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Optimization of solid-state medium for the production of inulinase by *Kluyveromyces* S120 using response surface methodology

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Abstract

The optimization of nutrient levels for the production of inulinase by a newly strain *Kluyveromyces* S120 in solid-state fermentation (SSF) was carried out using response surface methodology (RSM) based on Plackett–Burman design and Box–Behnken design. In the first optimization step, a Plackett–Burman design was used to evaluate the influence of related factors. Inulin, corn steep liquor and $(NH_4)_2SO_4$ were found to be more compatible supplement with the substrate of wheat bran and positively influenced on inulinase production. In the second step, the concentrations of the three nutrients above were further optimized using a Box–Behnken design. The final concentration of medium optimized with RSM was 12.72% inulin, 10.76% corn steep liquor and 1.61% $(NH_4)_2SO_4$ by employing wheat bran as the solid substrate. The average inulinase activity (409.8 U/g initial dry substrate) in triplicate under optimal medium was obtained.

Keywords: Response surface methodology; Optimization; *Kluyveromyces* S120; Inulinase production; Solid-state fermentation

1. Introduction

Inulin is a natural polysaccharide consisting of linear chains of fructose residues linked by β-2,1 bonds and terminated by a sucrose residue, and is stored in certain higher plants such as Jerusalem artichoke, chicory, and dahlia [1]. Inulinase has been used to produce high-content ultra-high-fructose syrup and oligosaccharides by hydrolyzing the β-2,1-glycosidic linkage of inulin. Inulinase from microorganisms has potential applications in reducing production costs and improving syrup quality compared with high-fructose corm syrup produced from starch. Interest in inulinase has increased over the last years mainly due to the application of ultra-high-fructose syrup in pharmaceutical industries [2].

Traditionally, inulinase has been produced by submerged fermentation (SmF) [2–4]. In recent years, however, there are a few reports on the production of inulinase by solid-state fermentation (SSF) [5]. The SSF has numerous advantages over SmF, including superior productivity, simple technique, low capital investment, low energy requirement and less water output, better product recovery and reported to be the most appropriate process for developing countries [6]. SSF holds tremendous potential for the production of enzymes and has been increasingly applied for the production of enzymes in recent years [7].

The optimal design of the culture medium is a very important aspect in the development of SSF processes. Statistical experimental design techniques are very useful tools for the selection of nutrient, as they can provide statistical models which help in understanding the interactions among the process parameters at varying levels and in calculating the optimal level of each parameter for a given target (i.e. maximal enzyme production) [8]. The application of statistical experimental design techniques in fermentation process development can result in improved product yields, reduced process variability, closer confirmation of the output response to nominal and target requirements and reduced development time and overall costs [9]. Response surface methodology (RSM) is a model, consisting of mathematical and statistical techniques, widely used to study the effect of several variables and to seek the optimum conditions for a multivariable system [8,10,11]. Successful application of RSM to enhance enzyme production by optimizing the culture media has been reported. On the other hand, studies regarding to optimization of culture medium in SSF for the production of inulinase are still few in the scientific literature.

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In the present study, inulinase was produced under SSF by a newly strain, *Kluyveromyces* S120. RSM was adopted here as a tool to obtain best SSF medium.

2. Materials and methods

2.1. Microorganism

Kluyveromyces S120, isolated from soil, was maintained on agar slants with the following medium (g/l): glucose 20, yeast extract 10, peptone 10, agar 20.

2.2. Solid-state fermentation

Inocula were prepared in a medium containing (g/l) yeast extract 10, peptone 20 and inulin 10. A loopful of cells from the slant were transferred into a 250 ml conical flask containing 20 ml culture medium and incubated to mid-log phase on a rotary shaker operating at 120 rpm at 30 °C. Twenty grams of wheat bran, supplemented with different nutrients and an acidified mineral solution containing 3.0 mg $MnSO_4{\cdot}H_2O$, 9.0 mg $FeSO_4{\cdot}6H_2O$, 2.5 mg $ZnSO_4{\cdot}7H_2O$ and 3.5 mg $CaCl_2$ for 100 g dry substrate, were set with initial pH 6.0 and moisture content 65% in 250 ml conical flasks, sealed with hydrophobic cotton and autoclaved at 121 °C for 20 min. The cooled substrates were inoculated with a 4% inoculum level, mixed carefully under strictly aseptic conditions with sterile glass rods, and then incubated in a chamber with relative humidity above 80% at 30 °C for 72 h in a static mode. By using Plackett–Burman design and Box–Behnken design to assess the effects of medium ingredients on production of inulinase, medium composition was various based on the experimental designs.

2.3. Screening of the supplemental nutrients using a Plackett–Burman design

Plackett–Burman design, an efficient technique for medium component optimization [12], was used to pick factors that significantly influenced inulinase production and insignificant ones were eliminated in order to obtain a smaller, more manageable set of factors. Wheat bran, purchased from a local market, was used as the solid substrate. The supplemental nutrients were screened by a Plackett–Burman design for eight variables at two levels.

2.4. Optimization of the supplemental nutrients using a Box–Behnken design

Once critical factors were identified via screening, a Box–Behnken design for three independent variables, each at three levels with three replicates at the centre points [10], was employed to fit a polynomial model:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

where Y was the yield of inulinase, β_0 the intercept term, β_1, β_2 and β_3 were linear coefficients, β_{12}, β_{13} and β_{23} were interactive coefficients, β_{11}, β_{22} and β_{33} were quadratic coefficients, and X_1, X_2 and X_3 were coded independent variables. SAS package (version 8.2, SAS Institute Inc., Cary, NC, USA) was used for the experimental design and regression analysis of the data obtained.

2.5. Extraction of inulinase

When fermentation was terminated, A weighed quantity of the fermented matter was transferred to 250 ml conical flasks, 10 volumes of distilled water was added (w/v, based on initial dry weight of the substrate) and the mixture was mixed at room temperature (20 ± 2 °C) on a rotary shaker (150 rpm) for 30 min. The whole contents were filtered through muslin cloth. After filtering twice using the same approach, the filtrates were pooled and the total volume was recorded.

2.6. Enzyme activity assay

The inulinase activity was determined by measuring the reducing sugars released by the hydrolysis of sucrose. For assay, 0.5 ml of suitably diluted enzyme extract was mixed with 4.5 ml of sucrose solution (2%, w/w) in 0.1 mol/l pH 4.8 acetate buffer. The reaction was carried out at 50 °C for 10 min and stopped by boiling for 10 min. The reaction mixture was assayed for reducing sugars as fructose by DNS method [3]. A calibration curve was prepared with fructose solutions of known strength and blanks were run simultaneously with enzyme and substrate solutions. One unit of inulinase activity was defined as the amount of enzyme, which produced 1 μmol of fructose under the assay conditions.

2.7. Plate count of Kluyveromyces

Fermented matter was adequately mixed with 10 volumes of sterilized water (w/v, based on initial dry weight of the substrate). The mixture was aseptically diluted to suitable concentration by tenfold dilution. A 0.1 ml volume of diluted mixtures in different concentrations was spread on malt extract medium in quintuplicate. After incubation for 48 h at 30 °C, the number of CFU/g (1 CFU = 1 colony-forming unit) was calculated according to the dilution factor and the number of colonies on the plates with 30–300 colonies.

3. Results and discussion

3.1. Screening of significant nutrients using a Plackett–Burman design

Based on the earlier studies, a total of eight variable components were analyzed for their effect on inulinase production using a Plackett–Burman design (Table 1). In the experimental design, each row represents an experiment and each column represents an independent variable. The signs + and − represent the two different levels (higher and lower) of the independent variable under investigation (Table 2). The yield of inuli-