Optimization of Xylanase Production by *Melanocarpus albomyces* IIS68 in Solid State Fermentation Using Response Surface Methodology

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Xylanase production by the thermophilic fungus, *Melanocarpus albomyces* IIS68, during solid state fermentation of wheat straw was studied and the effects of various variables were observed. Using the response surface methodology and the multivariate statistical approach, the optimum levels of the variables affecting xylanase production were determined. The optimum levels of the variables were 600–850 μm particle size, 43 h inoculum age, 1.37% Tween 80, 86% initial moisture content, 5.1% urea, 0.74% yeast extract and a harvest time of 96 h. Under these optimized conditions, xylanase activity of 7760 U/g initial dry substrate was obtained which was in very good agreement with the value predicted by the quadratic model (7890 U/g initial dry substrate).

[Key words: xylanase, solid state fermentation, response surface methodology, *Melanocarpus albomyces*, lignocellulosic residues, wheat straw]

Large quantities of agricultural residues accumulate every year which result not only in the deterioration of the environment but also in loss of potentially valuable material which can be processed to yield a number of value added products such as food, fuel, feed and a variety of chemicals (1). Cellulose, hemicellulose (xylan being the most abundant of the hemicelluloses) and lignin are the major contents of these residues. Over the years, xylanases have gained considerable importance in paper and pulp, feed and food industry. Resistance to thermal inactivation of the enzyme is a desirable property for many industrial applications. Xylanase has been successfully used in the prebleaching of kraft pulp (2) and improvements have been made by the use of xylanases that act at higher temperatures (3). Zamost *et al.* (4) have also listed xylanases to be useful in the manufacture of dissolving pulps yielding cellulose for rayon production and biobleaching of wood pulp while describing the thermostable enzymes for industrial applications. We have been working with *Melanocarpus albomyces* IIS68 as it has been found to be a good source of thermostable xylanase along with β-xylanidase and xylan debranching enzymes (5), with the aim of producing it on a large scale for prebleaching of pulp in paper industry.

Solid state fermentation (SSF) offers advantages over liquid cultivation, especially for the fungal cultures, as there is higher productivity per unit volume, reduced energy requirements, lower capital investment, lower waste water output, higher concentrations of metabolites obtained and low downstream processing cost (6–8). We, therefore, studied xylanase production by the fungus in SSF. The aim of this work was to apply fractional factorial design, such as the Box-Behnken design (9) followed by response surface methodology to investigate and optimize six variables (inoculum age, initial moisture, Tween 80 level, urea level, yeast extract level and the harvest time) which may affect xylanase production by *M. albomyces* IIS68 in solid state fermentation.

The thermophilic fungus *M. albomyces* IIS68 isolated from compost and soil (10) with an optimum growth temperature of 45°C and pH of 6.0 was used. It was maintained on yeast–phosphate–soluble starch agar (yeast extract 0.4%, KH₂PO₄ 0.1%, MgSO₄·7H₂O 0.05%, soluble starch 1.5%, agar 2%) and was subcultured once a month. The culture was grown on the slant for seven days at 45°C, and thereafter, stored at 4°C. Culture from a slant was transferred into 20 ml of glucose medium (glucose, 1%; KH₂PO₄, 0.06%; K₂HPO₄, 0.04%; MgSO₄·7H₂O, 0.05%; urea, 0.05%; yeast extract, 0.01%; pH 6.0) and grown for 36 h at 45°C, 220 rpm with 4 glass beads (4 mm diameter) in Erlentmeyer flask to avoid clumping of mycelia. This pre-inoculum (at 10% v/v level) was used to inoculate 180 ml of wheat straw medium (same as glucose medium except that glucose was replaced by wheat straw), and was grown for 48 h at 45°C, 220 rpm with 4 glass beads. This inoculum was then used to inoculate (at 10% v/v) fresh wheat straw medium and was again grown at 45°C, 220 rpm with 4 glass beads. Different ages of inoculum were used for carrying out SSF.

Finely ground wheat straw was sieved and particles that passed through 850 μm but not through 600 μm sieve were washed and dried overnight at 70°C. As per the experimental requirements, various additives (urea, yeast extract, KH₂PO₄, K₂HPO₄, MgSO₄·7H₂O and the surfactant Tween 80) were added to the wheat straw. The concentration of KH₂PO₄, K₂HPO₄ and MgSO₄·7H₂O was kept at 2.4%, 1.6% and 2% respectively (unless specified otherwise). This substrate was put in trays to form a thin layer of about 4 mm and was dried at 70°C. The trays were autoclaved at 121°C for 35 min. An appropriate amount of inoculum of suitable age (as per the experimental requirements) was uniformly spread over the substrate layer and the trays were kept at 45°C in a controlled humidity chamber at 92% relative humidity. The initial moisture content of wheat straw was adjusted by appropriate addition of inoculum and water.

After incubation, 0.05 M phosphate buffer of pH 7.0
was added to the tray. The buffer added was 20 ml/g initial dry substrate in the tray. After half an hour, it was filtered through a muslin cloth and the first extract was collected. The extract was again repeated to collect the second extract. The second extract contained about 10% of the activity present in the first extract. The activities reported are the sum of activities present in the two extracts. The xylanase activity was assayed using 1% oat spelt xylan (Sigma, USA) in 0.05 M phosphate buffer (pH 6.0) according to the method of Bailey et al. (11) at 70°C thermostat water bath with constant shaking (40 rpm). The release of reducing sugars was determined using the 3,5-dinitrosalicylic acid (DNS) method (12) using xylose as a standard. One unit of enzyme activity was defined as the amount of enzyme required to liberate one pmol of reducing sugars per minute. The results of the analysis are expressed as units per gram of initial dry substrate (U/g). In order to identify the optimum conditions, Box-Behnken design (9) was selected, as this is recommended over central composite design when the number of variables is large (more than four). This design evaluates the quadratic effects and two-way interactions among the variables and thus determines the non-linear nature of the response, if any. Each variable is experimented at ‘+1’, ‘0’ and ‘−1’ levels which represent the higher, middle and the lower levels of the variable. The coefficients of the quadratic model were calculated using standard regression techniques. The software, DesignExpert™, Version 5.0 of Stat-Ease Inc. (USA) was used to design the experiments, calculate the coefficients and generate the response surface. The possible variables that could be affecting the xylanase production were inoculum age, concentration of urea, concentration of yeast extract, concentration of Mg²⁺, initial moisture content, harvest time, extraction pH and the surfactant Tween 80. Initially the effect of each of these variables was independently observed keeping other variables constant in order to strike off the variables which did not have observable effect on the production of xylanase. Based on these preliminary experiments, it was observed that there was no noticeable effect of concentration of Mg²⁺ and the extraction pH on xylanase production. The remaining six variables (inoculum age, initial moisture, yeast extract concentration, urea concentration, surfactant concentration and harvest time) were optimized using the Box-Behnken design. The range and levels of variables investigated in this study are given in Table 1. The ‘0’ level indicates the values of variables obtained in the primary experiments (data not shown). The response of the variables was measured by assaying xylanase activity in two sets of independent experiments. The values reported are the average of the two, which differed by not more than ±5%.

The regression analysis of the data, generated by the Box-Behnken design experiments, was carried out to fit the response function. For checking the statistical significance of the quadratic model equation, the F-test was carried out. On analysis of the variance (ANOVA for Response Surface Quadratic Model), the ‘Prob>F’ value for the model was calculated to be less than 0.0001 i.e. 99.99% confidence level. The ‘Prob>|t|’ values for all variables were less than 0.01 and hence were acceptable. The significant terms have a small ‘Prob>|t|’ value.

The model also showed a high ‘R-Squared’ value of 0.97 (acceptable if above 0.94). The ‘ Adequate Precision’ value was also at a satisfactory level of 21.36 (desired >4). The Variance inflation factor (VIF) did not exceed 1.5 for any of the terms in the quadratic equation predicted. This reflects that there was no problem of multi-collinearity (since VIF<10). Also the leverage for each response was less than twice the average leverage and hence was acceptable. The maximum prediction variance was 0.562 and the average prediction variance was 0.519. The ‘G efficiency’ i.e. the average prediction variance as a percentage of maximum prediction variance was 92.2% (satisfactory since >50%).

The final equation in terms of the coded factors was as follows:


A response surface was generated by plotting the response (xylanase activity) on the Z-axis against any two independent variables while keeping the other four independent variables at their respective ‘0’ levels. Thus, 15 response surfaces (by considering all the possible combinations) were obtained. A plot of the response against each of the independent variables while keeping all other independent variables at their respective ‘0’ levels is given in Fig. 1. The plot revealed that the xylanase activity produced was most sensitive to inoculum age and moisture content. The solutions predicted by the software Design-Expert™, Version 5.0 of Stat-Ease Inc. are listed in Table 2. However, in all the solutions, except the solution 8, the predicted values of at least one of the variables was equal or very near to one of the extremes (i.e. ‘−1’ or ‘+1’ level). In such cases, as per the instructions of the software Design-Expert™, Version

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5.0, the solutions fall in areas of high extrapolation and the predicted value would not satisfy the 95% confidence level. This was also reflected in the standard error predicted by the software. Hence, a SSF experiment pertaining to solution 8 was carried out and a value of 7760 U/g was obtained, which was in good agreement with the predicted value of 7890 U/g. This experimental value of 7760 U/g was 19.8% higher than the experimental value of 6475 U/g, obtained when all the variables were set at ‘0’ level.

The activity obtained in the present investigation after optimization of process variables affecting the enzyme production is one of the highest reported in the literature (14, 15). Further, the investigation showed the applicability of the statistical theory for optimizing the production of a particular metabolite (xylanase) by SSF process.

REFERENCES


