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CONTROL OF MOLECULAR WEIGHT DISTRIBUTION OF THE BIOPOLYMER PULLULAN PRODUCED BY THE FUNGUS *Aureobasidium pullulans*

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This report summarizes one section of this study, the evaluation of environmental conditions and their influence on the biopolymer pullulan molecular weight distribution and yield. Films, fibers, and chemical derivatives made from these different molecular weight products, and the characterization of these materials will provide the bases for another report. In order to determine optimum growth conditions for the control of molecular weight distribution of extracellular pullulan, preliminary studies utilized cell suspensions of nine strains of Aureobasidium pullulans. One strain, NRRL-Y 6220 A. pullulans, was selected for further study. Carbon and nitrogen sources, along with phosphate concentration were evaluated for their effect on pullulan yield and molecular weight distribution. Batch systems, scale-up batch, and continuous fermentations of one liter and 10 liters were also evaluated. Processing variables, including solvents, extraction time, etc., were also studied. Pullulan biopolymer products with weight average molecular weights from 100 thousand to 4 million, with a dispersity of around two, were produced. The evaluation of chemical/physical properties of defined molecular weight fractions of pullulan is now under investigation. Keywords

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PREFACE

This report contains the results of a study performed to determine the control of molecular weight distribution of the biopolymer pullulan produced by the fungus Aureobasidium pullulans. This study was funded under the U. S. Army Natick Research, Development and Engineering Center (Natick) Program Element 61101A on Biopolymer Production for Varied Military Applications, Project No. 1L161101A91A, Task No. 07, Work Unit No. 144. The work was undertaken from March 1986 to September 1987.

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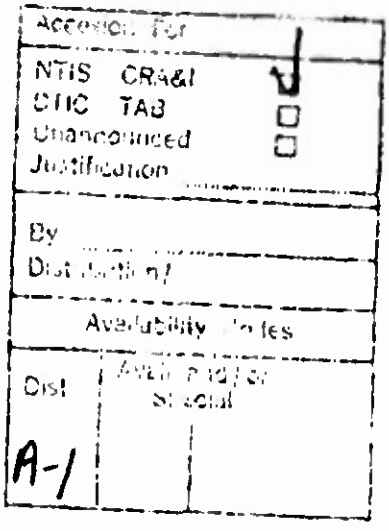

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CONTROL OF MOLECULAR WEIGHT DISTRIBUTION OF THE BIOPOLYMER PULLULAN PRODUCED BY THE FUNGUS *Aureobasidium pullulans*

INTRODUCTION

Pullulan is a biopolymer, which is released into the extracellular medium as a secondary metabolite by the dimorphic fungus *Aureobasidium pullulans* (de Bary) Arnaud during the yeast-like phase of the growth cycle but not used during routine metabolism.[1,2] Pullulan is a linear α-D-glucan, predominantly 1,4-linked maltotriose with some maltotetraose units, connected by 1,6-linkages between the terminal glucosidic residues of the trisaccharide.[3,4]

In this study, the term "biopolymer" refers to polymers synthesized by bacteria, fungi, or algae that are not part of the basic cellular structural or functional macromolecules common to most of these organisms. Thus, nucleic acids, carbohydrates and proteins produced during routine metabolism or for structural support in the cell are not considered under this definition of biopolymer. Biopolymers can be found intracellularly, extracellularly, or in the cell wall or membrane.

Extracellularly produced biopolymers are the most economical in terms of large-scale production due to the ease of purification, processing, and yield. General properties of many biopolymers include: an ability to form transparent films with low gas permeability, an ability to form an adhesive-like matrix, biodegradability, and excellent strength and flexibility characteristics.[5-7]

Biopolymers offer a number of potential advantages over synthetic polymers, including selective superior physical/chemical properties, biological compatibility, potential for genetic manipulation, control over polymer characteristics, an ability to produce defined molecular weight (MW) fractions, and nutritive value as desired. In addition, regulatory approval may be easier to obtain due to the natural source of these materials. The Japanese have commercialized the use of pullulan, producing films and powders for use in the food industry, and with potential applications as adhesives, laminates, fibers, and fabrics.[5-14]

The physiological requirements for pullulan production by *A. pullulans* have been studied extensively. Ueda *et al.*[15] studied the production of the polysaccharide by 16 strains of growing cells over time, and monitored the pH, dry weight of the cells, and the residual sugar in the medium. They also compared various sugars as carbon sources for the production of the polysaccharide, and found the MW of the polysaccharide to be approximately 250,000, when determined by light-scattering. Catley,[16-21] and others also did extensive studies on various physiological requirements for production of the biopolymer, and the influence of these constituents on biosynthesis and elaboration.[22-24]

The vegetative cycle of *A. pullulans* was studied extensively by Ramos and Garcia Acha.[22] They found that an inoculum containing a cell concentration of at least 2×10^7 cells/flask (150 mL) was required to maintain

blastospore (yeast cell) production. They also found that nitrate as a nitrogen source could cause the blastospores, after about 46 hours of incubation, to give rise to pseudomycelial forms with low viability that autolyzed as the culture aged. They found that chlamydospores were readily formed in a medium containing ammonium ion as a nitrogen source.

Catley[18,19] and Ono, et al.[23] found that the appearance of extracellular pullulan was not concomitant with an increase in cell mass, but that there was a lag in the rate of elaboration. Catley also studied the role of pH and nitrogen on the production of pullulan and found that the uptake of glucose at more acid pH was diverted to the synthesis of extracellular pullulan, and that high extracellular pH inhibited its production. He found that pullulan elaboration is dictated by depletion in nitrogen availability, and not carbon in the growth medium.[18] Ono et al.[23] found that maintaining a constant, controlled pH gave lower yields of pullulan than an uncontrolled culture. Lacroix et al.[24] described the development of a bistaged pH fermentation process, whereby the first stage of the fermentation was conducted at a very acidic pH (pH 2.0), then the pH was adjusted to a higher value (pH 5.5) to promote the production of pullulan. The amount of pullulan produced was determined by viscosity measurements. Molecular weights were not determined in their study.

Kato and Shiosaka described the effect of phosphate concentration on culture period and yield of pullulan.[10] They showed a marked decrease in mean MW of pullulan when comparing various concentrations of phosphate at pH 5.5, pH 6.0, and pH 6.5. Phosphate concentrations of 0.2%-0.4% yielded high MW pullulan at the lower pH level. They reported that using hydrolyzed starch as the carbon source resulted in yields of pullulan as high as 75% or more.[9]

To summarize the nutrient requirements for pullulan elaboration as reported in the literature: hydrolyzed starch yielded the largest amount of pullulan;[9] pH, nitrogen source, and nitrogen limitation affected pullulan elaboration,[18,22] and pH and phosphate concentration affected the MW of pullulan.[10] Culture incubation varied from a few hours to seven days, and most results were reported for batch studies. However, most of this information has not been assembled into a cohesive study. Only a few authors have reported the MW distribution of the pullulan elaborated in their studies.[10,15,16] In most reports, total carbohydrate was determined by a colorimetic procedure, estimation of glucose by glucose oxidase reagent, or by the viscosity of the medium.[15,23,24]

The objective of this study was twofold: first, to produce and characterize various MW's of pullulan, and second, to investigate the unique properties of pullulan for potential military applications, such as lower oxygen permeable films for food packaging, binders for material coatings, high performance fibers, nonwoven fabrics, derivatization for chemical agent applications, and edibility/digestibility properties for survivability applications. The first objective is the subject of this report.

METHODS AND MATERIALS

Cultures

Nine strains of Aureobasidium pullulans, A. pullulans var. melanigenum, or A. mansonii (Table 1) were evaluated in preliminary studies to compare pullulan elaboration. Strain NRRL-Y 6220 A. pullulans was chosen for further studies, based on product color and yield, when compared with the other cultures (Tables 2, 3, and 4). All cultures were maintained on potato dextrose agar slants.

Media

The following media were used, in grams per liter of distilled H_2O: (A) Ramos and Garcia Acha,[22] K_2HPO_4, 1.0; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $NaNO_3$, 2.0; $FeSO_4 \cdot 7H_2O$, 0.01; $(NH_4)_2SO_4$, 0.6; pH 5.3. (B) Ueda et al.,[15] K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; yeast extract (Oifco, Detroit, MI), 0.4; pH 5.5 and 6.0. (C) Kato and Shiosaka,[10] K_2HPO_4, 2.0; NaCl, 2.0; $MgSO_4 \cdot 7H_2O$, 0.4; $FeSO_4 \cdot 7H_2O$, 0.01; peptone, 2.0 (Difco); pH 5.4. Preliminary studies were performed to determine optimum sugar concentration (10%). The 10% sucrose (commercial grade) solution was prepared separately, and added aseptically after autoclaving, per liter of final volume.

Culture Conditions

For batch cultures, an Aquaferm® Water Bath Shaker (New Brunswick Scientific Co., Inc., Edison, NJ), temperature 25°C to 28°C, was shaken at 150 and 120 rpm. An Environ-Shaker® (Lab-Line Instruments, Inc., Melrose Park, IL), temperature 26°C to 27°C, was shaken at 120 rpm. A Model G25-R Incubator-Shaker® (New Brunswick), temperature 26°C ± 1°C, was shaken at 125 rpm. A BioFlo Model C30 Fermentor® (New Brunswick), temperature 26°C, was set at an agitation rate of 300 rpm, and aeration at 0.5 L/min. Media flow-rates were controlled with a Rabbit Peristaltic Pump® (Rainin Instrument Co., Inc., Woburn, MA) for the continuous fermentation studies. A Magnaferm Model MA-114 Fermentor® with an Automatic pH Controller and Pump Module (New Brunswick) with a Constant Speed Controller (Cole-Parmer Instrument Co., Chicago, IL) was used for the 10-liter studies and was set at a temperature of 26°C, an agitation rate of 1,200 rpm, and a variable oxygenation rate. A Model RC-5 Sorvall® Refrigerated Centrifuge (du Pont Instruments, Wilmington, DE) with a GSA rotor, 23,430 X g for 20 minutes, was used to remove the cells.

In preliminary studies to determine optimum growth conditions for pullulan elaboration, cell suspensions were made of each of the strains of A. pullulans. Ten mL of sterile distilled water was poured onto an agar slant culture, and growth was scraped from the agar surface using a sterile inoculating loop. Aliquots of the suspension were used to inoculate duplicate or triplicate 250 mL DeLong® flasks containing 25 mL or 50 mL of sterile medium. After culture comparisons were studied, all further work with shake flasks used 50 mL/250 mL DeLong flask with 2%

inoculum, or 500 mL medium per 2800 mL Fernbach flask and 2% inoculum. Inoculation of batch and continuous fermentations used two- or three-day old cell suspensions grown on the medium being studied.

Processing and Purification

The culture medium was neutralized using 1 N NaOH. Sometimes, the medium was diluted with concentrated ROCCAL II® (alkyl dimethyl benzyl ammonium chloride, EPA Registration No. 675-30-AA, Sterling Drug, Inc., Montvale, NJ) to a final volume of 1% ROCCAL II, then centrifuged at 13,180 X g to 23,430 X g for 20 min to remove the cells. The pullulan was then precipitated from the supernatant, using two volumes of acetone for each volume of supernatant, while stirring the suspension to eliminate lumping. After precipitation, the acetone was decanted, the precipitate was washed several times with acetone, and then filtered over vacuum. The pullulan was then dried over $CaSO_4$ in a desiccator.

Purification of the isolated pullulan was attempted, using two six-hour Soxhlet extractions. The first extraction was with 75% ethanol, 25% water, and the second was with 100% acetone. The first extraction solvent was used so as not to solubilize the pullulan, yet remove lower MW water-soluble contaminants. The dried pullulan was then extracted again with the second solvent. This procedure did not remove particulate impurities, so an alternative, less labor-intensive purification procedure was tried.

After the centrifugation of the culture medium, the supernatant containing the pullulan was purified using a Tangential Flow Filtration Unit with a Pellicon® Model Cassette System OM-141 (Millipore Corp., Bedford, MA). The pullulan solution was first passed through a 0.45 µm cassette to remove particulate impurities. Next, the filtrate was passed through a cassette with a 30,000 MW cut off. The selectively permeable system retained the higher MW biopolymer but allowed the lower MW impurities to pass through. The concentrated retentate was then precipitated with acetone and processed as described above. Occasionally, a precipitate formed in the concentrated retentate, which was then filtered through the 0.45 µm cassette again before the solution was acetone-precipitated.

Decolorization Procedure

A 1% (w/v) pigmented pullulan solution was treated by a variety of methods in an attempt to remove the unwanted color. The amount of decolorization was monitored by absorbance at 280 nm on a Lamda 3 UV-visible spectrophotometer® (Perkin-Elmer, Oak Brook, IL) before and after treatment. An unpigmented pullulan solution was used as a reference value, and samples prior to treatment served as controls. However, the absorbance of the pigmented control solutions at 1% was off-scale. Subsequently, all control and treated samples were diluted to 0.5% prior to reading the absorbance at 280 nm (0.681 units). The different treatment methods were:

Treatment with Activated Charcoal

Ten mL of a 1% pullulan solution was run through a column of activated charcoal (Sigma Chemical Co., St. Louis, MO) with either 1:1 (w/w) ratio of 14 mesh:20-40 mesh, or 1 g 20-40 mesh at a number of pH values. After the optimal pH was determined to be 8.9, 50 mL of a 1% solution at pH 8.9 was treated with 1 g fine charcoal powder (mesh size unknown), and shaken overnight at ambient temperature on a wrist-action shaker (Burrell Corp., Pittsburgh, PA).

Commercial "Mini-column"

Ten mL of a 1% pullulan solution, pH 6.9, was passed via syringe into three C-18 Sep-Pak® mini-columns (Waters Chromatography Div., Millipore Corp., Milford, MA).

Differential Precipitation by Solvent

Ten mL of a 1% pullulan solution, pH 8.9, was treated with the following solvent: a minimal amount of acetone necessary for pullulan precipitation, 4 mL (28.5% v/v); a mixture of 5 mL acetone and 5 mL toluene. After treatment, the precipitate was centrifuged and resolubilized to a 1% w/v) concentration.

Analytical Methods

Determination of pullulan weight average MW distribution and dispersity was performed on a Waters 150-C ALC/GPC Gel Permeation Chromatograph® (Waters Chromatography Div., Millipore Corp., Milford, MA). The system was calibrated using a series of polysaccharide standards ranging in MW between 12,200 and 853,000 (Polymer Laboratories Ltd., Church Stretton, UK). The standards were run through three Bio-Gel® (Bio-Rad Laboratories, Richmond, CA) columns; a TSK-60, separating in the 40,000 to 8,000,000 range, and two TSK-50 columns, effective from 4,000 to 800,000. The TSK-60 column preceded the two TSK-50 columns in line. A third order calibration curve was generated correlating MW distribution with retention time on the columns. The instrument automatically interpolated the calibration curve and these calculations were used to integrate the area under the sample MW distribution curve to determine weight average MW and dispersity. Standards and samples were solubilized at 0.1% in the carrier solvent, which consisted of an aqueous solvent of sodium acetate, 0.1 M; acetic acid, 2% (v/v), and sodium azide, 0.05% (w/v). The instrument was adjusted to 1.0 mL/minute flow-rate. The injection volume was 200 to 300 µL and the run time was 40 minutes.

RESULTS

Batch Culture

A series of preliminary experiments were conducted, using nine strains of the black yeast Aureobasidium pullulans, A. pullulans var. melanigenum, or A. mansoni (Table 1). Strains of A. pullulans produce the biopolymer pullulan which is released into the extracellular medium. Initially, the medium of Ramos and Garcia Acha[22] containing 5% sucrose was used to compare pullulan elaboration among the strains (Table 2). A five-day incubation period at a temperature of 28°C was chosen, based on previous observations of growth conditions of the microorganism on solid media. As shown in Table 2, the strains of A. pullulans varied in product color, yield, and MW distribution. The medium of Ueda, et al.,[15] and that of Kato and Shiosaka[10] were also evaluated, and the strain of NRRL-Y 6220 was chosen for further studies based on product yield and color. Differences among strains are also illustrated in Table 3. The effect of length of incubation on MW distribution and yield of pullulan obtained from QM 5752 is shown in Table 4.

Figures 1 and 2 illustrate the effect of incubation time on pullulan yield and MW distribution using NRRL-Y 6220. The highest MW distribution product was attained on the first day of incubation, but the greatest yield was afforded after seven days of incubation. These results agree with those reported by Kato and Shiosaka; however, they did not evaluate culture periods of less than four days.

The effects of carbon and nitrogen sources, and phosphate concentration on pullulan yield are shown in Tables 5, 6, and 7, using NRRL-Y 6220 and the medium of Kato and Shiosaka. Fructose used as a carbon source resulted in the highest MW distribution of pullulan, but the use of sucrose yielded a greater amount of product (Table 5). The use of soluble starch resulted in the highest yield of pullulan; however, the MW distribution of the product was relatively low. Various nitrogen sources were evaluated for use in pullulan production. A combination of peptone and ammonium sulfate gave the highest MW distribution of product, and yields varied from 5% to 9%. The use of urea, and urea with ammonium sulfate resulted in a high MW distribution of product, and yields were between 10% and 11% (Table 6).

As can be seen in Table 7, the phosphate concentration, within the range evaluated, seemed to have little effect on the MW distribution or the product yield, at least during the time period studied. Kato and Shiosaka reported, however, that using a medium containing glucose, phosphate concentration and initial pH value affected the yield, and MW distribution of pullulan. Their culture period was from four to seven days, whereas a time period of from two to three days was used in this study. We found that length of incubation and initial pH had more of an effect than phosphate concentration (see Table 10).

Table 8 shows the production of pullulan by 10-liter batch fermentation for two-day periods, using a 1% inoculum. The weight average MW was variable, but within the 2 to 3 million range, averaging about

2.2 million. For batches 4 and 7 alone, the weight average MW dropped to between 1.6 and 1.7 million. A 10-liter batch fermentation is shown in Table 9, where sampling was done from the fourth through the seventh day. A steady decline in MW was observed, corroborating the results shown in Fig. 1, although lower MW distribution pullulan was obtained from the flask study. Pigmentation and oxygenation were problems that occurred with the 10-liter fermentation studies. One factor that may have affected the results was the use of 1% inoculum for the 10-liter fermentations. Ramos and Garcia Acha[22] have reported that the initial concentration of cells used in the inoculum does have an effect on the amount of pullulan produced and the appearance of pigmentation in the culture. In the smaller batch studies, a 2% inoculum was always used, and pigmentation was not produced.

Continuous Culture

The production of pullulan by continuous fermentation is shown in Table 10. A modified Kato and Shiosaka medium (0.1% phosphate) with an aeration rate of 0.5 L/min, an agitation rate of 300 rpm, temperature of 26°C to 27°C, and a variable flow-rate was run for a total of seven days. After an initial culture incubation of two days, the media flow was begun. Yields were low, but the weight average MW of the product was 4.3 million. The use of urea and ammonium sulfate, and unmodified Kato and Shiosaka medium, are also shown in Table 10, as is the effect of varying the flow-rate of the medium. Weight average MW held within the 3 to 4 million range. Only after an extended period of cultivation (11 days) with a flow-rate of 15 to 20 mL/h was there a significant decrease in MW distribution. At this point, the culture began to decline, forming pigmented cells, and the fermentation was halted.

Extraction and Processing

The method of Ueda et al.[15] was first used to process the biopolymer. This involved diluting the culture medium with an equal amount of distilled water, neutralizing with 4% NaOH, and centrifuging at 7,000 X g for 15 min. The supernatant was diluted with an equal amount of 95% ethanol, then refrigerated overnight at 5°C. The mixture was centrifuged at 2,500 X g for 15 min. The precipitate was washed twice with 55% ethanol, then washed with absolute ethanol and twice with ether. The product was then dried. A simpler and easier method was devised (Fig. 3), using acetone, eliminating the second centrifugation step, and the petroleum ether drying. The use of ROCCAL II aided the processing of the culture medium and helped remove the discoloration in the older cultures. Acetone-precipitated polymer yielded a fine white powder when all excess moisture was removed.

Purification

The effect of further purification, using Soxhlet extraction and/or tangential flow filtration on MW distribution, is shown in Table 11. The GPC chromatograms are shown in Fig. 4. The initial weight average MW of

the sample was 2.3 million. Tangential flow filtration was more effective than Soxhlet extraction in removing contaminants, as can be seen by the size of the monomer peaks in Fig. 4. All purification methods lowered the MW distribution, and reduced the size of the monomer peak.

Decolorization

Table 12 summarizes the variety of methods that were tried in order to remove unwanted color from pullulan solutions. The amount of decolorization was monitored by absorbance at 280 nm on a UV-visible spectrophotometer. Treatment by activated charcoal on a column at various pH values yielded a product that removed 57.8% of the color at pH 8.9. The amount of pigment removed increased with increasing pH. However, only 42.5% of the color was removed at pH 10.9. Using fine activated charcoal (mesh size unknown), and shaking the solution overnight increased the surface area and the amount of pigment in contact with the charcoal. The percent decolorization was increased to 62.2%.

Treatment of the pigmented solution by passing it over three C-18 columns removed 50.3% of the pigment. The disadvantage of using this method was the long elution time needed.

The use of solvents met with mixed results. The acetone/toluene mixture caused absorbance of the resolubilized pullulan to increase to 1.153 units. The acetone/pullulan solution (28.5% v/v) removed 55.5% of the pigment.

The absorbance of an unpigmented pullulan solution was also determined. It yielded 0.191 units, far below that of the pigmented sample tested (0.681 units). When this sample was filtered over a 0.45 μm filter, the absorbance decreased further to 0.094 units.

DISCUSSION

In order to determine the best culture for pullulan production, nine strains of Aureobasidium pullulans were examined. Of these strains, only QM 3090 had been reported previously as being used for the study of pullulan production (cited erroneously as QM 3092),[17-19] except for the QM strains used by Dr. E. T. Reese of this Center (unpublished data, 1965). Tables 1, 2, and 3 detail the comparison of these strains. Three of the cultures produced an acid-soluble glycan, probably that first fractionated by Bouveng et al.[25,26] as discussed by Catley.[18,20] The fraction was not investigated further in this study. The criteria used in selecting the best culture were: product color, yield, and MW distribution. Both cultures of QM 5752 and NRRL-Y 6220 produced a white product, but the MW distribution of QM 5752 was low when compared over time (Table 4). The culture of NRRL-Y 6220 also produced a low MW product initially, but when the culture medium was changed, greater yields and higher MW distributions were obtained (Figs. 1 and 2).

Studies of carhon and nitrogen sources, and phosphate concentration, shown in Tables 5, 6, and 7, determined that 10% sucrose was the best carbon source, based on availability and economy, although the use of fructose produced the highest MW product, and soluble starch gave the best yield of low MW product. Combinations of peptone, ammonium sulfate, and urea were the best nitrogen sources evaluated, in terms of yield and MW distribution. Phosphate concentrations at pH 5.4 seemed to have little effect on MW distribution, at least during the incubation periods studied, in contrast to those reported by Kato and Shiosaka, as mentioned previously.

Ten-liter batch fermentation results are shown in Tables 8 and 9. In these studies, a 1% inoculum was used, in contrast to the smaller flask studies. The weight average MW was in the 2.2 to 2.8 million range, with about a 12% yield for the 10-liter studies (Table 8). With the smaller flask studies, weight average MW was in the 4 million range and yield was 15%. Pigmentation problems occurred with the large batch studies, and again, this can be attributed to the use of the smaller amount of inoculum.

The production of pullulan by continuous fermentation is shown in Table 10. Both 0.1% and 0.2% dipotassium hydrogen phosphate, and nitrogen sources of peptone, ammonium sulfate, and urea were studied, as well as two initial pH levels. After incubation of the culture for two days, the medium was dispensed into the fermentor jar at various flow-rates. With a flow-rate of ≈50 mL/h, a high MW product was obtained using 0.1% phosphate, although yields were low. Changing the medium to urea and ammonium sulfate affected both the MW distribution and the yield of the product when a higher flow-rate was used. When 0.2% phosphate was used at two different pH levels, MW distribution remained high, even at relatively high flow-rates. When the flow-rates were decreased, the turnover rate correspondingly decreased, and the cell mass and the extracellular polymer increased. The culture began to decline and hecame pigmented. This was probably due to lack of oxygen availability and the viscosity of the fermentor contents.

The processing procedure used in this study is shown in Fig. 3. The use of ROCCAL II (suggested by Dr. E. T. Reese, unpublished data) in processing the polymer was with reference to the work of Scott[27] and that of Bouveng *et al.*[25,26] who used long-chain quaternary ammonium compounds to precipitate acid-soluble glycans and β-linked glucans from α-linked glucans. The neutral α-linked glucans remain in solution and after the precipitated products are removed by centrifugation can then be precipitated by solvents such as ethanol or acetone. Very little of the quaternary compound precipitates with the polymer, and this compound can be removed by solubilizing the polymer in water and re-precipitation. Fuller's earth or activated carbon can also be used to adsorb the quaternary ammonium compound from the solubilized polymer; also, these substances aid in removing the discoloration products excreted into the medium by older cultures.[7,9,10,27]

In summary, defined MW fractions of pullulan were produced by varying fermentation conditions such as constituents of the culture medium, pH, length of incubation, etc. Pullulan biopolymer products with weight

average MW from 100 thousand to 4 million have been produced. The appropriate conditions were then established to produce sufficient quantities of low (below 500 thousand weight average MW), medium (1 to 2 million weight average MW), and high (above 2 million weight average MW) molecular weight pullulan products. Films and fibers, and chemical derivatives made from these different preparations are now under evaluation in terms of physical/chemical characterization.

CONCLUSIONS

In this study, fermentation and processing conditions were characterized for the production of the biopolymer pullulan. Parameters can be controlled so that quantities of defined MW product can be produced. The evaluation of physical/chemical properties of pullulan, and the investigation of specific military applications, such as low gas permeability films for food packaging and high performance fibers, has now begun.

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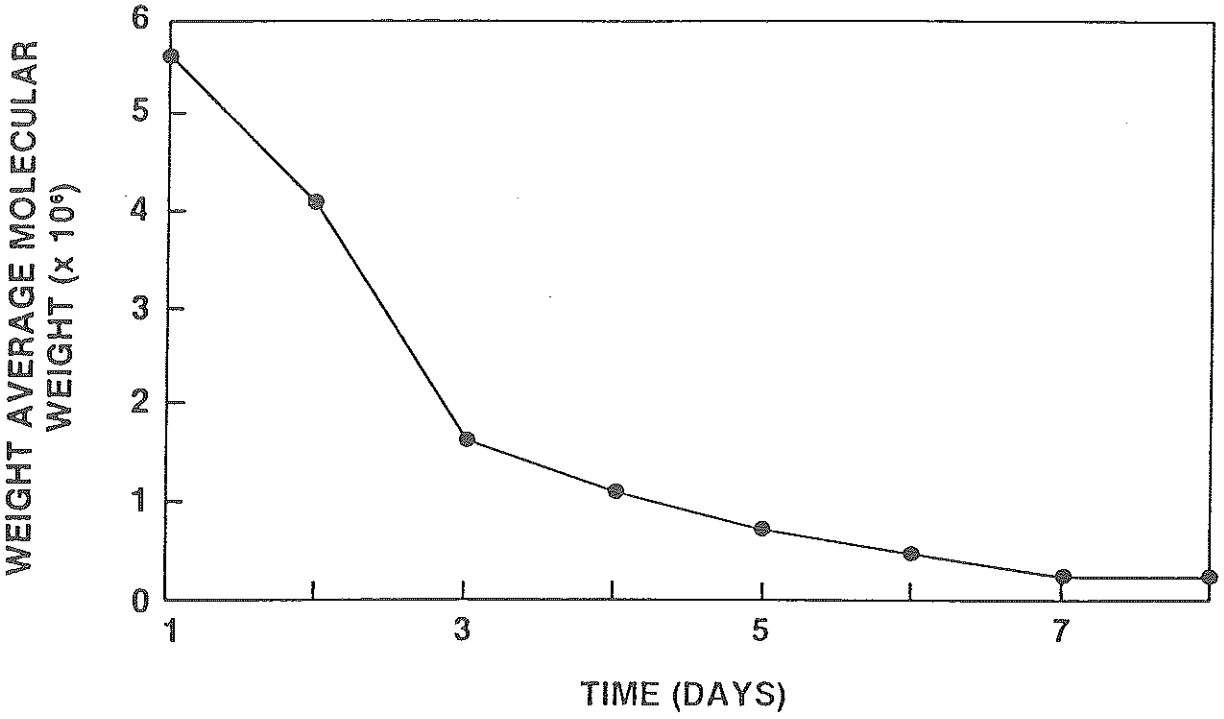

Figure 1. Weight average molecular weight of pullulan vs. time.

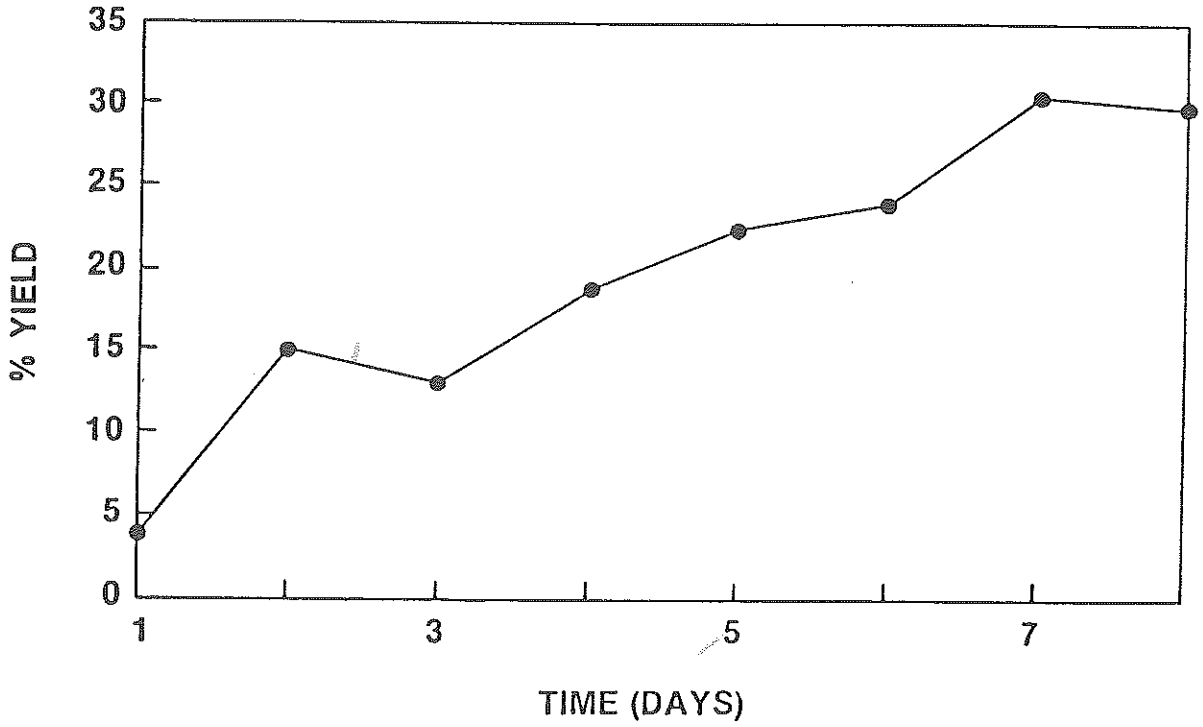

Figure 2. Percent yield of pullulan vs. time (NRRL-Y 6220).

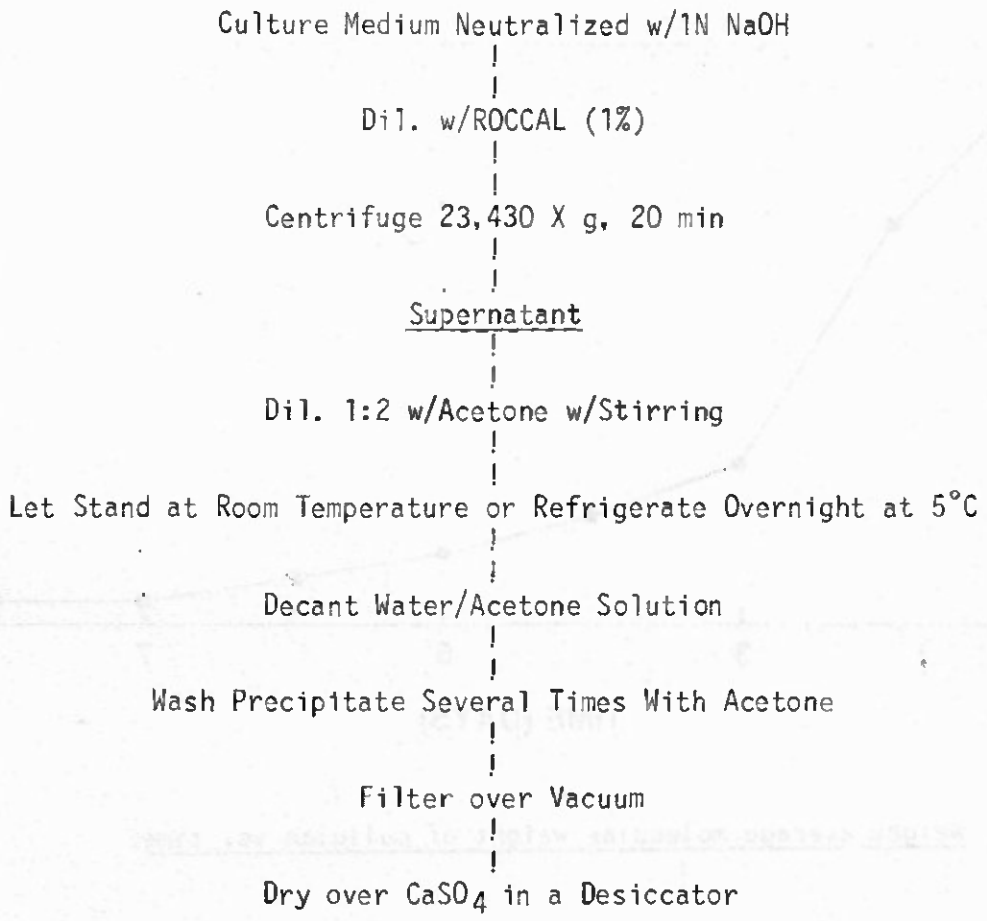

Figure 3. Pullulan processing conditions.

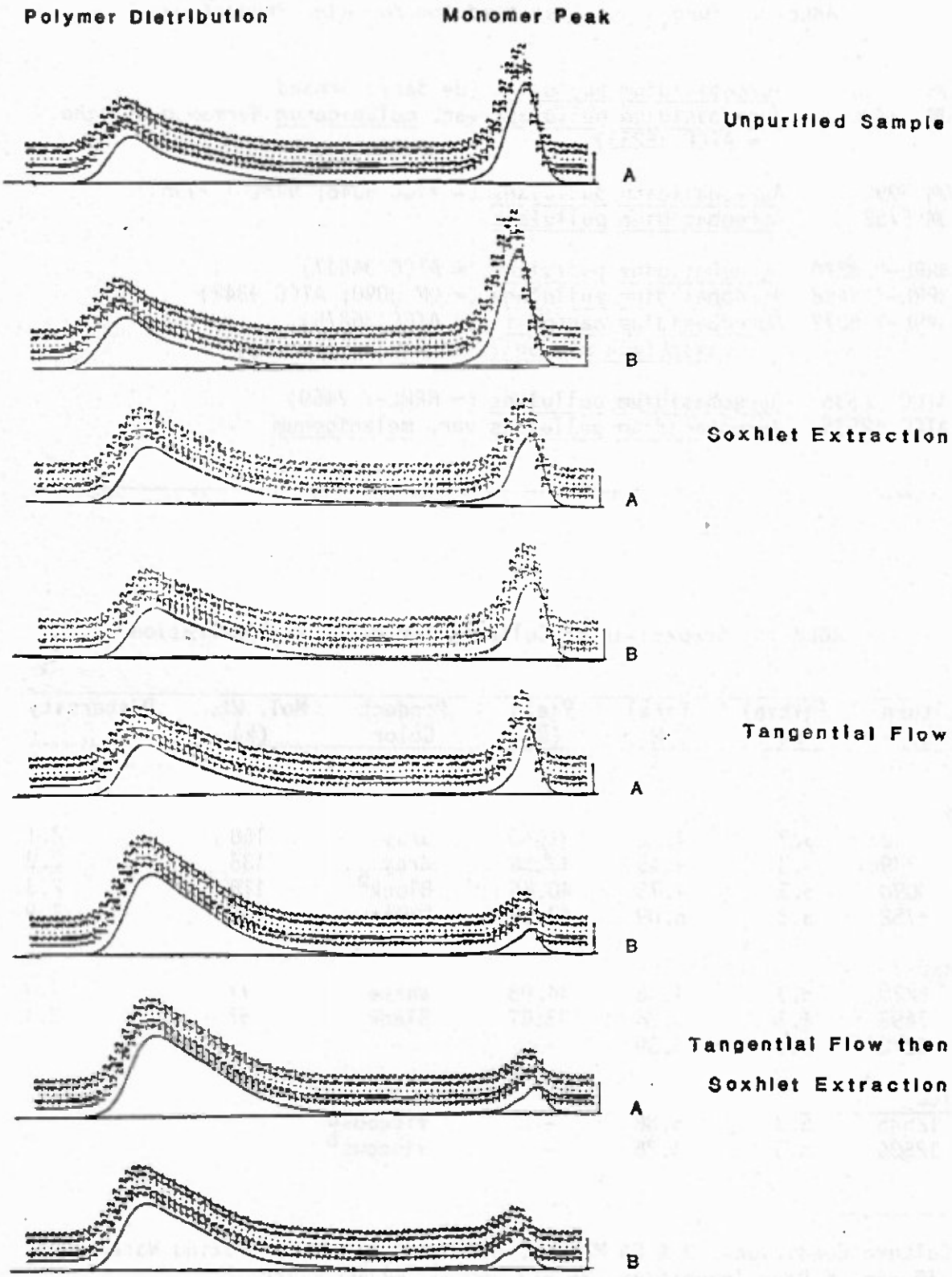

Figure 4. GPC chromatograms showing the effect of purification on MW distribution.

TABLE 1. Fungus Cultures Used for Pullulan Production

QM 72c	*Aureobasidium pullulans* (de Bary) Arnaud
QM 279c	*Aureobasidium pullulans* var. *melanigenum* Hermanides-Nijhof (= ATCC 15233)
QM 3090	*Aureobasidium pullulans* (= ATCC 9348; NRRL-Y 7498)
QM 5752	*Aureobasidium pullulans*
NRRL-Y 6220	*Aureobasidium pullulans* (= ATCC 34647)
NRRL-Y 7498	*Aureobasidium pullulans* (= QM 3090; ATCC 9348)
NRRL-Y 6272	*Aureobasidium mansonii* (= ATCC 36276) [= *Exophiala mansonii* (Castell.) de Hoog]
ATCC 12535	*Aureobasidium pullulans* (= NRRL-Y 7469)
ATCC 12536	*Aureobasidium pullulans* var. *melanigenum*

TABLE 2. Comparison of Cultures for Pullulan Elaboration[a]

Culture No.	Initial pH	Final pH	Yield (%)	Product Color	Mol. Wt. (k)	Dispersity
QM						
72c	5.3	4.42	31.50	Gray	168	2.1
279c	5.3	4.45	42.56	Gray	133	2.9
3090	5.3	4.75	40.66	Black[b]	118	2.3
5752	5.3	6.09	47.30	White	138	1.9
NRRL-Y						
6220	5.3	4.16	44.03	White	77	1.7
7498	5.3	4.26	33.07	Black	57	2.1
6272	5.3	6.39	-	-	-	-
ATCC						
12535	5.3	5.86	-	Viscous[b]	-	-
12536	5.3	3.26	-	Viscous[b]	-	-

[a]Culture Conditions: R & GA Medium, 5 % Sucrose, 28°C, Shaking Water Bath, 150 rpm, 5 Days Incubation, 25 mL/ 250 mL DeLong Flask.

[b]Heteropolymer Produced[25,26]

TABLE 3. Comparison of QM Cultures for Pullulan Elaboration[a]

Culture No.	Initial pH	Final pH	Yield (%)	Mol. Wt. (k)	Dispersity
72c	5.46	3.57	29.30	137	2.4
279c	5.46	3.67	19.40	653	2.5
3090	5.46	3.47	6.79[b]	1803	2.0
5752	5.46	5.93	31.17	94	2.3

[a]Culture Conditions: K & S Medium, 10 % Sucrose, 26.5°C, 120 rpm, Environ-Shaker, 4 Days Incubation, 50 mL/250 mL DeLong Flask, 2% Inoculum.

[b]Heteropolymer Produced[25,26]

TABLE 4. Effect of Incubation Time on Pullulan Yield Using QM 5752 Aureobasidium pullulans*

Culture Period	Initial pH	Final pH	Yield (%)	Mol. Wt. (k)	Dispersity
3	5.4	5.51	13.68	58.8	1.1
4	5.4	5.26	19.87	47.5	1.6
5	5.4	4.64	22.45	28.0	1.8
6	5.4	4.84	21.10	21.1	1.6

*Culture Conditions: Mod. K & S Medium, 10 % Sucrose, 27°C, Environ-Shaker, 75 rpm for 3 Days, 120 rpm 4th Day, unshaken 5th Day, 120 rpm 6th Day, 50 mL/250 mL DeLong Flask, 2% Inoculum.

TABLE 5. Effect of Carbon Source on Pullulan Yield Using NRRL-Y 6220 *Aureobasidium pullulans**

Carbon Source (10%)	Initial pH	Final pH	Yield (%)	Mol. Wt. (k)	Dispersity
Fructose	5.44	3.76	27.0	1122	2.5
Sucrose	5.44	3.69	34.4	895	2.4
Maltose	5.44	4.08	25.4	881	2.1
Corn Syrup	5.44	3.85	29.3	840	3.3
Dextrose	5.44	3.91	23.3	563	1.8
Lactose	5.44	3.79	5.8	518	1.6
Sol. Starch	5.44	4.24	70.3	137	1.4
Dextrin	5.44	2.99	21.6	9	1.1

*Culture Conditions: K & S Medium, 0.2% K_2HPO_4, 26.5°C, 120 rpm Environ-Shaker, 4 Days Incubation, 50 mL/250 mL DeLong Flask, 2% Inoculum.

TABLE 6. Effect of Nitrogen Source on Pullulan Yield Using NRRL-Y 6220 *Aureobasidium pullulans**

Nitrogen Source (%)	Initial pH	Final pH	Yield (%)	Mol. Wt. (k)	Dispersity
$NaNO_3$ (0.2)	5.31	6.67	2.00	78	1.1
$NaNO_3$ (0.2); Y. Ext. (0.25)	5.30	6.49	5.30	85	1.4
Peptone (0.2)	5.30	3.64	6.30	1935	2.3
Peptone (0.2); $(NH_4)_2SO_4$ (0.01)	5.50	3.50	9.00	1384	2.8
Peptone (0.1); $(NH_4)_2SO_4$ (0.01)	5.31	3.27	4.95	2940	4.9
$(NH_4)_2SO_4$ (0.14)	5.30	2.26	1.17	-	-
Urea (0.3)	5.30	4.93	10.20	2061	2.6
Urea (0.3); $(NH_4)_2SO_4$ (0.14)	5.32	5.08	11.02	1898	2.7
Y. Ext. (0.25)	5.31	4.16	18.80	859	6.7

*Culture Conditions: K & S Medium w/o Nitrogen, 10 % Sucrose, 26.5°C, Environ-Shaker 120 rpm, 3 Days incubation, 50 mL/250 mL DeLong Flask, 2% Inoculum.

TABLE 7. Effect of Phosphate Concentration on Pullulan Yield Using NRRL-Y 6220 *Aureobasidium pullulans**

Culture Period	Phosphate Conc. (%)	Initial pH	Final pH	Yield (%)	Mol. Wt. (k)	Dispersity
2 Days	K_2HPO_4					
	0.1	5.42	3.43	8.50	2704	2.0
	0.2	5.42	3.65	11.10	2659	2.1
	0.3	5.42	3.58	11.20	2646	1.8
	0.4	5.42	3.88	13.70	2165	2.3
	0.5	5.42	3.93	14.00	2477	2.2
2 Days	KH_2PO_4					
	0.2	5.42	3.50	9.20	2608	2.1
3 Days	K_2HPO_4					
	0.1	5.42	3.65	11.60	1968	2.2
	0.2	5.42	3.79	14.40	1551	2.3
	0.3	5.42	3.82	13.10	1629	2.2
	0.4	5.42	3.97	16.20	1369	2.3
	0.5	5.42	4.10	18.40	1169	2.4
3 Days	KH_2PO_4					
	0.2	5.42	3.77	11.00	1933	3.7

*Culture Conditions: Kato & Shiosaka Medium, 10% Sucrose, 26°C - 26.5°C, 125 rpm New Brunswick Shaker, 50mL/250 mL DeLong Flask, 2% Inoculum.

TABLE 8. Production of Pullulan by 10-Liter Batch Fermentation Using NRRL-Y 6220 *Aureobasidium pullulans*[a]

Batch No.	Oxygenation	Agitation (rpm)	Yield g	Yield (%)	Mol. Wt. (k)	Dispersity
1	45 (5L/min)	1200	113	11.3	2875	2.5
3	50	1200	75	7.5	2163	1.5
4	50	1200	100	10.0	1752	1.4
5[b,c]	60	1200	188	18.8	2425	2.4
6[b,c]	60	1200	150	15.0	2416	1.9
7[b,c]	60	1200	209	20.9	1663	1.6
8[c]	150	1200	-	-	2399	1.5

[a]Culture Conditions: Kato & Shiosaka Medium; 10% Sucrose; Temperature 26°C ±1°C, Two-Day Incubation, 1% Two-Day-Old Inoculum.

[b]Pigment Produced.

[c]New Culture Transfer.

TABLE 9. Effect of Incubation Time on Pullulan Yield Using NRRL-Y 6220 *Aureobasidium pullulans**

Incubation (Days)	Oxygenation	Agitation (rpm)	Yield g	Yield (%)	Mol. Wt. (k)	Dispersity
4	45 (5L/min)	1200		-	2262	2.0
5	45	1200		-	2013	2.5
6	45	1200		-	1586	1.6
7	45	1200		-	1438	2.5

*Culture Conditions: Kato & Shiosaka Medium; 10% Sucrose; Temperature 26°C ±1°C, 10-Liter Batch Fermentation, 1% Inoculum.

TABLE 10. Production of Pullulan by Continuous Fermentation Using NRRL-Y 6220 *Aureobasidium pullulans*[a]

Medium	Initial pH	Final pH	Volume (Product)	Flow-Rate (mL/h)	Yield g (%)	Mol. Wt. (k)	Disp.
0.1% K_2HPO_4	5.4	3.44	10.19 L	±50	43.5 (4.3)	4312	2.4
Urea/[b] $(NH_4)_2SO_4$	5.4	3.07	9.53 L	±120	20.0 (2.1)	2190	1.5
0.2% K_2HPO_4	6.0	4.01	3.57 L	100	9.0 (2.5)	4404	2.3
0.2% K_2HPO_4	6.0	4.68	5.88 L	75	27.0 (4.6)	3639	2.6
0.2% K_2HPO_4	6.0	4.47	1.33 L	Ferm.	11.1 (8.3)	2986	3.8
0.2% K_2HPO_4	5.4	3.70	0.53 L	15	4.1 (7.7)	4223	3.6
0.2% K_2HPO_4	5.4	3.56	1.46 L	15	17.5 (11.9)	4115	3.0
0.2% K_2HPO_4	5.4	3.37	0.85 L	15	9.2 (10.7)	3247	2.7
0.2% K_2HPO_4	5.4	3.79	1.45 L	20	23.9 (16.3)	1547	1.7
0.2% K_2HPO_4	5.4	4.05	2.14 L	20, 60[c,d] Ferm.	32.8 (15.4)	1258	1.8

[a] Culture Conditions: Modified K & S Medium; 10% Sucrose; Aeration 0.5 L/min; Agitation, 300 rpm; Temperature 26°C ± 1°C; Flow-Rates Variable, 2% Inoculum.

[b] Urea 0.03%, $(NH_4)_2SO_4$ 0.014%.

[c] Aeration 0.6 L/min, Agitation 400 rpm; 20 mL/h, 1 Oay.

[d] Aeration 1.0 L/min, Agitation 400 rpm; 60 mL/h, 1 Oay.

TABLE 11. Effect of Purification Procedures on the MW Distribution of Pullulan

Procedure (Sample)	Mol. Wt. (k)	Dispersity
Unpurified Sample		
(A)	2305	1.97
(B)	2296	2.18
Soxhlet Extraction		
(A)	1759	2.20
(B)	1796	2.02
Tangential Flow Filtration		
(A)	1778	1.95
(B)	1727	1.80
Tangential Flow then Soxhlet Extraction		
(A)	1644	1.89
(B)	1639	1.94

TABLE 12. Percent Pigment Removed from Pullulan

Treatment	Percent Removal
1:1 14:20-40 Mesh Charcoal Column	
pH 4.9	36.7
pH 6.9	42.2
pH 8.9	50.5
1 g 20-40 Mesh Charcoal Column	
pH 4.9	37.5
pH 6.9	49.2
pH 7.9	54.5
pH 8.9	56.5*
pH 9.9	49.9
pH 10.9	42.5
50 mL 1% Solution pH 8.9 Plus 1 g Fine Charcoal Shaken Overnight	62.6
1% Solution pH 6.9 C-18 Cartridge (3X)	50.3
10 mL 1% Solution pH 8.9 Plus 4 mL Acetone	55.5

*Repeated Twice