Extracellular Polysaccharide from the Black Yeast NRRL Y-6272: Improved Methods for Preparing a High-Viscosity, Pigment-Free Product

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When the extracellular polysaccharide from the black yeast NRRL Y-6272, composed of two parts N-acetyl-D-glucosamine and one part N-acetyl-D-glucosaminuronic acid, is isolated at maximum culture viscosity, adhering black pigment gives the polysaccharide preparations a gray-to-black appearance. Precipitation of the polysaccharide from cell-free culture supernatants with either ethanol or hexadecyltrimethylammonium bromide failed to remove the pigment. Various other methods were therefore tried for obtaining a high-viscosity polysaccharide product free of pigment. By systematically varying ingredients of defined and semidefined media, an improved medium was found that not only gave polysaccharide preparations of increased viscosity, but also increased yield. A key ingredient in this medium is L-asparagine. Also, adding autoclaved bovine serum albumin or egg albumin to this medium at the time of inoculation allowed a pigment-free polysaccharide to be isolated by standard procedures. None of several other proteins or synthetic polyamides tested were as effective as bovine serum albumin or egg albumin. In an alternate approach, pink mutants, obtained by irradiation of the parent black strain with ultraviolet light, apparently produce the same extracellular polysaccharide free of any pigment but in lower yields or inferior in quality.

The black yeast strain NRRL Y-6272 (15) produces an acidic extracellular polysaccharide that contains both N-acetyl-D-glucosamine and N-acetyl-D-glucosaminuronic acid (15, 27). This polysaccharide appears to have industrial potential, since it contains both acidic (carboxyl) and masked basic groups (N-acetyl) and since it disperses readily in water to give extremely viscous solutions.

When the polysaccharide was isolated at maximum viscosity of the culture after 4 days of incubation by precipitation with either ethanol or hexadecyltrimethylammonium bromide (28), the product was colored gray to black by adhering pigment(s). When the polysaccharide was isolated after 10 days of incubation, however, all the pigment was sedimented along with the cells when the culture was centrifuged (15). Incubation for 6 days beyond the time of maximum culture viscosity (5,000 to 6,000 centipoises) not only was impractically long, but also resulted in an irreversible decrease in viscosity to 2,000 to 3,000 centipoises.

The only extracellular polysaccharides characterized from the widely distributed black pigment-forming yeastlike fungi, other than polysaccharide Y-6272 (15), are the pullulans, a family of glucans (3, 5, 6) produced by Aureobasidium (Pullularia) pullulans (10). Although there are several studies on pigmentation of these organisms (16, 21–24), only one report (35) indicates that the extracellular polysaccharide, pullulan, was present and that pigment was found adhering to it.

Just as with pullulan (35), pigment can be removed from polysaccharide Y-6272 by the Sevag fractionation technique (32). This technique, however, is impractical because it has to be applied numerous times for complete pigment removal and because yield of pigment-free polysaccharide is low (35) (up to 15% of the polysaccharide is lost with each application).

We have found that the pigment contaminating polysaccharide Y-6272 is melanin-like, except for its apparent solubility in water. Reportedly, melanin binds many compounds (2) including chitin (4) and pullulan (35).

In seeking a practical method for obtaining not only a pigment-free polysaccharide, but also a product of high viscosity and in an apparent narrow range of molecular weight distribution, we tried to find: (i) methods that separate and
remove black pigment from the polysaccharide, (ii) methods that prevent formation of pigment, and (iii) mutants that would produce pigment-free polysaccharide.

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MATERIALS AND METHODS

Materials. NRRL Y-6272 is a black yeastlike organism (15) isolated initially from desert soil at Dugway Proving Ground, Utah, by G. F. Orr. The various biochemicals used, and their respective sources, are as follows: Vico P-300 (yeast autolysate paste), A. E. Staley Co.; bovine serum albumin (BSA) and egg albumin (EA), Schwarz/Mann; Promine-D (soy protein), Central Soya; sodium caseinate, Carnation Co.; gelatine, Atlantic Gelatine; gluten (extracted from Selkirk wheat with acetic acid by Floyd R. Huебner of the Northern Laboratory); and Polyclar AT (insoluble polyvinyl-pyrrolidone [PVP]), and Plasdone-C (soluble PVP, blood plasma expander grade), GAF Corp.

Culture media. All concentrations are for the whole medium. All parts were autoclaved separately at 121°C for 15 min, unless otherwise noted, and combined aseptically. Medium no. 1 (15) consisted of: part A, yeast extract (1%) and tryptone (3%), pH adjusted to 7.0; part B, glucose (5%). Medium no. 2 (11, 12) consisted of: part A, KH₂PO₄ (0.9%) and glucose (5%); part B, Wickerham’s nitrogen base (34) (10 times more concentrated than that used for carbon assimilation tests). Part B was filter sterilized (Seitz) and 7.5 ml was added aseptically per 67.5 ml of part A. Medium no. 3 consisted of: part A, KH₂PO₄ (0.5%), MgSO₄ (0.1%), ZnSO₄ (200 μg/75 ml of whole medium), and Vico P-300 (0.37%); part B, l-asparagine (0.75%); and part C, β-glucose (5%). Parts A and B were adjusted to pH 7.1 before autoclaving.

Culture maintenance and propagation. Stock cultures of strain Y-6272 were maintained on yeast-malt agar slants (14) which, after being streaked, were held at 25°C for 2 days and then stored at 4°C. Stock cultures were transferred at 1-month intervals. For experimental polysaccharide production, 300-ml Erlenmeyer flasks containing 75 ml of media (plus inoculum) were incubated at 25°C on a rotary shaker (200 strokes/min, 2-inch eccentricity).

To develop preinoculum, the stock culture was first transferred to fresh yeast-malt agar slants, incubated for 2 days, and then inoculated into yeast-malt broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 1.0% β-glucose). After 2 days of incubation, this preinoculum (7.5 ml or 10% by volume) was transferred to an inoculum flask containing 75 ml of test medium, and the culture was shaken for 1 day. Then this inoculum (3.75 ml or 5% by volume) was transferred to production flasks containing 75 ml of the test medium.

With the native strain, the culture liquor generally reaches maximum viscosity (in media 1 and 3) after shaking for 4 days and is highly cohesive andropy like egg white.

Recovery of polysaccharide Y-6272. Four-day cultures, having high viscosity (4,000 to 6,000 centipoises; Brookfield viscometer, model LVT, spindle no. 4, 30 rpm), were diluted with 3 volumes of water to reduce the viscosity (~175 centipoises) and then centrifuged (35,000 × g, 30 min) to remove cells. By addition of KCl (1%) and ethanol (95%, 2 volumes), the total polysaccharide separated as a stringy precipitate that wound around the stirrer. After this precipitate was dissolved in water, hexacycl trimethylammonium bromide (1%, Eastman Organic Chemical) (28) was added to remove the major fraction (acidic), again as a tough stringy precipitate that wound around the stirrer; the minor (neutral) fraction remained in solution. The acidic fraction was dissolved in 2 M KCl and, after dilution by addition of 1 volume of water, was reprecipitated by addition of 2 volumes of ethanol. The acidic polysaccharide (in K salt form) was then redissolved in deionized water and dialyzed until free of extraneous salt. The dialyzed solution was adjusted to pH 6.25 with dilute KOH and then freeze dried.

RESULTS

Characteristics of the soluble pigment. The absorption spectrum of the pigment in cell-free supernatants, which also contain the extracellular polysaccharide, has no unique features; the optical density at 400 nm was used as a measure of the amount of soluble pigment, as has been done for the black yeast Phalophora jeanselmei (11, 12).

Various physical, chemical, and enzymatic procedures were ineffective for separating pigment from polysaccharide. Physical treatments tried were: dialysis; addition of Celite, diethylaminoethy cellulose, or activated carbon followed by centrifugation; precipitation of the polysaccharide with ethanol from neutral, acidic, or basic solutions; precipitation with hexacycl trimethylammonium bromide; and extraction with several organic solvents.

Chemical treatments included the following. Addition of ferric salts, although expected to precipitate melanin (16), formed a water-insoluble polysaccharide-pigment complex; acidification with HCl precipitated pigment along with polysaccharide; NaOCl not only bleached the pigment to straw color, but also degraded the polysaccharide. Neither Pronase nor papain was effective in freeing polysaccharide from pigment. In its behavior under these various treatments, the pigment contaminant of polysaccharide Y-6272 appears to be melanin-like (13, 16, 30) except for its apparent water solubility.

Several compounds that were previously reported to inhibit tyrosinase or melanin formation in other organisms (7, 23, 24), when added to the culture medium of Y-6272, adversely affected either growth of the organism or poly-
saccharide biosynthesis. Attempts were also unsuccessful to inhibit pigment formation by complexing suspected phenolic precursors (18, 19) with such synthetic polymers as PVP.

Factors involved in pigment and polysaccharide formation. A chemically defined medium (no. 2) (11, 12) was selected, and the effect of systematic addition of other ingredients was observed. Growth of Y-6272 on this defined medium was slow; the yeastlike cells were brown; cell-free culture supernatants were straw colored and contained little polysaccharide, as indicated by both the low viscosity of culture liquors and the low yield of isolated material. Adding L-asparagine to the medium caused black pigment to occur in both the cells and cell-free culture supernatants and increased viscosity. Whereas addition of filter (Millipore Corp., 0.22-μm pore size)-sterilized BSA (11, 12), with L-asparagine either present or absent, resulted in highly pigmented cell-free culture supernatants of low viscosity, addition of heat-sterilized BSA plus asparagine resulted in moderate viscosity with less pigment than with asparagine alone. Addition of L-asparagine evidently stimulates polysaccharide Y-6272 biosynthesis, and addition of BSA, when autoclaved, diminishes soluble extracellular pigment.

The stimulative effect of L-asparagine on polysaccharide production noted with defined medium (no. 2) was an important factor in the development of the more practical medium, no. 3. Among the various media compositions we have tested, medium no. 3 was best for production of polysaccharide Y-6272 from D-glucose at 25°C. The viscosity of cultures grown on medium no. 3 is 5,000 to 6,000 centipoises in contrast to 2,000 to 3,000 centipoises obtained with medium no. 1 (15). When grown on either medium no. 1 or 3, cultures reach maximum viscosity in 4 days with essentially no change in pH (6.8 to 7.2). When shaking was continued to 10 days, cultures on medium no. 3 retained their viscosity and soluble pigment, whereas for those on medium no. 1 viscosity decreased and pigment became insoluble. To prepare both pigment-free and high-quality polysaccharide, further information on the combined roles of L-asparagine and BSA seemed necessary.

Effect of BSA on soluble pigment. After being heated under sterilizing conditions, BSA lowers the amount of pigment in cell-free supernatants of Y-6272 grown on medium no. 3, as well as on medium no. 2. The BSA may either be autoclaved with part A of the medium or heat sterilized separately and then added aseptically to the other sterile ingredients. In either case, however, the BSA is more effective when present in the medium at the time of inoculation rather than when autoclaved and added aseptically to the culture 2 or 4 days after inoculation. This effect is shown by optical density measurement of soluble pigment in cell-free supernatants when fermentation is terminated at 4 days (Fig. 1A).

Heat-sterilized BSA, whether present at inoculation or added later to the growing culture, does not affect viscosity (Fig. 1B). In contrast to the effect of heat-sterilized BSA, filter-sterilized BSA stimulates formation of soluble pigment (Fig. 1A) and lowers viscosity (Fig. 1B) throughout the course of fermentation.

Thus the polysaccharide having the most favorable quality, as compared with the control, is obtained under the conditions of points 1a and 1b in Fig. 1A and 1B, respectively. The optimum concentration of BSA and length of autoclaving (121°C) are 0.2% (wt/vol) and 5 to 15 min, respectively.

After culture fluids were centrifuged, the cell pad of cultures of Y-6272 to which heat-sterilized BSA had been added before inoculation had two visible layers: a large one at the bottom, which is a mixture of black yeast cells and insoluble BSA (white chunks), and a small gray-black one on top of the cell layer. This gray-black layer does not contain yeast cells and appears to be a pigment-BSA material. Control samples without added BSA do not have the top gray-black layer.

We have found that filtration (Millipore Corp., 0.65-μm pore size) is also effective in reducing the level of pigment in culture supernatants, primarily by removal of the last traces of suspended pigmented cells and debris. Filters of pore size less than 0.45 μm remove the polysaccharide and readily become clogged. Filtration of whole-culture broth is not practical, because filters rapidly become clogged with cells.

Effect of various proteins and synthetic polyamides on soluble pigment. To test their applicability as agents for removing soluble pigment, proteins other than BSA were added to part A of culture medium no. 3 before autoclaving (15 min) (Table 1). Of the materials tested, only EA was as effective as BSA, whereas almost all the materials tested actually increased the amounts of pigment in culture supernatants. After autoclaving, BSA and EA became insoluble. The amount of insoluble protein, as observed visually, was related in-
versely to the amount of soluble pigment in polysaccharide preparations; i.e., test media with the greatest amounts of insoluble protein gave the lowest amounts of pigment in cell-free supernatants. The optimum length of autoclaving (15 min) for both BSA and EA was established experimentally; however, for the other proteins optimum conditions were not sought. The yield of polysaccharide was not affected by the addition of either BSA or EA; when Promine-D, gelatine, or Plasdone was added, the apparently high yield probably was due to large amounts of contaminating pigment (Table 1).

The synthetic polyamide PVP, when added in an insoluble form (Polyclar AT), remained insoluble in the medium and appeared to be inert. Addition of PVP in a soluble form (Plasdone-C) resulted in low viscosity and high pigmentation of cell-free supernatants (Table 1).

**Pink mutant strains.** After irradiation of the black parent strain Y-6272 with ultraviolet light, two mutants were selected that are pink initially when streaked on yeast-malt agar slants (14). When these mutants were stored on slants at 4 C for 14 days, mutant Y-6272 pink (colony 10-2) remained pink, whereas Y-6272 brown (colony 10-1) gradually turned brown-black. After growth in medium no. 3, cell-free supernatants of both mutant strains were straw colored, and the extracellular polysaccharide when isolated was colorless. All the pink coloration remains with the cells.

To test whether L-aspartic acid, L-glutamic acid, or L-glutamine is a better nitrogen source than L-asparagine for polysaccharide production, the parent and two mutant strains were compared after 4 days of culture growth on medium no. 3 supplemented by each of these amino acids (Fig. 2). The samples fall into two distinct categories: (i) those from both mutants grown on L-asparagine or L-glutamine, additives which apparently provide unfavorable nutritive conditions (Fig. 2, lower right); and (ii) all the others, which fall on the same viscosity-yield line and for which nutritive conditions apparently were more favorable. After isolation, all samples of group (ii) gave nearly the same viscosity on a dry-weight basis and so are similar in nature but differ in yield.

The addition of L-asparagine, as compared with the other nitrogen additives tried, doubles the yield of polysaccharide with both the native and mutant strains (Fig. 2); however, the product from the two mutant strains appears to be degraded since the viscosity of these samples

**Table 1.** Effect of various proteins and synthetic polyamides on viscosity, pigment, and extracellular polysaccharide yield of NRRL Y-6272 cultures

| Polyamide added* (0.2%) | 4-Day culture viscosity* (centipoise) | Analysis of supernatant after centrifugation† and filtration‡ | Yield of polysaccharide (g)‡ |
|-------------------------|----------------------------------------|---------------------------------------------------------------|-----------------------------|
|                         |                                       | Viscosity* (centipoise)                                       | Pigment* (OD₄₅₀)            |                             |
|                         |                                       |                                                               |                             |                             |
| None                    | 5,240                                  | 168                                                           | 0.94                        | 0.76                        |
| BSA                     | 5,300                                  | 176                                                           | 0.31                        | 0.77                        |
| EA                      | 5,200                                  | 147                                                           | 0.44                        | 0.77                        |
| Polyclar AT             | 5,520                                  | 174                                                           | 0.83                        | 0.73                        |
| Glutin                  | 4,820                                  | 124                                                           | 1.13                        | 0.80                        |
| Promine-D               | 3,880                                  | 56                                                            | 1.85                        | 0.97                        |
| Gelatine                | 3,360                                  | 40                                                            | 1.98                        | 0.89                        |
| Casein                  | 2,290                                  | 20                                                            | 2.43                        | 0.76                        |
| Plasdone-C              | 1,780                                  | 6                                                             | 3.20                        | 0.88                        |

* Added to part A of medium no. 3 before autoclaving for 15 min.
† Brookfield model LVT, 30 rpm; column 2, spindle 4; column 3, spindle 3.
‡ Before centrifugation (35,000 × g, 30 min) 1 volume of culture fluid was diluted with 3 volumes of water.
§ Millipore Corp., 0.65-μm pore size.
|| Optical density at 400 nm (OD₄₅₀) of sterile uninoculated medium no. 3, diluted with 3 parts water, is ~0.3.
| Grams (dry weight) per 100 g of culture fluid.
Interrelated. The addition of known inhibitors of tyrosinase or melanin formation, culture conditions that preclude black pigment formation by the parent strain, either diminished or eliminated polysaccharide production. Mutant strains induced by ultraviolet irradiation, which contain pink but not black pigment, apparently produce a main (acidic) polysaccharide product that is compositionally the same as that from the black parent strain but varies in viscosity with the nitrogen source in the growth medium. Except for its water solubility, the black pigment appears to be melanin-like, as indicated by its chemical and physical behavior.

Failure to remove the pigment by dialysis indicates either that the molecular weight is so large that the pigment cannot pass through the membrane, or the pigment itself is small but interacts with the polysaccharide so strongly that the pigment-polysaccharide will not dialyze out. Removal of pigment from polysaccharide Y-6272 by the Sevag technique (32), generally used to separate noncovalently bound protein from polysaccharides, suggests that contaminating pigment is physically associated and has some characteristics of a protein. The low recovery of polysaccharide Y-6272 with the Sevag technique appears to be due mainly to its loss into the emulsion layer from which a considerable amount of polysaccharide can be removed. Characterization of fractions of this recovered polysaccharide indicates that most of it is equivalent to the main product. Loss of some polysaccharide would be expected if a small percentage of the polysaccharide molecules are firmly complexed with pigment and so are removed by the Sevag technique.

Further indication that the pigment has some proteinaceous character is the stimulation of soluble pigment formation by addition of soluble proteins, such as BSA, to culture media. With the black yeast *P. jeaneselmei* (11, 12), addition of soluble BSA solubilized the black pigment, normally found in mycelia, by attachment to the BSA molecule. These same studies also showed that the size of the BSA-pigment complex increased with incubation time until it was so large that the complex precipitated from solution. Similar behavior may take place when Y-6272 is grown on medium no. 1 since the soluble pigment seen at 4 days is removed by centrifugation when incubation is extended to 10 days. In this instance, soluble proteins in the medium may act like BSA.

Failure to remove pigment from polysaccharide Y-6272 by treatment with Pronase or papain before isolation by alcohol precipitation
may indicate either that the pigment is inhibitory to the enzymes or the proteinaceous portion of the pigment has been modified sufficiently to resist enzymatic cleavage.

The mechanism of action of BSA and EA is not established by our observations, but apparently autoclaving medium no. 3 for 15 min places BSA and EA in an optimum physical state for acting as an insoluble anchor to which pigment can be attached preferentially. There does not seem to be any correlation between the amount of tyrosine in the various proteins tried and pigment removal since gelatine and Proteamine-D, which have no tyrosine (31), stimulate extracellular pigment formation (see Table 1). Proteins, such as BSA, may be involved in the regulation of melanin biosynthesis (20).

The correlation between low viscosity and high pigmentation of polysaccharide supernatants is not due to the precipitation of the polysaccharide by the added protein, since we found that pH of culture fluids remains near neutrality (6.8 to 7.2) throughout the 4-day culturing period and that pH must reach pH 4.5 or below for BSA to carry a positive charge and thus be able to precipitate acidic polysaccharide. This behavior is similar to that reported for an α-1,4-linked poly-N-acetylgalactosaminuronic acid (Vi antigen) polymer (33) and is the basis for a turbidimetric assay of the Vi antigen and other acid polysaccharides (8, 25). The lowering of viscosity by added protein may be either due to the stimulation of biosynthesis of pigment, which binds the polysaccharide, or to the stimulation of an enzyme, such as the Vi antigen-degrading enzyme (1), which depolymerizes the polysaccharide.

The stimulation of polysaccharide Y-6272 production by addition of L-asparagine is not completely understood, especially in view of our finding that L-glutamine, not L-asparagine, is involved in the enzymatic biosynthesis of hexosamine; i.e., L-glutamine, but not L-asparagine, is a substrate in the assay of fructose 6-phosphate amidotransferase (EC 2.6.1.16) in washed-cell homogenates.

\[
\begin{align*}
\text{L-Glutamine} + \text{d-Fructose-6-PO}_4 & \rightarrow \text{EC 2.6.1.16} + \text{d-Glucosamine-6-PO}_4 \\
& \uparrow \text{Polysaccharide}
\end{align*}
\]

Two hypotheses have been proposed to account for the effect of L-asparagine: (i) L-asparagine may relate to the biosynthesis of a glycoprotein that is vitally related to polysaccharide synthesis, possibly as a bridge between protein and carbohydrate (17, 29); (ii) L-asparagine may induce in the microorganism a morphological change (5) that must take place before polysaccharide production can begin.

The inability to classify Y-6272 taxonomically is due mainly to our failure to induce sporulation. By an alternate approach of seeking taxonomic relationships through comparison of composition of polysaccharide products, we isolated extracellular polysaccharide from strains of a number of classified black yeasts. None of these produced extracellular polysaccharide comparable to that of Y-6272. The majority of strains were Aureobasidium pullulans, which produced glucans rather than amino sugar polysaccharides.

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