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Xylanase production from *Bacillus aerophilus* KGJ2 and its application in xylooligosaccharides preparation

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ABSTRACT

Xylanolytic enzyme was produced using a newly isolated *Bacillus aerophilus* KGJ2 and low cost lignocellulosic sources in solid state fermentation. Seven different agricultural residues (wheat bran, tea dust, saw dust, paper waste, cassava bagasse, rice straw and rice husk) and six nitrogen source namely yeast extract, beef extract, peptone, ammonium nitrate, ammonium sulphate, and ammonium chloride were examined for xylanase production. Upon initial screening, wheat bran and ammonium chloride were chosen as suitable carbon source and nitrogen source respectively. Plackett–Burman fractional factorial design was employed to screen the important process variables affecting enzyme production. Substrate concentration, nitrogen source, moisture content and $MgSO_4 \cdot 7H_2O$ were identified as statistically significant variables. Subsequently Box–Behnken method was used to optimize the process conditions to achieve maximum xylanase yield. Under optimized conditions xylanase yield was 45.9 U/gds. Best xylanase activity was obtained at 70 °C and pH 4.0. It retained more than 90% activity after incubation at 80–90 °C for 60 min. The hydrolytic efficiency of xylanase on xylan was examined and xylobiose, xylotriose and xylotetrose were obtained as hydrolytic products.

1. Introduction

Xylooligosaccharides (XOs) are sugar oligomers generated through hydrolysis of xylan. They find applications in a variety of industries including pharmaceutical, agriculture and feed industry. If orally consumed, XOs have prebiotic effects [1]. Xylooligosaccharides can be used to decrease cholesterol, sustain the gastrointestinal health, and enhance the biological availability of calcium etc. [2]. Xylooligosaccharides enhance the nutritional and sensory characteristics of food as they are modestly sweet, stable over a wide pH and temperature range and inhibit the starch retrogradation [3].

Enzymatic hydrolysis of xylan proved to be an efficient method in production of XOs as the enzymes are highly selective and do not produce by products. Though, enzymes of fungi, actinomycetes and bacterial origins are available, bacterial xylanases are preferred over other xylanases as they possesses better temperature and pH tolerance [4,5].

Recently solid state fermentation is widely adopted to reduce the cost of production of various biological molecules [6]. In solid state fermentation, various renewable xylan sources can be used for the production of xylanase as a substitute for hardwood xylan. Agricultural residues like wheat bran, paper waste, rice straw, cassava bagasse, tea dust, saw dust and rice husk have been investigated as possible low cost substrates. These are generated in large quantities through industrial processes [7]. Proper utilization of the agricultural substrates can be achieved through solid state fermentation (SSF) technique. The advantages of SSF in enzyme production are: (i) easy recovery of products, (ii) simple setup, (iii) high volumetric productivity, (iv) low energy demand, and (v) direct utilization of low cost substrate [8,9].

Thus in the present work, XO producing acidophilic xylanase was produced in solid state fermentation using a newly isolated *Bacillus aerophilus* strain KGJ2. Wheat bran was used as low cost carbon source in solid state fermentation. Statistical experimental design is widely adopted in bioprocess engineering for identification of influential process variables and for optimization of process variables with comparatively less number of variables than in conventional experimental design [10–14]. In the present work, this was performed in two stages. First, process variables influencing production of xylanase from wheat bran were first screened by Plackett–Burman fractional factorial design of experiments. After

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identifying the important variables optimization was carried out using Box–Behnken method in the second stage.

Properties of xylanase were also evaluated and their potential for XOs production was examined. This is the first report on production of xylanase from the new isolate *B. aerophilus* KGJ2 and its application for XO production.

2. Materials and methods

2.1. Microorganism

The strain isolated from paper mill effluent collected from a local pulp and paper industry, identified as *B. aerophilus* KGJ2 (NCBI Gene Bank Accession No. JX027507), was used in this work. The organism was preserved on xylan agar plates that contain (in g/l): birch wood xylan, 5.0 (Sigma Aldrich MA, USA); peptone, 5.0; NaCl, 1.0; K_2HPO_4, 2.0; $CaCl_2{\cdot}2H_2O$, 0.1; $MgSO_4{\cdot}7H_2O$, 0.1; yeast extract, 1.0; bacteriological agar, 15.0. Plates were maintained at 4 °C.

2.2. Production and extraction of xylanase in solid state fermentation

Xylanase production was carried out in solid state fermentation in 250 ml Erlenmeyer flasks using 10 g of finely powdered agro residue supplemented with basal salt solution. The composition of the basal salt solution was (g/l): NaCl, 1.0; KH_2PO_4, 2.0; NH_4Cl, 2.0; $MgSO_4{\cdot}7H_2O$, 0.5; $FeSO_4{\cdot}7H_2O$, 0.25; $MnSO_4$, 0.25 and $CaCl_2{\cdot}2H_2O$, 0.1. This was added to the substrate, with a moisture ratio of 1:1. The flasks were kept for sterilization at 121 °C for 15 min. Ten percent (w/v) (1.52×10^7 cfu/ml) of 18 h seed culture was added to each flask and was incubated at 30 °C for 24 h. Samples were taken from the flasks periodically and the enzyme was extracted using 0.1 M phosphate buffer, pH 7.0 (1:30 w/v) by mixing the content using an orbital shaker at 150 rpm for 30 min and then filtered through cheese cloth. The filtrate was subsequently centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatant, crude enzyme, was used for further studies [15].

2.3. Enzyme assay

Xylanase activity was measured DNS method [16]. 1 ml of 1% birch wood xylan solution was added with 0.5 ml enzyme solution in a test tube and incubated at 70 °C for 5 min in water bath. After 5 min 1.5 ml DNS reagent was added and the mixture was incubated for another 10 min in boiling water bath [16]. The blank (1.5 ml 20 mM sodium citrate buffer with 1.5 ml of DNS) and control (0.5 ml of enzyme, 1 ml of 20 mM sodium citrate buffer and 1.5 ml of DNS) were also run along with sample. The absorbance was measured at 540 nm. One unit of xylanase activity was defined as the amount of enzyme that liberates 1 μmol of reducing sugars equivalent to xylose per minute under the assay conditions described [1]. Total soluble protein concentration was determined using bovine serum albumin (BSA) as a standard according to Lowry et al. [17].

2.4. Screening of carbon source

Seven different agricultural residues (wheat bran, tea dust, saw dust, paper waste, cassava bagasse, rice straw and rice husk) were first chosen as possible low cost carbon source. To this, basal salt solution was added in 1:1 ratio and the mixture was autoclaved. Then 10% (w/v) inoculum was added to the flask and incubated at 30 °C. Crude enzyme samples were taken at regular time interval and the xylanase activities were analyzed. Effect of carbon sources was statistically examined using Tukey test and the one that showed maximum enzyme activity was chosen as best carbon source and the same was used in further studies.

2.5. Screening of nitrogen source

The nitrogen sources examined include yeast extract, beef extract, peptone, ammonium sulphate, ammonium nitrate, and ammonium chloride. The basal salt solution was mixed with 0.1% of the above nitrogen source and used for evaluation of xylanase activity. A control devoid of nitrogen source was used for comparison. Once again, using Tukey test results the best nitrogen source was identified.

2.6. Plackett Burman design

The carbon and nitrogen source screened in previous steps were added to the production medium. Nine variables namely substrate concentration, moisture ratio, inoculum size, concentration of nitrogen source, pH, fermentation time, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, and NaCl were considered as influential variables and included in the screening experiment. Nine factors were investigated in 14 experimental runs which included 3 replicates at central point as shown in Table 1. The first order model given in Eq. (1) was used here.

$$Y = \beta_o + \sum \beta_i x_i \quad (1)$$

where Y is the response variable (xylanase yield in U/gds), x_i is the level of independent variable, and β_o, β_i are the model intercept and linear coefficients respectively.

2.7. Box–Behnken design

Box–Behnken design of experiment was performed, with the important variables screened in previous step, to optimize the conditions favorable for xylanase production. Response surface method with the four factors, each varied at three levels was employed to optimize the response variable. The regression analysis was performed using Minitab 15 and experimental data were fitted to the following quadratic equation:

$$Y = a_0 + \sum_{i=1}^{n} a_i x_i + \sum_{i=1}^{n} a_{ii} x_i^2 + \sum_{i=1}^{n} \sum_{j=1+1}^{n} a_{ij} x_i x_j \quad (2)$$

where a_0 is the model constant term, a_i, a_{ii}, a_{ij} are the regression coefficients of linear, square and iteration terms [17].

2.8. Partial purification of enzyme

Crude enzyme produced under optimized conditions was partially purified by ammonium sulphate precipitation followed by dialysis [9]. The molecular weight of the protein was determined using SDS-PAGE electrophoresis. After electrophoresis, the gel was stained with coomassie brilliant blue R-250. A low molecular weight marker protein purchased from Bangalore Genei Pvt. Ltd. was used as the molecular weight standard.

Zymogram analysis was done using native PAGE (7%) with 1% xylan mixed with separating gel. After electrophoresis the gel was stained using 2% congo red for 20 min and further washed with 1 M NaCl solution for 10–20 min to visualize the band [1].

2.9. Effect of temperature and pH on xylanase activity and stability

Effect of temperature on xylanase activity was investigated by determining enzyme assay at different temperatures (30–90 °C) optimum pH 4 by using 20 mM sodium citrate buffer.

Thermostable nature of the xylanase was investigated by pre-incubation of the enzyme in 20 mM sodium citrate buffer (pH 4)