



Optimization of inulinase production by a newly isolated *Aspergillus tubingensis* CR16 using low cost substrates

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ABSTRACT

Production of an extracellular, thermostable inulinase was carried out by a newly isolated strain of *Aspergillus tubingensis* CR16 using wheat bran and corn steep liquor (CSL) under solid state fermentation (SSF). Response surface methodology (RSM) involving Box Behnken design (BBD) was employed for the optimization of process parameters viz. time period of fermentation, % moisture content, inoculum size and pH of the medium. Maximum yield of inulinase was 257 ± 11.4 U/g, obtained by inoculating 5 g of wheat bran with 10^9 spores/ml, at initial 71.2% moisture content and pH 6.1 after 103 h of fermentation along with 1358.6 ± 0.8 U/g of invertase activity. Crude inulinase showed maximum activity at 60 °C and pH 5.0. The enzyme was found to be thermostable retaining about 90% of its activity for 4.5 h at 60 °C. Fructose was produced as an end product of inulin hydrolysis proving that the enzyme produced was exoinulinase.

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1. Introduction

Inulin occurs as a reserved carbohydrate polymer mainly in the roots and tubers of jerusalem artichoke, chicory, dandelion, burdock and dahlia (Chen, Chen, Chen, Xu, & Jin, 2011; Pandey et al., 1999a). It consists of linear chains of β -2,1-linked D-fructofuranose molecules terminated by a glucose residue (Vandamme & Derycke, 1983). Inulin has recently received a great interest as a relatively inexpensive and abundant substrate for the production of high fructose syrup. D-Fructose is occupying an increasingly important position in the modern world as a sweetener because of its higher sweetening value, its physiological metabolism in human body and its insignificant insulinogenic effects (Pandey et al., 1999a). Fructose production by enzymatic inulin hydrolysis is more advantageous than conventional process based on starch, which requires the action of α -amylase; amyloglucosidase and glucose isomerase and yields only 45% of fructose in the final product, due to thermodynamical equilibrium of the reaction (Sguarezi et al., 2009). Thus microbial inulinases are important class of industrial enzymes that have gained much attention in recent times. Inulinases can be produced by many of the microorganisms including strains of *Aspergillus* sp., *Penicillium* sp. and *Kluyveromyces* sp. Owing to

the cost of pure inulin, alternate inulin containing raw, inexpensive substrates are preferred for microbial inulinase production. In recent years, inulinase production by solid state fermentation (SSF) has attracted much attention because of high productivity, simple operation, cost effectiveness and better product recovery (Singhania, Patel, Soccol, & Pandey, 2009). Moreover, the crude fermented products from SSF can be used directly as the enzyme source for biotransformation (Chen et al., 2011). Optimization of process by statistical experimental designs is very useful, as it helps in understanding the interactions among the process parameters at varying levels and in calculating an optimal level of each parameter for the maximal product yield (Reddy, Ramesh, Mrudula, Reddy, & Seenayya, 2003). 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 (Xiong, Jinhua, & Dongsheng, 2007).

Although microbial inulinase production has been reported by many researchers, studies on inulinase production under SSF are relatively less. Assessment of fermentation conditions for inulinase production is of relevance since many fermentation parameters may significantly affect the productivity of the enzyme and thus production cost. In this context, the present work was focused to study the optimization of process parameters for inulinase production on low cost substrate under SSF using statistical design employing a newly isolated fungi *Aspergillus tubingensis* CR16. Properties of crude inulinase were also evaluated to predict its end applications.

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2. Materials and methods

2.1. Materials

Dry chicory roots were procured from Pioneer Chicory Factory, Anand, India. Wheat bran and sugarcane bagasse were bought from local vendors. Corn steep liquor (CSL) was provided by Anil Starch Ltd., Ahmedabad, India. Pure inulin (Chicory) and potato dextrose agar (PDA) were obtained from Hi-media (India). All other chemicals were of reagent grade.

2.2. Identification of microorganism

A new fungal strain CR16 was isolated from chicory rhizosphere soil. Preliminary identification of isolate was done by study of growth characters on PDA plate and microscopic observations. The isolate was sent for molecular identification to Agharkar Research Institute, Pune, India. Molecular identification was done by partial sequencing i.e. ITS. Genomic DNA was isolated and about 500 bp rDNA fragments were amplified using universal primers. Sequencing PCR was done with ABI-Big DYE® Terminatorv3.1 Cycle Sequencing Kit (Part No. 4337455). Sequence data was aligned with publically available sequences and analyzed to reach identity. The strain was maintained on PDA slants at 4 °C.

2.3. Media

Fermentation medium-1 contained (g/l): peptone 15.0 and yeast extracts 15.0. Fermentation medium-2 contained (g/l): inulin, 10.0; K₂HPO₄, 1.0; MgSO₄·7H₂O, 0.5; NaNO₃, 1.5; KCl, 0.5; FeSO₄, 0.1; and NH₄H₂PO₄, 2.0.

2.4. Inoculum preparation

Spores of 72 h old culture of *A. tubingensis* CR16 were suspended in sterile distilled water containing 0.1% of Tween 80 and spore count was performed using Neuber's chamber.

2.5. Submerged fermentation using different carbon sources

Submerged fermentation was carried out in 50 ml of fermentation medium-1 containing different carbon sources in 1% concentration namely chicory root powder, wheat bran, sugarcane bagasse, inulin, sucrose, fructose and glucose. Medium was inoculated with 1% inoculum with 10⁸ spores/ml.

2.6. Solid state fermentation (SSF)

SSF was performed using each of the three different substrates viz. chicory root powder, wheat bran and sugarcane bagasse. Substrates were taken in 5 g quantity in 250 ml Erlenmeyer flask and autoclaved at 121 °C for 15 min. The substrates were moistened with 10 ml of the separately sterilized fermentation medium-1, along with 10⁸ spores/ml inoculum. Contents of the flasks were mixed thoroughly and were incubated at 30 °C under static conditions. To study the effect of different moistening agents, 5 g of wheat bran was moistened with 10 ml of different moistening agents which included tap water, 10% CSL, chicory root extract, fermentation medium-1 and fermentation medium-2, inoculated with 10⁸ spores/ml inoculum and incubated at 30 °C under static conditions. Flasks were mixed intermittently and were removed at regular intervals for analyses.

2.7. Statistical optimization of inulinase production

RSM using BBD was applied for the optimization of inulinase production which involves full factorial search by observing simultaneous, systematic and efficient variation of important components on the fermentation process. Wheat bran was selected as a substrate for optimization studies and 10% CSL was used as a moistening agent. Particle size of wheat bran (5–7 mm) was kept constant in all the experimental runs. Four important process parameters namely fermentation period (X_1), % moisture content (X_2), inoculum size (X_3) and pH of the medium (X_4) were selected as independent variables and inulinase activity (Y) was the dependent variable response. Each of these independent variables was studied at three different levels as per BBD in four variables. The number of experiments (N) required for the development of BBD is defined as

$$N = 2k(k - 1) + C_0$$

where k is the number of variables and C_0 is the number of central points (Ferreira et al., 2007). This was used to develop mathematical correlation between four variables on the production of inulinase with a total of 29 runs with five replicates at a central point to fit the polynomial model as per Eq. (1).

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{44}X_4^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{34}X_3X_4 + e \quad (1)$$

Specified range of four variables used for the optimization was selected as per shown in Table 2 along with the design of BBD in the coded and decoded levels of the four variables. Temperature was kept constant at 30 °C for all the experimental runs. Flasks were analysed for inulinase activity at specific time intervals as planned in BBD. Control reactors were also carried out to discount if enzyme activity already present prior fermentation.

Statistical analysis was done using the software MINITAB 16. Graphs were generated to highlight the roles played by various factors and to emphasize the roles played by physical constraints and biosynthetic aspects in the final yield of inulinase. Interpretation of optimum values of process parameters and curves showing their interactive effect was done using the same software.

2.8. Extraction of inulinase

After fermentation the enzyme was extracted in 0.2 M sodium acetate buffer (pH 5). Content of the flask was mixed thoroughly on a rotary shaker (150 rpm) at 30 °C for 30 min, filtered through muslin cloth and was centrifuged at 3000 rpm for 10 min at room temperature. Supernatant was considered as crude enzyme solution and was analyzed for inulinase, invertase and protein content.

2.9. Enzyme assays

Enzymes were assayed by measuring the reducing sugar concentration by DNS (Miller, 1939). Reaction mixture consisting of 0.1 ml of appropriately diluted enzyme and 0.9 ml of 1% inulin/sucrose in 0.2 M sodium acetate buffer (pH 5) was incubated at 60 °C for 20 min. The reaction was terminated by further incubation in boiling water bath for 10 min. One unit of inulinase activity was defined as the amount of enzyme necessary to release one micromole of fructose per minute under the above conditions. One unit of invertase activity was considered as the amount of enzyme which released one micromole of reducing sugar per minute under the above conditions.

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