ELSEVIER

Carbohydrate Polymers 52 (2003) 25–28

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Studies on downstream processing of pullulan

Dharmendra K. Kachhawa, Paramita Bhattacharjee, Rekha S. Singhal*

Food and Fermentation Technology Department, Institute of Chemical Technology, University of Mumbai, Matunga, Mumbai 400 019, India

Received 17 April 2002; revised 1 August 2002; accepted 24 August 2002

Abstract

Pullulan, an extracellular polysaccharide is obtained by fermentation using a fungus, *Aureobasidium pullulan*. To obtain the pure biopolymer from the fermentation broth, cell harvesting, removal of the melanin pigments co-produced during fermentation and precipitation of the polymer are essential. The present work reports on some of these aspects. Centrifugation of the fermentation broth (at optimized fermentation conditions) at 800 rpm for 30 min gave a cell pellet that was discarded and a greenish black supernatant containing the melanin pigments. The supernatant was subjected to demelanization by adsorption on activated charcoal, or by use of solvent/solvent blends, or by solvent/salt combinations, all after denaturation of the pullulanases at 80 °C/1 h. Among all these treatments, a combination of ethanol/ethyl methyl ketone in 60:40 ratios was found to be most effective. Melanin separated out in the solvent blend, while the pure pullulan that precipitated out was comparable in colour and texture to that of the commercial sample.

Keywords: Pullulan; Downstream processing; Charcoal adsorption; Solvent extraction; Melanin pigmentation

1. Introduction

Pullulan, a neutral glucan consisting of linear polymer of maltotriosyl units connected by α-(1 → 6) linkages, is an extracellular microbial polysaccharide produced by a yeast-like fungus, *Aureobasidium pullulans*. A number of potential applications have been reported for this biopolymer as a result of its good film forming properties. Pullulan can form thin films which are transparent, colourless, tasteless, odourless, tenacious, resistant to oil and grease and unaffected by small thermal variations. Besides, the films are also impermeable to oxygen, non-toxic, biodegradable and edible. It is insoluble in many solvents including methanol, ethanol and acetone, but soluble in water to form a transparent, colourless, viscous adhesive solution (Yuen, 1974).

Considerable attention has been paid to the microbiology of the pullulan producing organism (Guterman & Shabtai, 1996); production of pullulan with respect to the nutrient sources (LeDuy, Yarmoff, & Chagraoui, 1983; LeDuy & Boa, 1983; Deshpande, Rale, & Lynch, 1992), fermentation conditions such as pH (Ono, Kawahara, & Ueda, 1977; Lacroix, LeDuy, Noel, & Choplin, 1985), temperature (McNeil & Kristiansen, 1990), minerals such as Zn^{+2}, Fe^{+3}, Mn^{+2}, Ca^{+2} and Cu^{+2} (Reeslev & Jensen, 1995); and process technology for commercial manufacture of pullulan (Thibault & LeDuy, 1999).

The currently used commercial process for pullulan differs according to the intended application. For use in food processing applications, the fermentation broth is decolourized using activated charcoal, concentrated followed by spray or freeze drying, and finally pulverized. For pharmaceutical uses and film production, the protocol is similar except for additional steps of purification by alcohol fractionation or membrane filtration prior to decolourization and desalination after decolourization. For use as industrial adhesives, dispersants and coagulants, culture broth containing crude pullulan is concentrated, dried and pulverized (Thibault & LeDuy, 1999).

The major problems faced during the production of pullulan are high viscosity of the fermentation broth, melanin pigmentation and pullulanolysis during fermentation. After the completion of fermentation, the resulting fermentation broth consists of microbial cells and cellular debris, residual media components from culture medium and extracellular metabolites produced and excreted during the fermentation. The solids are normally separated by centrifugation. However, the liquid phase containing the pullulan also contains the melanin pigment, which makes the broth dark green to black in colour. It is because of this

* Corresponding author. Fax: +91-22-414-5614.
E-mail address: rekha@foodbio.udct.ernet.in (R.S. Singhal).

0144-8617/03/$ - see front matter © 2002 Elsevier Science Ltd. All rights reserved.
PII: S0144-8617(02)00261-8

reason that *Aureobasidium pullulans* is also called as black yeast. The melanin is synthesized both intracellularly as well as extracellularly by the pentaketide pathway (Siehr, 1981) during the last stages of the fermentation when the cell morphology changes from swollen cells to true chlamydospores. The synthesis of melanin depends on culture conditions, and the media is generally optimized to get least melanin pigmentation. Although the best solution is to use non-pigmented mutagenic strains, compromise on the productivity is often observed (Tarbasz-Szymanska & Galas, 1993). An appropriate downstream processing of the fermentation broth could alleviate this problem. This work attempts to comparatively evaluate the efficacy of decolourization of the fermentation broth by using activated charcoal, various solvents, solvent blends and combination of salts and solvent, so as to get melanin-free pullulan without compromising on yield.

2. Materials and methods

2.1. Materials

1. *Aureobasidium pullulans* NCIM 976 (PRL 1491) used in this work was obtained from National Collection of Industrial Microorganisms (NCIM), Pune. The organism was maintained on potato dextrose agar slants at 4 °C and sub cultured every two weeks. The cells for the inoculation were obtained from the culture grown at 28 °C for 48 h.
2. Media components such as sucrose, potassium monohydrogen phosphate, sodium chloride, magnesium sulphate, ammonium sulphate and potassium nitrate used in the optimization studies, ethyl methyl ketone and activated charcoal used for downstream processing were obtained from M/S S.D. Fine Chemicals, Mumbai. Other solvents such as methanol, ethanol and acetone were procured from M/S Merck India Ltd, Mumbai. Potato dextrose agar was purchased from M/S Himedia, Mumbai.

2.2. Methods

The media composition previously optimized with a view to obtain maximum pullulan with minimum pigmentation was: sucrose 5%, potassium monohydrogen phosphate 0.2%, sodium chloride 0.1%, ammonium sulphate 0.06%, magnesium sulphate heptahydrate 0.02% and potassium nitrate 0.04%, adjusted to a pH of 5.5. Similarly preliminary fermenter trials (carried out in a 7L Electrolab® fermenter of 30 cm height, 16 cm diameter having 2 turbine impellers; one at lower end with 6 blades, and other 10 cm apart at the upper end was a pitch blade impeller with an angle of 45°; the impellers were mounted on an impeller shaft mounted 3 cm above the fermenter bottom; it had 4 baffles of 3 cm breadth and 25 cm in length) had indicated 72 h fermentation as the optimum for pullulan production. This was then used for further studies on downstream processing (Kachhawa, unpublished work).

2.2.1. Cell recovery or harvesting

After 72 h of fermentation in a fermenter operating at 300 rpm and 2 VVM, the broth was neutralized to pH 7 using 1N NaOH, and transferred to 4 l plastic jar. 1000 ml of the broth was transferred to another 2 l flask of which 100 ml of the broth was centrifuged at 8000 rpm for 30 min at room temperature (~30–32 °C), after which the supernatant was cautiously transferred to flask, while the cell pellet separated from the centrifuge tubes was washed twice with distilled water. The first washing was transferred to the supernatant, while the cell pellet was autoclaved and discarded. The supernatant obtained was subsequently used for further steps in downstream processing.

2.2.2. Separating the melanin pigments

The supernatant obtained as above was heated to 80 °C for 1 h to deactivate the extracellular pullulanases, filtered through Whatman filter paper no. 2 using a vacuum of 400 mm Hg, and then cooled to 25 °C. The optical density of this supernatant was measured at 320 nm in a Hitachi Spectrophotometer as an indicator for melanin pigmentation. 10 ml of this suspension was precipitated using 20 ml absolute ethanol. The supernatant was decanted, and the residue obtained was dried till constant weight in an oven at 60 °C to give the crude pullulan. For the separation of melanin, different systems were attempted as follows:

2.2.2.1. Adsorption by activated charcoal. Different quantities of activated charcoal (0.2–2.0%) were used for the adsorption of the melanin pigments by shaking for 10 min at 250 rpm. The pigment adsorbed by the activated charcoal was removed by centrifugation at 8000 rpm followed by filtration through Whatman filter paper no. 2 using vacuum pump. The filtrate was then precipitated using 2 volumes of absolute alcohol and the resulting precipitate was dried in an air oven as above. The pullulan precipitate was further dissolved in distilled water to get 2.5% solution, and checked for optical density at 320 nm. A concentration of 2.5% was chosen, since the concentration of pullulan in the fermentation broth was 25 g/l.

The yield of pullulan after separation of pigments was calculated with reference to the initial weight of crude pullulan from the broth and the final weight of the precipitate obtained after separation of the pigments.

2.2.2.2. Adsorption of the pigments using solvents and solvent blends. 20 ml of the fermentation broth obtained after centrifugation was precipitated with different solvents (Table 2). During the precipitation, solvent was added until complete separation of the pullulan precipitate layer sank at the bottom of the flask and rendered the supernatant clear. The precipitated was decanted from the solvent and dried on

a preweighed filter paper at 60 °C in an oven till constant weight was obtained.

The optical density of the 2.5% solution of the purified precipitate was measured to check for the melanin pigments that may be present in the precipitate. The recovery and percentage yield of the resulting precipitate was calculated with reference to the initial concentration of pullulan as 2.5 g/l. This work was further extended to solvent blends using ethanol and ethyl methyl ketone in ratios of 10:90, 20:80, 30:70, 0:60 and 50:50.

2.2.2.3. Extraction of pigments using combination of solvents and salts. Varying combinations of potassium chloride and alcohol was used to extract polymer similarly as for solvents and solvent blends.

3. Results and discussion

The effect of amount of charcoal on the melanin pigmentation and recovery of pullulan is shown in Table 1. With increasing amounts of activated charcoal, the adsorption of the melanin pigments as well as the polymer increased. This rendered the separation of the polymer from the viscous broth very difficult. Viscosity of the broth can be decreased either by heating the broth to higher temperatures (80 °C) or by distilled water. These processes are impractical, since higher temperatures causes desorption of the melanin pigments from the surface of the activated charcoal and dilution of the broth adds to the cost of precipitation by increasing the quantity of the solvent required.

One percent activated charcoal was found to be the optimum for the separation of most of the pigments, and yield about 81.6% pullulan after precipitation. However, the remaining 18% adheres to the activated charcoal and is lost during the filtration. Adsorption was effective to separate the melanin pigments; almost 94.5% of the pigments were adsorbed by this method. It was further observed that increasing concentration of activated charcoal contaminated the precipitate with fine charcoal impurity that could not be separated even by centrifugation at 10,000 rpm. It also decreased the yield of pullulan.

Table 1
Effect of activated charcoal for the removal of melanin pigment by adsorption

Activated charcoal (g%) in the broth	Recovery (g/l)[a]	OD of the broth at 320 nm
0.0	25	0.66
0.2	22.4 (89.6)	0.58
0.4	22.1 (88.4)	0.51
0.6	21.6 (86.4)	0.43
0.8	20.8 (83.2)	0.26
1.0	20.4 (81.6)	0.12
1.2	19.0 (76.0)	0.48
1.4	18.6 (74.0)	0.79
2.0	16.6 (66.4)	2.2

[a] Values within parenthesis indicate the relative % yield.

Table 2
Effect of different solvents for the precipitation of pullulan and separation of melanin pigments

Solvent	Vol. of solvent (ml)[a,b]	Wt. of precipitate (g)	Pigments in 2.5% solution in water
Methanol	50	0.40	0.24
Ethanol	45	0.45	0.14
Isopropanol	45	0.42	0.21
Ethyl Methyl Ketone	42	0.44	0.02
Tetrahydrofuran	45	0.40	0.48
Acetone	45	0.42	0.22

[a] 20 ml supernatant was used for all sets.
[b] Volume of the solvent required for complete precipitation.

A comparison of different solvents on the efficiency of recovery of pullulan, and the removal of melanin pigments is shown in Table 2. While ethanol gave maximum yield of pullulan, ethyl methyl ketone was the most effective in the separation of melanin pigments. This was evident from the colour of the precipitate. All the other solvents included in this study gave yellow to brown coloured precipitate, which further darkened during storage. Hence further studies were done using blends of ethanol and ethyl methyl ketone.

An evaluation of blends of the two solvents viz. ethanol and ethyl methyl ketone in different proportions indicated a combination of 40:60 of ethyl methyl ketone/ethanol to be ideal for complete precipitation of pullulan with minimum melanin contamination. The results are documented in Table 3. This combination also gave an yield of 86% pullulan which was better than that obtained by treatment with the activated charcoal. The precipitate obtained by this method was brighter in colour and had a texture similar to the commercial polymer obtained by kind courtesy of M/S Hayashibara Biochemicals Ltd., Japan.

Salts such as potassium chloride are reported to be useful in the isolation of microbial polysaccharides such as xanthan (Smith & Pace, 1982). Use of 1% potassium chloride with different solvents indicated a marginal improvement in yield to 84.4% with isopropyl alcohol.

Table 3
Optimization of solvent blends for better pigment separation and precipitation of pullulan

% Blend		Recovery (g/l)[a]	Melanin in 2.5% solution of precipitate
Ethyl methyl ketone	Ethanol		
10	90	22.5 (88)	0.92
20	80	22.8 (88)	0.62
30	70	23.0 (86)	0.24
40	60	23.6 (86)	0.02
50	50	23.6 (86)	0.02

[a] Values within parenthesis indicate the relative % yield.

Table 4
Precipitation of pullulan using alcohol in combination with KCl

Solvent	Recovery (g/l)[a]	Melanin pigments
Ethanol	22.5	0.14
Ethanol + 0.5% KCl	21.6	0.16
Ethanol + 1% KCl	21.4	0.22
Ethanol + 1.5% KCl	20.2	0.22
Ethanol + 2% KCl	19.6	0.26
Methanol	20.0	0.24
Methanol + 0.5% KCl	19.4	0.24
Methanol + 1% KCl	18.6	0.26
Methanol + 1.5% KCl	18.1	0.27
Methanol + 2% KCl	16.2	0.32
Isopropyl alcohol	21.0	0.21
Isopropyl alcohol + 0.5% KCl	20.5	0.21
Isopropyl alcohol + 1% KCl	21.2	0.24
Isopropyl alcohol + 1.5% KCl	19.6	0.25
Isopropyl alcohol + 2% KCl	19.2	0.25

[a] Two vols of solvent, alone and that containing the salt/vol of supernatant.

However, with methanol and ethanol, use of potassium chloride decreased the yields. Hence attempts were made by using potassium chloride at different concentrations in combination with ethanol, methanol and isopropyl alcohol to separate the pullulan free of melanin pigments (Table 4). This may, however, be undesirable, since addition of potassium chloride to the polymer may require dialysis to remove the salts. This further adds to the cost of downstream processing.

4. Conclusions

Amongst the various downstream processing techniques evaluated to obtain a melanin-free pullulan, use of ethanol and ethyl methyl ketone in 60:40 ratio gave best results and is recommended for industrial use.

References

Deshpande, M. S., Rale, V. B., & Lynch, J. M. (1992). *Aureobasidium pullulans* in applied microbiology: A status report. *Enzyme and Microbial Technology*, *14*, 514–527.

Guterman, H., & Shabtai, Y. (1996). A self tuning-vision system for monitoring biotechnological processes. 1. Application to production of pullulan by *Aureobasidium pullulans*. *Biotechnology and Bioengineering*, *51*, 501–510.

Lacroix, C., LeDuy, A., Noel, G., & Choplin, L. (1985). Effect of pH on batch fermentation of pullulan from sucrose medium. *Biotechnology and Bioengineering*, *27*, 202–207.

LeDuy, A., & Boa, J. M. (1983). Pullulan production from peat hydrolysate. *Canadian Journal of Microbiology*, *29*, 143–146.

LeDuy, A., Yarmoff, J. J., & Chagraoui, A. (1983). Enhanced production of pullulan from lactose by adaptation and by mixed culture techniques. *Biotechnology Letters*, *5*, 49–54.

McNeil, B., & Kristiansen, B. (1990). Temperature effects on polysaccharide formation by *Aureobasidium pullulans* in stirred tanks. *Enzyme Microbiology and Biotechnology*, *12*, 521–526.

Ono, K., Kawahara, Y., & Ueda, S. (1977). Studies on pullulan elaboration by *Aureobasidium pullulans* S-1. Part II. Effect of pH on the content of glycolipids in *Aureobasidium pullulans* S-1. *Agricultural and Biological Chemistry*, *41*, 2313–2317.

Reeslev, M., & Jensen, B. (1995). Influence of Zn^{+2} and Fe^{+3} on polysaccharide production and mycelium/yeast dimorphism of *Aureobasidium pullulans* in batch cultivations. *Applied Microbiology and Biotechnology*, *42*, 910–915.

Siehr, D. J. (1981). Melanin biosynthesis in *Aureobasidium pullulans*. *Journal of Coating Technology*, *53*, 23–25.

Smith, I. H., & Pace, G. W. (1982). Recovery of microbial polysaccharides. *Journal of Chemical Technology and Biotechnology*, *32*, 119–129.

Tarabasz-Szymanska, L., & Galas, E. (1993). Two-step mutagenesis of *Pullularia pullulans* leading to clones producing pure pullulan with high yield. *Enzyme Microbiology and Biotechnology*, *15*, 317–320.

Thibault, J., & LeDuy, A. (1999). Pullulan, microbial production methods. In M. C. Flickeringer, & S. W. Drew (Eds.), (*Vol. 4*) (pp. 2232–2247). *Encyclopedia of Bioprocess Technology, Fermentation, Biocatalysis and Bioseparation*, New York: Wiley.

Yuen, S. (1974). Pullulan and its application. *Process Biochemistry*, *9*, 7–9.