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Utilization of corn steep liquor for biosynthesis of pullulan, an important exopolysaccharide

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ABSTRACT

Five different agricultural wastes viz. rice bran oil cake, soya bean oil cake, cotton seed oil cake, mustard seed oil cake and corn steep liquor (CSL) were evaluated for their use as nutrient along with 15% (w/v) glucose as carbon source for biosynthesis of pullulan using *Aureobasidium pullulans* RBF 4A3. Among the selected agricultural wastes, CSL was found to be the best and supported production of 77.92 g L^{-1} pullulan under un-optimized conditions. Single point optimization technique resulted in increase in 18% pullulan (88.59 g L^{-1}) production. The process was successfully validated in a 7-L fermenter and a process economic analysis has suggested that use of CSL as nutrient may result in 3-fold reduction of cost of raw materials for pullulan production as compared to a process where conventional nitrogen sources were used. These observations may be helpful in development of a cost effective process for pullulan production.

1. Introduction

Pullulan is an important exopolysaccharide having applications in several industrial sectors like pharmaceutical, food and cosmetic industries. This extra cellular homo-polymer of maltotriose subunits is produced by yeast-like fungus *Aureobasidium pullulans* and has unique linkage patterns of repeating units of α-1,4 and α-1,6 glucans which render it special physico-chemical properties like mechanical flexibility, oxygen impermeability, easy derivatibility, etc. and all these properties have made it potential candidate for several industrial applications. In spite of these unique properties and potential applications, this polymer is not very popular for industrial uses because of its cost. It is three times costlier than other biopolymers such as xanthan gum, which has similar application like pullulan in food and cosmetic industries. A suitable cost effective bioprocess for pullulan production may bring down the cost of pullulan and make it attractive for industrial applications.

Several reports have been published on production of pullulan via fermentation (Jiang, 2010; Ravella et al., 2010; Singh & Saini, 2008). Recently, we have also reported high pullulan production (66.79 g L^{-1}) using glucose, yeast extract and peptone as the substrate by an osmo-tolerant strain of *A. pullulans* RBF 4A3 (Choudhury, Saluja, & Prasad, 2011). Published reports indicate that media components used add significant cost to the production, and it may even reach up to 30% of the total production cost (Miller & Churchill, 1986). Therefore, it is utmost important to find cheap substrates for production of pullulan which will make the process economically viable. Alternative nitrogen sources like urea, ammonium salts, etc. have been used with limited success for pullulan production (West & Reed-Hamer, 1991, 1994).

There are also reports of using cheap raw materials like soybean pomace (Seo et al., 2004), hydrolysed potato starch for pullulan production (Ksungur, Uzunoğulları, & Dağbağlı, 2011). However, in those cases, the yield was not significantly high and hence the economics of the process was also not much favorable. Therefore, it is required to evaluate low cost substrates to develop a cost effective process. Agri-industrial residues are used as feed stock for production of different chemicals and biochemicals like ethanol, citric acid, lactic acid due to their easy availability of large quantity, low cost and high nutrient content (Leathers, 2003). Hence, it may also be possible to develop a cost-effective process for production of pullulan by using agri-industrial residues.

The aim of the present study was to examine potential of agri-industrial residues as nutrient in place of conventional media components like yeast extract and peptone for production of pullulan by *A. pullulans* RBF-4A3. Previously, soyabean pomace was utilized as nitrogen source for production of pullulan, in which the yield was very low (Seo et al., 2004). In the present

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study, five different agri-industrial wastes namely rice bran oil cake, soya bean oil cake, cotton seed oil cake, mustard seed oil cake and corn steep liquor (CSL) were examined for their use as nutrient sources for production of pullulan using *A. pullulans* RBF 4A3. Among the selected agri-industrial residues CSL was found to be the most suitable as nutrient and the pullulan produced was also high as compared to earlier published reports (Ksungur et al., 2011; Shengjun, Jin, Tong, & Chen, 2009).

2. Materials and methods

2.1. Media components

Media components like glucose, yeast extract, peptone, agar, etc. were obtained from Hi Media Laboratories (Mumbai, India) and standard pullulan was procured from Sigma (St. Louis, USA). Corn steep Liquor was obtained from Bharat Starch Industries Limited (Yamunanagar, India) and rice bran oil cake, soya bean oil cake, cotton seed oil cake and mustard seed oil cake were obtained from local market.

2.2. Yeast strain, culture conditions and inoculum development

The strain, *A. pullulans* RBF 4A3, used in this study was isolated from inflorescence of *Caseulia axillaries* (Choudhury et al., 2011). It was maintained at 28 °C using yeast peptone dextrose (YPD) agar media and for long term preservation, glycerol vials were stored at −70 °C.

Inoculum development was carried out by using fresh cultures grown on YPD agar plate for 24 h followed by incubation at 28 °C for 24 h at an agitation speed of 200 rpm on a YPD (1% yeast extract, 2% peptone and 2% dextrose) broth. This inoculum (2.5 mL) was used to inoculate 50 mL of production medium in a 250 mL conical flask.

2.3. Screening of different agri-industrial wastes for their use as nutrient in production media

Five different agri-industrial wastes, namely, rice bran oil cake, mustard oil cake, soybean oil cake, cotton seed oil cake and corn steep liquor were evaluated as nutrients for pullulan production. The production media composed of 2% of the agricultural waste, and 15% dextrose in each case. Fermentation was carried out in 250 mL shake flask containing 25 mL media at 28 °C in an orbital shaker at 200 rpm for 120 h. Samples were taken at 24-h interval and pullulan content was examined.

2.4. Optimization of pullulan production in shake-flask

The yield of product in case of any fermentation process may be enhanced by optimizing various parameters. In present study single point optimization technique was used to optimize CSL concentration, incubation temperature, agitation speed, inoculum size and initial pH of the media for pullulan production by *A. pullulans* RBF 4A3.

2.4.1. Effect of concentration of corn steep liquor on pullulan production

Among the selected agri-industrial residues CSL was found to be the best. In further experiments CSL was used as nutrient along with dextrose. The concentration of corn steep liquor was varied in the range of 1% (v/v) to 8% (v/v) in the production media and the dextrose concentration was maintained at 15% (w/v) in all cases. The initial pH was maintained at 4.5 and the media was inoculated using 20.8 mg fresh weight biomass per mL of the production medium. The flasks were incubated at 28 °C and 200 rpm. Samples were withdrawn periodically (at every 24-h interval up to 120 h) and analyzed for pullulan content.

2.4.2. Effect of incubation temperature on pullulan production

Effect of incubation temperature on pullulan production was studied by varying the same in the range of 15–30 °C using a production media consisting of 2% CSL and 15% dextrose. All other process conditions were maintained same as described earlier. Samples were withdrawn at every 24 h and analyzed for pullulan production, residual sugar content and pH of the media.

2.4.3. Effect of agitation speed

In aerobic fermentation processes agitation plays a key role in product formation. The agitation speed was varied from 100 to 350 rpm to understand its effect on pullulan production. All other parameters were maintained as optimized in earlier experiments. The fermentation was carried out till 120 h and periodic samples were obtained to determine pullulan production.

2.4.4. Effect of inoculum size

Inoculum size is another factor that substantially affects the production of the product in fermentation processes. In the present study the production media was inoculated using an inoculum containing 41.6 mg mL^{-1} biomass (on fresh weight basis) and varying the inoculum size from 2% to 10% (v/v) level. All other parameters were kept same as optimized during earlier batches.

2.4.5. Effect of initial pH

pH plays a very important role in product formation in all fermentation processes. In case of present study, the initial pH of the medium was varied from 3.5 to 6.5 to obtain optimum pullulan production. The remaining process conditions were same as optimized during earlier experiments.

2.5. Analysis, purification and characterization of pullulan

The fermentation broth was centrifuged at 16,000 × *g* for 20 min at 4 °C using a Sigma 6K-15 centrifuge. This cell free broth was subjected to solvent precipitation using 2 volumes of ethanol at 4 °C. The precipitate thus obtained was once again separated by centrifugation at 16,000 × *g* for 20 min at 4 °C and was re-dissolved in de-ionized water and subjected to dialysis (MWCO 20,000) for removal of small molecular weight compounds. Then the polymer was re-precipitated using 2 volumes of ethanol at 4 °C and the precipitate was separated by centrifugation and dried at 80 °C till constant weight. Pullulan content in the exopolysaccharide was determined by enzymatic method (Chi & Zhao, 2003). The dried and purified precipitate was dissolved in de-ionized water (3 mL) and used as substrate for enzymatic hydrolysis. Reaction was carried out at 40 °C for 2 h in 1 mL volume (0.5 mL of the substrate; 0.4 mL of 0.2 (M) phosphate buffer (pH 5.0) and 0.1 mL (0.84 U) of pullulanase enzyme (Sigma, USA) solution. The residual sugar released during enzymatic hydrolysis was measured by the method of Miller (1959) using a Hitachi U-2900 UV-visible spectrophotometer. This was compared with the residual sugar released during hydrolysis of standard pullulan obtained from Sigma, USA. The pullulan content was expressed in terms of grams of pullulan (dry weight) produced per liter of fermentation broth.

The EPS produced was characterized by using FT-IR spectroscopy as described earlier (Choudhury et al., 2011). The sample for FT-IR was prepared by blending 2 mg of EPS with 60 mg of potassium bromide powder. The mixture was desiccated overnight at 50 °C under reduced pressure. Pullulan from Sigma, USA was used as a standard. Fourier transform infrared (FTIR) spectra were recorded with a Perkin Elmer spectrophotometer over a range of