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Optimization of fermentation conditions for the production of ethanol from sago starch by co-immobilized amyloglucosidase and cells of *Zymomonas mobilis* using response surface methodology

Veera Venkata Ratnam Bandaru [a,*], Subba Rao Somalanka [b,1], Damodara Rao Mendu [c,2], Narasimha Rao Madicherla [b,3], Ayyanna Chityala [b,4]

[a] *Department of Neurology, Johns Hopkins University School of Medicine, 600 North Wolfe Street, Pathology 233/Meyer 222, Baltimore, MD 21287, USA*
[b] *Center for Biotechnology, Department of Chemical Engineering, College of Engineering, Andhra University, Visakhapatnam 530003, India*
[c] *Department of Pediatrics, Division of Infectious Diseases, Johns Hopkins University School of Medicine, 720 Rutland Avenue, Ross 1135B, Baltimore, MD 21205, USA*

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Abstract

Statistical experimental design was used to optimize the conditions of simultaneous saccharification and fermentation (SSF), viz. temperature, pH and time of fermentation of ethanol from sago starch with co-immobilized amyloglucosidase (AMG) and *Zymomonas mobilis* MTCC 92 by submerged fermentation. Maximum ethanol concentration of 55.3 g/l was obtained using a starch concentration of 150 g/l. The optimum conditions were found to be a temperature of 32.4 °C, pH of 4.93 and time of fermentation of 17.24 h. Thus, by using SSF process with co-immobilized AMG and *Z. mobilis* cells MTCC 92, the central composite design (CCD) was found to be the most favourable strategy investigated with respect to ethanol production and enzyme recovery.

Keywords: Co-immobilization; Amyloglucosidase; Sago starch; Ethanol; Response surface methodology

1. Introduction

The new developments in biotechnology will play an important role in resolving part of the energy and food problems that lie ahead. One important development, which has stimulated worldwide interest is the utilization of renewable carbohydrate sources for the production of ethanol as a liquid fuel [1–3]. Sago starch is an agricultural material abundantly produced in India and other tropical countries [4] and is an alternative source of energy. The starch is a product extracted from the seeds or tubers, stems of palms and cycads such as *Metroxylon sagu* and is a mixture of 27% (w/w) amylose and 73% (w/w) amylopectin [5]. There are a variety of products that can be obtained from starch biomass via hydrolysis. Alcohol is one of the largest volume of products that can be produced from biomass. Recently, there has been active research aimed at attaining an increase in ethanol yield by immobilized techniques.

Zymomonas mobilis cells and AMG were co-immobilized in the form of alginate beads and SSF was carried using the co-immobilized cells and enzyme. The SSF process combines enzymatic hydrolysis of starch to glucose and ethanol fermentation into a single operation. Consequently, this process offers a great potential of increased rate of hydrolysis, reduction of fermentation time, decreased capital cost [6] and removing end point inhibition as well as eliminating the need

* Corresponding author. Tel.: +1 443 287 3717; fax: +1 410 502 6736.
E-mail addresses: vbandar2@jhmi.edu, bvvratnams@yahoo.co.in (V.V.R. Bandaru), dmendu1@jhmi.edu (D.R. Mendu), mnrmd_au@yahoo.com (N.R. Madicherla), ayyanna123@rediffmail.com (A. Chityala).
[1] Tel.: +91 891 284 4890/2534032.
[2] Tel: +1 410 614 0058.
[3] Tel.: +91 891 284 4883/2537641, mobile: +91 9440403268.
[4] Tel.: +91 893 3222959/891 2552405.

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for separate reactors. SSF process has attracted many investigators [7,8].

The traditional 'one-factor at a time' technique used for optimizing a multivariable system is not only time consuming but also often easily misses the alternative effects between components. Also, this method requires to carry out a number of experiments to determine the optimum levels, which are untrue. These drawbacks of single factor optimization process can be eliminated by optimizing all the affecting parameters collectively by CCD using response surface methodology (RSM). Recently, many statistical experimental design methods have been employed in bioprocess optimization. Among them, RSM is the one suitable for identifying the effect of individual variables and for seeking the optimum conditions for a multivariable system efficiently. This method has been successfully applied to optimize alcoholic fermentation and other fermentation media [9–16]. A detailed account of this technique has been outlined [17]. Basically, this optimization process involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model and predicting the response and checking the adequacy of the model. Hence, the authors report the application of the RSM using the Box–Wilson design [18] of experiments to develop a mathematical correlation between the temperature, pH and time of fermentation and concentration of ethanol.

In the present study, the optimal conditions of temperature, pH and time of fermentation for maximum ethanol yield have been quantified from the Box–Wilson CCD.

2. Materials and methods

2.1. Substrate

Sago starch was collected from cultivators, East Godavari District, Andhra Pradesh, India.

2.2. Organism

Z. mobilis MTCC 92 obtained from IMTECH, Chandigarh, India, was used throughout this study.

2.3. Enzymes

α-Amylase from *Bacillus licheniformis* and AMG from *Aspergillus niger* were obtained from Sigma Chemical Co. (St Louis, MO). The activities of the two enzymes were 60 KNU/g and 200 AGU/ml, respectively.

2.4. Growth conditions

The *Z. mobilis* MTCC 92 was maintained on agar slants having composition (g/l): glucose, 100; yeast extract, 10; KH_2PO_4, 1; $(NH_4)_2SO_4$, 1; $MgSO_4{\cdot}7H_2O$, 0.5 and the cells were grown at a temperature of 35 °C and pH of 5.5.

2.5. Co-immobilization

The enzyme was immobilized on powdered chitin using the procedures of Stanley et al. [19]. Exponentially growing *Z. mobilis* cells (8 g dry cell weight) were centrifuged and resuspended with the immobilized glucoamylase on chitin in 50 ml physiological saline. The suspension was carefully mixed with 50 ml 4% sodium alginate solution. The slurry was then added drop wise to a 0.05 M $CaCl_2$ solution with continuous stirring using a 5 ml disposable pipette tip. Beads of 3–4 mm diameter were formed in this solution. The total volume of beads was approximately 68 ml. The concentrations of the chitin immobilized glucoamylase and *Z. mobilis* cells in the beads were 77.2 and 93.2 g dry weight/l beads, respectively.

2.6. Production media and fermentation

Starch liquefaction was carried out by adding 0.2% (v/w) α-amylase to the slurry at pH 6.5 and heating at 95 °C for 1 h. No problem was faced during the solubilization of starch because of the reduction in viscosity of fermentation mashes by enzymes. Fermentation media were composed of 150 g sago starch, 10 g yeast extract and co-immobilized enzyme and cell beads (120 ml bead volume) in 1 l water. Fermentation was carried out in a Biostat M fermentor supplied by B. Braun Co., Germany, with all necessary controls. The reactor was of 2 l capacity and the working volume was 1 l. The operating conditions were maintained at a temperature of 30 °C and pH 5.0. The reactor was maintained under anaerobic conditions.

2.7. Analytical methods

Ethanol was estimated by GLC in which a flame ionization detector and stainless steel column (2.0 m length, 3.0 mm i.d.) packed with Porapak-Q (50–80 mesh, manufactured by Nucon Engineers, India) were used. The column oven was operated isothermally at 150 °C and the detector and injection ports were kept at 170 °C. Nitrogen was used as carrier gas at a flow rate of 30 cm^3/min and the combustion gas was a mixture of hydrogen and air [13]. Sugars were determined using Miller's method [20].

2.8. Experimental design and optimization

CCD [18] was used in the optimization of ethanol production. Temperature (X_1, °C), pH (X_2) and time of fermentation (X_3, h) were chosen for the independent variables shown in Tables 1 and 2. Ethanol concentration (Y_i, g/l) was used as the dependent output variable. For statistical calculations the variables X_i were coded as x_i according to Eq. (1):

$$x_i = \frac{X_i - \bar{x}_i}{\Delta x_j}, \quad i = 1, 2, 3, \ldots, k \tag{1}$$