Biocatalysis and Agricultural Biotechnology 1 (2012) 232–237

Contents lists available at SciVerse ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Application of response surface methodology to understand the interaction of media components during pullulan production by *Aureobasidium pullulans* RBF-4A3

A. Roy Choudhury [a,*], M.S. Bhattacharyya [a], G.S. Prasad [b]

[a] *Biochemical Engineering Research & Process Development Centre (BERPDC), CSIR-Institute of Microbial Technology (IMTECH), Council of Scientific and Industrial Research (CSIR), Sector-39A, Chandigarh 160036, India*
[b] *Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology (IMTECH), Council of Scientific and Industrial Research (CSIR), Sector-39A, Chandigarh 160036, India*

ARTICLE INFO

Article history:
Received 11 November 2011
Received in revised form
7 February 2012
Accepted 8 February 2012
Available online 16 February 2012

Keywords:
Exopolysaccharide
Pullulan
Aureobasidium pullulans
Central composite design
Response surface methodology
Effect of nitrogen sources

ABSTRACT

Response surface methodology was applied to understand the interactions amongst the components of a complex medium for pullulan production by a novel *Aureobasidium pullulans* strain using a second order quadratic model. A total of 20 experimental runs were carried out and three dimensional response surfaces were generated to study the interaction. Results indicated that the concentration of yeast extract plays a critical role and has significant influence in pullulan production compared with peptone. Understanding the effect of complex nitrogen sources in the media using statistical methods has resulted in 70.43 g/L pullulan production which is higher as compared to earlier published reports.

1. Introduction

Pullulan is a water soluble homopolymer composed of maltotriose subunits. It is essentially a linear glucan containing α-1,4 and α-1,6 linkages in the ratio of 2:1. The unique structural and physical properties of pullulan provide it structural flexibility, easy derivatibility and superior solubility. This is also responsible for its certain physico-chemical properties like low oxygen permeability, film and fiber forming capacity, biodegradability, etc. All these properties have made pullulan a potential candidate for many applications in the food, pharmaceutical and other industries (Singh et al., 2008; Leathers, 2003; Shingel, 2004). It is used extensively in the food industry as a food ingredient for over 20 years in Japan, and has obtained 'Generally Regarded as Safe' (GRAS) status in the USA (USFDA., 2002). It is a slow digesting macromolecule which is tasteless as well as odorless and hence used as a low-calorie food additive providing bulk and texture (Wolf, 2005). Recently, pullulan is also being investigated for its biomedical applications in various aspects like targeted drug and gene delivery, tissue engineering, wound healing and in diagnostic imaging using quantum dots (Rekha and Sharma, 2007).

Despite these applications, pullulan is costlier ($ 25 Kg^{-1}) as compared with other exopolysaccharides which is major limiting factor for cost effective applications (Tibault et al., 2007). The high price of pullulan is attributed to several factors including low product yield. Therefore, it is important to enhance the yield of pullulan during fermentation which will make the process economically viable. Yield of product during fermentation depends on several factors and one of them is interaction among the media components. Classically, single point optimization technique has been used for enhancement of yield during fermentation (Chi and Zhao, 2003; Lee et al., 2001). However, these classical single variable or one point optimization methods are tedious and time consuming and tend to overlook the interaction among different factors and therefore, may often lead to misinterpretation of results (Francis et al., 2003).

Statistical approaches are ideal alternatives to enhance the yield of product during fermentation when compared to classical one point optimization techniques (Gupta and Lorenz, 2002; Haaland, 1989). These methods not only reduce the number of experiments to be carried out, but also consider the interaction of several factors which may affect the yield of the product during fermentation. Response Surface Methodology (RSM) is one such

* Corresponding author. Tel.: +91 172 6665328; fax: +91 1722695215.
E-mail address: anirban@imtech.res.in (A.R. Choudhury).

1878-8181/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bcab.2012.02.003

collection of mathematical and statistical techniques useful for modeling and analysis. This can be used to identify the effective factors and to study interactions for identifying critical factors which have significant influence on the yield of final product. It can also be utilized to quantify the relationship between one and/or more major responses, by carrying out a limited number of experiments and has been widely used in optimization of media and process conditions, in different bioprocesses (Oskouei et al., 2008; Sen and Swaminathan, 1997; Smith et al., 1997; Teruel et al., 1997). Central Composite Design (CCD) can be used for determining the key factors out of large number of media components by carrying out small number of experiments (Prakash and Shrivastva, 2005). This design helps to estimate coefficient of quadratic model and has been extensively used for bioprocess optimization (Box and Wilson, 1951; Puri et al., 2002).

Earlier, Singh and Saini (2008) have successfully applied RSM for media optimization to produce pullulan using a synthetic medium containing sucrose as carbon source. Cheng et al. have demonstrated enhanced pullulan production in a biofilm reactor using RSM (Cheng et al., 2010). There are few other reports where RSM was successfully applied to optimize pullulan production (Gibson and Coughlin, 2002; Tarabasz-Szymanska et al., 1999).

Previously our group has reported pullulan production by an osmotolerant yeast like fungus *A. pullulans* RBF 4A3 using glucose as the carbon source (Choudhury et al., 2011). In the present study RSM has been used to examine the interactions among three media components, namely glucose, yeast extract and peptone for pullulan elaboration by *A. pullulans* RBF-4A3. Yeast extract was found to have more significant effect as compared to peptone on the yield of pullulan production by *A. pullulans* RBF-4A3. To the best of our knowledge, this is the first report on the statistical analysis to understand the effect of complex nitrogen sources in the media on pullulan elaboration.

2. Materials and methods

2.1. Microorganisms and culture maintenance

The organism *Aureobasidium pullulans* RBF 4A3 was a wild strain isolated from flowers and was identified based on biochemical tests, morphology study and sequencing of D1/D2 domain of large subunit ribosomal RNA gene (Choudhury et al., 2011). Stock cultures of the isolate were stored in 20% glycerol at −80 °C. Prior to each experiment, the organism was subcultured from the frozen stocks onto YPD Agar medium plates containing: dextrose 2% (w/v), yeast extract 1% (w/v), peptone 2% (w/v) and agar 1.8% (w/v) at 28 °C.

2.2. Inoculum preparation

For pullulan production, freshly grown culture was inoculated into 250 ml Erlenmeyer flasks containing 50 ml of sterile inoculation medium containing yeast extract, glucose, peptone-1%, 2%, 2% (w/v) respectively. The culture was grown at 28 °C on a shaker at 200 rpm for 20 h.

2.3. Shake flask fermentation

The shake flask fermentation was carried out using a media containing glucose, yeast extract and peptone. Concentration of media components was varied according to the experimental design. In all cases a 250 ml conical flask containing 50 ml of media was inoculated with 5% (v/v) inoculum and incubated at 28 °C in a rotary shaker with 250 rpm for 96 h.

2.4. Determination of biomass and residual sugar

The flasks were harvested after 96 h of fermentation and centrifuged in a Sigma 6 K-15 centrifuge for 20 min at 10,000 rpm. The supernatant was collected for further study and pellet was washed twice using distilled water and re-centrifuged and dried overnight in an oven at 80 °C. The biomass was expressed as mg per ml broth. The residual sugar concentration was measured in the cell free broth using Miller's method (Miller, 1959).

2.5. Recovery, purification and characterization of pullulan

The recovery of pullulan was carried out following earlier reported methods (Choudhury et al., 2011) with minor modifications. Samples were withdrawn after 96 h and subjected to centrifugation at 12000 rpm for 10 min at 4 °C using Sigma 6 K-15 table top centrifuge to make the broth cell free. Pullulan was precipitated by adding 2 volumes of ethanol to the cell free broth and kept at 4 °C for 12 h for complete precipitation. The precipitate was separated by centrifugation (12000 rpm, 4 °C, 10 min). Pullulan was further purified by re-precipitating it with 2 volumes of ethanol at 4 °C and dried at 80 °C till constant weight was obtained.

Pullulan content in the precipitate was determined by enzymatic method (Leathers et al., 1988). The dried precipitate was re-dissolved in 0.05 M sodium acetate buffer (pH 5.0) at a concentration of 1 mg/ml. To 1 ml of this solution, 0.1 U/ml pullanase enzyme (Sigma; EC 3.2.1.41) was added and incubated at 25 °C for 21 h. A standard of pullulan obtained from Sigma was also incubated in the similar fashion. The enzymatic reaction was stopped by adding 2 ml of 0.1 N HCl after the incubation is over. The contents of maltotriose units were determined using DNSA method as described by Miller (Miller, 1959) in both the cases. The pullulan content in the fermentation broth is expressed as gm of pullulan produced per liter of cell free fermentation broth.

The pullulan obtained was characterized using FT-IR spectroscopy using a Perkin-Elmer spectrophotometer. In a typical analysis, 2 mg of purified pullulan sample was mixed with 60 mg of 95% potassium bromide powder. The mixture was desiccated overnight at 50 °C under vacuum. The FT-IR spectra was taken using potassium bromide pallets of purified pullulan and standard pullulan obtained from sigma over a range of 4000–400 cm^{-1} at a rate of 16 scans with a resolution of 2 cm^{-1}.

2.6. Experimental design and optimization

Response surface methodology was used to study the interaction among complex media components and their contribution towards pullulan production. The Central Composite Design (CCD) with 3 factors and 5 levels, including 6 replicates, all the center point has used for a second order response surface.

The CCD was developed as an imbedded factorial matrix with center points and star points pointed (replicate of axial point) around the center point which allows estimation of the curvature. One unit was designated to the distance from the center of the design space, to a factorial point, while α unit designated to the distance of the center to the design space, to a star point. The star points represent the extreme values (both low and high) for each factor in this design and hence in case of full factorial design α is equal to $(2^k)^{1/4}$. In our case k is equal to 3 i.e. glucose, yeast extract and peptone, hence value of α will be 1.68179. Each variable was studied into five different levels and these are listed in Table 1. According to this, a set of 20 experiments which include 6 center points, 6 axial points with α value 1.68179 were carried out (Table 2).