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STATISTICAL OPTIMIZATION OF MEDIA COMPONENTS FOR THE ENHANCED B-XYLANASE AND B-MANNANASE PRODUCTION FROM *ASPERGILLUS NIGER* C-5 VIA SOLID STATE FERMENTATION OF WHEAT BRAN

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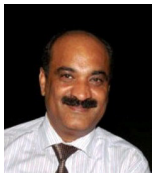
ABSTRACT

Hemicellulases are the key enzymes in the production of alternative fuels and chemicals from lignocellulosic biomass. The present study dealt with the statistical optimization of various fermentation parameters for enhancing the yields of β-xylanase and β-mannanase from *Aspergillus niger* C-5 using wheat bran based basal media. Plackett-Burman was first employed to screen out the important process parameters that have significant effect on the production of enzymes. Out of 23 variables screened, four variables including tryptone, SDS, NH_4Cl, NaCl were selected to further study the interactive effects and optimum level of these variables in central composite design of response surface methodology. This combinatorial statistical approach led to the fine tuning of productivities revealing 5200 and 200 IU/g of β-xylanase and β-mannanase yields respectively. Hence, this two step optimization approach led to 3.72 and 2.08 fold increase in the activities of enzymes as compared to under unoptimized conditions.

KEYWORDS: **Solid State Fermentation (SSF), Plackett-Burman Design (PBD), Response Surface Methodology (RSM), *Aspergillus niger* C-5, Wheat bran**



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INTRODUCTION

Hemicellulose is the second most abundant polymer (20–50% of lignocellulose biomass) after cellulose and differs from cellulose in that it is not chemically homogeneous. It represents the major renewable resource present on earth comprising of branched, heterogenous polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) and acetylated sugars. Xylan, after cellulose, is the most abundant hemicellulose present in wood, agricultural and several agro-industrial wastes. This complex hetero-polysaccharide consists of a main chain of 1,4-β-D-xylose monomers containing different substituents or ramification[1-3]. Several hydrolytic enzymes are involved in the complete breakdown of this hemicelluloses fraction. Among them, xylanase is the most predominant enzyme which catalyzes the hydrolysis reaction of xylan[4] producing oligosaccharides that are eventually degraded by 1,4-β xylosidase to produce xylose units[2]. Mannan and heteromannans, also form a part of the hemicellulose fraction in plant cell walls. They consist of monomers of glucose, mannose and galactose and require mannanases and galactomannanases for their hydrolysis. In recent years, hemicellulases have emerged as key enzymes in the rapidly growing biotechnology industry, owing to their multifaceted properties, which find usage in a wide array of industrial applications. Most studies on hemicellulases have focused until now on enzymes that hydrolyse xylan. Enzymes that hydrolyse mannan have been largely neglected, even though it is an abundant hemicellulose, therefore the application of mannanase for catalyzing the random hydrolysis of β-D-1,4 mannopyranoside linkages in β-1,4 mannans is as important as the application of xylanases. At industrial level, the production of hemicellulases is restricted due to its high cost and low enzyme yields. Hence, there is a need to develop a simple low cost production medium for the enhanced production of enzymes. Among the various fermentation routes, solid-state fermentation (SSF) hold tremendous potential for increased enzyme productivities and offers additional advantages of low capital investment, higher reactor volume and ease of product recovery. Several reports exist where SSF has been employed for the production of industrially important enzymes such as cellulases, polygalacturonase, xylanase, pectinase and mannanase[5-7]. It is generally understood that 30-35% of the production cost of industrially important enzymes is due to the expenses of the medium. The enzymes obtained from microorganisms are generally extracellular and their production is highly affected by the cultural and environmental factors, such as carbon and nitrogen ratio, inorganic nutrients, temperature, pH, aeration and agitation. Therefore, development of an economically viable production medium requires selection of process parameters and their optimization strategies. Optimization of media components by using one variable at a time approach (OVAT) is not only tedious and cumbersome but also becomes infeasible, in cases where large numbers of variables are to be screened. The combinatorial statistical approaches of Plackett-Burman and response surface methodologies (RSM) can be employed to screen large number of factors and then design a simple low cost medium for enhancing the product yields. Plackett-Burman based statistical designs are first employed for screening various cultural and environmental factors in order to understand their significance on the product formation and then few better factors that show a significant role are selected for subsequent optimization studies by response surface methodology. Response surface methodology (RSM) then provides models and graphs showing the effects of independent variables on enzyme yield and also gives the predictive responses of each combination, the interactive effects of variables and the optimum levels of each independent variable in the growth medium. The aim of the present study was to bring down the cost of hemicellulases by statistically optimizing the media constituents of wheat bran based solid media, thereby enhancing the productivities of xylanase and mannanase from *Aspergillus niger* C-5 that are really an important class of enzymes from industrial point of view.

MATERIALS AND METHODS

(i) Microorganism

The xylanolytic and mannanolytic fungal strain of *Aspergillus niger* C-5 used in the present study was isolated from the soil samples of Chandigarh city[7]. It was grown and maintained on potato dextrose agar plates at 28°C for 4 days to allow the development of spores and then stored at 4°C until use.

(ii) Enzyme production by solid state fermentation

The production of xylanase and mannanase was carried out under solid state cultivation conditions in 250 ml Erlenmeyer flasks containing 5 g wheat bran 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°C in stationary state for 4 days. The enzymes were extracted by adding 200 ml of distilled water to each flask and churning the contents in a blender. After churning, the contents were filtered through metallic sieve and the solid residue was thoroughly pressed to extract the remaining liquid. The suspension from each flask was then centrifuged at 10,000×g for 10 min at 4°C, and the supernatant analysed for enzyme activity. Hemicellulase activity was determined in terms of endo-β-1,4-xylanase and endo-β-1,4-mannanase activities using xylan[8] and guar gum[9], respectively, as the substrates and determining the μmoles of xylose and mannose liberated/min, respectively using dinitrosalicylic acid reagent[10]. The enzyme activities were expressed in terms of International units (IU). One international unit (IU) of xylanase and mannanase activity was defined as equivalent to the enzyme that releases one μmole of xylose from xylan, mannose from guar gum, in one min under standard assay conditions.

(iii) Statistical optimization of media components for enzyme(s) production by Plackett-Burman design

Enzyme production is highly influenced by many factors including media constituents and other cultural and environmental parameters. For screening the effect of these parameters on enzyme productivities, 23 different process variables were chosen and examined, in one block, at two levels using first order Plackett-Burman factorial design:

$$Y = \beta_O + \sum \beta_i X_i$$

Where, Y is the response, β_o is the model intercept, β_i is the linear coefficient, and X_i is the level of the independent variable. This design was applied for preliminary screening of medium components as it aids in evaluating the relative importance as well as the contribution of various factors within a complex medium especially when large numbers of factors are to be screened[11].

(iv) Standardization of important screened parameters for further optimization of enzyme(s) production by Aspergillus niger C-5 using response surface methodology (RSM):

In order to determine the optimal concentrations of most significant factors screened during Plackett Burman design, four independent variables including Tryptone (X_1), SDS (X_2), NH4Cl (X_3), NaCl (X_4) affecting enzyme(s) production were chosen to investigate the first- and higher-order main effects of each factor and interactions amongst them for further optimization through RSM. All other factors which showed positive behavior during screening by Plackett-Burman model were kept constant. A 2^4 factorial central composite experimental design resulted in 30 experimental runs was generated by Design Expert, Version 9.0, Stat-Ease Inc., Minneapolis, MN). The relation between coded and actual values is described according to equation:

$$x_i = (X_i - X_{0i}) / \Delta X_i$$

i = 1,2,3… j

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

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

X_{0i} = the value of Xi at the center point,

ΔX_i = the step change value.

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

$$Y = \beta_O + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X^2_i + e$$

Where Y= measured response; β_o, β_i, β_{ij}, β_{ii} are constant and regression coefficients of model; X_i and X_j are levels (codes values) of independent variables; e is random error.

(v) Statistical analysis of data

The software package, Design-Expert trial version 9 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 and F- values to evaluate the significance of experimental design. Contour plots were also obtained to illustrate the relationship between the variables. Accuracy and general ability of the model was evaluated by coefficient of determination (R^2). The statistical significance of model coefficient was evaluated by ANOVA.

RESULTS AND DISCUSSION

The cost of substrate contributes more than 40% of the total cost of enzyme production. Hence utilization of the cheaper substrates for enzyme production can help lower down the cost of hydrolytic enzymes to some extent. Reducing the cost of enzyme production by utilizing cheaper substrates and optimizing fermentation and cultivation conditions for enhanced microbial growth and increased productivities is the goal of basic research for industrial application of these enzymes. 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 enzyme production. *Aspergillus* spp. are known to utilize broad range of lignocellulosic substrates[5,12,13] for the production of hydrolytic enzymes. Several agricultural crop residues, in the form of flours, brans, straws, hulls, residues of the fruit processing industries, waste of the oil processing mills have been successfully used in solid state fermentation by many workers[14,15]. Wheat bran has been the prime among many solid state fermentation processes, which have been developed for the production of bulk chemicals and value added fine products[13,16]. This has been attributed mainly due to its particularly rich nutritional composition: vitamin B, about 14% proteins, 27% carbohydrates (64% cellulose and 36% hemicellulose), 6% lipids, 5% minerals, and around 64% digestible nitrogen [6, 17-19]. When used as a solid state fermentation substrate, in the present study also, wheat bran was able to remain loose in moist conditions. The organism colonized well on this substrate and produced high xylanase and mannanase yield corresponding to 1396±27.90 and 96±2.28 IU/gds respectively, when cultivated on a wheat bran based solid medium.

Statistical optimization of media components for enzyme(s) production by Plackett-Burman design

Plackett-Burman is a set of small and efficient experimental design which is very powerful, widely applicable and especially well suited for biotechnology research and development[20]. It has been recently employed by many research groups aiming to enhance various enzyme yields utilizing different agro-industrial substrates. The usefulness of the design lies in the fact that is determining the effect of one variable, the net effect of changing other variables cancel out so that the effect of each variable on the system can be independently determined. Based upon our preliminary studies and literature survey, a set of 23 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 Table 1 and 2. The main effects of the examined variables on xylanase and mannanase production were calculated as the difference between the average measurements were made at higher level (+1) and lower level (-1) of that factor, as represented in Fig.1.

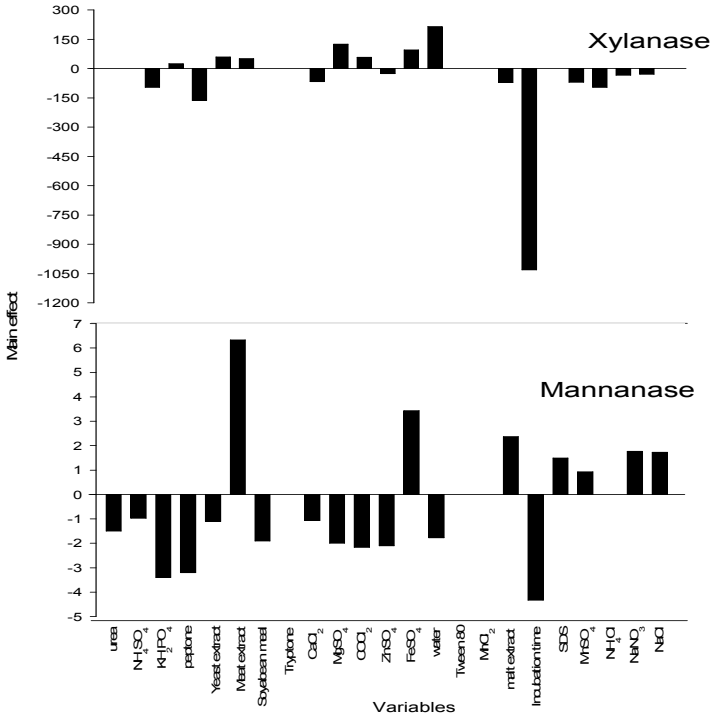

Figure 1
Main effects of independent variables on xylanase and mannanase production by Aspergillus niger C-5 in Plackett- Burmann model.

Table 1

Randomized Plackett-Burmann experimental design for evaluating the factors influencing the co-production of various components of enzyme cocktail.

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})	$FeSO_4$ (X_{13})	Water (X_{14})	Tween 80 (X_{15})	$MnCl_2$ (X_{16})	Malt Extract (X_{17})	Incubation Time (X_{18})	SDS (X_{19})	$MnSO_4$ (X_{20})	NH_4Cl (X_{21})	$NaNO_3$ (X_{22})	NaCl (X_{23})	Xylanase (IU/gds)	Mannanase (IU/gds)
1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	-1	+1	-1	+1	2273	148
2	+1	-1	-1	+1	+1	-1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	568	112.8
3	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	-1	+1	-1	+1	-1	5328	156.8
4	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	-1	+1	-1	+1	-1	-1	+1	-1	+1	+1	+1	+1	+1	4256	124.8
5	-1	+1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	-1	+1	-1	+1	-1	-1	-1	-1	5864	100.8
6	-1	-1	+1	+1	-1	-1	+1	+1	-1	+1	+1	+1	+1	-1	-1	-1	+1	+1	+1	+1	+1	+1	-1	568	106.4
7	-1	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	-1	+1	-1	+1	+1	-1	-1	-1	+1	+1	+1	5472	124.8
8	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	+1	-1	-1	-1	-1	-1	-1	5472	139.2
9	-1	-1	+1	-1	-1	+1	+1	-1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	568	133.6
10	+1	-1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	+1	5648	160
11	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	+1	-1	2416	118.4
12	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	5048	109.6
13	+1	+1	+1	-1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	-1	5824	176
14	+1	+1	-1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	+1	+1	-1	+1	+1	-1	-1	1600	100.8
15	+1	+1	-1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	288	106.4
16	+1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	+1	-1	+1	+1	-1	-1	+1	5544	135.2
17	-1	-1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	-1	5760	97.6
18	+1	-1	+1	+1	-1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	568	100.8
19	-1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	-1	+1	+1	+1	-1	-1	-1	-1	+1	424	118.4
20	-1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	3200	104
21	+1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	+1	-1	+1	-1	+1	-1	-1	4800	109.6
22	+1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	+1	-1	-1	-1	2256	92
23	-1	+1	-1	-1	+1	+1	-1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	496	144.8
24	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	-1	+1	-1	+1	-1	+1	-1	-1	+1	+1	+1	+1	5688	160

Table 2

Levels of independent variables used for medium optimization in Plackett Burmann 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	100 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}:$FeSO_4$	0	0.03 mg
X_{14}:Water	5	12 ml
X_{15}:Tween 80	0	0.01 mg
X_{16}:$MnCl_2$	0	0.5 mg
X_{17}:Malt extract	0	100 mg
X_{18}:Incubation time	72 h	144 h
X_{19}:SDS	0	0.6 mg
X_{20}:$MnSO_4$	0	0.5 mg
X_{21}:NH_4Cl	0	1.5 mg
X_{22}:$NaNO_3$	0	5.0 mg
X_{23}:NaCl	0	1.5 mg

1. *Xylanase production*

In the case of xylanase yields, it was seen that potassium dihydrogen orthophosphate, yeast extract, meat extract, magnesium sulphate, cobalt chloride, iron sulphate, water exerted a positive effect while ammonium sulphate, peptone, calcium chloride, zinc sulphate, malt extract, incubation time, manganese sulphate, ammonium chloride, sodium nitrate, sodium chloride exerted a negative effect (Fig. 1). Of the variables promoting the enzyme activity, presence of water was found to have the most significant effect followed by $MgSO_4$ and yeast extract. Of the variables inhibiting the enzyme synthesis, potassium dihydrogen orthophosphate was found to have pronounced effect followed by zinc sulphate and sodium chloride. It may be mentioned that the presence of magnesium ions plays very important role in some regulatory functions through increased adenosine triphosphate metabolism and nucleic acid synthesis during the microbial bioconversion[21] which may be the reason of promotion of enzyme synthesis in the presence of $MgSO_4$.

2. *Mannanase production*

In case of mannanase production, presence of meat extract, malt extract, $FeSO_4$, SDS, manganese sulphate, sodium nitrate and sodium chloride exerted a positive effect whereas urea, ammonium sulphate, kotassium dihydrogen orthophosphate, peptone, yeast xtract, soyabean meal, calcium chloride, magnesium sulphate, cobalt chloride, zinc sulphate, water, incubation time, exerted a negative effect. Of the variables promoting the enzyme activity, meat extract was found to have the most significant effect followed by iron sulphate. Of the factors inhibiting the enzyme synthesis, ammonium sulphate had the pronounced effect followed by calcium chloride (Fig 1). Peptone as an organic nitrogen source which was highly utilized by microorganisms for growth and development had a negative effect on enzyme production in the present study, this effect can be linked with what was reported by Rajendran and Thangavelu[22] where high concentrations of peptone resulted in enhanced protease production causing proteolysis of other enzymes in the medium.

3. *Standardization of important screened parameters for further optimization of enzyme(s) production by solid statecultures of Aspergillus niger C-5 using response surface methodology (RSM) designs*

Response surface methodology (RSM) is an empirical modeling technique used to evaluate the relationship between a set of controllable experimental factors and observed results[23, 24]. A second order model like central composite design (CCD) is widely used in RSM because it can take on wide variety of functional forms and this flexibility allows it to predict the true response surface more closely. This approach has been successfully employed to maximize enzyme production in SSF [25, 26]. In the present study, to determine the optimum response regions for the maximum production of xylanase and mannanase, a 2^4 factorial central composite experimental design was generated by Design Expert, Version 9.0 (Stat-Ease Inc., Minneapolis, MN) to evaluate the nature of the response surface in the experimental region and to identify optimal values for the most significant variables including Tryptone (X_1), SDS (X_2), NH_4Cl (X_3), NaCl (X_4). The experimental designs resulting in 30 experimental runs with the observed responses for enzyme(s) production is presented in Table 3. All other factors which showed positive behavior during screening by Plackett Burmann model including X_1, X_2, X_3, X_4, X_5, X_6, X_7, X_9, X_{10}, X_{11}, X_{12}, X_{13}, X_{14}, X_{16}, X_{17}, X_{18}, X_{20}, X_{21}, at the levels of 0.60 mg, 0.65 mg, 0.17 mg, 81.42 mg, 10.28 mg, 99.97 mg, 96.26 mg, 1.06 mg, 0.05 mg, 0.002 mg, 0.006 mg, 0.025 mg, 8.85 ml, 0.006 mg, 74.05 mg, 3 days, 0.58 mg, 0.44 mg, 1.4 mg, were kept constant. The flasks were inoculated with 2.5 ml of fungal spore suspension having 2.8×10^7 spores/ml, incubated at 30^oC, pH 4.0 in stationary state for 72 h. The regression equation was developed using RSM, allowing for the analysis of interacting factors by identifying which significant factors were contribute to the regression model and determining the optimal values of the most significant independent variables[27].

Table 3
Central composite design matrix of Response Surface Methodology with experimental values obtained for Xylanase, Mannanase, production by Aspergillus niger C-5.

Run	A-Tryptone* (X_1) (-2) = 100 mg (-1) = 200 mg (0) = 300 mg (+1) = 400 mg (+2) = 500 mg	B-SDS* (X_2) (-2) = 0.40 mg (-1) = 0.60 mg (0) = 0.80 mg (+1) = 1.00 mg (+2) = 1.20 mg	C-NH_4Cl* (X_3) (-2) = 1.00 mg (-1) = 1.50 mg (0) = 2.00 mg (+1) = 2.50 mg (+2) = 3.00 mg	D-NaCl* (X_4) (-2) = 1.00 mg (-1) = 1.50 mg (0) = 2.00 mg (+1) = 2.50 mg (+2) = 3.00 mg	Xylanase (IU/gds)	Mannanase (IU/gds)
1	-1	+1	+1	+1	4954.95	205.8
2	0	0	0	0	5250	216
3	0	0	-2	0	4374.75	200
4	+1	+1	-1	+1	4537.5	193.74
5	-1	+1	-1	-1	4412.82	202
6	0	0	0	+2	4710	205
7	-1	+1	-1	+1	4320	201.2
8	+1	-1	+1	+1	4792.2	195
9	0	0	0	0	5268	216
10	-2	0	0	0	4521.39	196
11	-1	-1	+1	+1	4897.5	195.2
12	0	0	0	0	5272.5	221.6
13	-1	+1	+1	-1	5077.5	184
14	0	0	0	0	5262	220
15	-1	-1	-1	-1	4501.5	198.92
16	-1	-1	+1	-1	4822.5	186
17	0	0	0	0	5259	220
18	0	-2	0	0	4792.5	185.2
19	0	0	+2	0	5217	200.38
20	+1	-1	+1	-1	4668	186.4
21	-1	-1	-1	+1	4584	192.6
22	+2	0	0	0	4522.5	190.82
23	+1	+1	-1	-1	4518	194
24	+1	-1	-1	+1	4570.44	181
25	+1	+1	+1	+1	5050.5	209.8
26	+1	-1	-1	-1	4437	191.8
27	0	0	0	-2	4644	192
28	+1	+1	+1	-1	5023.5	188
29	0	0	0	0	5263.5	218
30	0	+2	0	0	4935	197.6

The values are per 5 g of the wheat bran used in each of the experimental runs

To decide about the adequacy of each of the model for enzyme production, different tests were carried out[28]. The statistical significance of the ratio of mean square variation due to regression and mean square residual error was tested using the analysis of variance (ANOVA). The goodness of fit for each of the model was tested by analyzing their F value, p-value. Accuracy and general ability of polynomial model was evaluated by coefficient of determination (R^2). P- values greater than 0.1000 indicated the model terms are not significant. A determination coefficient R^2 value close to 1 indicates that the model describes and represents the experimental data well. The value of Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The graphical representation of the regression equation for each of the response surface model has been presented in terms of iso response contour plots. From the contour plots, it is 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.

3.1. Xylanase production

Various models including linear, 2FI, quadratic and cubic were tested in the present study for enhancing xylanase yield. Of all the models analysed, the quadratic regression model was found to be significant. The ANOVA analysis indicated a linear relationship between the main effects of the tryptone, SDS, ammonium chloride, sodium chloride, the interaction between tryptone and SDS, tryptone and ammonium chloride, tryptone and sodium chloride, SDS and ammonium chloride, SDS and sodium chloride, the quadratic relationship with tryptone, SDS, ammonium chloride, sodium chloride. However, some regression coefficients i.e A and CD were found to be unnecessary having p values >0.05 suggesting their insignificance and hence were removed by backward elimination step. Thus, by neglecting the insignificant terms, the final model equation for xylanase in terms of coded factors may be written as:

Xylanase = +5262.50+1.19×A+37.78×B+212..08×C+15.76×D+43.88×A×B-28.93×A×C+22.63×A×D+76.93×B×C-36.50×B×D-187.91×A^2-102.46×B^2-119.43×C^2-149.15×D^2

Where A, B, C and D are 'Tryptone', 'SDS', 'NH_4Cl', 'NaCl' respectively.

The ANOVA test for the response surface quadratic model is shown in Table 4. The resulting model F-value of 817.40 implies that the model is significant, with only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms were significant. In this case B, C, D, A^2, B^2, C^2, D^2, AB, AC, AD, BC, BD are significant model terms. The goodness of the fit of the model was checked by the 'determination coefficient' R^2 which was calculated to be 0.9985, indicating that 99.85 % of variables fit the response. The "Pred R-Squared" of 0.9932 is in reasonable agreement with the "Adj R-Squared" of 0.9973." The value of Adeq Precision 81.874 which indicates an adequate signal. This model can be used to navigate the design space. Coefficient estimates in the reduced quadratic model indicate that all the factors B, C and D had a positive effect on Xylanase yield with highest improvement with C. The t- test values of variables also showed a higher positive effect of ammonium chloride and higher negative effect of SDS on xylanase production. The graphical representation of the regression equation is presented as the contour graphs indicating the interactions between two factors for the optimization of conditions for Xylanase production. The contour graph obtained as a function of tryptone concentration versus SDS concentration indicated that xylanase production increased with the increase of both tryptone as well as SDS but at higher concentration of both the factors, the enzyme productivity decreased.

Table 4

Statistical analysis of central composite design showing sum of squares, F-value, p-value, F value, coefficient estimate, t-test, confidence level for each variable affecting xylanase activity after backward elimination regression analysis.

Source	Sum of squares	F- value	P value	Coefficient estimate	Standard error	t- test	Confidence level	
Model	2922535	817.40	< 0.0001	5262.5	6.77	777.2	99.99	Significant
A-Tryptone	34.05784	0.12	0.7295	1.19	3.38	0.35	27.05	
B-SDS	34249.08	124.53	< 0.0001	37.77	3.38	11.15	99.99	
C-NH_4Cl	1079458	3924.85	< 0.0001	212.07	3.38	62.64	99.99	
D-NaCl	5962.00	21.68	0.0003	15.76	3.38	4.65	99.97	
AB	30808.15	112.02	< 0.0001	43.88	4.14	10.58	99.99	
AC	13390.54	48.69	< 0.0001	-28.92	4.14	-6.97	99.99	
AD	8190.70	29.78	< 0.0001	22.62	4.14	5.45	99.99	
BC	94690.06	344.29	< 0.0001	76.92	4.14	18.55	99.99	
BD	21316.73	77.51	< 0.0001	-36.50	4.14	-8.80	99.99	
A^2	968542.90	3521.57	< 0.0001	-187.91	3.16	-59.34	99.99	
B^2	287958.90	1047.00	< 0.0001	-102.46	3.16	-32.35	99.99	
C^2	391234.30	1422.51	< 0.0001	-119.43	3.16	-37.71	99.99	
D^2	610165.80	2218.53	< 0.0001	-149.15	3.16	-47.10	99.99	

Std. Dev. 16.58; R-Squared 0.9985; Mean 4815.34; Adj R-Squared 0.9973; C.V. % 0.34; Pred R-Squared 0.9932; PRESS 19768.81; Adeq Precision 81.874

The maximum production of Xylanase corresponding to 5266.05 IU/gds was obtained in the wheat bran based optimized medium where the concentrations of supplemented tryptone and SDS were 310 mg and 0.84 mg respectively (Fig 2a) while NH_4Cl and NaCl were held at 0, 0 coded levels equivalent to 2 and 2 mg respectively. The contour graph obtained as a function of tryptone concentration versus NH_4Cl concentration showed that the enzyme productivity increased with the increase in the concentration of tryptone but at higher concentration, the productivity decreased. Similarly, with enzyme yields increased upon increasing the NH_4Cl but at higher levels (higher than 2.50 mg), the productivity began to decline. The interactive effect of both the parameters predicted a maximum yield of 5357.03 IU/gds at a point where the concentration of tryptone and NH_4Cl were 297.61 mg and 2.44 mg respectively with SDS and NaCl held at 0, 0 coded levels equivalent to 0.80 and 2 mg respectively (Fig 2b). Fig 2c shows the effect of Tryptone and NaCl on xylanase production. Increase in the concentration of both Tryptone and NaCl promoted the xylanase production, but at high level of both Tryptone and NaCl (higher than 300 mg and 2.0 mg), enzyme production decreased. The maximum production of xylanase corresponding to 5262.61 IU/gds was obtained at 299.15 mg and 2.04 mg concentrations of Tryptone and NaCl respectively while NH_4Cl and SDS, were held at 0,0 coded levels equivalent to 2 and 0.80 mg respectively. Fig 2d shows the effect of SDS and NH_4Cl on xylanase production. Increase in the concentration of both SDS as well as NH_4Cl increased the xylanase production upto a certain level, thereafter with further an increase in the concentration of both the parameters, the productivity decreased. The maximum productivity of 5387.09 IU/gds was obtained at a concentration of 0.91 mg and 2.50 mg SDS and NH_4Cl respectively with Tryptone and NaCl held at 0, 0 coded levels equivalent to 300 and 2 mg

respectively. The contour graph obtained as a function of NH_4Cl concentration versus NaCl concentration indicated that xylanase production increased with the increase of both SDS and NaCl concentration, but at higher concentration of both the parameters, the enzyme production decreased. The model predicted a maximum yield of 5266.11 IU/gds in the wheat bran based solid medium when the concentration of SDS and NaCl was 0.84 mg and 2.01 mg respectively with tryptone and NH_4Cl held at 0,0 coded levels corresponding to 300 and 0.80 mg respectively (Fig 2e). To evaluate the accuracy of statistical model of response surface methodology design for xylanase production, attempts were made to maximize the yields using significant variables. Numerical optimization for xylanase production attempted with Design Expert using A (Tryptone 312.85 mg), B (SDS 0.81 mg), C (NH_4Cl, 2.19 mg), D (NaCl, 1.72 mg), inoculated with 2.5 ml of fungal spore suspension having 2.8×10^7 spores/ml, incubated at 30°C in stationary state for 72 h in 5g wheat bran based medium containing urea, ammonium sulphate, potassium dihydrogen orthophosphate, peptone, yeast extract, meat extract, soyabean meal, calcium chloride, magnesium sulphate, cobalt chloride, zinc sulphate, water, manganese chloride, malt extract, manganese sulphate, sodium nitrate at the levels of 0.60 mg, 0.65 mg, 0.17 mg, 81.42 mg, 0.28 mg, 99.97 mg, 96.26 mg, 1.06 mg, 0.05 mg, 0.002 mg, 0.006 mg, 0.025 mg, 8.85 ml, 0.006 mg, 74.05 mg, 0.44 mg, predicted the yield of 5273.34 IU/gds.

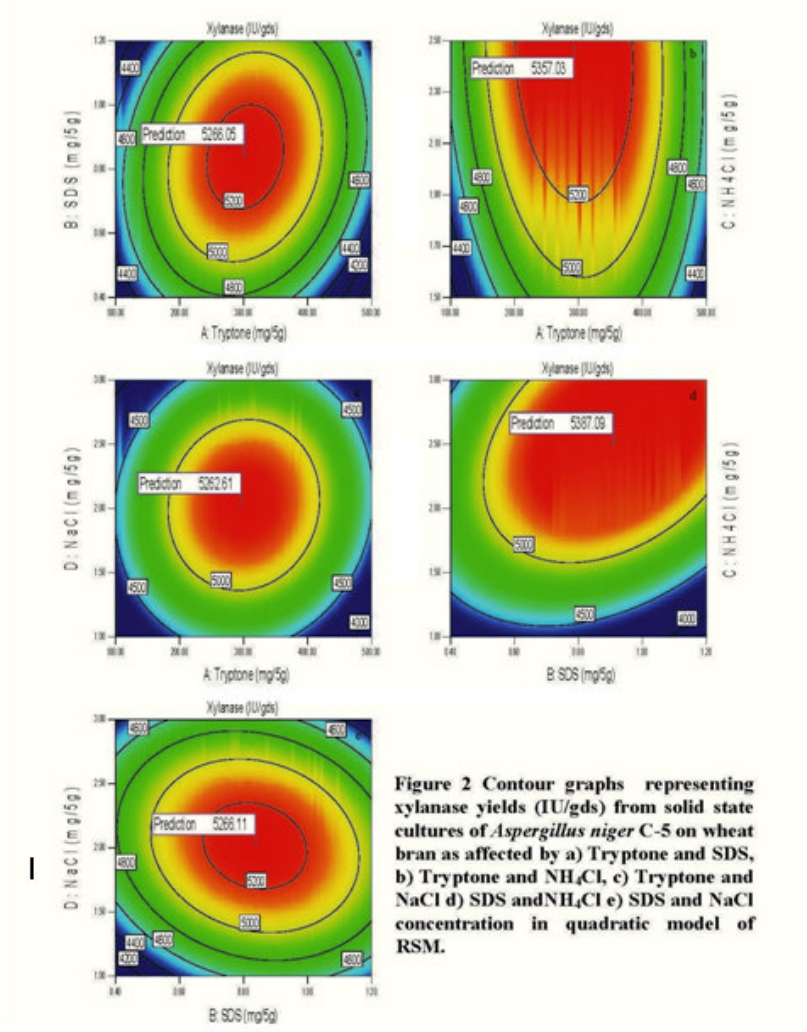

Figure 2 Contour graphs representing xylanase yields (IU/gds) from solid state cultures of *Aspergillus niger* C-5 on wheat bran as affected by a) Tryptone and SDS, b) Tryptone and NH_4Cl, c) Tryptone and NaCl d) SDS andNH_4Cl e) SDS and NaCl concentration in quadratic model of RSM.

To validate the optimum concentrations, an experiment with the above specified conditions was performed and the result was 5200 IU/gds which is quite close to the predicted value, hence validating that the model chosen for optimization was correct.

3.2. Mannanase production

Various models including linear, 2FI, quadratic and cubic tested in the present study for enhancing mannanase production were analysed on the basis of their F value, P value, determination coefficient (R^2), standard deviation and PRESS values. Of all the models analysed, the quadratic regression model was found to be significant. The ANOVA test for the response surface quadratic model is shown in Table 5. The ANOVA analysis for Mannanase model indicates a linear relationship between the main effects of the tryptone, SDS, ammonium chloride, sodium chloride, the interaction between tryptone and ammonium chloride, SDS and sodium chloride, ammonium chloride and sodium chloride, the quadratic relationship with

tryptone, SDS, ammonium chloride, sodium chloride. However, some regression coefficients i.e BC, C, AD and AB were found to be unnecessary having p values >0.05 suggested their insignificance and hence were removed by backward elimination step. Thus, by neglecting the insignificant terms, the final model equation for xylanase in terms of coded factors may be written as:

Mannanase = +218.60-1.51×A+3.18×B-0.18×C+2.88×D+2.65×A×C+2.62×B×D+4.97×C×D-6.59×A^2-7.10×B^2-4.90×C^2-5.32 ×D^2

Where A, B, C and D are 'Tryptone', 'SDS', 'NH_4Cl', 'NaCl' respectively.

The resulting model F-value of 96.62 implies that the model is significant, with only a 0.01% chance that a "Model F-Value" this large could occur due to noise. In this case A, B, D, A^2, B^2, C^2, D^2, AC, BD, CD are significant model terms. The "Lack of Fit F-value" of 0.58 implies the Lack of Fit is not significant relative to the pure error. There is a 80.23% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit. The goodness of the fit of the model was checked by the 'determination coefficient' R^2 which was calculated to be 0.9833, indicating that 98.33 % of variables fit the response. The "Pred R-Squared" of 0.9532 is in reasonable agreement with the "Adj R-Squared" of 0.9732." The value of Adeq Precision" is 29.298 indicating an adequate signal. This model can be used to navigate the design space. Coefficient estimates indicate that among all the factors, B and D had a positive effect while A and C had negative effect on mannanase yield. The interactions between the factors AC, BD, and CD had positive effects on enzyme yields with highest improvement by CD. The combined effect of sodium chloride and ammonium chloride had a strong positive effect whereas ammonium chloride alone exerted a negative effect on mannanase production as indicated by the values of t–test.

Table 5

Statistical analysis of central Composite design showing sum of squares, F-value, p-value, F value, coefficient estimate, t-test, confidence level for each variable affecting mannanase activity after backward elimination regression analysis.

Source	Sum of squares	F- value	P- value	Coefficient estimate	Standard error	T- test	Confidene level	
Model	3964.63	96.62	< 0.0001	218.6	0.78	277.23	99.99	Significant
A-Tryptone	55.02	14.75	0.0012	-1.51	0.39	-3.84	99.88	
B-SDS	243.33	65.23	< 0.0001	3.18	0.39	8.07	99.99	
C-NH_4Cl	0.77	0.21	0.6549	-0.17	0.39	-0.45	34.51	
D-NaCl	199.64	53.52	< 0.0001	2.88	0.39	7.31	99.99	
AC	112.25	30.09	< 0.0001	2.64	0.48	5.48	99.99	
BD	109.51	29.36	< 0.0001	2.61	0.48	5.41	99.99	
CD	395.81	106.11	< 0.0001	4.97	0.48	10.30	99.99	
A^2	1192.90	319.78	< 0.0001	-6.59	0.36	-17.88	99.99	
B^2	1381.62	370.37	< 0.0001	-7.09	0.36	-19.24	99.99	
C^2	658.50	176.53	< 0.0001	-4.89	0.36	-13.28	99.99	
D^2	776.96	208.28	< 0.0001	-5.32	0.36	-14.43	99.99	

Std. Dev. 1.93; R-Squared 0.9833; Mean 199.47; Adj R-Squared; 0.9732; C.V. % 0.97; Pred R-squared 0.9530; PRESS 189.68; Adeq Precision 29.298

The graphical representation of the regression equation is presented in the form of contour graphs indicating the interactions between two factors for the optimization of conditions for mannanase production. The contour graph obtained as a function of tryptone concentration versus NH_4Cl concentration indicated that mannanase production increased with the increase of both tryptone and NH_4Cl but at higher concentration of both the parameters the productivity began to decline. The maximum production corresponding to 218.55 IU/gds was obtained in the wheat bran based optimized medium where the concentrations of supplemented tryptone and NH_4Cl were 283.45 mg and 2.05 mg respectively (Fig 3a) while SDS and NaCl were held at 0, 0 coded levels equivalent to 0.80 and 2 mg respectively. The contour graph obtained as a function of SDS concentration versus NaCl concentration showed that the enzyme productivity increased with the concentration of both SDS and NaCl but at higher concentrations of both, the productivity began to decline. The maximum mannanase productivity of 219.52 IU/gds occurred at a concentration of .87 mg of SDS and 2.21 mg of NaCl with Tryptone and NH_4Cl held at 0, 0 coded levels equivalent to 300 and 2 mg respectively (Fig 3b). Fig 3c shows the effect of NH_4Cl and NaCl on mannanase production. Increase in the concentration of both NH_4Cl and NaCl promoted the mannanase production, but at high level of NH_4Cl and NaCl (higher than 2.0 mg of both), enzyme production decreased. The maximum production of mannanase corresponding to 219.04 IU/gds was obtained at 2.11 mg and 2.16 mg of NH_4Cl and NaCl respectively while concentrations of Tryptone and SDS, were held at 0,0 coded levels equivalent to 300 and 2 mg respectively. To evaluate the accuracy of statistical model of response

surface methodology design for mannanase production, attempts were made to maximize the yields using significant variables. Numerical optimization for mannanase production attempted with Design Expert using A (Tryptone 292 mg), B (SDS 0.86 mg), C (NH_4Cl, 2.0 mg), D (NaCl, 2.2 mg), inoculated with 2.5 ml of fungal spore suspension having 2.8×10^7 spores/ml, incubated at 30°C in stationary state for 72 h in 5g wheat bran based medium containing urea, ammonium sulphate, potassium dihydrogen orthophosphate, peptone, yeast extract, meat extract, soyabean meal, calcium chloride, magnesium sulphate, cobalt chloride, zinc sulphate, water, manganese chloride, malt extract, manganese sulphate, sodium nitrate at the levels of 0.60 mg, 0.65 mg, 0.17 mg, 81.42 mg, 10.28 mg, 99.97 mg, 96.26 mg, 1.06 mg, 0.05 mg, 0.002 mg, 0.006 mg, 0.025 mg, 8.85 ml, 0.006 mg, 74.05 mg, .44 mg, predicted the yield of 219.74 IU/gds.

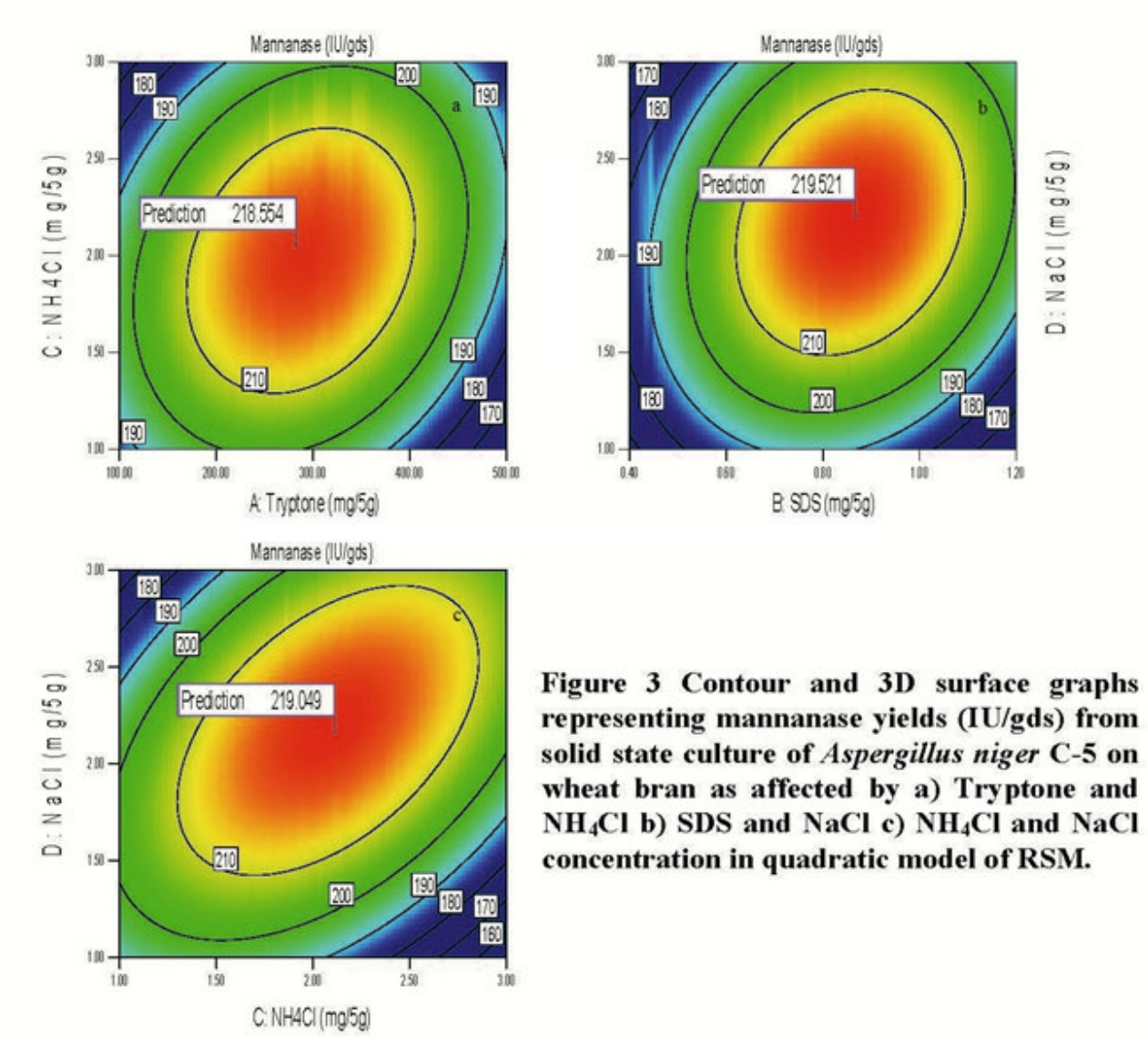

Figure 3 Contour and 3D surface graphs representing mannanase yields (IU/gds) from solid state culture of *Aspergillus niger* C-5 on wheat bran as affected by a) Tryptone and NH_4Cl b) SDS and NaCl c) NH_4Cl and NaCl concentration in quadratic model of RSM.

To validate the optimum concentrations, an experiment with the above specified conditions was performed and the result was 200 IU/gds which is quite close to the predicted value, hence validating that the model chosen for optimization was correct. The yields obtained in the present study on wheat bran based medium are quite appreciating as compared to the published reports. A comparison of the yields of xylanases and mannanases studied in the present work with already published reports is depicted in Tables 6 and 7.

Table 6
Comparison of the productivities of xylanases from Aspergillus niger C-5 with the yields of solid state cultures of other fungal species on various lignocellulosic substrates.

Microorganism	Substrate	Xylanase (IU/gds)	References
Sporotricum thermophile	Wheat bran	334	34
Aspergillus niger LBB326	Sugarcane bagasse + soyabean meal	3099	3
*Thermomyces lanuginosus*195	Wheat bran	2335	35
Penicillium echinulatum 9A02S1	Sugarcane bagasse +wheat bran	36	36
T. aurantiacus	Wheat straw	1315.9	37
T. aurantiacus	Sugarcane straw	1679.5	37
T. aurantiacus	Sugarcane bagasse	978.1	37
T. aurantiacus	Corn cobs	672.9	37
Trichoderma reesei RUT-C30	Wood chips	52.1	38
Trametes versicolor	TP	50	39
Aspergillus niger F3	Carrot peels	65	40
A. oryzae + T.reesei+P. chrysosporium	Dried distillers grains	399.2	41
Trichoderma reesei SAF3	Wheat bran	299.7	42
A. Terrus K1	Palm kernel cake	262.57	43
Fusarium oxysporum SS-25	Brewers spent grain	5874	29
Aspergillus niger C-5	Wheat bran	5200	Present study

Table 7
Comparison of the productivities of mannanase from Aspergillus niger C-5 with the yields of solid state cultures of other fungal species on various lignocellulosic substrates.

Microorganism	Substrate	Mannanase (IU/gds)	References
Aspergillus wentii TISTR 307	Palm kernel meal	380	44
Aspergillus niger USM F4	Palm kernel cake	918	27
A. niger	Wood shavings of G. arborea	25.938	45
Aspergillus niger SN-09	Apple pomace	561.03	46
Aspergillus niger C-5	Wheat bran	200	Present study

The yield of xylanase from *Aspergillus niger* C-5 are quite high as compared to the various published reports except for those reported[29]. Similarly to the best of our knowledge, the yield of mannanases obtained in the present study is quite appreciable. Hence, application of statistical tools in the present study proved to be useful in augmenting the enzyme yields on wheat bran based medium. Several earlier reports have also indicated the effective role of statistical modeling by Plackett-Burman and RSM tools in enhancing the enzyme productivities[30,31,32,33]. This approach of enhancing the enzyme yield by developing a low cost culture medium will certainly reduce the cost of overall production of hemicellulases that are also an important class of enzymes from the industrial point of view.

CONCLUSION

In this study, wheat bran was used as raw material for the production of a mixture of xylanase and mannanase enzyme under solid state fermentation. Statistical optimization of media components and other process variables by employing Plackett-Burmann and response surface methodology designs was carried out that led to an improvement in the yields of xylanase and mannanase revealing 3.72 and 2.08 fold increase in the activities respectively as compared to under unoptimized conditions. Therefore, the availability of low cost hemicellulases will enable more efficient utilization of lignocellulosic fraction by allowing the easy extraction of hemicellulosic sugars from the waste biomass, hence it is worth investigating

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CONFLICT OF INTEREST

No conflict of interest declared.

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