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A Study on Downstream Processing for the production of Pullulan by *Aureobasidium pullulans*-SB-01 from the Fermentation broth

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Abstract

Pullulan, which is made up of linear α-D-glucan maltotriose and maltotetrose repeating units interconnected by α (1→6) and α (1→4) linkages, is a water-soluble homopolysaccharide produced extracellularly by Aureobasidium pullulans. Although the production of this bio-polymer is commercially going on still the establishment of the cost effective downstream processing has not attained up to the mark. It is necessary to harvest cells, remove the melanin pigments co-produced during its fermentation followed by its precipitation, concentration and drying. The present work reports on some of these aspects. Centrifugation of the fermentation broth at 8,000 rpm for 20 min gave cell pellets that were discarded and a greenish black supernatant containing melanin pigment which was subjected to the heat treatment at 80°C for 30 min in order to remove the protein (mainly Pullulanase) in the fermentation broth. The supernatant was demelanized by with hydrogen peroxide and activated charcoal, solvent-solvent blends, or by solvent-salt combinations in which hydrogen peroxide treatment shows better result for the removal of melanin pigments. For the precipitation of the exopolysachride the cold Isopropanol was used followed by its drying process at 60°C for 40 min. This methodology produced high purity pullulan that was comparable in colour and texture to the commercial samples which was characterized by the HPLC and FT-IR analysis.

Keywords: *Aureobasidium pullulans*, melanin, greenish black, demelanized, downstream processing.

Introduction

Apart from plant and animal systems, several micro-organisms such as fungi, bacteria and algae, produce polysaccharides and of all the microbial polysaccharides Pullulan is the one of the best most potent bio-compatible polymer which is synthesized by the *Aureobasidium pullulans*[1,2] . This polymer appears to be a linear α-glucan of maltotriose units with occasional branching of glucosyl or maltosyl substitution. The regular alternation of α(1→4) and α(1→6) bonds results in structural flexibility and enhanced its hydrophilicity. So many pharmaceuticals, cosmetics and food industries are using the surface-modified pullulan in which the hydrophilic pullulan can be converted to hydrophobic[3-5]. The major step in the process and production of pullulan is the proper cost-effective downstream processing for its use in the various pharmaceutical formulations. Although the current downstream processing for the production of pullulan is well established still some problem like production of the proteins and melanin pigment co-produced during the fermentation process is a major drawback. Moreover due to production of the melanin pigments the culture broth become greenish-blue colour and to decolorize this the activated charcoal is generally used by which some amount of the pullulan is lost with it [6,7].

In this study we have taken an attempt to remove the protein and melanin pigments with concentrating the fermentation broth before precipitating with the organic solvent in order to optimize the recovery of the purified pullulan from the fermentation broth.

Materias and Methods

Microorganism: The microorganism *A. Pullulans*-SB-01 was screened from the leaves of the *Brassica oleracea* by the selective enrichment procedure in our previous report and was preserved in the YEPD media at 4°C and was subcultured in every 15 days in PDA medium[8].

Inoculum medium Preparation: The inoculum medium was prepared with following composition (g/l) which consists of sucrose, 50.0; yeast extract, 2.0; K_2HPO_4, 5.0; $(NH_4)_2$ SO_4, 0.6; $MgSO_4.7H_2O$, 0.2; NaCl, 1.0 and distilled water l litre. The medium was autoclaved at 121°C for 20 min and the pH was adjusted to 4.5 [9,10].

Fermentation process: 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 minimal salt broth medium and subsequently incubated at 30°C for 48 hours with shaking at 200 rpm. 2.5 ml of the seed culture were transferred into the 250 ml flask containing 50 ml of the inoculum media. The culture was shaken at 28°C and with 200 rpm for 120 hours [11,12].

Removal of Proteins from the fermentation broth: After 4 days of fermentation, the fermentation broth was centrifuged at 8,000 rpm for 20 min at 25°C to separate the cell pellet. The separated cell pellet was again washed twice with distilled water centrifuging at 8,000 rpm for 20 min at 25°C. Supernatant from

the washing was treated at different temperature (65°C, 70°C, 75°C, 80°C, 85°C and 90°C) and from these temperatures 80°C was found to be maximum temperature for the protein precipitation. The next parameter for this protein precipitation is time, so the supernatant was held at 80°C for the different time limit (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 with distilled water as the method described above.

Melanin Pigment removal from the fermentation broth: ifferent level of hydrogen peroxide (1–12%, v/v) was added to the supernatant collected from the centrifuged broth at room temperature and slowly stirred for 15 min to determine the optimum amount of hydrogen peroxide required for the oxidation process with the removal of the melanin pigments. The OD at 320 nm was checked for the each concentration of the utilised H_2O_2 in order to find the amount of the melanin pigment.

Recovery of Pullulan: The Pullulan recovery was done first by centrifuging the cells (2500×g, 10 minutes) and then the resulted supernatant was kept at 4°C following the adding of twice volumes of cold isopropyl alcohol. The residual hydrogen peroxide which was utilized for the melanin removal from the fermentation broth was discarded with the supernatant after the precipitation of pullulan. The precipitate was washed twice with distilled water and dried at 80°C till a constant weight. Before adding the isopropanol the biomass was separated by centrifugation and taken as pellet. The pellet was placed in aluminium foil and dried overnight at 80°C. The recovered pullulan was estimated as grams of pullulan (dry weight) produced per 100 ml of fermented broth.

HPLC Analysis: The purity of the recovered pullulan was studied by HPLC (Perkin- Elmer model) with the parameters as follows: 1 mg sample, 2µl sample volume and run time was 30 min. The precipitated pullulan sample and standard pullulan (50 kDa, Sigma) were dissolved in water. The sample peak and the standard peak were analysed [13].

FT-IR Analysis: Fourier transform infrared (FT-IR) spectra were recorded with a Perkin–Elmer Spectrometer with the following parameters: 32 scans; resolution, 2 cm^{-1} over the KBr pellet. Pullulan sample (2 mg) was manually well blended with 80 mg of KBr powder. These mixtures were then desiccated overnight at 50°C under reduced pressure prior to FTIR measurement [14].

Results and Discussion

Proteins removal from the fermentation broth with heat treatment: Heat treatment to the fermentation broth denatures the protein (generally thermo-sensitive proteins). Therefore, it was of our interest to investigate the effects of heating temperature for the precipitation of the different thermo-sensitive proteins.

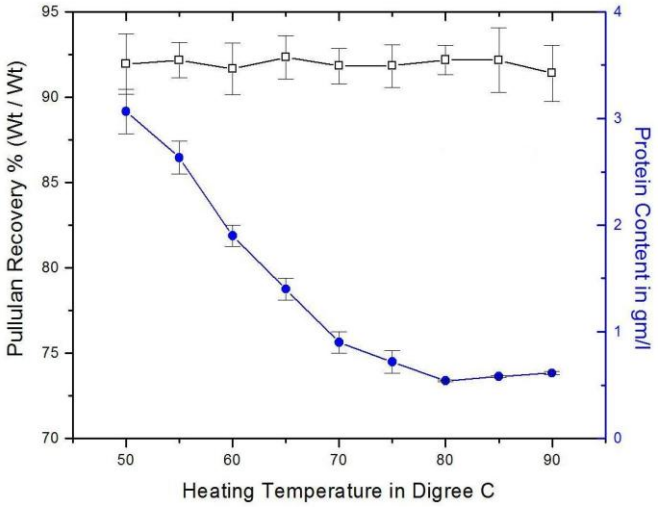

Figure-1
Effect of temperature for the protein precipitation with recovery of Pullulan

It was found that from 70°C to 80°C the protein content in the culture supernatant was very less and after 80°C this has increased slightly (Figure.1). This increase is due to denaturation of some of the enzymes (may be Pullulanase).

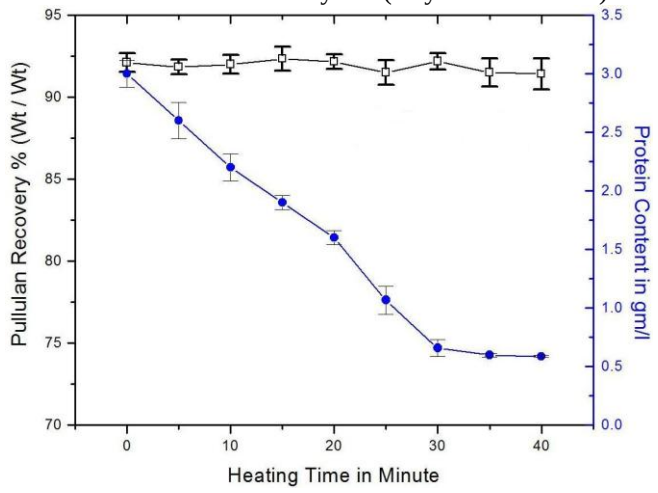

Figure-2
Effect of time for the protein precipitation and Pullulan recovery

Therefore the 80°C was taken as the temperature for the maximum protein precipitation and at this temperature we have kept for different time limits from which 30 min was the optimum time for the maximum protein precipitation. But the precipitation rate was decreasing gradually up to 20 min (Figure.2).

Melanin Pigment removal from the fermentation broth: The melanin removal by the treatment of activated charcoal is an

effective methods in adsorbing pigments, but the major drawback with this activated charcoal treatment that it adsorbs pullulan so there is a decrease in the recovery of pullulan from the fermentation process. Sometimes the highly powered form of activated charcoal causes the problem in the separation of the pullulan also [16]. So here we have used Hydrogen peroxide as the decolorizing agent which can be again separated from the fermentation broth by centrifugation.

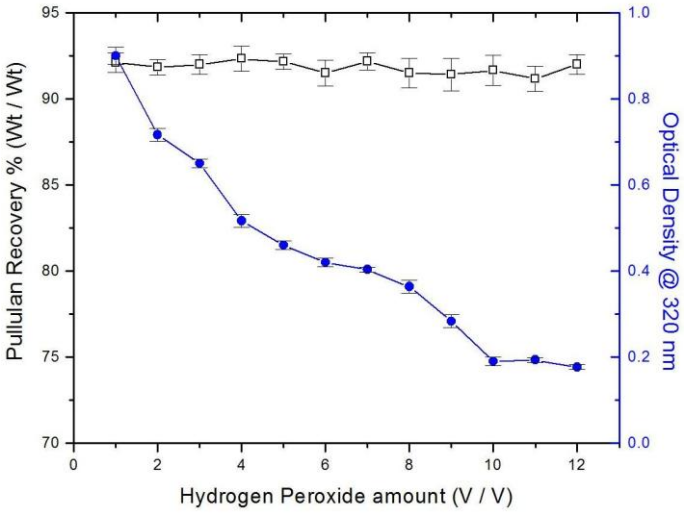

Figure-3
Effect of H_2O_2 for the removal of melanin pigments

Here we have used different concentration of H_2O_2 ranging from 2% to 12% (V/V) which have been directly added to the supernatant after the centrifugation and it was found that at 10% of hydrogen peroxide the melanin content was very less. Here we have taken the OD of the culture filtrate at 320 nm in order to find the melanin content in the supernatant [17,18]. At the 10% or more than 10% of the addition of hydrogen peroxide there is no change in the OD of the supernatant which suggests the complete absence of the melanin pigments.

HPLC Analysis: HPLC analysis (Figure.4) has shown that the sample peak was matched with a standard pullulan 50 kDa (1mg/ml; Sigma). In the fig.4 (A) is for the test sample that was obtained after the heating and H_2O_2 treatment (B) is for the standard pullulan. In the (A) the noise in the HPLC. Profile is very less which suggests that the pullulan sample is purified and can be used as that of commercial pullulan.

FT-IR Analysis: The strong absorption at 3448 cm^{-1} indicated that all the pullulans had some repeating units of –OH as in sugars. The other strong absorption at 2926 cm^{-1} indicated a SP3-hybridisation of C–H bond, 1641 cm^{-1} for the O-C-O bond, 1384 cm^{-1} for C-O-H bond, 992 cm^{-1} for the C-O bonds in the alkane compounds existed in all the samples (Figure.5). The sample (A) peaks and the standard (B) peaks do possess no shift at all suggesting that any types of chemical bonds has form newly due to the treatment of H_2O_2 in the purification process.

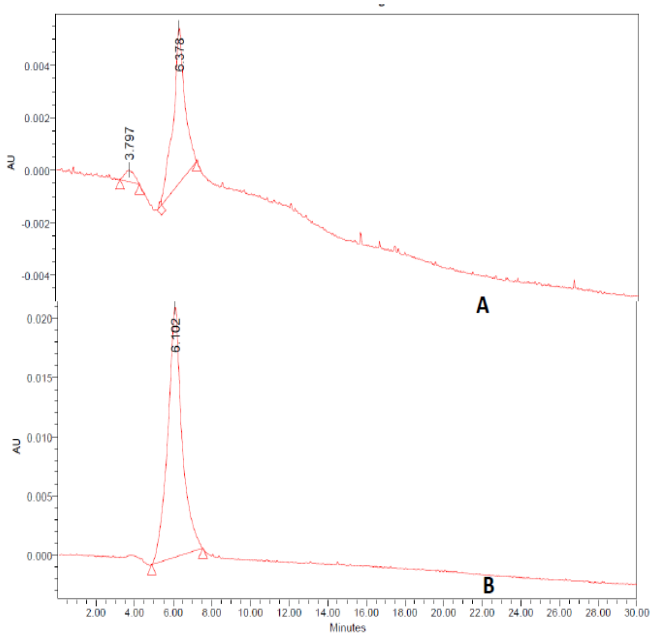

Figure-4
HPLC Profile of the standard pullulan (B) and test pullulan (A)

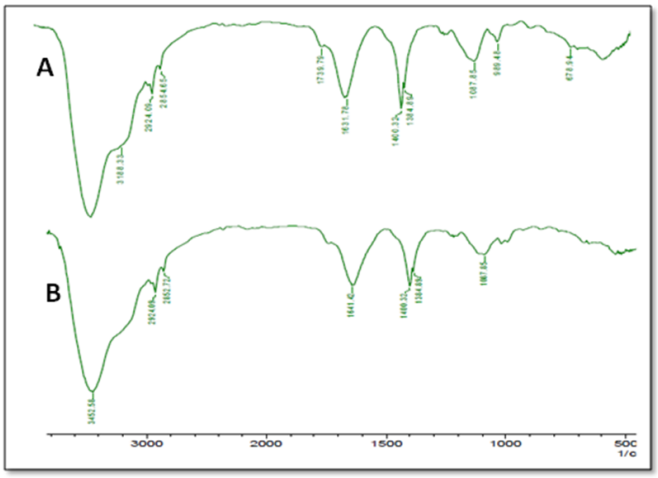

Figure-5
FT-IR Combined plots for standard (B) and test (A) Pullulan

Conclusion

By heat treatment to the fermentation broth most of the thermo-sensitive protein can be precipitated without affecting the recovery of pullulan. It was found that most of the proteins can be precipitated at 80°C with the time duration of 30 min. The productivity of the purified pullulan was found to be more after the heat treatment and 12% (V/V) of hydrogen peroxide addition to the fermentation broth. More over the 12% of hydrogen peroxide can able to de-melanin the fermentation broth easily. The HPLC profile shows that there is very less

impurities in the obtained pullulan and FT-IR peaks give the organic structural configurations of the pullulan which is same as the standard pullulan. From all these studies it can be concluded that the above methodology can produce highly purified pullulan comparable in colour and texture to the commercial pullulan.

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