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Downstream processing of pullulan from fermentation broth

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ABSTRACT

pullulan, a water soluble extracellular polysaccharide, was produced by downstream fermentation employing the strain *Aureobasidium pullulans*. To obtain pure biopolymer from the fermentation broth, it is necessary to harvest cells, heat the broth, remove the melanin pigments co-produced during fermentation, concentration, precipitate and dry. Centrifugation of the fermentation broth at 10,000 rpm for 15 min gave cell pellets that were discarded and a green–black supernatant containing melanin pigment was subjected to the heat treatment at 80 °C for 20 min in order to remove the protein in the fermentation broth. The supernatant was demelanized by oxidation with hydrogen peroxide, concentrated under vacuum, precipitated with ethanol and dried at 60 °C for 30 min. This procedure produced high purity pullulan that was comparable in color and texture to the commercial samples.

1. Introduction

Pullulan is an exocellular homopolysaccharide produced by the strain of *Aureobasidium pullulans*. It is a linear mixed linkage of α-D-glucan consisting mainly of maltotriose repeating units interconnected by α-(1 → 6) linkages (Saha & Zeikus, 1989). The regular alternation of α-(1 → 4) and α-(1 → 6) bonds results in structural flexibility and enhanced solubility (Leathers, 1993). Pullulan can form thin films that are transparent, oil resistant and impermeable to oxygen and can be used as a coating and packaging material, a sizing agent for paper or a starch replacer in low-calorie food formulations, cosmetic emulsions and other industrial applications (Deshpande, Rale, & Lynch, 1992). In addition, many pharmaceutical and chemical industry usages of modified pullulan have been reported in recent years (Akiyoshi et al., 1998; Alban, Schauerte, & Franz, 2002; Masci, Bontempo, & Crescenzi, 2002; Shibata, Asahina, Teramoto, & Yosomiya, 2001; Sivakumar & Rao, 2003).

At present, the pullulan preparation process differs according to the intended application. For use as industrial adhesives, dispersants and coagulants, the fermentation broth is simply concentrated, dried and pulverized. For use in food applications, the culture is decolorized using activated charcoal, concentrated, dried and pulverized. As for pharmaceutical uses, the fermentation broth is purified by alcohol fractionation or membrane filtration, decolorized using activated charcoal followed by desalination, concentration, drying and pulverization (Thibault & LeDuy, 1999).

Although these processing methods can produce pullulan in different purity, they are not the suitable ways for pullulan production because removal of protein co-produced during fermentation and the optimal concentration of the fermentation broth before drying should be taken into consideration. In fact, drying is not a proper process for pullulan production due to the retention of most of the impurities in final product, while ethanol precipitation can partly resolve this problem because some of the impurities can be dissolved in the water–ethanol system and can be separated from the product after ethanol precipitation. Moreover, decoloration with activated charcoal can result in considerable lost of pullulan (Kachhawa, Bhattacharjee, & Singhal, 2002).

Therefore, this study attempts to remove protein, decolor the melanin pigments and concentrate the fermentation broth before precipitating with ethanol in order to optimize the recovery of the pullulan.

2. Materials and methods

2.1. Microorganism

Aureobasidium pullulans AP329 was kindly supplied by Professor Qunyi Tong in the School of Food Science and Technology, Jiangnan University. The microorganism was maintained on potato dextrose agar at 4 °C and subcultured every 2 weeks.

2.2. Preparation of inoculum medium

The inoculum medium contained (g/l), sucrose, 50.0; yeast extract, 2.0; K_2HPO_4, 5.0; $(NH_4)_2SO_4$, 0.6; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 1.0 and distilled water l L. The medium was autoclaved at 121 °C

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for 15 min and the initial pH of the medium was adjusted to 5.0 (Vijayendra, Bansal, Prasad, & Nand, 2001).

2.3. Fermentation

Seed cultures were prepared by inoculating cells grown on a potato dextrose agar slant into a 250-ml flask that contained 50 ml of the inoculum medium and subsequently incubated at 28 °C for 48 h with shaking at 200 rpm. 2.5 ml of the seed culture were transferred into the 250-ml flask containing 50 ml of the fermentation media. The culture was shaken at 28 °C and with 200 rpm for 120 h (Roukas, 1998).

2.4. Heat treatment to remove protein

After 120 h of fermentation, the fermentation broth (FB) was centrifuged at 10,000 rpm for 20 min at room temperature (about 25 °C) to separate the cell pellet. The cell pellet separated was washed twice with distilled water by suspending in 30 ml distilled water and centrifuging at 10,000*g* for 20 min at room temperature. Supernatant from the washing was transferred to the centrifuged FB (this solution is called SCFB in this paper) to be concentrated as seen in the followings. The cell pellet was autoclaved and discarded. To begin, the SCFB was treated at different temperature (65 °C, 70 °C, 75 °C, 80 °C, 85 °C and 90 °C). When the maximum temperature (80 °C) for heating to precipitate the protein was selected, SCFB was held at 80 °C for different time (5 min, 10 min, 15 min, 20 min, 25 min and 30 min) in order to find out the heating time for the maximum protein precipitation. After heat treatment, the precipitate was washed according to the method described above. The efficacy of the treatment was evaluated by quantifying the recovered pullulan and protein content in the supernatant after centrifuging.

2.5. Concentration of the protein-removed-SCFB, removal of the melanin pigments and pullulan separation

The protein-removed-SCFB was concentrated (CSCFB) in a rotary evaporator at 100 rpm to different extent at 50 °C. Different level of hydrogen peroxide (1–6%, v/v) was added to the CSCFB at 50 °C and slowly stirred for 30 min in order to determine the optimum amount of hydrogen peroxide for the oxidation and consequently the removal of the melanin pigments. The residual hydrogen peroxide was discarded as well as supernatant after ethanol precipitation. The oxidized melanin pigments, which became colorless by hydrogen peroxide, can be dissolved in the water–ethanol system after ethanol precipitation of the pullulan and discarded with the broth.

Melanin-removed CSCFB was cooled to 4 °C, subsequently added with 2 volumes of cold ethanol under violent agitation and left until the complete separation of the pullulan layer occurred, sinking at the bottom of the flask at temperature about 4 °C. The precipitate was then dried on a preweighted filter paper at 60 °C in an oven until a constant weight was obtained, and the recovered pullulan was determined.

2.6. Removal of the melanin pigments

2.6.1. Analytical methods

The pullulan content in the ethanol precipitate was determined by the coupled-enzyme assay technique described by Israilides, Scanlon, Smith, Harding, and Jumel (1994). The recovery of pullulan was calculated with reference to the initial weight of pullulan in the broth and the final weight of the pullulan obtained after heat treatment, concentration and removal of the pigments. Ash, moisture and protein content of the samples were determined as per standard methods (Anon, 1984). The optical density at 320 nm was used to determine the melanin pigments that may be present in the fermentation broth. The oligosaccharide content was analyzed by Water600 HPLC equipped with a double column system. The first column (Sugarpark 1, 6.5 mmid × 300 mm) used pure water as mobile phase at a flow rate of 0.5 ml/min and the column temperature was maintained at 85 °C. The second column (SpherisorbNH2, 4.6 mmid × 250 mm) used acetonitrile/water (70/30, v/v) as mobile phase at a flow rate of 1 ml/min and the column temperature was at 30 °C. The detector sensitivity was 4 and the inject volume was 10 μl.

3. Results and discussion

3.1. Heat treatment

Although Sevag method is being used as the universal method for removing protein from polysaccharide (Staub, 1965), it is not suitable for industrial production due to the toxicity of chloroform. Heat treatment can denature and precipitate the thermo-sensitive protein with other insoluble material. Therefore, it was of our interest to investigate the effects of heating temperature (Fig. 1) and time (Fig. 2) on protein precipitation. In Fig. 1, when the SCFB was heated, the protein content decreased sharply up to 80 °C and then increased slightly at above 80 °C. The slight increase in protein content was probably due to the slight destruction of some enzyme at the temperature above 80 °C. Therefore, 80 °C was chosen for heating temperature to precipitate the protein.

In order to find out the heating time for the maximum protein precipitation, SCFB was heated to 80 °C and held for different time. In Fig. 2, protein content decreased sharply within 15 min, moderately from 15 min to 20 min and then was not changed much from 20 min to 30 min.

The results in Fig. 1 and Fig. 2 suggested that heating time for 20 min at 80 °C was the most optimal condition for removing of protein from the fermentation broth. As can be seen in Figs. 1 and 2, heat treatment did not affect the recovery of pullulan.

3.2. Concentration, removal of the melanin pigments and pullulan separation

Two-volume of cold ethanol is often used for precipitating pullulan in water solution (Rho, Mulchandani, Luong, & LeDuy, 1988; Roukas, 1998; Youssef, Roukas, & Biliaderis, 1999). However, a small amount of the polysaccharide can still remain in the water–ethanol system and can be wasted when the supernatant after precipitation with ethanol is discarded if the concentration of the polysaccharide is low in the SCFB. Therefore, it is necessary to concentrate the protein-removed-SCFB before ethanol precipitation.

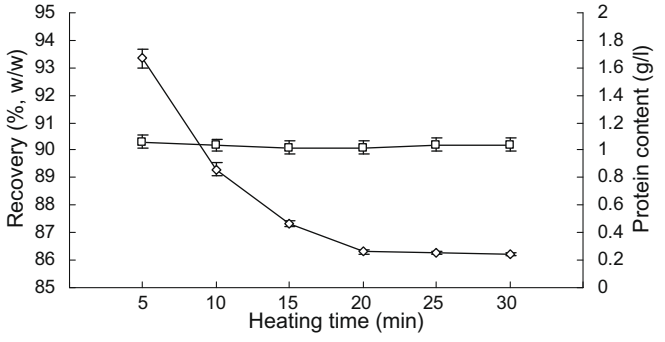

Fig. 1. Effect of heating time on the recovery of pullulan (□) and the protein content (◇) of the broth (the results are from three replicate experiments).

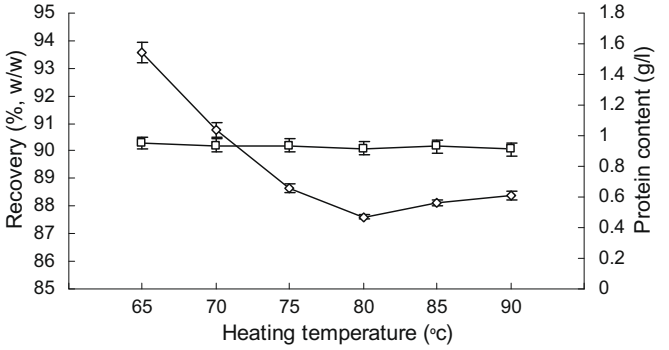

Fig. 2. Effect of heating temperature on the recovery of pullulan (□) and the protein content (◇) of the broth (the results are from three replicate experiments).

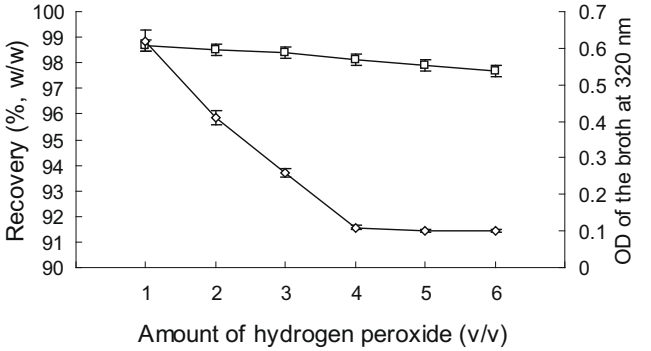

Fig. 4. Effect of the level of H_2O_2 on the recovery of pullulan (□) and OD at 320 nm of the broth (◇) (the results are from three replicate experiments).

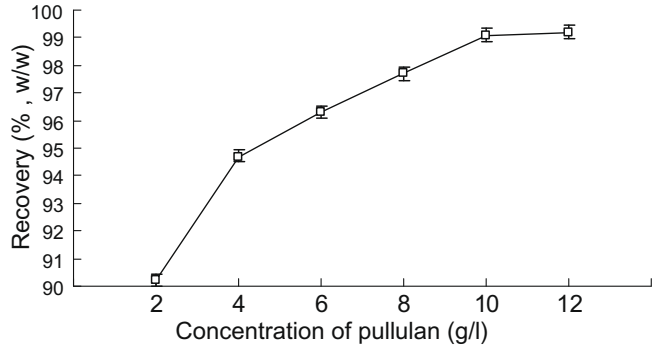

Fig. 3. Effect of concentration of SCFB on the recovery of pullulan (the results are from three replicate experiments).

As seen in the Fig. 3, the recovery of pullulan increased as SCFB is concentrated. But the recovery of the polysaccharide increased only slightly when the concentration of pullulan exceeded 10% (w/v). In addition, high viscosity of the broth made the concentration process difficult. The results we observed here suggested that the most optimal concentration of pullulan in the CSCFB before ethanol precipitation was 10% (w/v).

Though activated charcoal is effective in adsorbing pigments and is often used in removing of melanin before concentration for pullulan production, it also adsorbs pullulan at the same time and decreases the recovery of pullulan. Furthermore, the fine powders of activated charcoal as well as high viscosity of the broth make separation process very difficult (Kachhawa et al., 2002).

Hydrogen peroxide is a decolorant for its ability to oxidize pigments. Moreover, in this experiment, there is no need to separate hydrogen peroxide from the broth by filtration or centrifugation. It can be discarded with the broth just by heating after ethanol precipitation. The effect of amount of hydrogen peroxide on removing of melanin was investigated and the results are shown in Fig. 4. As the amount of hydrogen peroxide increased, the OD of the broth decreased first. When the amount of hydrogen peroxide increased higher than 4% (w/v), no further decrease in OD was observed, indicating all melanin pigments were oxidized and removed. Therefore, it was considered that 4% hydrogen peroxide was the most optimal amount for the removal of the pigments.

3.3. Characterization of the pullulan sample

The samples of pullulan prepared in this experiment and obtained from Japan Pharmacopoeia were characterized. As shown in Table 1, the pullulan purity of the sample prepared in this experiment (93.2%, w/w) was higher than that obtained from Japan Pharmacopoeia (89.7%, w/w). There was no difference in the content of ash and moisture between the two samples, but both protein and oligosaccharide in the sample prepared in this experiment were much lower than those in the sample from Japan Pharmacopoeia. Both samples were comparable in color, texture, water soluble and viscosity (1% water solution).

Table 1
Composition of the samples of pullulan prepared in this experiment and obtained from Japan Pharmacopoeia.

Composition (%, w/w)	Source of Pullulan	
	Prepared in this experiment	Pharmacopoeia Obtained from Japan
Pullulan	93.2 ± 1.45[a]	89.7 ± 1.52
Protein	0.06 ± 0.01	0.13 ± 0.04
Oligosaccharide	3.2 ± 0.57	7.4 ± 0.68
Ash	0.1 ± 0.01	0.1 ± 0.01
Moisture	2.6 ± 0.23	2.5 ± 0.22

[a] Mean + SD from three independent experiments.

4. Conclusions

Heat treatment of the fermentation broth can precipitate most of the protein without affecting the recovery of pullulan. The optimum condition for removing protein from the fermentation broth was heating the broth to 80 °C and hold it for 20 min. It is necessary to concentrate the fermentation broth before ethanol precipitation and the most optimal concentration of pullulan solution before ethanol precipitation is 10% (w/v). Addition of hydrogen peroxide at 4% (w/w) to the broth is effective in removing the melanin pigments.

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