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Development of culture medium using extruded bean as a nitrogen source for yeast growth

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ABSTRACT

In this study extruded bean was used as a nitrogen source substitute in culture medium formulation. A 3-factor simplex-lattice mixture design was used to establish better growth conditions. Completely substituted medium resulted in 43% of increase in the growth of *Saccharomyces cerevisiae*. Mixtures containing 1% extruded bean and 1% yeast extract, or 1% extruded bean and 1% peptone presented growths of 76–79% higher than the commercial YPD medium for *S. cerevisiae*. *Pichia pastoris* (GS115) growth was enhanced by 20% using a completely substituted medium. The protein expression patterns in *P. pastoris* (GS115) remained unchanged when growth was conducted in a medium containing extruded bean as unique nitrogen source. The total amount of recombinant protein expressed in extruded bean medium was 88.5% higher than in control expression medium. These results evidenced that extruded bean can be successfully used as a substitute of peptone and yeast extract in culture media for *S. cerevisiae's* and *P. pastoris'* (GS115) growth.

1. Introduction

Yeasts play a prominent role in wine and other beverage fermentations, which can strongly affect the quality and flavor of the final product. *Saccharomyces cerevisiae* is an important microorganism present during the fermentation process (Arroyo-López et al., 2009; Pretorius, 2000). However, recent studies showed that other yeasts are also important for the production of esters during fermentation, which contribute to the fruity flavor of wines and other fermented beverages. Among the *non-S. cerevisiae* yeasts, the genus *Pichia* figures as a high producer of esters such as ethyl acetate, isobutyl acetate and isoamyl acetate (Domizio et al., 2011; Li et al., 2012).

S. cerevisiae and *Pichia pastoris* are also yeasts widely used for the production of recombinant proteins as they merge the advantages of unicellular organisms with the ability to perform eukaryotic post-translational modifications. Culture medium composition is a key factor in maximizing the growth and the expression of proteins and secondary metabolites of these microorganisms (Alberghina et al., 2012; Çelik and Çalik, 2012; Li et al., 2011; Rughoonundun et al., 2012; Ye et al., 2010).

It is estimated that the costs of protein production in the industrial scale are 30–40% dependent on the growth medium used in the process (Hajii et al., 2008). In order to reduce medium costs, hard-to-cook bean, a by-product of industrial bean processing, was used as a component of a culture medium. After thermoplastic extrusion processing, the hard-to-cook (HTC) beans provide a rich but inexpensive source of nutrients. Moreover, thermoplastic extrusion increases the bioavailability of proteins and carbohydrates (Batista et al., 2010; Ruiz-Ruiz et al., 2008).

In the last decades, statistical methods have been applied to optimize the composition of culture media for industrial purposes. Statistical designs are used mainly because they provide the tools for simultaneously analyzing the large quantity of variables considered in the formulation of culture media (Coello et al., 2002; Didier et al., 2007; Mohamad et al., 2011; Nowruzi et al., 2008; Vaidya et al., 2003). Main effects, as well as factor interactions, can be predicted by using such statistical methods, making their use a fundamental part of large scale biotechnological processes (Chang et al., 2006; Fábregas et al., 2000; Myers and Montgomery, 1995).

In this study, an experimental mixture design (Statistical 7.0) was used to determine the effect of the extruded hard-to-cook bean as a substitute of peptone and yeast extract in culture media for the growths of *S. cerevisiae* and recombinant *P. pastoris*. A comparison between the original medium and the experimental medium for growth and expression of a recombinant protein is presented.

2. Materials and methods

2.1. Microbial strains

S. cerevisiae was obtained from the Laboratório de Química de Proteínas culture collection and *Pichia pastoris* (GS115) was obtained from the Laboratório de Bioquímica e Engenharia Genética culture collection at the Universidade Federal de Goiás. The cultures were

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cultivated and maintained by weekly transfers on YPD agar (HiMedia Laboratories, India).

2.2. Flour bean preparation and extrusion

The HTC beans (*Phaseolus vulgaris*) utilized for the extrusion process were provided by EMBRAPA Arroz e Feijão, Santo Antônio de Goiás, Goiás, Brazil. The grains were grounded in a Tecnal mill-grinder, sifted in a screen 0.42 mm and then extruded. The extrusion process was carried out in a Cerealtec International extruder (CT-L15) according to the methodology described by Batista et al. (2010). The extrudates were ground, sealed in plastic bags and refrigerated at 4 °C until their use.

2.3. Experimental design for substitution assays

A 3-factor simplex-lattice design was used to optimize the substitution of peptone and yeast extract by extruded HTC bean flour. The mixture design was used to study the relationship between the proportion of the different nitrogen sources and their respective responses in the optical density. The design was implemented by using Statistica 7.0 software (StatSoft Inc. Tulsa, OK, USA) and the factors defined as independent variables were peptone (HiMedia Laboratories, India), yeast extract (HiMedia Laboratories, India) and extruded bean (Table 1).

The response function (Y) of the mixture model was explained by the following quadratic equation:

$$y = \sum_{i=1}^{q} \beta_i x_i + \sum \sum_{i<j}^{q} \beta_{ij} x_i x_j. \quad (1)$$

Geometrically, in Eq. (1) the parameter β_i represents the expected response of the pure mixture $x_i = 1, x_j = 0, j \neq i$. The term given by $y = \sum_{i=1}^{q} \beta_i x_i$ represents the response of the linear blending terms of all the mixture components. It describes the relative effect of the pure components in the mixture. The quadratic term $\beta_{ij} x_i x_j$ represents the excess response over the linear model due to an interaction between two components (Scheffé, 1963).

2.4. Growth profiles of microorganisms

The growths of *S. cerevisiae* and *P. pastoris* (GS115) were evaluated by using a commercial YPD medium (HiMedia Laboratories, India) as a reference medium and the experimental media with a composition determined by the mixture design. Pre-inoculums were prepared as follows: a 5 mL culture YPD medium was inoculated from a single colony and incubated at 150 rpm for 24 h at 30 °C or 37 °C. Cells were centrifuged at 5000 *g* for 10 min and transferred to 5 mL of 0.15 mol/L saline solution. 250 μL of cell suspension of *S. cerevisiae* or *P. pastoris* (GS115) strains were transferred to 250 mL of the different media, incubated at 37 °C for 24 h and 30 °C for 42 h, respectively, under shaking (150 rpm). Aliquots of 1 mL were withdrawn every 2 h for *S. cerevisiae* and every 4 h for *P. pastoris*. Collected aliquots were centrifuged at 5000 *g* for 10 min and the cell mass was ressuspended in 1 mL of 0.15 mol/L saline solution. The absorbance was determined at 600 nm by using a UV–VIS spectrophotometer.

Table 1
Assigned concentrations of each compound at different levels of the mixture design.

Compound	Variable	Levels		
		0	0.5	1
Peptone	X_1	0%	1%	2%
Yeast extract	X_2	0%	1%	2%
Extruded bean	X_3	0%	1%	2%

2.5. Recombinant protein expression in P. pastoris (GS115)

The recombinant protein expression using *P. pastoris* (GS115) was carried out as recommended by the supplier (Invitrogen, Carlsbad, CA). Starter cultures were inoculated in YPD medium and incubated at 30 °C, 150 rpm, for 24 h. After this initial growth phase, 20 mL of the recombinant culture was harvested by centrifugation (10,000 *g*, 5 min) and cell pellet was washed twice with 100 mmol/L sodium phosphate buffer (pH 6.0). The washed recombinant *P. pastoris* (GS115) cells were re-suspended in 20 mL of the expression medium supplemented with 1% methanol. In this study two media were tested for expression: a control medium (1% yeast extract, 2% peptone, 2% dextrose and glycerol) and an experimental medium containing extruded bean (2% extruded bean, 2% dextrose (Invitrogen, Carlsbad, CA) and glycerol (Invitrogen, Carlsbad, CA)). The expression systems were incubated at 30 °C for 96 h at 150 rpm, with the addition of methanol (Sigma Aldrich, St. Louis, USA) every 24 h. Culture samples were collected every 24 h and analyzed for the production of extracellular protein.

2.6. Precipitation and analysis of recombinant protein

The cell-free supernatant from the expression medium was recovered by centrifugation at 5000 *g* for 10 min. Total proteins were subsequently precipitated with cold acetone (1:2 v/v), incubated at −80 °C for 2 h, centrifuged at 5000 *g* for 5 min and re-suspended in 100 mmol/L sodium phosphate buffer (pH 6.0).

Total protein concentration was determined following the method described by Bradford (1976). The precipitated proteins were analyzed for purity by using 10% denaturating polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Electrophoresis was run at 25–40 mA for 2 h at room temperature. Gels were stained with Comassie Blue R-250. Pre-stained molecular weight markers (New England Biolabs, MA) ranging from 7 to 175 kDa were used as a running standard.

The image analysis of the gel was performed by using the program ImageJ 1.46r (Wayne Rasband, National Institute of Health, USA). The parameters for obtaining/recording the gel image were 300 dpi-resolution, 100%-zoom, and 16 bits per pixel-depth.

2.7. Statistical analysis

The statistical analysis of the experimental mixture design was performed by multivariate analysis. The model was simplified to exclude terms that were not considered statistically significant ($p > 0.05$) by analysis of variance (ANOVA). The quality of the polynomial model equation was judged by using the coefficient of determination R^2. The mixture design and all subsequent tests were conducted in triplicate and the level of significance was 95%. All analyses were carried out by using the software Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA).

3. Results and discussion

3.1. Model establishment

The composition of culture media is an important factor for growth and expression in yeasts, especially nutrient contents such as nitrogen and carbon (Rughoonundun et al., 2012). Commercial media for growth and expression in yeast usually contain glucose as a carbon source and peptone and/or yeast extract as a nitrogen source. However, peptone and yeast extract are expensive media components when considering industrial scale protein production (Ye et al., 2010). Thus, aiming to develop a cheap industrial medium, extruded bean was tested as a substitute for peptone and/or yeast extract.

To study the feasibility of using extruded beans as a low cost nutrient substitute for *S. cerevisiae's* and *P. pastoris'* (GS115) growths, a mixture-model design was implemented and the amount of cellular growth was