turned black, so that after precipitation with IPA the pullulan was dark olive. The less pigmented cultures were various shades of light yellow, olive-grey, and combinations and gave rise to pullulan with an off-white tint. The intensity and color depended on the culture conditions (i.e., nitrogen source and concentration, carbon source and concentration, phosphate concentration, initial pH, aeration, inoculum source and amount, trace minerals, and temperature [results not shown]). All these variables were held constant for meaningful comparisons of color. Isolate AP27 produced the least pigment in liquid culture, and the IPA-precipitated pullulan was white.

Relatedness of repeated DNA sequences. We confirmed the visual identifications and distinguished the new *A. pullulans* isolates from preexisting strains by the pattern of restriction fragments generated from repetitive sequences of DNA (Fig. 2). For *S. cerevisiae* (19), *Candida* spp. (15, 20), and other eukaryotes, relatively intense bands of repeated DNA are visible over a background of fainter unique restriction fragments. Five bands generated by *Eco*RI cleavage of DNA from *S. cerevisiae* were prominent and corresponded to the expected fragments of ribosomal DNA of 2.79, 2.46, 2.02, 0.66, and 0.59 kb (Fig. 2A, lane j). The additional expected bands at 0.35 and 0.22 kb migrated off the bottom of the gel. In Fig. 2A we compared the preexisting strains, AP1 through AP9, with our new strains AP11 and AP30. The majority of bands appeared to be in the same relative positions, suggesting that all the strains were probably closely related. However, a few bands were unique. The banding pattern for AP1 was like that for AP4, AP2 was like AP7, and AP11 was like AP30. Likewise, in Fig. 2B we compared the other new strains with AP11 and AP30. Two patterns were evident: one for strains AP11, AP24, AP30, AP33, AP34, AP35, and AP36, and another for strains AP31 and AP32. Members of these two groups were found in close physical proximity: strains AP31 and AP32 were isolated from different plant species separated by only about 12 m from AP30.

Pigment reduction following treatment with ethidium bromide. Since it appeared that the melaninlike pigment was accumulated mainly by filaments and chlamydospores, and not by the yeast forms, we attempted to enrich for yeast cells. Kelly and Catley (9) described the use of ethidium bromide for inducing the formation of yeasty cultures of *A. pullulans*. This mutagenic method affects the mitochondrial genome and had previously been used with *S. cerevisiae* (22) to decrease respiration and promote yeasty or fermentative metabolism. Although we were not sure that the treatment with ethidium bromide was the direct cause of our yeasty derivative strains, we recovered mutants that not only produced fewer filaments on agar plates but also accumulated less pigment. Strain AP24 was a reduced-pigment mutant derived from AP11. Treatment of AP24 with UV light

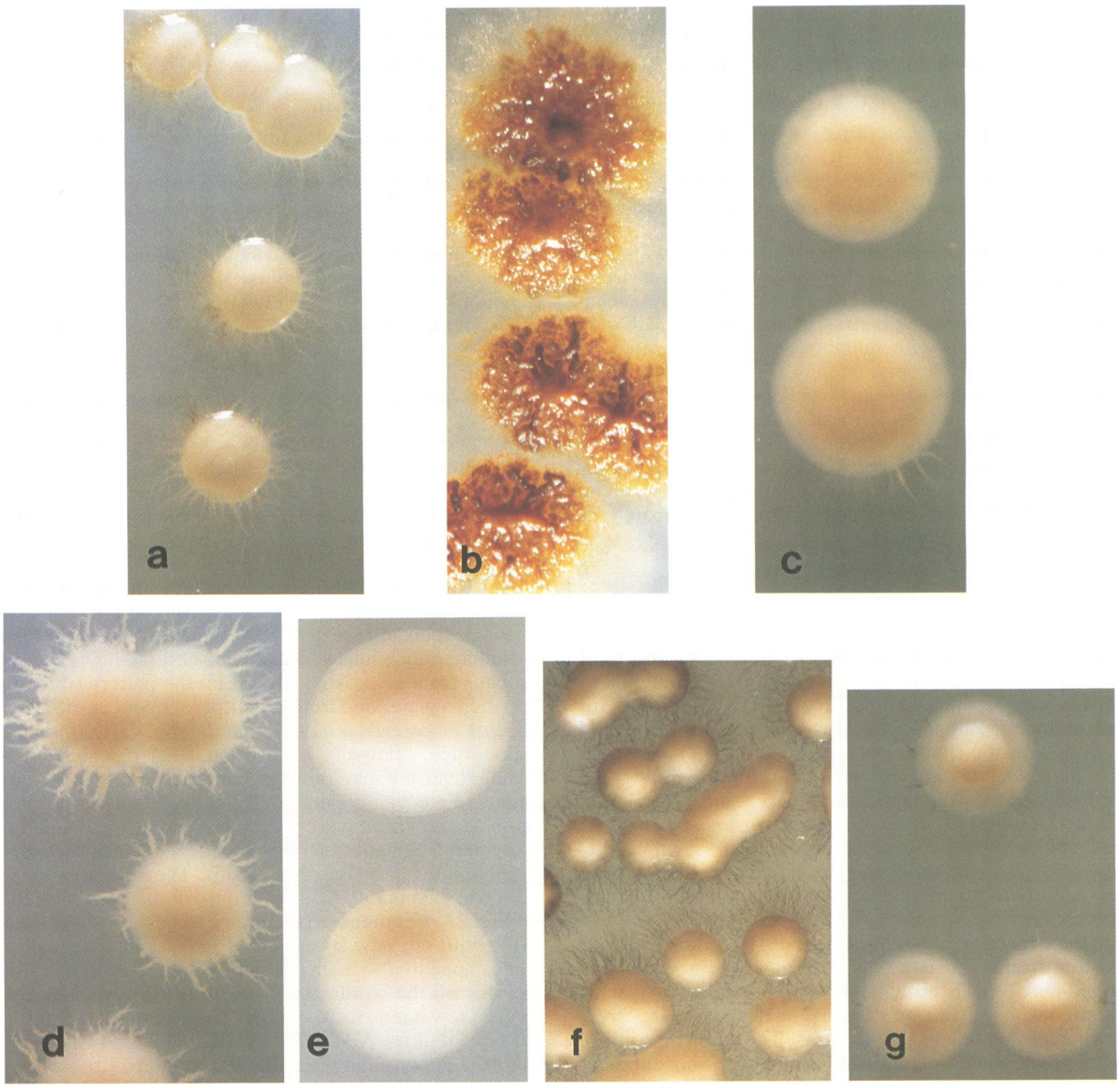

FIG. 1. Colony morphology and color of *A. pullulans* strains. The strains are as follows: (a) AP1, (b) AP2, (c) AP27, (d) AP11, (e) AP41, (f) AP30, and (g) AP42.

resulted in the isolation of AP41, which was even less strongly pigmented. Also, a nonpigmented strain, AP42, was obtained from AP30 after exposure to ethidium bromide followed by a mild treatment with UV light. These colony morphologies are shown in Fig. 1. The pullulan recovered from these mutants was essentially free of pigment and formed a white precipitate in IPA, compared with off-white or slightly tan precipitates for other strains.

Pullulan yield, intrinsic viscosity, and composition. Duplicate shake flask cultures for each isolate were analyzed for yield and molecular weight of extracellular polysaccharides. As shown by the matrix in Fig. 3, we observed an inverse relation between pullulan yield and intrinsic viscosity, a measure of molecular weight. The intrinsic viscosities for pullulan samples from cultures of strains AP2, AP11, AP27, and AP30 were considerably higher than those of any other pullulan samples studied to date (1).

To test our viscosity and molecular weight determinations, we measured the intrinsic viscosities of two commercial pullulan samples with reported molecular weights of about 100,000 (PF10) and 200,000 (PF20) (26). We obtained intrinsic viscosities of 0.32 and 0.70, respectively. The relation determined by Buliga and Brant (1) indicated corresponding weight-average molecular weights of 61,000 and 207,000, close to the reported values.

Viscosities were also measured for more concentrated solutions of polysaccharide samples obtained from strains AP1, AP24, and AP30 and from commercial sample PF20. These samples were prepared from cultures that were independent from those described above. As shown in Table 2,

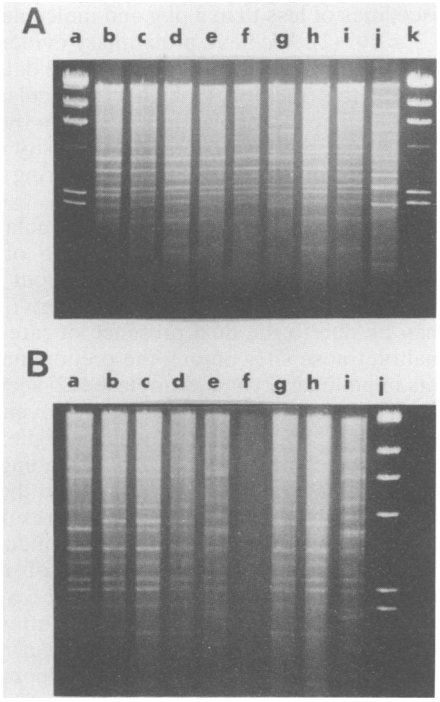

FIG. 2. Electrophoretic patterns for repeated DNA after digestion with *Eco*RI. (A) Lanes: a and k, bacteriophage λ DNA digested with *Hin*dIII; b, AP1; c, AP2; d, AP3; e, AP4; f, AP7; g, AP9; h, AP11; i, AP30; j, *S. cerevisiae*. (B) Lanes: a, AP1; b, AP11; c, AP24; d, AP30; e, AP31; f, AP33; g, AP34; h, AP35; i, AP32; j, bacteriophage λ DNA digested with *Hin*dIII.

strain AP30 accumulated pullulan, which, as before, showed significantly higher viscosities than the other strains or sample PF20.

The compositions of the IPA-precipitable polysaccharides for the same samples (AP1, AP24, AP30, and PF20) were determined in three ways. First, we found that less than 1% of the IPA-precipitated material in the culture supernatants was precipitated by a cationic detergent, cetyltrimethylammonium bromide, at pH 7 (data not shown). This meant that the pullulan was essentially free of contaminating acidic polysaccharides. Second, we tested the same material for sensitivity to the enzyme pullulanase (E.C. 3.2.1.41) of *E. aerogenes*. Less than 1% of the polysaccharide was resistant to digestion with pullulanase (data not shown). Lastly, we separated the products of digestion with pullulanase by thin-layer chromatography. The digests of all three strains (AP1, AP24, and AP30) and a sample of commercial pullulan are shown in Fig. 4. The chromatogram indicated that only maltotriose was generated. The identity of the IPA-precipitable polysaccharide in these samples was also confirmed by nuclear magnetic resonance spectroscopy (data not shown).

TABLE 2. Viscosity as a function of polysaccharide concentration

Sample	Concn (g/100 ml)	Viscosity[a] (*cP*)
AP1	5.0	1,080
	2.0	38
	1.0	7
AP24	5.0	13,200
	2.0	460
	1.0	43
AP30	5.0	30,000
	2.0	1,660
	1.0	180
PF20	5.0	20
	2.0	4

[a] Measured at a shear rate of 6.5/s with spindle 18.

DISCUSSION

Our initial objective was to isolate new strains of *A. pullulans* for the production of nonpigmented pullulan, a neutral polysaccharide secreted in large amounts by the fungus during submerged stirred fermentation. By using

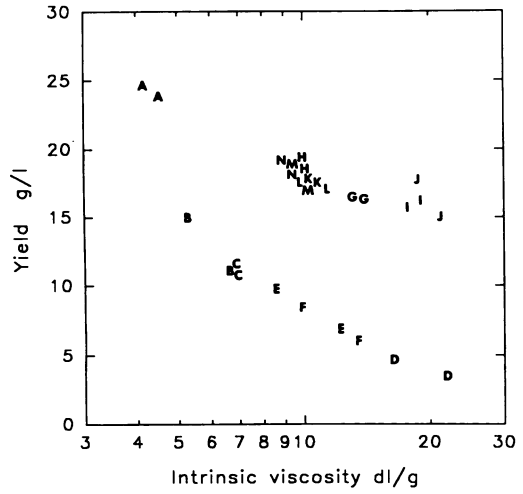

FIG. 3. Matrix of yield versus intrinsic viscosity for duplicate shake flask cultures. Viscosities were measured at a shear rate of 36.6/s with a UL adaptor. Abbreviations: A, AP1; B, AP9; C, AP4; D, AP2; E, AP31; F, AP32; G, AP11; H, AP26; I, AP27; J, AP30; K, AP33; L, AP34; M, AP35; N, AP36.

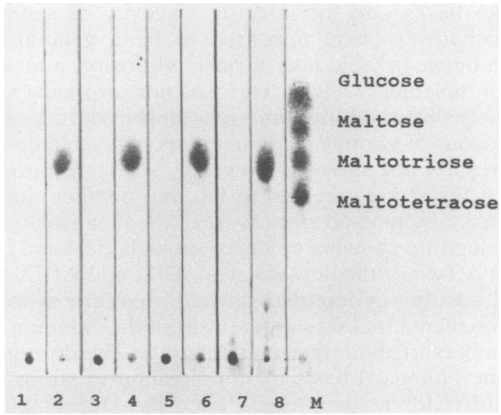

FIG. 4. Separation by thin-layer chromatography of pullulanase-treated (lanes 2, 4, 6, and 8) and untreated (lanes 1, 3, 5, and 7) polysaccharides. Lanes: 1 and 2, AP1; 3 and 4, AP24; 5 and 6, AP30; 7 and 8, commercial PF10 pullulan; M, maltooligosaccharide position markers.

semiselective culture conditions, many new strains were isolated from leaves. We concluded that these polymorphic fungal isolates were *A. pullulans* on the basis of colony morphology; microscopic observation of yeastlike cells, filaments, and chlamydospores; comparison of electrophoretic profiles of repetitive DNA sequences; and, lastly, observation of the synthesis of pullulan. A similar electrophoretic analysis of cellular proteins (results not shown) led to the same conclusion. One new strain in particular, AP27, was especially free of pigment and produced nonpigmented pullulan.

At the outset we thought that the elimination of pigment production by this fungus might be amenable to standard strain improvement methods involving mutagenesis and screening of colonies for appearance. However, this was not the case, for two reasons. First, variable colony morphologies were observed: among 100 colonies on a typical plate, there were often 1 or 2 unusual colonies, whose appearances changed upon replating. Second, mutagenesis with nitrosoguanidine caused an unexpectedly nonrandom distribution of rare auxotrophic mutations: three of three independent amino acid auxotrophs appeared to carry a defect in a single step in the lysine biosynthetic pathway (unpublished results). A similar situation was previously observed for the dimorphic fungus *Candida albicans* (24), which is now considered to be diploid and heterozygous for a few genetic loci. Therefore, the previous assumption of haploidy (6) for *A. pullulans* may be incorrect. To avoid the difficulty in obtaining mutants, we tried instead to treat the mitochondrial genome with ethidium bromide in the manner previously used successfully with *A. pullulans* (9) and *S. cerevisiae* (22) to obtain respiration-deficient petite mutants. From ethidium-treated cells, we isolated mutants that not only tended to grow more as yeast forms instead of as filaments, but also accumulated less pigment than the parental strains. However, we lack proof that the mutants arose directly from the ethidium treatment, since similar derivatives arose spontaneously or after additional treatments with UV light.

We were fortunate to discover that AP27 and other new strains accumulated pullulan of higher molecular weight than was previously observed by others. Too little was known about the mechanism of synthesis of pullulan to suggest a rapid screening method for strains that produce high-molecular-weight pullulan. Therefore, a small number of new and preexisting natural isolates were screened for pullulan yield in shake flasks and for intrinsic viscosity of semipurified polysaccharide. There appeared to be a general inverse relation between yield and intrinsic viscosity, a measure of polymer molecular weight. This was not surprising since we previously observed the same relationship during extensive comparisons of various medium components and concentrations (results not shown). However, we were intrigued to find that the strains seemed to fall into distinct clusters on the matrix of yield and size. Most of the new isolates tested were in a group showing moderate to high yield and intrinsic viscosity. Two of the new isolates, AP31 and AP32, that did not fall into this group also showed a pattern of repetitive DNA fragments that was different from the common pattern for members of the larger matrix group. We do not understand the biological basis for the position of strains on the matrix of yield versus intrinsic viscosity. One simple possibility is that the inverse relation results from interference by viscous pullulan with nutrient uptake, and, accordingly, the higher-yielding cluster may be more efficient in uptake.

The relation between intrinsic viscosity and molecular weight for pullulan was established with samples having intrinsic viscosities of less than 3 dl/g and molecular weights lower than 3×10^6 (1). We have preliminary evidence based on light-scattering measurements (unpublished data) that an extrapolation of this relation to higher molecular weights may not be valid. The extrapolation leads to an overestimation of molecular weight: where intrinsic viscosity suggested a molecular weight of 40×10^6, light scattering indicated only about 10×10^6.

The molecular weight of pullulan that accumulates in the culture medium depends largely on the time of harvest. Pullulan decreases in molecular weight from about 3×10^6–6×10^6 to 1×10^5–2×10^5 after 7 days in culture (2, 7). The decrease may be due to the dual presence of rare amylase-sensitive maltotetraose sites among the predominantly maltotriose units in pullulan (2) and α-amylase (12) secreted into the medium. However, it has not yet been proven that this amylase causes the reduction in molecular weight for pullulan. We have recently found that the degrading activity contaminates pullulan even after recovery from the medium by precipitation with alcohol (unpublished results). This causes instability during storage of semipurified pullulan suspensions. Therefore the reproducibility of molecular weight measurements depends not only on the age of the culture but also on how the material is treated after harvest. The instability could explain differences in lower molecular weights observed previously by others. At this point one can only speculate whether the molecular weight of pullulan is controlled primarily by synthetic or postsynthetic events.

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