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ABSTRACT

This study concerned with the optimization of fermentation parameters for the hyper production of mannanase from *Fusarium oxysporum* SS-25 employing two step statistical strategy and kinetic characterization of crude enzyme preparation. The Plackett-Burman design was first used to screen out the important factors in the culture medium which were found to be: 20% (w/w) wheat bran, 2% (w/w) each of potato peels, soybean meal, malt extract, 1% tryptone, 0.14% NH_4SO_4, 0.2% KH_2PO_4, 0.0002% $ZnSO_4$, 0.0005% $FeSO_4$, 0.01% $MnSO_4$, 0.012% SDS, 0.03% NH_4Cl, 0.1% $NaNO_3$ in brewer's spent grain based medium with 50% moisture content, inoculated with 2.8×10^7 spores and incubated at 30°C for 6 days. Out of twenty seven factors, four variables including soybean meal, $FeSO_4$, $MnSO_4$ and $NaNO_3$ were selected to study the interactive effects and optimum level of these variables in central composite design of response surface methodology. The final mannanase yield was 193 IU/g which was active at broader temperature and pH range and could result in 26.6% reduction in kappa number with 4.93% higher tear index and 1% increase in brightness when used to treat the wheat straw based kraft pulp. The hydrolytic potential of enzyme was demonstrated on both locust bean gum and guar gum.

Keywords: Mannanase, Solid State Fermentation, Brewer's Spent Grain, Plackett-Burman, Response Surface Methodology, *Fusarium oxysporum*

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INTRODUCTION

Hemicellulose is a class of complex polysaccharides and constitutes one of the basic components of plant cell wall. It also represents one of the major renewable biomass on earth. The major components present in the hemicellulose part of soft woods are mannan and heteromannans. These components are also present as part of the hemicellulose in hardwoods, in beans and also in the seed coat many species of legumes (*Buckeridge et al., 2000; Capoe et al., 2000; Lundqvist et al., 2002; Handford et al., 2003*). The mannans present in hardwood are composed of mannopyranose and glucopyranose units joined together in β-1, 4-linkages, whereas in softwood two different types of acetylated galactomannan and glucomannans are present. These mannans made up of glucose, galactose and mannose in the ratio 1:1:3 and 1:0.1:4 respectively (*Lundqvist et al., 2002*). The galactomannan which is main storage carbohydrate present in the leguminous seeds, comprising 18- 20% of the total dry weight of the seed material (*McCleary, 1988*).The D-mannose is the main component of mannan, but because of complex structure (physical and chemical) of plant mannans, different enzymes are required to break down this heterogeneous polymer (*Moreira et al., 2008*). The complete hydrolysis of mannans into monomer sugars that can be easily available source of energy by the group of particular microorganisms, requires the synergistic action of both exo acting β-mannosidases (E.C 3.2.1.25) and endo-1, 4-β-mannanases (E.C 3.2.1.78, mannan endo-1, 4-β-mannosidase). There are some enzymes such as β-glucosidases (EC 3.2.1.21), α-galactosidases (EC 3.2.1.22) and acetyl mannan esterases are needed for the removal of the individual sugars and sugars units which are present at several points on mannans (*Tenkanen, 1998*).

There are number of industrial processes in which mannanases play a key role, such as biobleaching of softwood samples in the paper and pulp industries, to reduce the viscosity of coffee extracts and improving the quality of food and feed (*Dhawan & Kaur, 2007*). β-mannanases are used in pulp and paper industry for modifying the existing technologies like bleaching the pulp samples and to reduce the harmful impact of mill effluents by effluent treatment. If kraft pulps are prebleached with mannanases, then it lowers the chlorine requirement to bleach the kraft pulps, which ultimately will lead to reduce chloro-organic discharges (*Viikari et al., 1991*). There are some feed components such as corn and soybean meal

in which mannan is present in significant quantity which hinder the digestion and absorption of these contents in the small intestine of domestic animals due to the absence of mannanases in their digestive system. They affect the digestion and absorption of gastrointestinal contents by increasing the viscosity of medium and also can cause diarrhoea to the livestock (*Jackson, 2001*). However, these adverse effects of the mannan can be overcome by the use of β-mannanase in their feed. β-mannanase (EC 3.2.1.78) can breakdown the mannans into mannooligosaccharides, thus reducing the viscosity of these contents into stomach as well as in the intestine of livestock and improving the digestion and easily absorption of nutrients in their feed (*Burke & Cairney, 1997*). Further, the oligosaccharides which are formed from mannan hydrolysis can play an important role in the regulation of number of metabolic processes in the animal's intestine (*Wu et al., 2005*).

At industrial level, the production of mannanases is restricted due to its high cost and low yields. So, there is a great need to develop a simple production medium with low cost substrates which provides a high mannanase activity. Among the existing technologies in the enzyme production, solid-state fermentation (SSF) offers many advantages over submerged state fermentation, such as low capital investment and much higher reactor volume (*Grajek, 1987*). There are wide number of application of SSF process in the food, pharmaceutical and agricultural industries. There are a large number of reports available in literature in which they have been using the SSF processes for producing industrially important enzymes such as cellulases, polygalacturonase, xylanase, pectinase and mannanase (*Soni et al., 2010; Bansal et al., 2011*). It is generally understood that 30-35% of the production cost of industrially important enzymes is due to the expenses of growth medium of microorganism (*Laxman et al., 2005*). Therefore, development of an economically viable enzyme production medium requires selection of process parameters and their optimization strategies. The enzymes obtained from microorganisms are generally extracellular and their production is highly affected by cultural and environmental factors, such as carbon and nitrogen ratio, inorganic nutrients, temperature, pH, aeration and agitation (*Mudau & Setati, 2006; Li et al., 2006; Lin et al., 2007*). The medium optimization by one factor at a time approach is laborious, especially for those in which large numbers of variables are involved and also it does not ensure perfect desirable conditions for the microorganism to grow. The statistical experimental designs such as Plackett-Burman and subsequent response surface methodologies (RSM) can collectively overcome the difficulties of a one variable at a time

optimization process. Plackett-Burman design (*Plackett and Burman, 1946*) is a statistical technique used in optimization of fermentation conditions (*Salihu et al., 2011; Cui et al., 2010; Chen et al., 2011*). According to Pareto's law, the screening of cultural and environmental factors is done to understand the significance of their effects on the product formation and then few better factors are selected for subsequent optimization studies (*Naveena et al., 2003*). The response surface methodology (RSM) is a mathematical tool which provides models and graphs showing the effects of independent variables on enzyme yield and also give the predictive responses of each combination, the interactive effects of each variable to another and the optimum levels of each independent variable in the growth medium (*Dobrev et al., 2007; Senthilkumar et al., 2005; Kim et al., 2007*). Therefore, the aim of this study was to optimize the nutrient medium with Brewer's spent grain (BSG) for hyperproduction of mannanase by *Fusarium oxysporum* SS-25 via SSF, applying statistical experimental designs and analysis methods and subsequently kinetic characterization of this crude enzyme preparation and its use in biobleaching of kraft pulp and hydrolysis of locust bean and guar gum.

MATERIALS AND METHODS

Microorganism

The mannanolytic fungal strain of *Fusarium oxysporum SS-25* used in this study was isolated from the soil samples of Chandigarh city. It was grown and maintained on potato dextrose agar plates at 28^{o}C for 4 days to allow the development of spores and then stored at 4^{o}C until use. Macroscopic and microscopic studies of the fungus revealed it to be a strain of *Fusarium* sp. hence tentatively named as *Fusarium* sp. SS-25. Complete identification of the strain was carried out by 28S rDNA sequencing by taking the services of Xcelris Labs Ltd, India. Molecular identification revealed it to be a strain of *Fusarium oxysporum,* hence named as *Fusarium oxysporum* SS-25**.**

Solid state fermentation of brewer's spent grain for the production of mannanase

The production of mannanase was carried out under solid state conditions in 250 ml Erlenmeyer flasks containing 5 g brewer's spent grain moistened with 5 mL of distilled water. The flasks

were autoclaved and inoculated in triplicate with 2.5ml of fungal spore suspension (2.8×10^7 spore/mL) and incubated at 30°C in stationary state for 4 days. The extraction of enzyme was done by adding 100 mL of distilled water to each flask churning the contents in a blender. After churning, the contents were filtered through metallic sieve and remaining solid residue was thoroughly pressed to extract the remaining liquid. The suspension from each flask was centrifuged at 10,000×g for 10 min at 4°C, and the supernatant analysed for mannanase activity.

Enzyme assays

The activity of mannanase was determined by monitoring the liberation of reducing sugars. The reaction mixture, containing 1% locust bean gum (Himedia, India) prepared in 0.1M acetate buffer (pH 4.5), with properly diluted enzyme solution, was incubated at 50°C for 15 min (*Miller, 1959*). The amount of reducing sugars was determined by dinitrosalicylic acid method with a mannose standard curve. One unit of enzyme activity was defined as the amount of enzyme producing 1 µmol of mannose per minute under the given assay condition. The mannanase yield was expressed in terms of IU/g dry substrate.

Statistical optimization of mannanase production by Plackett-Burman design

Mannanase production is highly influenced by many factors including media components and environmental parameters. For screening the effect of these parameters on enzyme production, 27 different process variables were chosen and examined in one block, at two levels using first order Plackett-Burman factorial design:

$$Y = \beta o + \sum \beta i Xi \quad \text{----------} \quad (1)$$

Where, Y is the response, βo is the model intercept, βi is the linear coefficient, and Xi is the level of the independent variable.

Statistical analysis of data

The software package, Design-Expert trial version 8 from Stat-Ease which provides highly efficient design of experiments was employed. Multiple linear regression analysis was carried out to estimate t-values, p-values to evaluate the significance of experimental design and to screen out the factors affecting enzyme production.

Optimization of screened nutrient sources for mannanase production by *Fusarium oxysporum* SS-25 using Response Surface Methodology

On the basis of Plackett-Burman results, four independent variables including soyabean meal (X_7), FeSO4 (X_{14}), $MnSO_4$ (X_{24}) and $NaNO_3$ (X_{26}) were selected to know the first- and higher-order main effects of each nutrient factor and interactions between them for subsequent optimization studies through response surface methodology. The 2^4 factorial central composite design (CCD), constituting the 30 experimental runs was generated by Design Expert, Version 8.0, Stat-Ease Inc., Minneapolis, MN. The relationship between coded and actual values in the experiment is described according to equation:

$$xi = (Xi - X0i)/\Delta Xi \quad \text{----------} \quad (2)\, i = 1,2,3\ldots j$$

Where xi = coded (dimensionless) value of the variable Xi,

Xi = actual value of the i^{th} variable

X_0 = the value of Xi at the center point,

ΔX = the step change value.

The behavior of the system was explained by the following second order polynomial equation:

$$Y = bo + \sum bi\, xi + \sum\sum bij\, xi\, xj + \sum bii\, x2i + e. \quad \text{----------} \quad (3)$$

Where Y = measured response; bo, bi, bij, bii are the constant and regression coefficients of the proposed model; xi and xj are the two levels (codes values) of each independent variables; e is the random error in experimental conditions.

The software was used for regression analysis of the obtained data and also to estimate the coefficients occurs in experiment during the regression equation analysis. The contour graphs were also obtained to depict the relationship and interactions among variables. The general ability and accuracy of polynomial model was assessed by coefficient of determination (R^2). The statistical significance and accuracy of model coefficient values was assessed by ANOVA.

Kinetic characterization of crude enzyme preparation

Mannanase obtained from solid state culture of *Fusarium oxysporum SS-25* on brewer's spent grain was characterized in terms of pH and temperature activity profiles and effects of various metal salts. To study the effect of pH and temperature on enzyme activity, enzyme assays were

carried out using buffers of different pH ranging between 3.0-9.0 (acetate buffer pH 3.0–5.0, phosphate buffer pH 6.0–7.0, and tris–HCl, pH 8.0–9.0) and at different incubation temperature (30–100℃) respectively. The effect of various metal salts on enzyme activity was measured by incubating the enzyme preparations with 5 mM of different salt solutions at optimum pH and temperature conditions.

Biobleaching of kraft pulp by crude mannanase from *Fusarium oxysporum* SS-25

The in-house produced mannanase was evaluated for its potential use in the biobleaching of Kraft Pulp. The kraft pulp (unbleached) prepared from wheat straw by kraft process was provided by M/S Shreyans paper mill, Rupnagar, India. The parameters those determined the quality of paper such as kappa number and brightness of the pulp were assessed according to Tappi (Technical Association of pulp and paper industry) test methods T 236 and T 452, respectively (*Anonymous, 1991*). Kappa number is defined as the volume (in mL) of 0.1N $KMnO_4$ solution consumed by one gram of moisture free pulp under standard assay conditions and is equal to approximately seven times the mass percentage of lignin. Tearness index of the paper was assessed using the facilities available at paper mill. The enzymatic treatment of the kraft pulp was carried out under optimized conditions (5 g oven-dried pulp, 90 IU/g crude enzyme, pH 8, at 50 °C, for 45 min with 5% consistency) in triplicate runs. The enzyme treated pulp was washed number of times with tap water till the paper sheets could be developed with a Buchner funnel. The paper sheets were air dried before testing for brightness and kappa number.

Hydrolysis of locust bean gum and guar gum by mannanase from *Fusarium oxysporum* SS-25

The hydrolysis experiments were carried out with 1 mL (6.98 U/mL) of mannanase preparation, incubated with both locust bean gum and guar gum (each substrate of 10% w/v consistency) in separate flasks with acetate buffer (pH 4.5), agitated at 150 rpm in water bath shaker at 50ºC. The samples were withdrawn from the reaction mixture from each flask after 24, 48, 60 and 72 h. The rate of hydrolysis of both locust bean gum and guar gum was calculated by measuring the amount of reducing sugars released during the hydrolysis, using dinitrosalicylic acid method (*Miller, 1959*). All experiments were carried out in triplicate so that mean can be deduced.

RESULTS AND DISCUSSION

During the past decades, numerous β-mannanases have been purified and characterized from bacteria, fungi, actinomycetes, plants and molluscs. Among the filamentous fungi, such as *Trichoderma reesei* (*Juhasz et al., 2005*) and *Aspergillus nidulans* (*Bauer et al., 2006*) were considered to possess great potential for the industrial production of β-mannanases. Many researches and R & D efforts have been done on exploiting the β-mannanases with many superior characteristics, increasing β-mannanase yields by optimizing the production medium and resulting β-mannanases production on an industrial level by both submerged and solid-state fermentation (*Lin & Chen 2004; Heck et al., 2005; Ozturk et al., 2010*). However, the β-mannanase yields reported in literature were so low as to restrict its commercial use in industry.

The cost of substrate contributes more than 40% of the total cost of enzyme production, hence utilization of the cheaper substrates is the need of hour. There are large numbers of agro-industrial residues which are produced from diverse economic activities for almost throughout the year. These agro industrial residues constitute one of the large energy rich resources available on the earth and when not properly managed, causing environmental pollution (*Francis et al., 2003*). Reducing the costs of enzyme production by utilizing cheaper substrates and optimizing fermentation and cultivation conditions is the goal of basic research for industrial application. Solid-state fermentation (SSF) is experiencing a new gush of interest, primarily due to the increase in production and prospects of using a large number of agro-industrial residues for mannanase production. In solid state fermentation process, the solid substrate serves as anchorage sheet for the microbial cells and supplies nutrients to the microorganisms growing in it.

A number of agro-waste residues including wheat bran (*Bansal et al., 2011; Kar et al., 2012*), palm kernel cake (*Lee et al., 2011*), empty palm fruit bunch fiber (*Kim & Kim, 2012*), sugarcane beet pulp (*Nasab & Nasab, 2010*), apple pomace (*Sun et al., 2010*), pea peels (*Verma et al., 2011*) have already been tried for the cultivation of microorganisms to produce industrial enzymes. Chen et al. (2013) have also used the palm kernel expeller as a solid substrate for mannanase production by *Aspergillus terreus* K1 and achieved the yield of 41.24 IU/gds. In another study, *Gmelina arborea* was used as a substrate for mannanase production from *Aspergillus niger* under solid state fermentation with the yield of 25.93 IU/gds **(***Adesina et al.,*

2012). Brewer's spent grain (BSG) is one such residue which has gained attention for the production of enzymes under SSF (*Francis et al., 2003; Xiros et al., 2008*) by acting as a substrate and growth medium for microorganisms capable of utilizing the complex carbohydrates present in them. It is the major by-product of brewing industry also known as distiller's dried grains with soluble (DDGS), representing around 85% of the total by-products generated. BSG is a lignocellulosic material containing about 17% cellulose, 28% non-cellulosic polysaccharides, chiefly arabinoxylans, and 28% lignin (*Aliyu & Bala, 2011*). BSG is available in large quantities throughout the year, but its main application has been limited to animal feeding. Considering the substantial availability of brewers spent grains at very low prices, it was used as a substrate in the present study for the low cost production of mannanase by *Fusarium oxysporum* SS-25 under solid state fermentation (SSF). The organism colonized well on this substrate and produced high mannanase yield corresponding to 76 IU/gds.

Screening of factors affecting mannanase production by *Fusarium oxysporum* SS-25 employing Plackett-Burman design

Based upon our preliminary studies and literature review, a set of 27 independent variables, designated as X_1, X_2, X_3 …….X_{27}, were chosen and examined in the present study with their respective responses as shown in (Tables 1 and 2).The main effects of the examined variables on mannanase production were calculated as the difference between the average measurements made at higher level (+1) and low level (-1) of that factor, as represented in Fig.1. Incubation time found to have the maximum positive effect on mannanase production followed by the presence of soybean meal (X_4), $FeSO_4$ (X_{14}), $MnSO_4$ (X_{17}) and $NaNO_3$ (X_{26}) while Urea (X_1),Meat extract (X_6), CaCl2 (X_9) and $CoCl_2$ (X_{11}) exerted significant inhibitory effect (Fig. 1). In the model, some regression coefficients were found to be unnecessary having p values > 0.05 suggesting their insignificance. Therefore, by omitting the insignificant terms in the model, the final model equation for mannanase activity in terms of coded factors may be written as:

$$\text{Mannanase} = +72.56 - 8.02 \times X_1 + 2.89 \times X_2 + 2.81 \times X_3 - 3.46 \times X_4 - 1.72 \times X_5 - 6.81 \times X_6 + 10.86 \times X_7 + 0.34 \times X_8 - 6.43 \times X_9 - 2.20 \times X_{10} - 8.33 \times X_{11} + 1.61 \times X_{12} + 0.7 \times X_{13} + 5.28 \times X_{14} - 3.35 \times X_{15} - 1.13 \times X_{17} + 1.09 \times X_{18} + 21.04 \times X_{19} - 3.96 \times X_{20} - 1.84 \times X_{21} + 2.52 \times X_{22} + 1.33 \times X_{23} + 4.27 \times X_{24} + 1.93 \times X_{25} + 3.98 \times X_{26} - 0.95 \times X_{27} \quad (4)$$

Where X_1, X_2, X_3,X_4,X_5 ,X_6, X_7, X_8 ,X_9,X_{10}, X_{11},X_{12}, X_{13}, X_{14}, X_{15}, X_{17}, X_{18}, X_{19}, X_{20}, X_{21},X_{22}, X_{23}, X_{24},X_{25}, X_{26}, X_{27} are urea, NH_4SO_4, KH_2PO_4, peptone, yeast extract, meat extract, soyabean meal, tryptone,$CaCl_2$,$MgSO_4$,$CoCl_2$, $ZnSO_4$,wheat bran, $FeSO_4$, moisture content, $MnCl_2$, malt extract, incubation time,Tween 20, SDS, potato peels, $MnSO_4$,NH_4Cl,$NaNO_3$,NaCl respectively.

The model was examined for the goodness of fit by analyzing the common indicators including p-value, coefficient of determination (R^2), standard deviation and predicted sum of square (PRESS). The associated p-values were used to estimate probability whether F values are large enough to indicate statistical significance. The F values corresponding to 22729.99 observed in the present study indicate the significance of the model with p-values < 0.05 (Table 3). The adequate precision which measures the signal to noise ratio (*S/N)* is 431.04. The desirable value for adequate precision should be more than 4, in the favors of the fitness of the model (*Muthukumar, 2003*). The coefficient of variation (CV) values determines the degree of precision with which the experimental treatments are compared. Generally, the lower the value of coefficient of variation, the higher is the authenticity of given experiment. In the present study, lower CV value corresponding to 0.29, signify a greater reliability of the experiments performed. The statistical analysis showed that the form of the model selected to define the relation between the experimental parameters and the responses is correct. Further, the "Adj R-Squared" values of 1.00 was found to be close to "Pre R-Squared values of 0.9987 (Table 3). The t-test for an individual effect provides an assessment of the likelihood of finding the observed effect in an experiment purely by chance and some authors have found that confidence level more than 70% are justifiable for further studies (*Stowe and Mayer, 1966*). Thus, in this case parameters with confidence levels greater than 99% were chosen as significant. Moreover, the quality of fit for the factorial model was defined in terms of R^2, which is coefficient of determination and found to be 1.00 for mannanase model. After first step of optimization study, the twenty seven nutrient factors were lessen to four, chosen on the basis of their maximum positive effect in Plackett-Burman design, proposing that this is a mathematical tool to screen out the important fermentation parameters. The proper optimal values of the individual parameters were still undefined but could be explained by subsequent central composite design (CCD).

Optimization of screened nutrient sources for mannanase production by *Fusarium oxysporum* SS-25 using Response Surface Methodology

The central composite design (CCD) was created as shown in Table 4 to investigate the individual and interactive effect of each significant independent variable. The responses for mannanase production are also listed in Table 4. Each independent variable was studied at five different levels. On the basis of p value, R^2 and standard deviation, the need of the quadratic regression model was come out to be considerable for efficient mannanase production. The analysis of variance (ANOVA) which is a useful statistical parameter that subdivides the total variation in a group of data into its individual parts joined with the specific sources of variation for the testing of given hypotheses on the parameters of the given model (*Huiping et al., 2007*). The associated p-value is used to estimate the F value so that it is large enough to show statistical significance in the given model. If p-value is lower than 0.05, then it shows that the given model is statistically significant (*Segurola et al., 1999*).

The model F-value of 435.96 for mannanase production showing that the given model is significant (Table 5).The p-values lower than 0.005, show model terms are significant. The value of correlation coefficient (R^2) which is 0.9975, indicates 99.7% variability can be explained by the model. The adequate precision which measures the signal to noise ratio (*S/N)* is 61.702. The desirable value for adequate precision should be more than 4, in the favors of the fitness of the model (*Muthukumar, 2003*). The coefficient of variation (CV) values determines the degree of precision with which the experimental treatments are compared. Usually, lower the value of CV, higher is the reliability of experiment. In this experiment, a lower value of CV (%) corresponding to 1.00 indicates a greater reliability of the experiments performed. The statistical analysis showed that the form of the model which was selected to define the relation between the experimental parameters and the responses is correct.

The analysis of variance study showed a linear relationship between the significant effects of soyabean meal, $FeSO_4$, $MnSO_4$ and $NaNO_3$, the interactive effect between soyabean meal and $FeSO_4$, soyabean meal and $MnSO_4$, soyabean meal and $NaNO_3$, $FeSO_4$ and $MnSO_4$, $FeSO_4$ and $NaNO_3$, $MnSO_4$ and $NaNO_3$ and the quadratic relationship with soyabean meal, $FeSO_4$, $MnSO_4$ and $NaNO_3$. The multiple regression analysis was applied to given set of experimental data, the following second order polynomial equation was obtained that explains the mannanase production by omitting insignificant terms and is shown below:

$$\text{Mannanase} = +193.67 - 0.50 \times X_1 - 0.50 \times X_2 + 8.42 \times X_3 + 0.50 \times X_4 - 1.87 \times X_1 \times X_2 - 2.87 \times X_1 \times X_3 + 1.25 \times X_1 \times X_4 - 2.50 \times X_2 \times X_3 + 8.63 \times X_2 \times X_4 - 1.37 \times X_3 \times X_4 - 16.06 \times X_1 \times X_1 - 11.94 \times X_2 \times X_2 - 5.81 \times X_3 \times X_3 - 11.81 \times X_4 \times X_4 \quad \text{-------- (5)}$$

Where X_1, X_2, X_3, X_4 are soyabean meal, $FeSO_4$, $MnSO_4$ and $NaNO_3$ respectively.

Interactions among the factors

The student's t–test was used to know the error mean square that is important in investigating the significance of the estimated coefficient values of the multiple regression equation in the given model. The coefficient having the smaller p value and larger value of t- test show the greater significance of that coefficient in the given model (*Liu et al., 2004*). The values of coefficients and values of t-test in the given quadratic model as showed in Table 5 depicted that parameters X_3 and X_4 had positive effects on mannanase activities with $MnSO_4$ influencing the highest individual effect. The interactive effects between X_1X_2, X_1X_3, X_2X_3 and X_3X_4 illustrates the negative impact on mannanase yields while the interactive effects between X_1X_4 and X_2X_4 had positive impact on mannanase yields with highest impact by X_2X_4. The contour graphs revealing the interactive effect between two parameters for the optimization of fermentation conditions for mannanase production Fig. 2(a-f). From the contour graphs, it was easy to understand the interactions between two variables and also to locate the optimum levels of two variables. The contour graph between the concentration of soyabean meal versus $FeSO_4$ concentration illustrates that mannanase yield increased with the increase of both soyabean meal and $FeSO_4$ but at high concentration enzyme productivities decreased. The highest yield of 193.63IU/g was obtained where the respective concentrations of supplemented soyabean meal and $FeSO_4$ were 5.74 % w/w and 0.0012% w/w in the brewer spent grain based optimized medium (Fig. 2a) while other parameters such as $MnSO_4$ and $NaNO_3$ were held at 0, 0 coded level. Fig. 2b shows the effect of soyabean meal and $MnSO_4$ on mannanase production. The enzyme yield increased with the increase in the concentration of soyabean meal and $MnSO_4$. The maximum mannanase productivity of 196.71 IU/g obtained at respective concentration of 5.78% w/w of soyabean meal and 0.019% of $MnSO_4$ with other parameters such as $FeSO_4$ and $NaNO_3$ were held at 0, 0 coded levels. Fig. 2c illustrates the effect of soyabean meal and $NaNO_3$ on mannanase yield. Increase in the concentration of both the factors improve the mannanase yield, but at high concentration of both (higher than 5.9% and 0.162%), mannanase yield decreased. The highest mannanase yield of 193.63 IU/g was obtained at 5.9% w/w and 0.162% w/w concentrations of soyabean meal and $NaNO_3$ respectively with other parameters such as $MnSO_4$ and $FeSO_4$, were held at

respective 0, 0 coded levels. Fig 2d depicts the interactive effect of $FeSO_4$ and $MnSO_4$ on mannanase yield. Increase in the concentration of both $FeSO_4$ and $MnSO_4$ increased the mannanase yield, but at high concentration of $FeSO_4$ (greater than 0.0012%), mannanase yield decreased. The maximum mannanase yield of 196.71IU/g was obtained at 0.0012% w/w and 0.019% w/w respective level of $FeSO_4$ and $MnSO_4$ while soyabean meal and $NaNO_3$ were held at 0, 0 coded levels respectively. The contour graph between the $FeSO_4$ concentration and $NaNO_3$ concentration depicted that mannanase yield increased with the increase of both $FeSO_4$ and $NaNO_3$ but at high concentrations of both $FeSO_4$ and $NaNO_3$ (more than 0.0012% and 0.16%), mannanase yield decreased. The highest yield of 193.67 IU/g was obtained in the brewer's spent grain based solid medium when the concentration of $FeSO_4$ and $NaNO_3$ was 0.0012% w/w and 0.16% w/w respectively with soyabean meal and $MnSO_4$ were occur at 0,0 coded levels respectively (Fig. 2e). Fig. 2f illustrates the effect of $MnSO_4$ and $NaNO_3$ on mannanase yield. Increase in the concentration of both variables led to the increase in mannanase yield, but at high $NaNO_3$ concentration (more than 0.158%), enzyme productivity decreased. The highest mannanase yield was obtained when the levels of $MnSO_4$ and $NaNO_3$ were 0.018% w/w and 0.158% w/w respectively resulting 196.71 IU/g while soyabean meal and $FeSO_4$ were held at 0,0 coded levels respectively.

Model validation

In order to evaluate the accuracy of statistical experimental model of response surface methodology (RSM), attempts were made to formulate a medium for maximizing the mannanase yield. Point type optimization for mannanase production attempted with Design Expert using X_2 ($(NH_4)SO_4$,7 mg), X_3(KH_2PO_4,10 mg), X_7 (soyabean meal,300 mg), X_8 (Tryptone,100 mg), X_{12} ($ZnSO_4$,0.01 mg), X_{13} (wheat bran,1 gm), X_{14} ($FeSO_4$, 0.06 mg), X_{15} (water,5 ml), X_{18} (malt extract,100 mg), X_{22} (SDS,0.6 mg), X_{23} (potato peels, 100 mg), X_{24} ($MnSO_4$,0.8 mg), X_{25}(NH_4Cl,1.5 mg), X_{26}($NaNO_3$,8 mg), inoculated with 1 mL of fungal spore suspension having 2.8×10^7 spores, incubated at 30°C in stationary state for 6 days in 5 g brewer's spent grain based medium predicted the yield of 193.12 IU/g. An experiment with the above mentioned conditions was performed to validate the optimum level of each variable and the result was 193 IU/g which is 0.062% less than the predicted value of mannanase production. Therefore, the results obtained with this accuracy confirm the validity of the proposed model with small disparity due to the

some fluctuations in experimental conditions. Statistical evaluation of culture conditions thus enhanced the production of mannanase to an appreciable amount.

Kinetic characterization of crude enzyme preparation

The enzyme preparation was active at broader pH (3-10) and temperature range (30-100^{0}C) with greater than 45% activity remained till pH 8 and 90^{o}C (Fig. 3 a-b).The effect of various metal salts on enzyme activity was studied by incubating enzyme preparation in various salt solutions individually. Figure 3c showed that salts such as $MgSO_4$, $FeSO_4$ and KCl caused promotory effect while $CaCl_2$, $MnSO_4$ and $CuSO_4$ exhibited negative effect on mannanase activity. Further the, temperature stability profile was studied by incubating the enzyme preparation at optimum temperature. The residual activity was measured from 2h continuously upto 216h and results are shown in Fig. 3(d).

Biobleaching of kraft pulp by mannanase produced in-house

At industrial level, mannanases were used as process aids in bleaching process. Large number of attempts has been made towards the replacement of elemental Cl_2 by ClO_2 and O_2 based bleaching chemicals and has resulted in the use of number of modified kraft cooking processes. The potential of mannanase from *Fusarium oxysporum SS-25* for the pre-treatment process in kraft pulp (obtained from wheat straw by kraft process) bleaching have been evaluated. 5 g of oven dried pulp was treated with 90 U/g of mannanase for 45 min. The mannanase treated pulp samples were properly washed several times, dried in an oven and the weight losses were determined. The results are shown in Table 6. Kappa number decreased from 15 to 11 on treatment with mannanase for 45 min indicating the removal of mannan. There was 1% and 4.93% increase in brightness and tearness index in mannanase treated pulp samples respectively.

Hydrolysis of locust bean gum and guar gum by mannanase produced in-house

The rate of hydrolysis of both locust bean gum and guar gum by mannanase was rapid and exponential during the period 0 to 24 h and after that it followed a somewhat stationary pattern except for guar gum (Fig. 4). After 72 h of hydrolysis the total reducing sugars were 6.37 g/100 ml and 5.29 g/100 ml for locust bean gum and guar gum respectively.

CONCLUSION

In this study, brewer's spent grain was used as raw material for the production of mannanase enzyme to make the better use of brewery waste as substrate for enzymes production and reduce the production cost of mannanase. Statistical optimization of media components employing Plackett-Burman and response surface methodology designs led to an improvement in mannanase yield revealing 3.21-fold increase in activity as compared to unoptimized conditions. Temperature and pH activity profiles revealed that the enzyme was quite active over a broader pH and temperature range, exhibiting its wide applicability. The crude enzyme preparation proved to be quite effective in the biobleaching of kraft pulp revealing 1% increase in brightness, 4.93% increase in tearness index with 26.6% decrease in kappa number in pulp samples. This will reduce the ClO_2 demand for prebleaching of kraft pulp. The hydrolysis of mannanase substrates like locust bean gum and guar gum showed the industrial application of mannanase to reduce the viscosity of these substrates so that partially hydrolysed gum can be used in foodstuffs as a water soluble dietry fiber.

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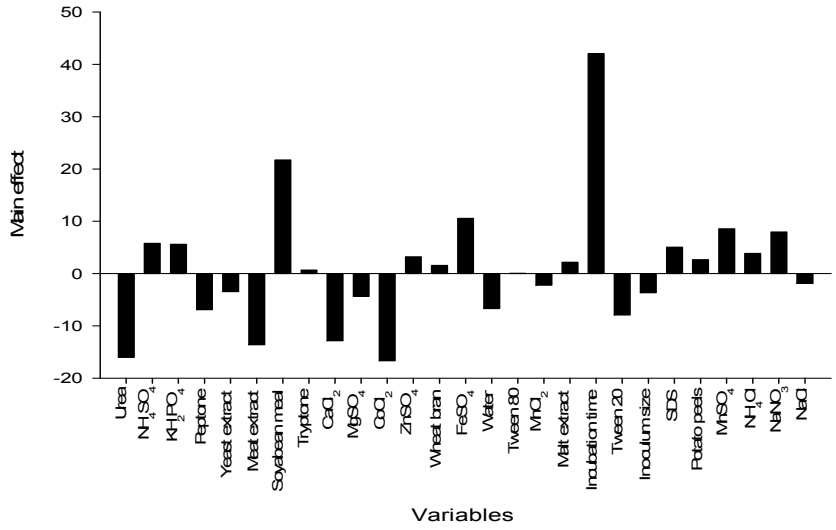

Figure 1 Effect of various parameters on mannanase production

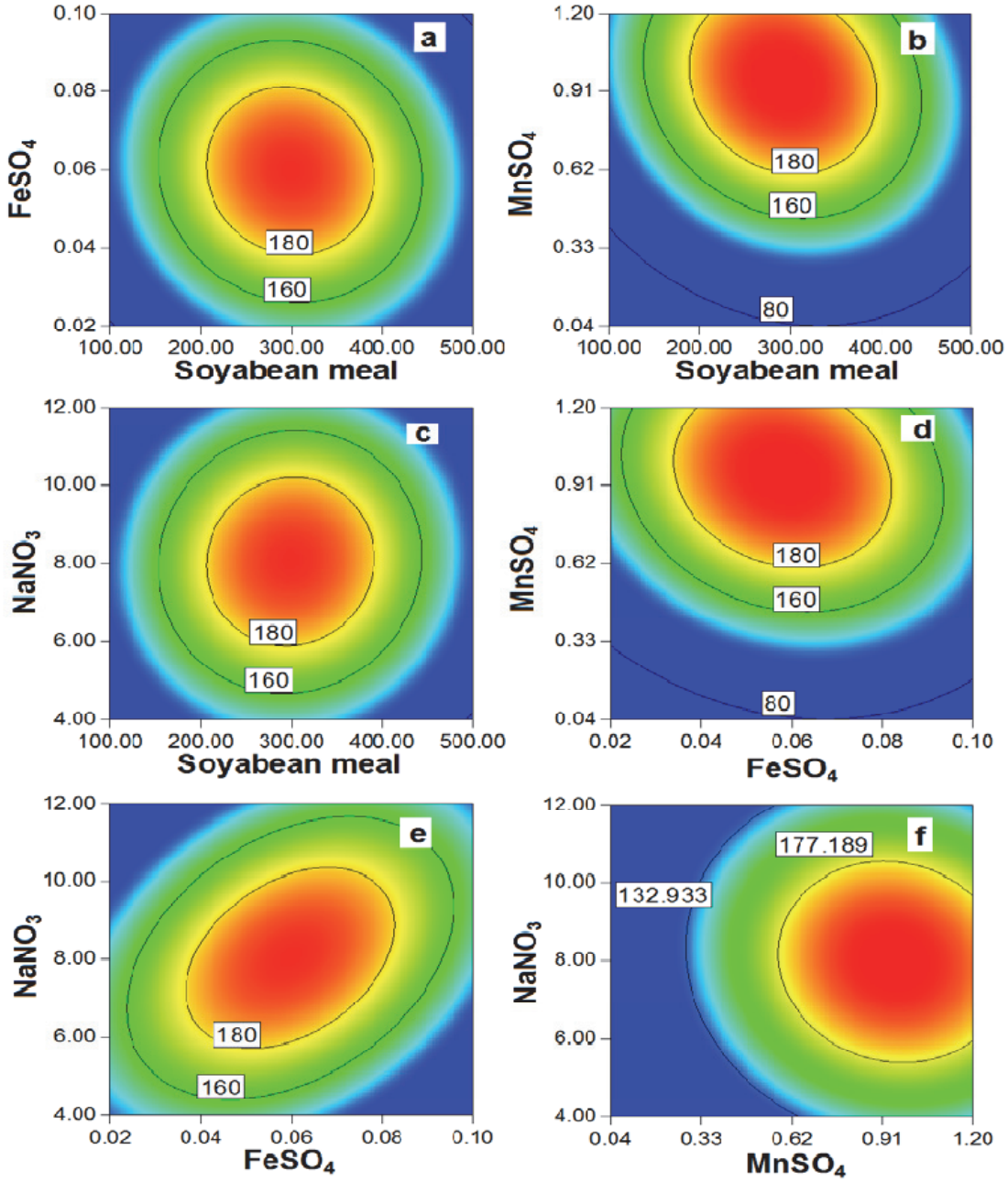

Figure 2 Contour plots representing mannanase yields from solid state culture of *Fusarium oxysporum SS-25 as* affected by cultural conditions (a) soyabean meal and $FeSO_4$ (b) soyabean meal and $MnSO_4$ (c) soyabean meal and $NaNO_3$ (d) $FeSO_4$ and $MnSO_4$ (e) $FeSO_4$ and $NaNO_3$ (f) $MnSO_4$ and $NaNO_3$. All values are expressed in terms of mg.

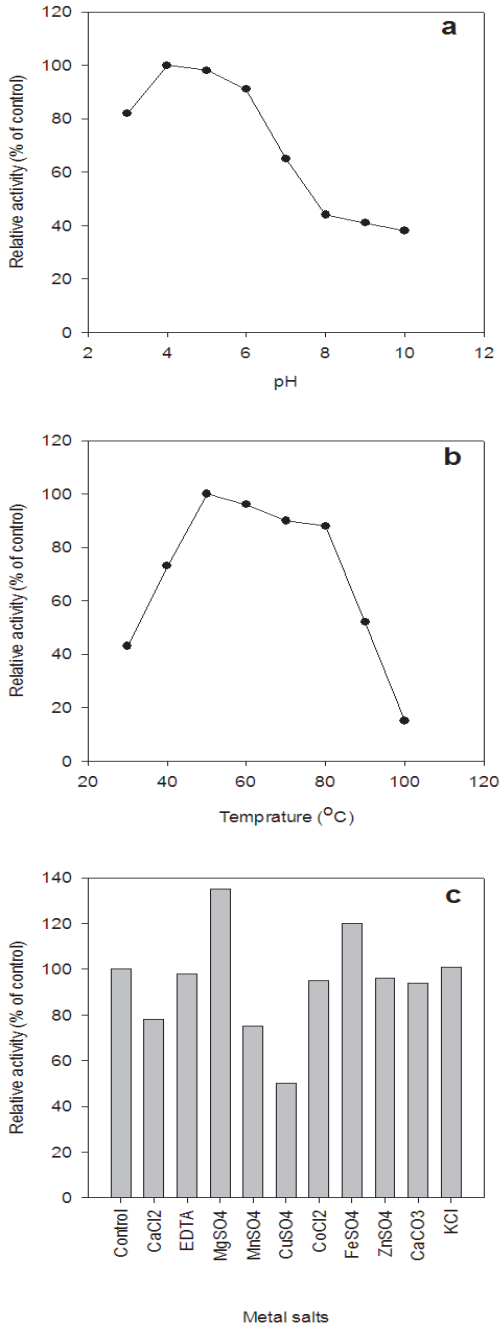

Figure 3 pH versus activity profile (a), temperature versus activity profile (b), and effect of various metal salts and EDTA (c), of crude mannanase preparation from solid state culture of *Fusarium oxysporum* SS-25 at temp 50°C.

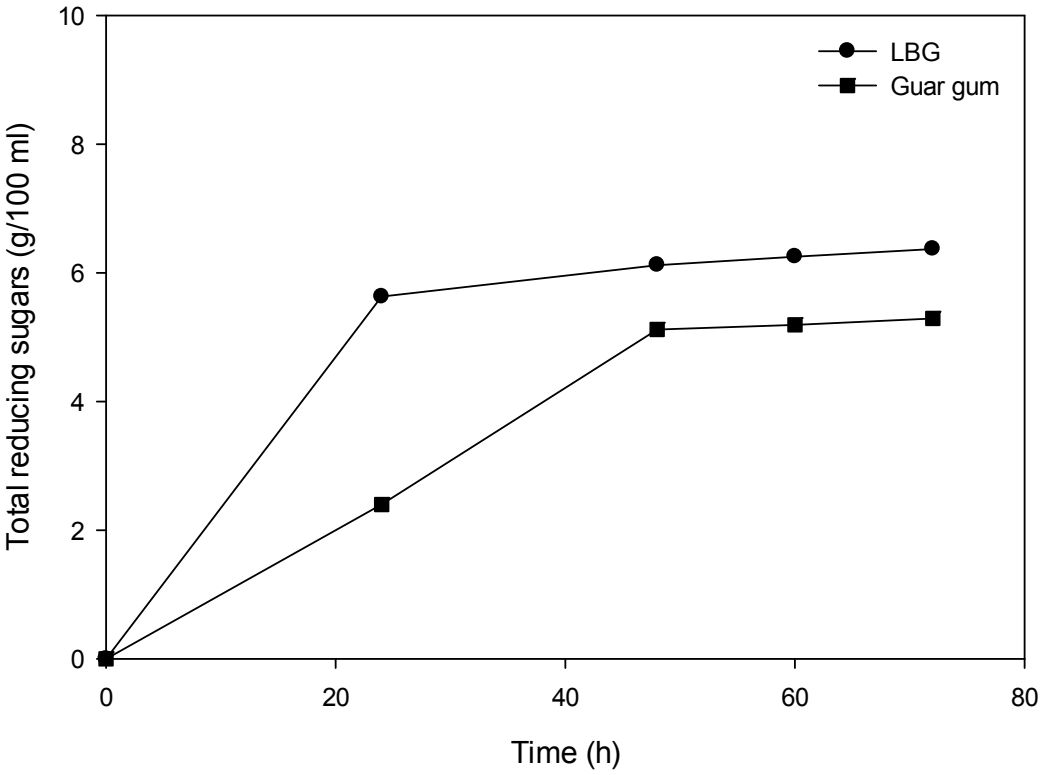

Figure 4 Hydrolysis of locust bean gum and guar gum with crude mannanase preparation from solid state culture of *Fusarium oxysporum SS-25* at 50°C, pH 4.5

Table 1 Randomized Plackett-Burman experimental design for evaluating factors influencing mannanase production.

Run	Urea (X_1)	NH_4SO_4 (X_2)	KH_2PO_4 (X_3)	Peptone (X_4)	Yeast Extract (X_5)	Meat Extract (X_6)	Soyabean meal (X_7)	Tryptone (X_8)	$CaCl_2$ (X_9)	$MgSO_4$ (X_{10})	$CoCl_2$ (X_{11})	$ZnSO_4$ (X_{12})	Wheat Bran (X_{13})	$FeSO_4$ (X_{14})	Water (X_{15})	Tween 80 (X_{16})	$MnCl_2$ (X_{17})	Malt Extract (X_{18})	Incubation Time (X_{19})	Tween 20 (X_{20})	Inoculum size (X_{21})	SDS (X_{22})	Potato Peels (X_{23})	$MnSO_4$ (X_{24})	NH_4Cl (X_{25})	$NaNO_3$ (X_{26})	NaCl (X_{27})	Response (IU/g)
1	-1	+1	-1	+1	-1	-1	-1	+1	-1	-1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	62
2	+1	-1	-1	-1	-1	+1	+1	-1	-1	+1	+1	-1	-1	+1	+1	+1	+1	-1	-1	-1	-1	+1	+1	-1	+1	+1	+1	50.30
3	+1	-1	+1	+1	-1	+1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	-1	-1	+1	-1	-1	+1	-1	+1	-1	48.68
4	+1	+1	+1	-1	-1	-1	+1	+1	-1	+1	-1	-1	+1	-1	-1	-1	-1	+1	+1	+1	-1	+1	+1	-1	-1	+1	+1	125
5	-1	+1	+1	-1	+1	+1	+1	-1	+1	+1	+1	+1	-1	-1	-1	-1	+1	+1	-1	+1	-1	-1	+1	-1	+1	-1	-1	43.88
6	+1	-1	+1	-1	+1	+1	-1	+1	+1	-1	+1	+1	+1	+1	+1	-1	-1	-1	+1	-1	-1	-1	+1	-1	-1	+1	-1	65
7	-1	-1	+1	-1	-1	+1	-1	+1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	76.61
8	-1	+1	-1	-1	-1	+1	-1	-1	+1	+1	+1	-1	+1	-1	+1	+1	-1	+1	+1	-1	+1	+1	+1	+1	-1	-1	-1	70
9	+1	+1	-1	-1	+1	+1	+1	+1	-1	-1	-1	-1	+1	+1	-1	+1	+1	+1	+1	-1	-1	-1	-1	+1	+1	-1	-1	121.69
10	-1	-1	+1	-1	+1	-1	-1	-1	+1	+1	-1	+1	+1	+1	-1	+1	-1	+1	-1	-1	-1	+1	-1	+1	+1	+1	+1	86
11	-1	+1	+1	+1	+1	-1	+1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	-1	-1	+1	+1	-1	-1	+1	-1	-1	73
12	+1	+1	-1	+1	+1	-1	-1	+1	+1	+1	+1	+1	-1	-1	-1	+1	+1	-1	+1	-1	-1	+1	-1	-1	-1	-1	+1	52.39
13	-1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	-1	-1	-1	+1	-1	47.60
14	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	60
15	+1	-1	+1	+1	+1	-1	+1	-1	+1	-1	-1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	-1	+1	-1	-1	-1	+1	40
16	+1	-1	-1	+1	-1	-1	-1	-1	+1	+1	+1	-1	+1	+1	-1	-1	+1	+1	+1	+1	+1	-1	-1	-1	+1	+1	-1	57.46
17	+1	-1	+1	+1	+1	+1	-1	-1	-1	-1	+1	-1	-1	-1	+1	-1	-1	+1	+1	+1	-1	+1	-1	+1	+1	-1	+1	54.13
18	+1	+1	+1	-1	-1	-1	-1	+1	+1	-1	+1	-1	-1	+1	-1	+1	-1	-1	-1	+1	+1	-1	+1	+1	+1	-1	+1	38.70
19	-1	-1	-1	+1	+1	-1	+1	+1	+1	+1	-1	-1	-1	-1	+1	+1	-1	-1	+1	+1	-1	-1	+1	+1	+1	+1	-1	105.35
20	+1	+1	+1	-1	-1	-1	+1	-1	+1	-1	-1	+1	-1	-1	+1	-1	+1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	122.24
21	-1	-1	+1	+1	-1	-1	+1	-1	-1	-1	+1	+1	+1	+1	-1	+1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	127.32
22	+1	+1	-1	+1	+1	+1	-1	-1	-1	-1	-1	+1	+1	-1	-1	+1	-1	-1	-1	+1	+1	+1	+1	-1	+1	+1	-1	38.53
23	+1	-1	-1	-1	+1	-1	-1	+1	-1	+1	-1	+1	-1	+1	+1	-1	+1	+1	-1	+1	+1	+1	+1	+1	+1	-1	-1	48.44
24	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	-1	-1	-1	+1	-1	-1	+1	+1	+1	-1	+1	-1	+1	+1	43.14
25	+1	+1	-1	+1	-1	+1	+1	-1	+1	+1	-1	+1	+1	+1	+1	-1	-1	-1	-1	+1	-1	-1	-1	+1	-1	-1	+1	41
26	-1	+1	-1	-1	+1	-1	+1	-1	-1	-1	+1	+1	-1	+1	+1	+1	-1	+1	+1	+1	+1	-1	-1	-1	-1	+1	+1	113.23
27	-1	+1	+1	+1	+1	+1	-1	-1	-1	+1	-1	-1	-1	+1	-1	-1	+1	-1	+1	-1	+1	-1	+1	+1	-1	+1	+1	107
28	-1	-1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	-1	+1	-1	-1	-1	+1	+1	-1	+1	+1	+1	-1	+1	-1	+1	113

Table 2 Levels of independent variables used for media optimization in Plackett-Burman design.

Variables	Levels	
	Low (-1)	High (+1)
X_1:Urea	0	1.5 mg
X_2:NH_4SO_4	0	7.0 mg
X_3:KH_2PO_4	0	10 mg
X_4:Peptone	0	100 mg
X_5:Yeast extract	0	100 mg
X_6:Meat extract	0	100 mg
X_7:Soyabean meal	0	100 mg
X_8:Tryptone	0	100 mg
X_9:$CaCl_2$	0	1.5 mg
X_{10}:$MgSO_4$	0	1.5 mg
X_{11}:$CoCl_2$	0	0.01 mg
X_{12}:$ZnSO_4$	0	0.01 mg
X_{13}:Wheat bran	0	1.0 g
X_{14}:$FeSO_4$	0	0.025 mg
X_{15}:Water	5	12 mL
X_{16}:Tween 80	0	5µL
X_{17}:$MnCl_2$	0	0.5 mg
X_{18}:Malt extract	0	100 mg
X_{19}:Incubation time	3 days	6 days
X_{20}:Tween 20	0	5µL
X_{21}:Inoculum size	1 ml	2.5 mL
X_{22}:SDS	0	0.6 mg
X_{23}:Potato peels	0	100 mg
X_{24}:$MnSO_4$	0	0.5 mg
X_{25}:NH_4Cl	0	1.5 mg
X_{26}:$NaNO_3$	0	5.0 mg
X_{27}:NaCl	0	1.5 mg

Table 3 Statistical analysis of Plackett-Burmann design showing sum of squares,coefficient values, t-test, F-value, p-value,confidence level for each variable affecting Mannanase activity after backward elimination regression analysis.

Variables	Sum of squares	Coefficients	t-test	F-value	p-value	Confidence level (%)
Model	26005.22	72.56	1814	22729.99	0.0052	99.48
X_1:Urea	1801.13	-8.02	-200.5	40931.49	0.0031	99.69
X_2:NH_4SO_4	233.34	2.89	72.1	5302.73	0.0087	99.13
X_3:KH_2PO_4	220.81	2.81	70.2	5018.00	0.0090	99.10
X_4:Peptone	334.44	-3.46	-86.4	7600.38	0.0073	99.27
X_5:Yeast extract	82.73	-1.72	-42.9	1880.12	0.0147	98.53
X_6:Meat extract	1297.03	-6.81	-170.1	29475.63	0.0037	99.63
X_7:Soyabean meal	3299.92	10.86	271.4	74992.10	0.0023	99.77
X_8:Tryptone	3.23	0.34	8.4	73.40	0.0740	92.60
X_9:$CaCl_2$	1159.33	-6.43	-160.8	26346.26	0.0039	99.61
X_{10}:$MgSO_4$	134.95	-2.20	-54.8	3066.76	0.0115	98.85
X_{11}:$CoCl_2$	1942.72	-8.33	-208.2	44149.20	0.0030	99.70
X_{12}:$ZnSO_4$	72.16	1.61	40.1	1639.89	0.0157	98.43
X_{13}: Wheat bran	16.99	0.78	19.4	386.07	0.0324	96.76
X_{14}: $FeSO_4$	780.07	5.28	131.9	17727.36	0.0048	99.52
X_{15}: Water	314.83	-3.35	-83.8	7154.72	0.0075	99.25
X_{17}:$MnCl_2$	35.55	-1.13	-28.1	807.89	0.0224	97.76
X_{18}:Malt extract	33.29	1.09	27.2	756.50	0.0231	97.69
X_{19}:Incubation time	12396.3	21.04	526	281712	0.0012	99.88
X_{20}:Tween 20	439.32	-3.96	-99.02	9983.79	0.0064	99.36
X_{21}: Inoculum size	95.20	-1.84	-46.09	2163.51	0.0137	98.63
X_{22}: SDS	177.46	2.52	62.93	4032.82	0.0100	99.00
X_{23}: Potato peels	49.82	1.33	33.34	1132.23	0.0189	98.11
X_{24}:$MnSO_4$	511.63	4.27	106.86	11627.06	0.0059	99.41
X_{25}: NH_4Cl	104.49	1.93	48.29	2374.59	0.0131	98.69
X_{26} :$NaNO_3$	442.97	3.98	99.43	10066.78	0.0063	99.37
X_{27} : NaCl	25.44	-0.95	-23.83	578.16	0.0265	97.35

Table 4 Central composite design matrix with experimental values of mannanase production by *Fusarium oxysporum SS-25.*

Runs	Soyabean meal (mg)*	$FeSO_4$ (mg)*	$MnSO_4$ (mg)*	$NaNO_3$ (mg)*	Mannanase activity (IU/g)
1	200.00	0.08	0.60	6.00	132
2	300.00	0.06	0.80	8.00	192
3	300.00	0.02	0.80	8.00	147
4	200.00	0.08	0.60	10.00	151
5	300.00	0.06	0.80	4.00	145
6	200.00	0.08	1.00	6.00	154
7	400.00	0.04	0.60	10.00	137
8	300.00	0.06	0.80	8.00	194
9	300.00	0.06	0.80	8.00	194
10	300.00	0.06	1.20	8.00	187
11	400.00	0.08	1.00	10.00	160
12	100.00	0.06	0.80	8.00	130
13	200.00	0.04	1.00	10.00	151
14	400.00	0.04	1.00	10.00	148
15	300.00	0.06	0.80	8.00	193
16	400.00	0.08	1.00	6.00	137
17	300.00	0.10	0.80	8.00	144
18	300.00	0.06	0.80	12.00	147
19	200.00	0.04	0.60	10.00	126
20	400.00	0.08	0.60	10.00	153
21	200.00	0.04	1.00	6.00	171
22	300.00	0.06	0.80	8.00	195
23	300.00	0.06	0.40	8.00	153
24	200.00	0.04	0.60	6.00	141
25	400.00	0.08	0.60	6.00	132
26	500.00	0.06	0.80	8.00	128
27	400.00	0.04	0.60	6.00	147
28	200.00	0.08	1.00	10.00	164
29	400.00	0.04	1.00	6.00	168
30	300.00	0.06	0.80	8.00	194

*Values of the variables are per 5 g of brewer's spent grain

Table 5 ANOVA results for mannanase production under response surface quadratic model and model coefficients estimated by multiple linear regressions.

Variables	Sum of squares	Coefficients	t-test	F-value	p-value	Confidence level (%)
Model	14953.42	193.67	302.60	435.96	< 0.0001	99.99
X_1:Soyabean meal	6.00	-0.50	-1.56	2.45	0.1385	86.15
X_2: $FeSO_4$	6.00	-0.50	-1.56	2.45	0.1385	86.15
X_3: $MnSO_4$	1700.17	8.42	26.30	693.95	< 0.0001	99.99
X_4: $NaNO_3$	6.00	0.50	1.56	2.45	0.1385	86.15
$X_1 \times X_2$	56.25	-1.87	-5.85	22.96	0.0002	99.98
$X_1 \times X_3$	132.25	-2.87	-8.98	53.98	< 0.0001	99.99
$X_1 \times X_4$	25.00	1.25	3.90	10.20	0.0060	99.40
$X_2 \times X_3$	100.00	-2.50	-7.81	40.82	< 0.0001	99.99
$X_2 \times X_4$	1190.25	8.63	26.95	485.82	< 0.0001	99.99
$X_3 \times X_4$	30.25	-1.37	-4.29	12.35	0.0031	99.69
$X_1 \times X_1$	7076.68	-16.06	-50.19	2888.44	< 0.0001	99.99
$X_2 \times X_2$	3908.68	-11.94	-37.30	1595.38	< 0.0001	99.99
$X_3 \times X_3$	926.68	-5.81	-18.16	378.24	< 0.0001	99.99
$X_4 \times X_4$	3827.25	-11.81	-36.91	1562.14	< 0.0001	99.99

Std.Dev. =1.57, R^2 =0 .9975, Mean =157.17, Adj R^2 = 0.9953, C.V. % = 1.0, Pred. R^2 = 0.9874, PRESS = 188.64, Adeq Precision =61.702