Statistical Modeling for Enhanced Xylanase Production by *Fusarium oxysporum* SS-25 *via* Solid State Fermentation of Brewer's Spent Grain

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Abstract: The objective of the present study was to optimize the nutritional factors in the low cost brewer's spent grain based culture medium for the hyperproduction of xylanase from *Fusarium oxysporum* SS-25 in solid state fermentation employing statistical Plackett-Burman and Central composite designs.The important nutritional factors identified by Plackett-Burman design were: 20% (w/w) wheat bran, 2% each of potato peels, peptone, soyabean meal, malt extract, 0.14% NH_4SO_4, 0.0006% $FeSO_4$, 0.01% $MnCl_2$, 0.012% SDS, 0.03% urea, 0.03% NaCl in brewer's spent grain based medium,70% moisture content, inoculum size of 2.8×10^7 spores and incubation at 30°C for 3 days. On the basis of maximum positive effect on xylanase production in Plackett-Burman design, four variables of NH_4SO_4, peptone, $FeSO_4$ and $MnCl_2$ were chosen and their interactive effects were studied in Central composite design. The xylanase activity reached 5874 IU/gds revealing 4.28-fold increase in xylanase activity in comparison to the basal medium containing only brewer's spent grain.

Keywords: Brewer's spent grain, Plackett-Burman design (PBD), Response Surface Methodology (RSM), *Fusarium oxysporum* SS-25.

INTRODUCTION

Lignocellulosic residues appear to be promising substrates for meeting the energy requirements of the society due to high proportion of renewable cellulose fraction. However, this is not easily hydrolysable due to its highly crystalline nature and its integration with hemicelluloses and lignin [1]. To hydrolyse cellulosic fraction into glucose and subsequently into cellulosic alcohol, the disintegration of lignin and hemicelluloses with some suitable thermochemical or biological pretreatment is required [2]. Hemicellulosic fraction is heterogeneous with D-Xylans as the most abundant polysaccharides. The basic structure of xylans is a main chain of (1→4)-linked β-D-xylopyranosyl residues. Typically, these linear chains carry short side chains to a varying extent, whereas pure unsubstituted xylans are extremely rare. Due to the structural heterogeneity of the xylans, xylan-degrading enzyme systems include several hydrolytic enzymes including endoxylanase (1,4-β-D- xylan xylanohydrolase, E.C. 3.2.1.8) and β-xylosidase (1,4-β-xylan xylohydrolase, E.C. 3.2.1.37) [3]. Endoxylanase (EC 3.2.1.8), also commonly known as xylanase, primarily cleaves β-1, 4-linked xylan back bone and β-xylosidase (EC 3.2.1.37) hydrolyses xylo-oligomers. From a commercial viewpoint, xylanases are an important group of carbohydrases and have a worldwide market of around $200 million each year [4]. Xylanases have been widely applied in food, animal feed, bioenergy, textile, paper and pulp industries [5]. Apart from the use of xylanases in the production of cellulosic alcohol, another promising application of xylanases is in the prebleaching of kraft pulp for the production of a good quality paper. The pulp and paper industry is modifying its pulping, bleaching and effluent treatment technologies to reduce the environmental impact of mill effluents. If kraft pulps are prebleached with xylanases, then lower chlorine charges are required to bleach the kraft pulps, which reduce chloro-organic discharges [6]. Tremblay and Archibald [7] reported the delignification of unbleached softwood and hardwood kraft pulps thus reducing the Cl_2 required to achieve a given degree of bleaching. Oksanen *et al*. [8] has reported the changes in fiber properties by treating recycled pulps with purified *T. reesei* cellulases and hemicellulases. Qy *et al*. [9] reported the enzymatic treatment of birch kraft pulp, which resulted in a brightness of 6.8% more than untreated one using the same chlorine dosage.

The high cost and low yields of xylanase have been the main problems for its industrial production [10]. Therefore, there is urgent need to develop a new fermentation medium with inexpensive substrates that provides a high xylanase yield. Among existing technologies in the fermentation industry, solid-state fermentation (SSF) has many advantages over fermentation with submerged culture, such as lower cost and much higher reactor volume [11]. There is a great deal of literature available regarding the use of SSF process for producing enzymes with industrial

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importance, such as protease, cellulase, polygalacturonase, xylanase, pectinase, amylase, and glucoamylase [1, 2]. It is well known that 30–40% of the production cost of industrial enzymes is taken up by the cost of growth medium [12]. Carbon and nitrogen sources together with fermentation time have been reported to play significant roles in the determination of the final morphology of the culture [13]. Therefore, it is significant to optimize these conditions for low-cost enzyme production using powerful statistical techniques.

Conventional single dimensional search involves changing one independent variable at a time while fixing the others at a constant level, which gives unreliable results, inaccurate conclusion, and even less frequent interactions of two or more factors. Statistical experimental designs including Plackett-Burman and response surface methodologies (RSM) can collectively eliminate these limitations of a single factor optimization process. Plackett-Burman design [14] is a powerful statistical technique for screening medium components in solid-state fermentation (SSF) and has been widely used in fermentation optimization [15, 16]. This technique can not only determine the exact quantity but also can provide indication and tendency regarding the necessity of each factor in relatively few experiments. The following response surface methodology (RSM) can provide mathematical models showing the dependence of the enzyme activity on independent variables (the concentration of the separate components of the nutrient medium or operating parameters), and even give predictive results of responses and the possible levels of related independent variables. Response surface methodology has been used as a successful statistical tool for optimization of medium components in a fermentation process for enzyme production [17, 18]. Therefore, the aim of this study was to statistically optimize the nutritional parameters for the hyperproduction of xylanase by *Fusarium oxysporum* SS-25 *via* SSF of low cost brewer's spent grain.

MATERIALS AND METHODS

Microorganism

The xylanolytic fungal strain used in the present 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 identification 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 Xylanase Production

The xylanase production 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 2.5 mL of fungal spore suspension (2.8×10^{7} spore/mL) and incubated at 30^{o}C in stationary state for 96 h. The enzyme was extracted by adding 100 mL of deionised water to each flask and churning the contents in a laboratory blender. The contents of the flask were then filtered through a metallic sieve and the solid residue was pressed to release remaining liquid. The suspension from each flask was analysed for xylanase activity. The yield has been expressed as IU/g dry solids.

Enzyme Assay

Xylanase activity was assayed using 1% birchwood xylan (Sigma, USA) in 0.5 M acetate buffer (pH 4.0), according to the method of Bailey *et al.* [19]. The release of reducing sugars was determined using the 3, 5- dinitrosalicylic acid method [20]. One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 µ mol of reducing sugars, measured in terms of xylose per min.

Statistical Optimization of Xylanase Production by Plackett-Burman Design

Xylanase 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:

$$Y = \beta o + \sum \beta i Xi \quad (1)$$

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 (Inc, Minneapolis, MN) 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 Xylanase Production by *Fusarium oxysporum* SS-25 Using Response Surface Methodology

Based upon the results of Plackett-Burman, four independent variables including NH_4SO_4 (X_2), peptone (X_4), $FeSO_4$ (X_{14}) and $MnCl_2$ (X_{17}) were chosen to investigate the first- and higher-order main effects of each factor and interactions amongst them for further optimization through RSM keeping the other factors having significant positive effect on enzyme production as constant. A 2^4 factorial central composite experimental design resulting in 30 experimental runs was generated by Design Expert. The relation between coded and actual values is described according to equation:

$$xi = (Xi - X^0i) / \Delta Xi \qquad i = 1,2,3\ldots j \qquad (2)$$

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:

$$Y = bo + \sum bi\ xi + \sum\sum bij\ xi\ xj + \sum bii\ x^2i + e. \qquad (3)$$

Where Y = measured response; bo, bi, bij, bii are constant and regression coefficients of model; xi and xj are levels (codes values) of independent variables; e is random error. The Design Expert was used for regression analysis of the data obtained and to estimate the coefficients of the regression equation. Contour graphs were also obtained by using Design Expert software to illustrate the relationship between the variables. Accuracy and general ability of polynomial model was evaluated by coefficient of determination (R^2). The statistical significance of model coefficient was evaluated by ANOVA.

RESULTS AND DISCUSSION

Fungi are the most potent producers of various industrially important enzymes used for the degradation of various agrowaste residues [21] including pectinases [22], invertase [23], feruloyl esterases [24], cellobiase [25], cellulases and hemicellulases [26], xylanase [27]. Large amounts of agro-industrial residues are generated every year from diverse economic activities. These residues represent one of the most energy-rich resources available on the planet and when not properly discharged or used, add to environmental pollution [28]. On the other hand, the cost of an enzyme is one of the main factors determining the economics of process. Reducing the costs of enzyme production by optimizing fermentation and cultivation conditions is the goal of basic research for industrial application. Most of the reports concerning xylanases are dealt with the purification and characterization of these enzymes, with very few studies regarding optimizing their production [29]. Solid-state fermentation (SSF) is receiving a renewed surge of interest, primarily because of increased productivity and prospects of using a wide range of agro- industrial residues for xylanase production. In a SSF process, the solid substrate not only supplies nutrients to the microbial culture growing in it but also serves as an anchorage for the cells.

A number of agro-waste residues including wheat bran [1], palm kernel cake [30], empty palm fruit bunch fiber [31], sugar cane baggase [32], sugarcane beet pulp [33], apple pomace [34], pea peels [35] have already been tried for the cultivation of microorganisms to produce industrial enzymes. Brewer's spent grain (BSG) is one such residue which has gained attention for the production of enzymes under SSF [28, 36] 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, 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 [37]. BSG is available in large quantities throughout the year, but its main application has been limited to animal feeding. Considering the substantial availability of brewer's spent grains at very low prices, it was used as a substrate in the present study for a low cost production of xylanase by *Fusarium oxysporum* SS-25 under solid state fermentation (SSF). The organism colonized well

Table 1: Randomized Plackett-Burman Experimental Design for Evaluating Factors Influencing Xylanase 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 (U/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	2665.5
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	2930.3
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	1774.8
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	1270
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	1598.4
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	950
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	728.3
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	1740
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	1264.2
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	1650.7
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	2659.6
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	1259.5
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	2851.4
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	1370
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	3343.2
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	1163.6
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	1249.5
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	1765.4
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	513.5
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	1390
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	1638.9
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	1631.3
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	2454.3
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	1670.7
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	3085.5
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	1212.4
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	1474.2
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	1330.1

on this substrate and produced high xylanase yield corresponding to1370 IU/gds. BSG has also been evaluated as a substrate for xylanase production in SSF [38].

Screening of Parameters Affecting Xylanase 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 xylanase 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 Figure **1**. Moisture content in the medium was found to have the maximum positive effect on xylanase production followed by the presence of peptone (X_4), $MnCl_2$ (X_{17}), $FeSO_4$ (X_{14}) and NH_4SO_4 (X_2) while incubation time (X_{19}), NH_4Cl (X_{25}), Tween 20 (X_{20}) and yeast extract (X_5) exerted significant inhibitory effect (Figure **1**). In the model, some regression coefficients were found to be unnecessary having p values > 0.05 suggesting their insignificance. Thus, by neglecting the insignificant terms, the final model equation for xylanase activity in terms of coded factors may be written as:

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 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	0.03 mg
X_{15}:Water	5	12 mL
X_{16}:Tween 80	0	5 µL
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	5 µL
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

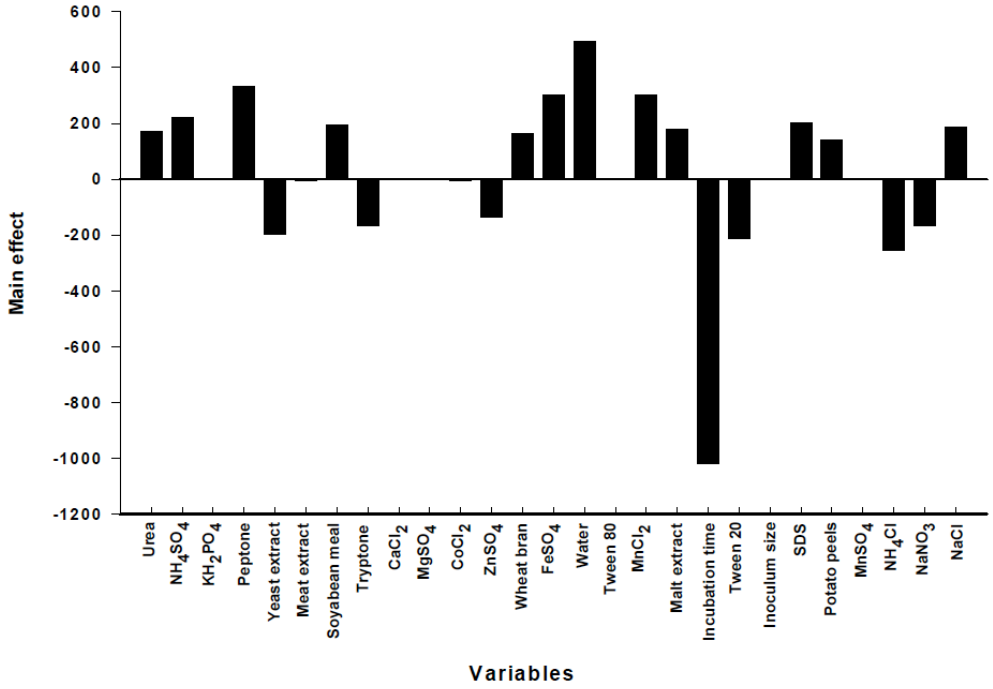

Figure 1: Effect of various parameters on Xylanase production.

$$\text{Xylanase} = +1737.01+86.71\times X_1+110.70\times X_2+165.93\times X_4-99.01\times X_5-2.79\times X_6+97.43\times X_7-82.8\times X_8-2.79\times X_{11}-67.71\times X_{12}+81.71\times X_{13}+150.93\times X_{14}+246.84\times X_{15}+151.05\times X_{17}+89.32\times X_{18}-509.53\times X_{19}-106.02\times X_{20}+100.62\times X_{22}+70.53\times X_{23}-126.96\times X_{25}-83.51\times X_{26}+94.12\times X_{27} \quad (4)$$

Where X_1, X_2, X_4, X_5,X_6, X_7, X_8, X_{11},X_{12}, X_{13}, X_{14}, X_{15}, X_{17}, X_{18}, X_{19}, X_{20}, X_{22}, X_{23}, X_{25}, X_{26}, X_{27} are urea, NH_4SO_4, peptone, yeast extract, meat extract, soyabean meal, tryptone, $CoCl_2$, $ZnSO_4$, wheat bran, $FeSO_4$, moisture content, $MnCl_2$, malt extract, incubation time, Tween 20, SDS, potato peels, 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 are used to estimate probability whether F values are large enough to indicate statistical significance. The F values corresponding to 14774.54 observed in the present study indicate the significance of the model with p- values < 0.05 (Table **3**). The value *S/N*, which calculates signal to noise ratio and is a measure of the adequate precision is 466.28. A value greater than 4 is desirable in support of the fitness of the model [39]. The coefficient of variation (CV) indicates the degree of precision with which the treatments are compared. Usually, the higher the value of CV, the lower is the reliability of experiment performed i.e. there are chances ascertaining the inaccuracy of experiments performed and results obtained. In the present study, lower CV value corresponding to 0.39 indicates a greater reliability of the experiments performed. The analysis showed that the form of the model chosen to explain the relationship between the factors and the responses is correct. Further, the "Adj R-Squared" values of 0.9999 was found to be close to "Pre R-Squared values of 0.9996 (Table **3**). A t-test of an individual effect allows an evaluation of the probability of finding the observed effect purely by chance and some investigator have found that confidence level greater than 70% are acceptable [40]. Thus in this case variables with confidence levels exceeding 99% were considered as significant. Moreover, the quality of fit for the factorial model equation was expressed by the coefficient of determination R^2, which was 1.00 for xylanase model.

After initial optimization, the nutrient sources were reduced to four major variables, chosen on the basis of their maximum positive effect during the Plackett-Burman experimental design, suggesting that Plackett-Burman design is a powerful tool for screening important fermentation factors. The exact optimal values of the individual factors were still unknown but could be determined by the subsequent Central composite design (CCD).

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 Xylanase Activity after Backward Elimination Regression Analysis

Variables	Sum of squares	Coefficients	t-test	F-value	p-value	(%)*
Model	14480625	1737.01	1346.51	14774.54	< 0.0001	99.99
X_1:Urea	210526	86.71	67.21	4510.80	< 0.0001	99.99
X_2:NH_4SO_4	343127	110.70	85.81	7351.94	< 0.0001	99.99
X_4:Peptone	770960	165.93	128.62	16518.79	< 0.0001	99.99
X_5:Yeast extract	274481	-99.01	-76.75	5881.11	< 0.0001	99.99
X_6:Meat extract	218.57	-2.79	-2.16	4.68	0.0737	92.63
X_7:Soyabean meal	265810	97.43	75.52	5695.32	< 0.0001	99.99
X_8:Tryptone	192309	-82.87	-64.24	4120.48	< 0.0001	99.99
X_{11}:$CoCl_2$	218.68	-2.79	-2.16	4.69	0.0736	92.64
X_{12}:$ZnSO_4$	128382	-67.71	-52.48	2750.75	< 0.0001	99.99
X_{13}:Wheat bran	186941	81.71	63.34	4005.44	< 0.0001	99.99
X_{14}:$FeSO_4$	637827	150.93	117	13666.24	< 0.0001	99.99
X_{15}:Water	1707054	1246.84	191.34	36554.33	< 0.0001	99.99
X_{17}:$MnCl_2$	638865	151.05	117.09	13688.50	< 0.0001	99.99
X_{18}:Malt extract	223391	89.32	69.24	4786.43	< 0.0001	99.99
X_{19}:Incubation time	7269372	-509.53	-394.98	155755	< 0.0001	99.99
X_{20}:Tween 20	314737	-106.02	-82.18	6743.64	< 0.0001	99.99
X_{22}:SDS	283460	100.62	78	6073.50	< 0.0001	99.99
X_{23}:Potato peels	139292	70.53	54.67	2984.52	< 0.0001	99.99
X_{25}:NH_4Cl	451304	-126.96	-98.41	9669.76	< 0.0001	99.99
X_{26}:$NaNO_3$	195288	-83.51	64.73	4184.29	< 0.0001	99.99
X_{27}:NaCl	248053	94.12	72.96	5314.85	< 0.0001	99.99

Std.Dev. = 6.83, R^2 = 1.0000, Mean = 1737.01, Adj R^2 =0 .9999, C.V. % = 0.39, Pred. R^2 = 0.9996 , PRESS = 6098.44, Adeq Precision = 466.28,*Confidence level.

Optimization of Screened Nutrient Sources for Xylanase Production by *Fusarium oxysporum* SS-25 Using Response Surface Methodology

To determine the optimum response regions for xylanase yield, central composite design (CCD) was created as shown in Table **4** to study the combined effect of each significant independent variable. Each variable was studied at five different levels. The results of the Y (response) for xylanase production are listed in Table **4**. To decide about the adequacy of model for xylanase production, two different tests viz., Sequential model Sum of Squares and Model Summary Statistics were carried out. On the basis of their p value, R^2, standard deviation and predicted sum of square (PRESS) values, the adequacy of the quadratic regression model was found to be significant for xylanase production. The statistical significance of the ratio of mean square which is a statistical technique that subdivides the total variation in a set of data into component parts associated with specific sources of variation for the purpose of testing hypotheses on the parameters of the model [41]. The associated p-value is used to estimate whether F value is large enough to indicate statistical significance. If p-value is lower than 0.05,then it indicates that the model is statistically significant [42]. The ANOVA result for the xylanase production shows the model F-value of 163.10 indicating that the model is significant (Table **5**). There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. The p-values less than 0.005 indicate model terms are significant. Of all the regression coefficients, the larger p-values (>0.05) of the regression $FeSO_4$ (X_4) suggest their insignificance in the model. The value of correlation coefficient (R^2= 0.9935) indicates 99.3% variability can be explained by the model. The value *S/N* is 41.924. In this experiment, a lower value of CV % corresponding to 1.95 indicates a greater reliability

Table 4: Central Composite Design Matrix with Experimental Values of Xylanase Production by *Fusarium oxysporum* SS-25

Runs	Peptone (mg)	NH_4SO_4 (mg)	$MnCl_2$ (mg)	$FeSO_4$ (mg)	Response* (Y)
1	300.00	8.50	0.80	0.06	5716
2	200.00	7.00	0.60	0.08	4264
3	200.00	10.00	1.00	0.08	4029
4	300.00	5.50	0.80	0.06	4427
5	300.00	8.50	0.80	0.10	3441
6	300.00	8.50	0.80	0.06	5715
7	300.00	8.50	0.80	0.06	5712
8	400.00	7.00	1.00	0.08	4648
9	300.00	8.50	1.20	0.06	5812
10	200.00	7.00	1.00	0.08	4032
11	200.00	10.00	0.60	0.04	4054
12	300.00	8.50	0.80	0.02	3702
13	300.00	8.50	0.80	0.06	5713
14	300.00	11.50	0.80	0.06	3935
15	300.00	8.50	0.40	0.06	5094
16	500.00	8.50	0.80	0.06	3765
17	400.00	10.00	0.60	0.04	3201
18	400.00	7.00	0.60	0.08	3985
19	300.00	8.50	0.80	0.06	5715
20	400.00	7.00	0.60	0.04	3690
21	200.00	10.00	0.60	0.08	4613
22	400.00	10.00	0.60	0.08	4730
23	400.00	10.00	1.00	0.08	5041
24	400.00	10.00	1.00	0.04	4149
25	400.00	7.00	1.00	0.04	4991
26	100.00	8.50	0.80	0.06	4003
27	200.00	10.00	1.00	0.04	4108
28	200.00	7.00	0.60	0.04	4919
29	300.00	8.50	0.80	0.06	5714
30	200.00	7.00	1.00	0.04	5346

*The response (Y) is expressed as xylanase activity (IU/gds).

of the experiments performed. The analysis shows that the form of the model chosen to explain the relationship between the factors and the response is correct.

The ANOVA analysis indicates a linear relationship between the significant effects of peptone, NH_4SO_4, $MnCl_2$, $FeSO_4$, the interaction between peptone and NH_4SO_4, peptone and $MnCl_2$, peptone and $FeSO_4$, NH_4SO_4 and $MnCl_2$, NH_4SO_4 and $FeSO_4$, $MnCl_2$ and $FeSO_4$, the quadratic relationship with peptone, NH_4SO_4, $MnCl_2$, $FeSO_4$. By applying multiple regression analysis on the experimental data, the following second order polynomial equation was found to explain the xylanase production by only considering the significant terms and is shown below:

$$\text{Xylanase}=+5714.33-58.58\times X_1-122.25\times X_2+180.17\times X_3+15.08\times X_4 97.75\times X_1\times X_2+222.37\times X_1\times X_3+241.37\times X_1\times X_4-89.38\times X_2\times X_3+307.37\times X_2\times X_4-160.75\times X_3\times X_4-442.56\times X_1\times X_1-368.31\times X_2\times X_2-50.31\times X_3\times X_3-520.69\times X_4\times X_4 \quad (5)$$

Table 5: ANOVA Results for Xylanase Production Under Response Surface Quadratic Model and Model Coefficients Estimated by Multiple Linear Regression

Variables	Sum of squares	Coefficients	t-test	F-value	p-value	(%)*
Model	18407094	5714.33	155.87	163.10	< 0.0001	99.99
X_1:Peptone	82368	-58.58	-3.19	10.22	0.0060	99.40
X_2:NH_4SO_4	358681	-122.25	-6.66	44.49	< 0.0001	99.99
X_3: $MnCl_2$	779040	180.17	9.82	96.63	< 0.0001	99.99
X_4: $FeSO_4$	5460	15.08	0.822	0.68	0.4234	57.66
$X_1 \times X_2$	152881	97.75	4.35	18.96	0.0006	99.94
$X_1 \times X_3$	791210	222.37	9.90	98.14	< 0.0001	99.99
$X_1 \times X_4$	932190	241.37	10.75	115.63	< 0.0001	99.99
$X_2 \times X_3$	127806	-89.38	-3.98	15.85	0.0012	99.88
$X_2 \times X_4$	1511670	307.37	13.69	187.50	< 0.0001	99.99
$X_3 \times X_4$	413449	-160.75	-7.16	51.28	< 0.0001	99.99
$X_1 \times X_1$	5371191	-442.56	-25.82	666.35	< 0.0001	99.99
$X_2 \times X_2$	3719956	-368.31	-21.48	461.51	< 0.0001	99.99
$X_3 \times X_3$	69316	-50.31	-2.93	8.61	0.0102	98.98
$X_4 \times X_4$	7435120	-520.69	-30.37	922.37	< 0.0001	99.99

Std.Dev. =89.79, R^2 =0 .9935, Mean = 4608.83, Adj R^2 = 0.9874, C.V. % = 1.95, Pred. R^2 = 0.9624, PRESS = 696509, Adeq Precision = 41.924,*Confidence level.

Where X_1, X_2, X_3, X_4 are peptone, NH_4SO_4, $MnCl_2$, $FeSO_4$ respectively.

Interactions Among the Factors

Student's t–test was employed to determine the knowledge of the error mean square that is essential in testing the significance of the estimated coefficient of the regression equation. The larger magnitude of t value and smaller p value, the more significant is the corresponding coefficient [43]. Coefficient estimates and t values in the quadratic model as depicted in Table **5** indicate that factors X_3 and X_4 had positive effects on xylanase yield with $MnCl_2$ exhibiting the highest effect. The interactions between the factors X_2X_3 and X_3X_4 showed negative effects on enzyme yields while the interactions between X_1X_2, X_1X_3, X_1X_4 and X_2X_4 had positive effects on xylanase yields with highest improvement by X_2X_4. Figure **2a-f** shows the contour graphs showing the interactions between two factors for the optimization of conditions for xylanase production. From the plots, it was easy and convenient to understand the interactions between two variables and also to locate the optimum levels. Each curve represents an infinite number of combinations of two test variables with the other variables maintained at constant level. The contour graph obtained as a function of peptone concentration versus NH_4SO_4 concentration indicated that xylanase production increased with the increase of both peptone and NH_4SO_4 but at high concentration enzyme productivities decreased. The maximum production of xylanase corresponding to 5727 U/gds was obtained in the brewer spent grain based optimized medium where the concentrations of supplemented peptone and NH_4SO_4 were 5.84 % w/w and 0.165 % w/w respectively (Figure **2a**) while $MnCl_2$ and $FeSO_4$ was held at 0, 0 level. The contour graph obtained as a function of peptone concentration versus $MnCl_2$ concentration showed that the enzyme productivity increased with the concentration of peptone but decreased with the increase in the concentration of $MnCl_2$. The maximum xylanase productivity of 5956 U/gds occurred at a concentration of 6.92% w/w of peptone and 0.024% of $MnCl_2$ with NH_4SO_4 and $FeSO_4$ held at 0, 0 levels respectively (Figure **2b**). Figure **2c** shows the effect of peptone and $FeSO_4$ on xylanase production. Increase in the concentration of both peptone and $FeSO_4$ promoted the xylanase production, but at high level of peptone and $FeSO_4$ (higher than 6% and 0.0012%), enzyme production decreased. The maximum production of xylanase corresponding to 5714 U/gds was obtained at 6% w/w and 0.0012% w/w concentrations of peptone and $FeSO_4$ respectively while NH_4SO_4 and $MnCl_2$, were held at 0,0 coded levels respectively. Figure **2d** shows the effect of NH_4SO_4 and $MnCl_2$ on xylanase production. Increase in the concentration of both NH_4SO_4 and $MnCl_2$

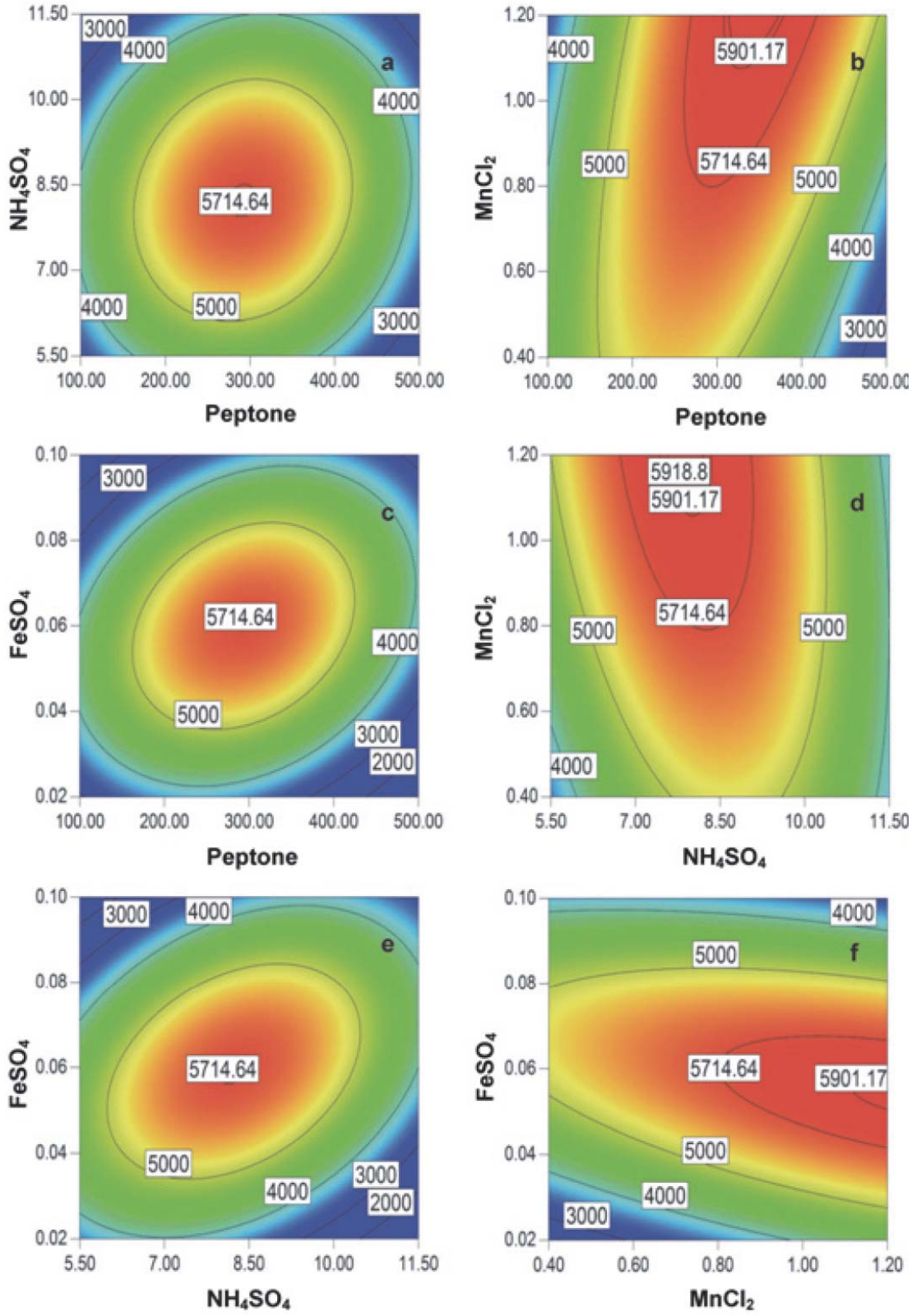

Figure 2: (**a-f**): Contour plots representing xylanase yield from solid state culture of *Fusarium oxysporum* SS-25 on 5g Brewer's spent grain as affected by cultural conditions (**a**) peptone and NH_4SO_4 (**b**) peptone and $MnCl_2$ (**c**) peptone and $FeSO_4$ (**d**) NH_4SO_4 and $MnCl_2$ (**e**) NH_4SO_4 and $FeSO_4$ (**f**) $MnCl_2$and $FeSO_4$. All values are expressed in terms of mg.

increased the xylanase production, but at high level of NH_4SO_4 (higher than 0.162%), enzyme production decreased. The maximum productivity of 5934 U/gds was obtained at 0.159 % w/w and 0.024% w/w

concentration of NH_4SO_4 and $MnCl_2$ respectively with peptone and $FeSO_4$ held at 0,0 coded levels respectively. The contour graph obtained as a function of NH_4SO_4 concentration versus $MnCl_2$ concentration indicated that xylanase production increased with the increase of both NH_4SO_4 and $MnCl_2$ but at but at high concentration of NH_4SO_4 and $MnCl_2$ (higher than 0.164% and 0.016%), enzyme production decreased. The maximum production of 5724.71U/gds was obtained in the brewer spent grain based solid medium when the concentration of NH_4SO_4 and $FeSO_4$ was 0.164 % w/w and 0.016% w/w respectively with peptone and $MnCl_2$ held at 0,0 coded levels respectively (Figure **2e**). Figure **2f** shows the effect of $MnCl_2$ and $FeSO_4$ on xylanase production. Increase in the concentration of both increase the xylanase production, but at high $FeSO_4$ level (higher than 0.0012%), enzyme production decreased. Highest production of xylanase was obtained when the concentration of $MnCl_2$ and $FeSO_4$ was 0.023% w/w and 0.0012% w/w respectively revealing 5853.55 U/gds while peptone and NH_4SO_4 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 xylanase yield. Point optimization for xylanase production attempted with Design Expert using X_1 (urea, 1.5 mg), X_2 (NH_4SO_4, 8.1 mg), X_4 (peptone, 300 mg), X_7 (soyabean meal, 100 mg), X_{13} (wheat bran, 1 gm), X_{14} ($FeSO_4$, 0.06 mg), X_{15} (water, 12 mL), X_{17} ($MnCl_2$, 1.0 mg), X_{18} (malt extract, 100 mg), X_{22} (SDS,0.6 mg), X_{23} (potato peels, 100 mg), X_{27} (NaCl, 1.5 mg), inoculated with 1 mL of fungal spore suspension having 2.8×10^7 spores, incubated at 30°C in stationary state for 3 days in 5 g brewer's spent grain based medium predicted the yield of 5874.50 IU/g. To validate the optimum concentrations, an experiment with the above specified conditions was performed and the result was 5874 IU/g which is 0.002% less than the predicted value.

CONCLUSIONS

The locally isolated strain of *Fusarium oxysporum* SS-25 proved to be a potential candidate for xylanase production under solid state fermentation of brewer's spent grain, a low cost otherwise unattended waste of brewery industry. Further, statistical optimization of media components and process conditions employing Plackett-Burman and Response surface methodology designs led to an improvement in xylanase productivity yielding 5874 IU/g and revealing 4.28-fold increase in activity as compared to unoptimized conditions containing brewer's spent grain only.

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