Statistical optimization of media components for xylanase production by solid state fermentation and its application in fruit juice clarification

Abstract: Xylanases are enzymes that convert xylan into xylose, xylobiose, and xylotriose. The present study deals with the production and optimization of xylanase through Solid-State Fermentation (SSF) using different agricultural wastes by *Aspergillus spp*. The Placket Burman (PB) design was used to screen significant media components affecting the xylanase production. The carbon sources screened were wheat bran, rice bran, sugarcane bagasse, corn cob, and orange peel. The nitrogen sources screened were yeast extract, peptone, $(NH_4)_2SO_4$, Na_2NO_3, and urea. Also, nine different salts such as KCl, $MgSO_4$, Na_2HPO_4, $CaCl_2$, $FeSO_4$, $ZnSO_4$, Na_2CO_3, KH_2PO_4, and NaH_2PO_4 which act as trace elements were screened. The results showed that wheat bran, yeast extract, Na_2NO_3 and KCl are the significant factors that affect xylanase production. A 3^3 Full Factorial Design (FFD) was performed to optimize the significant media components (wheat bran, KCl, yeast extract) obtained from PB design using Response Surface Methodology (RSM). Statistical analysis of results showed that wheat bran, KCl, yeast extract, and interaction between wheat bran and yeast extract were found to be significant. The optimum concentration of wheat bran, KCl, yeast extract was 8 g/L, 0.1 g/L, 3 g/L. The Partial purification of xylanase was carried out using ammonium salt precipitation and dialysis. Gel filtration chromatography was performed to optimize the elution time, which was found to be 6 minutes. Application of xylanase in orange juice clarification was studied at 40 °C, 50 °C, and 60 °C. The optimum temperature obtained was 60 ºC.

Keywords
Juice clarification, Partial purification, Placket Burman design, Solid state fermentation, Statistical Optimization, Xylanase.

INTRODUCTION

Xylan is the key structural polysaccharide found in the plant cell walls, and it is the next most widespread in the environment after cellulose. Xylan is a heterogeneous polymer which consists of a linear β-(1,4)-D-xylose backbone, with side chains of α-D-glucuronosyl and α-L-arabinosyl units [1,2]. Xylanase (EC 3.2.1.8) is the key enzyme that depolymerizes the xylan molecules into monomers. These monomers are utilized by microbes as a major carbon source[2,3]. Xylanase is of immense research importance because of its important applications in the field of biotechnology such as the production of ethanol from lignocellulosic waste materials, clarification of juices and beverages, and bread making [4]. Xylanases are useful in the bio-bleaching of wood pulp and in rayon production[5,6].

The enzyme production cost can be reduced by optimizing the process parameters and media components during the fermentation process [7,8]. The conventional approach of optimization is costly and time-consuming [9,10]. Hence statistical methods such as PB design are advantageous to study the effect of a various parameters by performing a minimum set of experiments [11].

Solid-state fermentation (SSF) is preferred over Submerged Fermentation (SmF) because it requires less investment, less wastage of water, more product recovery, high product concentration, lower production cost, and simple cultivation equipment [12,13]. In SSF, various agricultural wastes such as orange peels, sugar cane bagasse, wheat bran, lemon peels and soy bran are used as substrates for fermentation [14]. SSF is used in the production of various commercially important products, such as enzymes, fuels, pesticides, organic acids, secondary metabolites, and aromatic compounds [15].

The present study deals with screening of media components by PB design, statistical optimization of xylanase production by FFD and RSM, partial purification of the enzyme, and application of enzyme in orange juice clarification.

MATERIALS AND METHODS

Isolation of xylanolytic microbes

Several soil samples from different locations of Hubballi and Dharwad were collected and processed for the isolation of microorganisms using standard microbiological spread plate method. The media used for isolation was xylan agar consisting of (g/l): Xylan from Birchwood, 5.0; yeast extract,

5.0; peptone, 5.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.2 and agar,20.0. Different bacterial and fungal strains were isolated and those that are capable of producing xylanase were screened on xylan agar media for xylanolytic activity[16].

Screening for xylanase producing strain

Screening of the bacterial and fungal strains for their ability to produce xylanase was carried out by point inoculation method. The media used was Czapek's agar which contains xylan as the main source of carbon [17]. After inoculation the plates were kept for incubation for 48 h at 37 ^oC for bacterial cultures and for 7 days at 30 ^oC for fungal cultures. To observe zone of clearance formed, 0.1% (w/v) Congo Red was flooded on the plates and incubated for 30 min. Further it was washed with 1 M NaCl. The results indicated that fungal strains showed a higher xylanase activity when compared to bacterial strains and therefore were selected for the production of xylanase by SSF.

Establishment of SSF

SSF was established by considering wheat bran as the substrate. Mineral salt solution was used as a moisturizing agent whose composition is as follows: KCl, 0.5; $CaCl_2.2H_2O$, 0.01; $MgSO_4.7H_2O$,0.5; $ZnSO_4.7H_2O$,0.002; $(NH_4)2HPO_4$, 2.5; $FeSO_4.7H_2O$, 0.01; NaH_2PO_4, 0.5 and birch wood xylan, 1.0 at pH 7. The Salt solution was prepared by constantly heating to dissolve the minerals. An approximate amount of salt solution was added to conical flask containing 5 g of wheat bran to get desired moisture content (V/W %). The salt solution and the substrate was appropriately mixed and autoclaved at 121^oC for 20 minutes [18].

Selection of Fungal strain

Two fungal strains P11 and P15, were considered for SSF. SSF was carried out using wheat bran (substrate) and varying incubation periods. The results indicated, P15 fungal strain was a potent producer of xylanase compared to P11 and therefore was considered for further studies.

Inoculum preparation

Inoculum preparation was carried out by inoculating a loopful P15 fungal culture in 100 mL of inoculation media containing (g/L) glucose, 20.0 ; yeast extract, 3.0 ; Peptone, 5.0 ; NaCl, 15.0 ; Na_2HPO_4 ,11.0 ; NaH_2PO_4 ,6.0 ; KCl, 3.0 ; $MgSO_4$, 0.1 and xylan, 10.0 which was incubated for 7 days at 26-28 °C for the development of spores.

Xylanase production by SSF

SSF was conducted by adding carbon sources, nitrogen sources, and salts as per the high and low values generated in PB design (Table 1). The moisture content was set to 100% using salt solution. After sterilization of the flasks, inoculum of 1% was added aseptically, and it was incubated at 27^oC for 5 days.Substrates in SSF:Wheat bran, sugarcane bagasse, rice bran, corncob and orange peel were taken as substrates [19,20]. These substrates were cut into small pieces and ground to fine powder of maximum particle size limit of 2 mm and dried at 60±5 ^oC for 24 h.

Enzyme extraction and enzyme activity

The incubated flasks were treated with a volume equal to ten times the mass of substrate of 0.1% tween 80 solution and kept in a orbital shaker for 3h at 100 rpm. It was then filtered with muslin cloth. The filtrate obtained was then centrifuged at 8000 rpm for 12 minutes. The supernatant collected was used for the estimation of xylanase activity and protein concentration. The enzyme assay was done using DNS (3, 5-dinitrosalicylic acid) method [21]. Birchwood xylan (1%) added in 0.1M Phosphate buffer (pH 5.0) was used to determine crude enzyme activity. Substrate along with the enzyme was incubated at 37˚C for 1h. The color developed was estimated at 540 nm using a Spectrophotometer (make: Elico). One unit (U) of enzyme activity is expressed as the amount of enzyme releasing 1mmol of reducing sugar equivalent per minute under the assay conditions. The Protein concentration was estimated using Lowry's method [22]. The standard used was Bovine Serum Albumin (BSA). The color developed was measured at 660 nm with the help of Spectrophotometer.

Experimental Design: Screening of media components using PB design

PB design was performed using the statistical tool (Minitab 16) to screen media components: wheat bran (X_1), rice bran (X_2), sugarcane bagasse (X_3), corncob (X_4), orange peel (X_5), yeast extract (X_6),

peptone (X_7), $(NH_4)_2SO_4$ (X_8), Na_2NO_3(X_9) and Urea(X_{10}), KCl(X_{11}), $MgSO_4$(X_{12}), Na_2HPO_4(X_{13}), $CaCl_2$(X_{14}), $FeSO_4$(X_{15}), $ZnSO_4$(X_{16}), Na_2CO_3(X_{17}), KH_2PO_4(X_{18}), NaH_2PO_4(X_{19}) in SSF for the production of xylanase. These nineteen factors were screened in twenty experimental runs. To evaluate the linear effects of various factors, two different levels (Low level: -1 & High level: +1) were considered as shown in table 1. The enzyme activity (U/mL) was determined by taking the average of triplicate experimental values. The significant factors ($p < 0.05$) which affect the enzyme activity were determined from the regression analysis [23].

Table 1: Different media components and their levels in PB Design

Parameters(g/l)	Symbols	Levels	
		Low	High
Wheat bran	X1	2	10
Rice bran	X2	2	10
Sugarcanebaggase	X3	2	10
Corn cob	X4	2	10
Orange peel	X5	2	10
Yeast extract	X6	0.5	5
Peptone	X7	0.5	5
$(NH)_2S0_4$	X8	1	5
Na_2NO_3	X9	1	5
Urea	X10	1	5
KCl	X11	0.1	2
$MgSO_4$	X12	0.1	2
Na_2HPO_4	X13	0.1	2
$CaCl_2$	X14	0.01	0.1
$FeSO_4$	X15	0.01	0.1
$ZnSO_4$	X16	0.01	0.1
Na_2CO_3	X17	0.1	2
KH_2PO_4	X18	0.1	2
NaH_2PO_4	X19	0.01	0.1

Optimization by Response Surface Methodology

The significant factors obtained from PB design were selected and used for optimization using RSM [14]. The optimal conditions for the enzyme production was determined using 3^3 full factorial design with 27 experimental runs. Enzyme activity (U/mL) was chosen as a dependent variable for this study, whereas wheat bran, yeast extract and KCl were considered as independent variables whose levels are shown in table 2.

Partial Purification of Xylanase

Ammonium sulphate precipitation: Ammonium sulphate (85% saturation) was added to 50ml of the supernatant and left overnight. The next day, the supernatant was centrifuged at 8000rpm for 15 minutes to get the precipitate. The above procedure was performed at 4 °C [15].

Dialysis: The precipitate obtained after ammonium sulphate precipitation was suspended in 0.05M potassium phosphate buffer (pH: 7.0) and then dialyzed against the same buffer at 4 ºC for about 3 h on a magnetic stirrer by changing the buffer every hour [16,18].

Gel filtration Chromatography: The dialyzed sample (5 mL) was chromatographed on sephadex G-50 column equilibrated, and eluted with 0.05 M phosphate buffer of pH 7.0, flowing at 0.375 mL/h. The protein content of the fractions (1.5 mL) was estimated using spectrophotometer at 280nm. The DNS method was used to determine the xylanase activity [15,16].

Application of Xylanase in Fruit juice clarification

The clarification process of orange juice was performed using xylanase. A comparative study of clarification was performed using crude enzyme, purified enzyme, and standard commercial xylanase enzyme. The extracted orange juice was filtered using muslin cloth. To determine % clarification at various temperatures, the enzyme, and the orange juice were mixed in the ratio of 1:10. The clarification was studied at 40,50, and 60 °C for 90 minutes. Further the samples were heated in water bath at 100°C for 5 minutes for inactivation of the enzyme. After boiling, the samples were centrifuged at 8000 rpm for 15 min. The supernatants were studied for yield, clarity and reducing sugar. The juice clarity was determined using spectrophotometer at 660 nm. The juice yield was determined by measuring its volume after centrifugation. DNS assay was used to determine the reducing sugars. Untreated fruit juices were considered as control [17,18,19].

RESULTS AND DISCUSSION

Screening of Fungal Strains

Based on the batch studies, the xylanase enzyme production by P11 and P15 strains were found to be higher on the 7th day when compared to 5th and 6th day. The strain P15 showed higher xylanase production compared to P11 strain on 7th day. Thus P15 strain was considered to be a potent producer of xylanase and was used for further studies. The results of which are represented in figure 1.

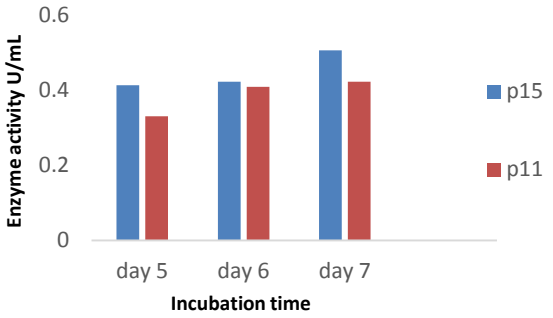
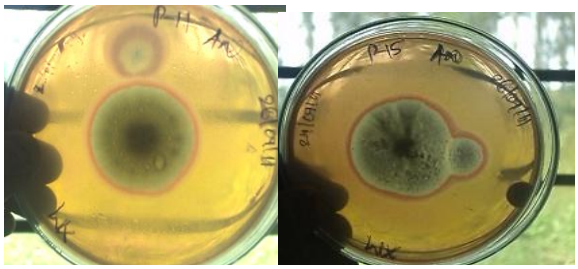


Figure 1: Production of xylanase enzyme using P11 and P15 strains.

The screening of the isolates was performed by estimating the zone of clearance formed on xylan agar plates. The results of which are depicted in figure 2.



(a) (b)

Figure 2 (a). *Aspergillus*species (strain P11) (b) *Aspergillus* species (strain 15) on xylan agar plate showing xylanolytic activity (Orange hallows).

Screening by Placket Burman design

The variables significantly affecting the response with two-factor interactions were analyzed in PB design. The design matrix with the response (Enzyme Activity) is shown in table.2. The Pareto chart shows the effect of the media components on enzyme activity. The results indicated that except $MgSO_4$, NaH_2PO_4, sugarcane, and $(NH_4)_2SO_4$ all other components have a significant effect on xylanase activity as shown in figure 3. Among them, wheat bran (P-value 0.006), yeast extract (P-value 0.011), KCl (P-value 0.008) showed a major effect on xylanase production as shown in table.3. Xylanase activity was found to be in the range of 15.792 U/mL to 30.66 U/mL. Similar results were obtained by other researchers [12,18]

The maximum enzymeactivityobserved from screening the process parameters was found to be 30.66 U/mL which was the 10^{th} run consisting of wheat bran, yeast extract, peptone, urea, Na_2HPO_4, $CaCl_2$, $FeSO_4$, $ZnSO_4$ K_2HPO_4. In addition, the R^2 value predicted was 99.15% which indicates the model is of good quality.

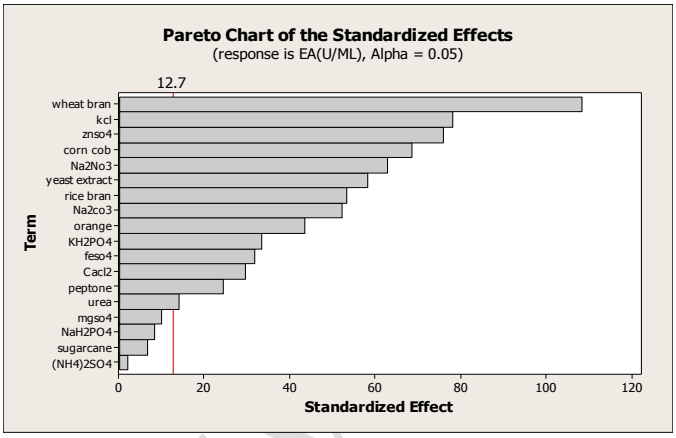


Figure 3. Pareto Chart showing the effect of media components for xylanase production using PB design.

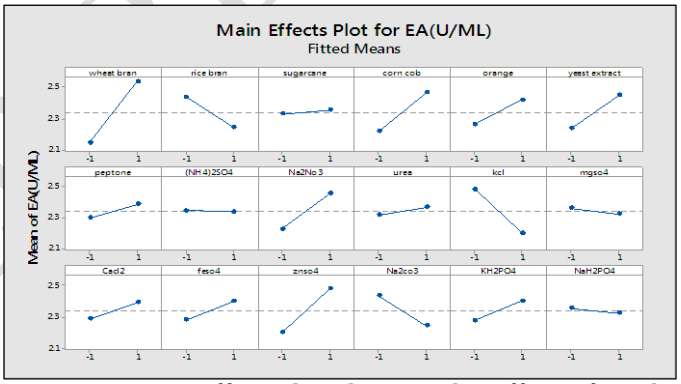


Figure 4. Main effect plot showing the effect of media components for xylanase production using PB design.

Main effect plots aim to analyse the average outcome (xylanase activity) for individual parameters. In the present study, it was observed that the average outcome for wheat bran was highest at a higher level in comparison with all other factors considered. Thus, we can conclude wheat bran potentially influences the xylanase activity. Corn cob, Orange, yeast extract, Na_2NO_3, $ZnSO_4$ show higher activity at higher levels. Also,Rice bran, KCl, Na_2CO_3 show higher activity at low levels indicating that they are required in low quantities for a higher xylanase activity. While, the main effect plot for Sugar cane, peptone, $(NH_4)_2SO_4$, Urea, $MgSO_4$, NaH_2PO_4 are almost horizontal indicating they have no significant effect on xylanase activity.

Table 2: PB Design matrix in coded form with the response (enzyme activity in U/mL)

Run no	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{II}	X_{12}	X_{13}	X_{14}	X_{15}	X_{16}	X_{17}	X_{18}	X_{19}	EA U/mL	protein µg/mL
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	29.84	242.06
2	-1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	19.01	239.41
3	-1	-1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	20.53	112.83
4	1	-1	-1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	27.41	137.08
5	1	1	-1	-1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	21.62	304.00
6	-1	1	1	-1	-1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	20.82	168.75
7	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	17.19	202.00
8	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	22.85	263.04
9	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	1	-1	1	-1	1	22.65	272.29
10	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	1	-1	1	-1	30.66	152.20
11	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	1	-1	1	15.79	61.12
12	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	1	-1	26.22	169.66
13	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	1	22.13	110.83
14	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	20.49	69.20
15	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	22.43	95.58
16	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	23.79	176.70
17	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	22.96	126.31
18	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	25.95	157.79
19	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	27.45	176.45
20	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	28.28	246.35

X_1: wheat Bran, X_2:Rice bran, X_3: Sugarcane Bagasse, X_4:Corn Cob, X_5:Orange peel, X_6:Yeast Extract, X_7:Peptone, X_8:Ammonium Sulphate, X_9:Sodium Nitrate, X_{10}:Urea, X_{11}:KCl, X_{12}:$MgSO_4$, X_{13}:Na_2HPO_4, X_{14}:$CaCl_2$, X_{15}:$FeSO_4$, X_{16}:$ZnSO_4$, X_{17}:Na_2CO_3, X_{18}:K_2HPO_4, X_{19}:NaH_2PO_4

Optimization by Response Surface Methodology

In order to optimize the media components, three factors were considered. 3^3 experiments were performed to check the effect on xylanase activity. The Full factorial design matrix with the response (Enzyme Activity) is shown in table 4. After performing the full factorial design, P-Value was analyzed to check the effect of factors. The factors were considered to be significant whose P-Value is less than 0.15. ANOVA table 5 shows the following P-Values (wheat bran: 0.096, KCl: 0.000, Yeast extract: 0.14). The highest xylanase activity of 26.15 U/mL was observed for 13^{th} run, which consisted of wheat bran and yeast extract at mid-levels of 8 g/L and 3 g/L respectively. While KCl is at low level of 0.1 g/L. The results indicated that wheat bran significantly affects xylanase production at mid-level and decreases at high and low levels. KCl significantly affects xylanase production at a low level, and its effect decreases at a high level. Yeast extract significantly affects Xylanase production at midlevel, and its effect decreases at high and low levels.

Table 3: Different levels of media components in FFD

Independent variables	Symbol	Levels (g/L)		
		Low	Mid	High
Wheat bran	X1	2.0	8.0	14.0
KCl	X2	0.1	1.1	2.1
Yeast Extract	X3	0.5	3.0	5.5

Table 4: FFD matrix with the response (EA)

Run No.	Wheat bran	KCl	Yeast extract	EA (U/mL)
1	2	0.1	0.5	15.05
2	2	1.1	0.5	10.74
3	2	2.1	0.5	09.25
4	8	0.1	0.5	24.00
5	8	1.1	0.5	23.61
6	8	2.1	0.5	20.96
7	14	0.1	0.5	21.49
8	14	1.1	0.5	17.62
9	14	2.1	0.5	14.56
10	2	0.1	3	18.90
11	2	1.1	3	14.36
12	2	2.1	3	12.64
13	8	0.1	3	25.51
14	8	1.1	3	24.12
15	8	2.1	3	23.29
16	14	0.1	3	18.62
17	14	1.1	3	17.94
18	14	2.1	3	15.67
19	2	0.1	5.5	20.68
20	2	1.1	5.5	12.28
21	2	2.1	5.5	16.08
22	8	0.1	5.5	23.71
23	8	1.1	5.5	20.45
24	8	2.1	5.5	20.29
25	14	0.1	5.5	14.08
26	14	1.1	5.5	11.25
27	14	2.1	5.5	09.24

The result showed an average optimum enzyme activity of 27.51 U/ml at 13th run order, where wheat bran is at mid-level,KCl at low-level and yeast extract at mid-level. The lowest enzyme activity was 9.24U/ml at 27th run order, where all media components were at a high levels.

Regression Equation in Uncoded Units

EA = 6.18 + 4.210 Wheat bran - 5.44 KCl
+ 3.333 Yeast extract
- 0.2244 Wheat bran*Wheat bran
+ 1.182 KCl*KCl
- 0.3623 Yeast extract*Yeast extract
+ 0.0269 Wheat bran*KCl
- 0.1839 Wheat bran*Yeast extract
+ 0.097 KCl*Yeast extract

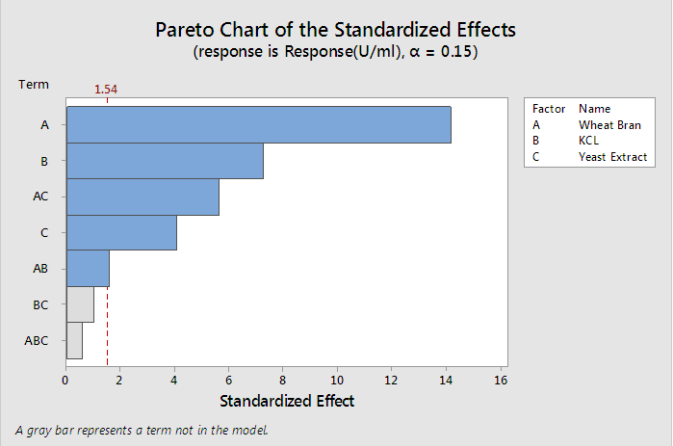


Figure 5. Pareto Chart for RSM showing the interaction effect of different factors on xylanase production.

The Pareto chart distinguishes the high significant factors by identifying the factor affecting the affect response in a significantly. In the present study Pareto chart results indicate that wheat bran potentially affects the xylanase activity in comparison with KCl and Yeast extract. And theinteraction effect between wheat bran and Yeast extract arefound to be more significant than the interaction effect between Wheat bran and KCl. Similar results were obtained by other researchers [15, 16].

Table 5: ANOVA table for RSM

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	632.253	70.250	35.59	0.000
Linear	3	109.116	36.372	18.43	0.000
Wheat bran	1	6.113	6.113	3.10	0.096
KCl	1	98.280	98.280	49.79	0.000
Yeast extract	1	4.723	4.723	2.39	0.140
Square	3	430.817	143.606	72.75	0.000
Wheat bran*Wheat bran	1	391.665	391.665	198.43	0.000
KCl*KCl	1	8.386	8.386	4.25	0.055
Yeast extract*Yeast extract	1	30.766	30.766	15.59	0.001
2-Way Interaction	3	92.320	30.773	15.59	0.000
Wheat bran*KCl	1	0.314	0.314	0.16	0.140
Wheat bran*Yeast extract	1	91.301	91.301	46.26	0.000
KCl*Yeast extract	1	0.706	0.706	0.36	0.558
Error	17	33.556	1.974		
Total	26	665.809			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.40494	94.96%	92.29%	85.49%

From ANOVA Table 5, the following inferences were drawn. The regression model is significant (P-value < 0.15 level of significance).All the main effects and interaction effects were found to be significant except the interaction of KCl *Yeast Extract (P-value< 0.15 level of significance). The third order interaction between Wheat bran* KCl *Yeast Extract is found to be insignificant.The adequacy of the model can be verified by the coefficient of determination. From the regression analysis, the coefficient of determination(R^2) was found to be 94.96% (0.9496) which is very close to 1. This indicates 94.96% of total variability is explained by the regression model. The adjusted R^2 value is 92.96% and the predicted R^2 value is 85.49%.

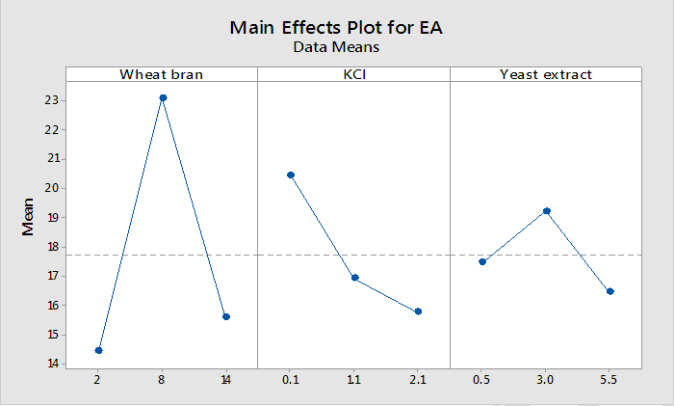


Figure 6:Main effect plot for xylanase activity

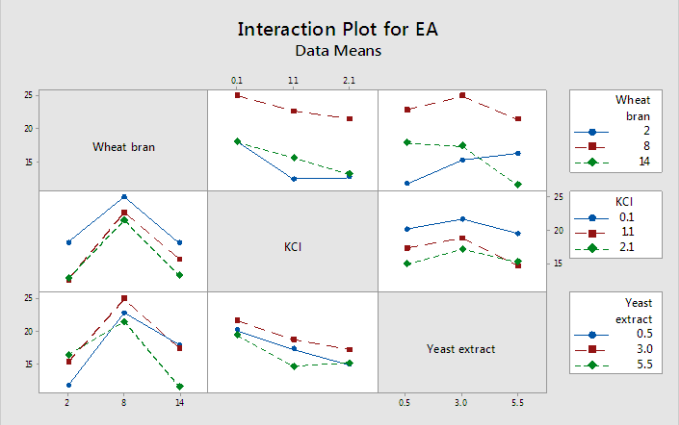
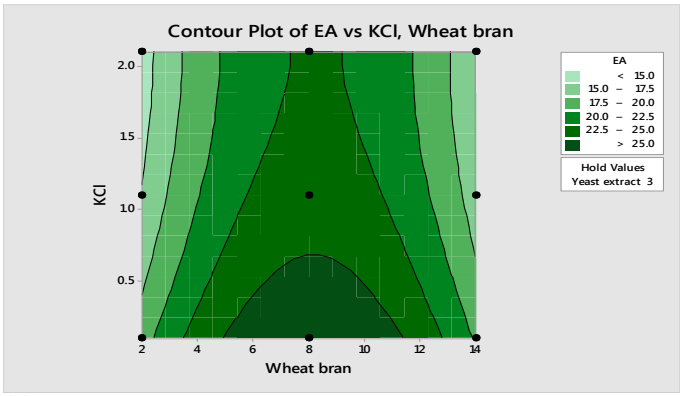
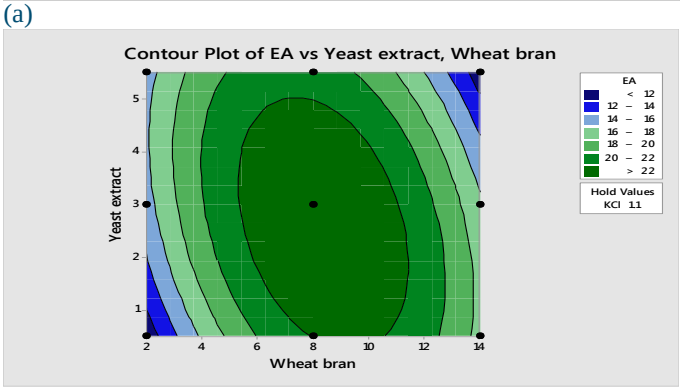


Figure 7: Interaction effect plot for xylanase activity

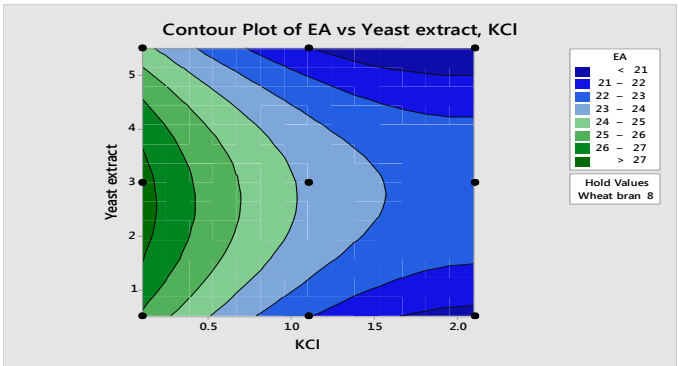
Through main effect plots (Figure6), it was observed that as the quantity of wheat bran increases from low (2g/L) to mid-level (8g/L) the enzyme activity increases from 14 U/mL to 23U/mL and then decreases to 15 U/mL as wheat bran is further increased to 14 g/L.Similarly, it was observed that as KCl increases from low (0.1 g/L) to mid-level (1.1 g/L) the enzyme activity decreases from 20U/mL to 17 U/mL and then further decreases to 15U/mL as KCl is further increased to 2.1 g/L.Similarly, it was observed that as yeast extract increases from low (0.5g/L) to mid-level (3.0 g/L) the enzyme activity increases from 17.5 U/mL to 19.5 U/mL and then decreases to 16.5 U/mL as yeast extract is further increased to 5.5 g/L. From the Interaction Plot (Figure 7) it can be inferred that there is considerable interaction among themedia parameters (wheat bran* Yeast extract).Similar results were obtained from other workers [16,18].



(a)



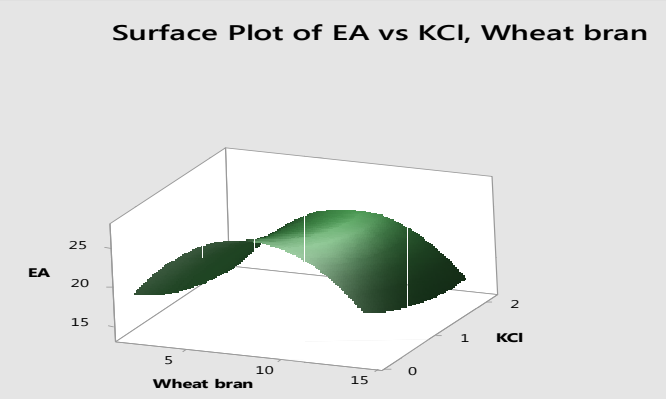
(b)



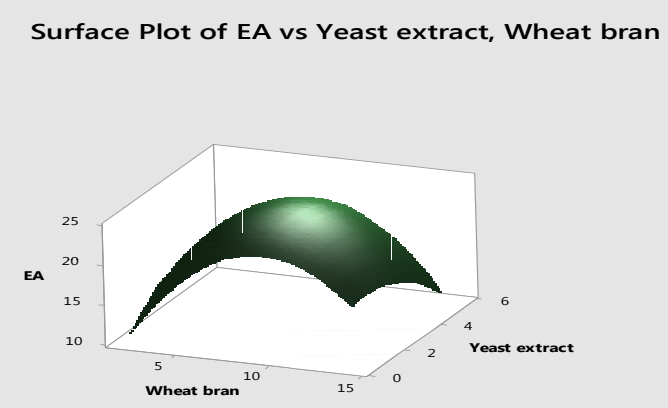
(c)

Figure 8: Counter plots representing interaction effect of (a) KCl and Wheat bran, (b) Yeast extract and Wheat bran, (c) Yeast extract and KCl.

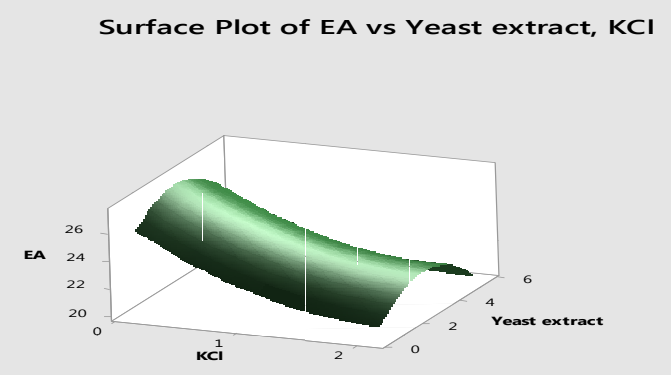
From contour plots, as shown in figure 8(a) it was observed that maximum enzyme activity was seen for low level of KCl and mid-level of wheat bran. Similarly, in figure 8(b), maximum enzyme activity was observed at mid-level of yeast extract and wheat bran. In figure 8 (c), the maximum enzyme activity was observed at mid-level of yeast extract and low level of KCl.



(a)



(b)



(c)

Figure 9: Surface plots representing interaction effect of (a) wheat and KCl, (b) wheat bran and yeast extract, (c) KCl and yeast extract

3D surface plots as shown in figure 6 were plotted to study the interaction effects of media components on enzyme production. In surface plots, the vertical axis represents Enzyme Activity (EA), and two horizontal axes representing the levels of two independent variables, by keeping other variable at its control level. From the plots, it can be inferred that there is a nonlinear effect of the factors on enzyme activity.

Model validation

Experimental validation of the regression model was performed by carrying out experiments at the optimum settings predicted by RSM optimizer as shown in figure 10. The experiments were performed in triplicates as per the optimum settings as shown in the table 6 and enzyme activity was found to be 26.15 U/ml, which is in good agreement with the model predicted value of 27.43U/ml. Hence Model is validated as model output is in line with observed data.

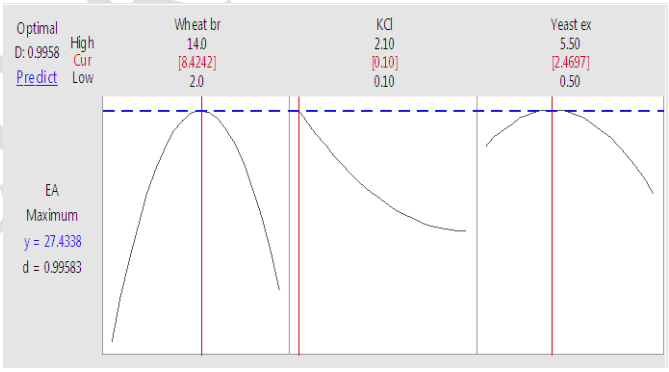


Figure 10: RSM Optimizer showing optimized values of media components

Table 6: Optimal Settings of media components

Parameter	Parameter Settings from RSM Optimizer	Model predicted value (U/mL)	Experimental value (U/mL)
Wheat bran(g/L)	8.42		
KCl (g/L)	0.1	27.43	26.15
Yeast Extract(g/L)	2.47		

Partial purification of Xylanase enzyme

Ammonium sulphate precipitation

The enzyme produced in solid state fermentation was purified by ammonium sulphate fractionation followed by dialysis. The results indicated that maximum enzyme activity was observed after

dialysis 3.231 U/mL with specific activity of 0.0237 U/µg.

Gel filtration Chromatography

Purification of xylanase was performed by gel filtration Chromatography on Sephadex G-50. At 6th minute the maximum xylanase activity was obtained. The activity was high from volume fraction 2 to 7.Hence it was concluded that the elution time of 6 min is ideal for the purification of xylanase enzyme.

Table 7: Partial purification of xylanase enzyme

Purification step	Total Protein (µg/ml)	Total Enzyme Activity (U/ml)	Specific activity (U/µg)	Purification fold
Crude Enzyme	13104.3	130.65	0.00997	1.0
$(NH_4)_2SO_4$	2203.2	27.54	0.0125	1.25
Dialysis	1363.29	32.31	0.0237	2.37

Application of xylanase in fruit juice clarification

The orange juice clarification carried out at 60°C liberated high reducing sugar for pure enzyme (10.477 µM/mL) and higher %clarity was achieved at 60°C for pure enzyme (85%). The results indicated that the samples treated with pure enzyme showed maximum % clarity and high reducing sugar were liberated. The results of which are indicated in Table 8.

Table 8: Results of orange juice clarification

Temperature ˚C	Type of Enzyme	%clarity	Reducing Sugar (µM/mL)
R T (Control)	-	-	8.827
40	Pure	83	9.235
	Crude	66	8.996
	Partially purified	80	9.0669
50	Pure	83	10.447
	Crude	68	10.0105
	Partially purified	79	10.072
60	Pure	85	10.477
	Crude	79	10.2669
	Partially purified	80	10.1662

CONCLUSIONS

In the present work, optimization of xylanase production was carried out using solid state fermentation by PB Design and RSM-FFD. The applied Statistical tools proved to be efficient for optimizing xylanase enzyme production by locally isolated *Aspergillus* species. Plackett-Burman design was used to test the relative importance of medium components on xylanase production. Among the several variables, Wheat bran,Rice bran,Sugar cane bagasse,Corn cob, Orange peel, Yeast extract, Peptone, Na_2NO_3, Urea, KCl, Na_2HPO_4, $CaCl_2$, $FeSO_4$, $ZnSO_4$, Na_2CO_3 and KH2PO4werefound to be significant.Response surface methodology using Full Factorial designwas proved to be a powerful statistical tool for optimization of media components for the enhanced production of xylanase. Maximum production of enzyme was obtained with the media composition of wheat bran (8.4g/L), KCl (0.1g/L) and yeast extract (2.5g/L). Under optimal conditions of independent variables, the experimental responses showed close agreement with predicted responses, confirming the validity of regression model.Partial purification of xylanase produced was carried out using ammonium sulphate precipitation and dialysis. Application of xylanase in orange juice clarification was studied and optimum temperature was found to be 60ºC.

REFERENCES

1. Pooja K. Mahale, Desai SV, Hombalimath VS, Sharanappa Achappa. Isolation, screening and characterization of lipase producing strain from oil contaminated Soil of Hubballi, Karnataka; International Journal of Basic and Applied Biology. 2015;2(4):198-201.
2. Veeranna S. Hombalimath, Basavaraj B. Udapudi, Laxmikant R. Patil, Anil R. Shet**,** Deepak A. Yarguppi. Isolation and Characterization of Lipolytic microorganisms from oil-contaminated soil. International Journal of Advances in Engineering Science and Technology. 2012;2(3):293-297.
3. Sharanappa Achappa, Patil LR, Hombalimath VS, Anil R. Shet. Implementation of Project-Based-Learning (PBL) Approach for Bioinformatics Laboratory Course, Journal of Engineering Education Transformations. 2020;33 (Special issue):247-252.

4. Sharanappa A, Anil R. Shet, Laxmikant R. Patil, Veeresh S. Hombalimath, Santosh Kadapure. Biosynthesis of silver nanoparticles using Citrus sinensis peel extract and their application as antibacterial agent, International Journal of Research in Pharmaceutical Sciences. 2020;3:4726-4732.
5. Laxmikant R. Patil, Anil R. Shet, Arati G. Lohar, Gururaj B. Tennalli, Sharanappa A, Hombalimath VS. Optimization of Process Parameters for Synthesis of Silver Nanoparticles Using Leaf Extract of Tridax Procumbent and Its Biotechnological Applications. International Journal of Scientific & Technology Research. 2020; 9(6):1050-1056.
6. Sharanappa Aachapa, Veeranna S Hombalimath, Jayshree R. Kamaraddi, Shivalingsarj V Desai, Anil R Shet, Laxmikant R Patil, Jagadish R PatilStatistical Optimization of Lipase Production from Bacillus Species by Submerged Fermentation, Bioscience Biotechnology Research Communications. 2021;14(1):264-269.
7. Anil R. Shet, Sanjana More, L. R. Patil, Sharanappa A, V. S. Hombalimath and Gururaj Tennalli. Immobilization of Xylanase in PVA-Alginate Matrix and its Characterization, World Journal of Pharmaceutical and Life Science. 2020; 6(3):88-91.
8. Sharanappa A, Wani KS, Pallavi Patil. Bio processing of Food Industrial waste for α-amylase Production by Solid-State Fermentation, International Journal of Advanced Biotechnology and Research. 2020;2(4):473-480.
9. Aishwarya Kulkarni, Desai SV, Anil R. Shet. Isolation and characterization of pigment producing actinomycetes from different sources. Research Journal of Pharmaceutical, Biological and Chemical Sciences. 2012;8(3):101-109.
10. Sharanappa A, Patil LR, Hombalimath VS, Deepak Yaraguppi, Anil R Shet. Application of Statistics in Bioprocess Engineering Laboratory to Reinforce Students' Ability in Data Collection, Analysis and Interpretation. Journal of Engineering Education Transformations. 2018;Special Issue. eISSN: 2394-17.
11. Laxmikant Patil, Gururaj Bhadri, Shivalingsarj Deasi, Anil Shet, Veeresh Hombalimath. Application of Statistical Modeling and Hypothesis Testing to Reinforce Model Validation Concepts in Bioprocess Control Laboratory. Journal of Engineering Education Transformations. 2021;Special issue. eISSN: 2394-1707.
12. Bhavikatti S, Saikrishna Rahul M, Bodducharl, Rahul S. Kamagond, Shivalingsarj V. Desai, Anil R. Shet. Statistical optimization of protease production using a freshwater bacterium *Chryseobacterium cucumeris* SARJS for multiple industrial applications. Journal of 3 Biotech. 2020; 10:279.
13. Anil R Shet, Laxmikant R Patil, Veeranna S Hombalimath, Sharanappa Achappa. Parametric optimization of oil extraction and lipase catalyzed biodiesel production from rice bran. Journal of Bioscience Biotechnology Research Communications. 2021 14(1):340-345.
14. Adriana Knob,SusanMichelzBeitel,DianaFortkamp,César Rafael FanchiniTerrasan, and Alex Fernando de Almeida. Production, Purification, and Characterization of a Major *Penicilliumglabrum* Xylanase Using Brewer's Spent Grain as Substrate. BioMed Research International.2013,8-16.
15. Petroskatapodis,vassilikichristakopoulou,Dimitris kekos paulchristakopoulos,Optimization of Xylanase Production by *chaetomiumthermophilum* in wheat straw Using Response Surface Methodology. Biochemical Engineering Journal, 2008;35(2):136-141.
16. SomeetNarang, Vikram Sahai, Virendra S. Bisaria. Optimization of Xylanase Production by *Melanocarpusalbomyces* IIS68 in Solid State Fermentation Using Response Surface Methodology. Journal of Bioscience and Bioengineering. 2001; 91(4):425-7.
17. UttamBudhathokiPanna Thapa, Ellaiah Poluri. Medium optimization of production of xylanase by solid state fermentation from *Brevibacillusborstelensis* – MTCC 9874 isolated from soil sample of eastern Nepal. Malaysian Journal of Microbiology. 2011;7(2):83-91.
18. Tony J.Fang,Bo-Chin Liao, Shu-ChihLee. Enhanced Production of Xylanase by *Aspergillus carneus* M34 in Solid – State Fermentation with Agricultural Waste Using Statistical Approach. New Biotechnology. 2010; 27.
19. Reeta Rani Singhania,Anil Kumar Patel ,Carlos R. Soccol, Ashok Pandeya. Recent advances in solid-state fermentation. Biochemical Engineering Journal. 2009; 44:13–18.
20. Marcelo A. Umsza-Guez, Ana B. Díaz, Ignacio de Ory' Ana Blandino 'Eleni Gomes, Ildefonso Caro. Xylanase production by *Aspergillus awamori* under solid state fermentation conditions on tomato pomace. Brazilian Journal of Microbiology. 2011; 42.
21. G.L Miller,Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Analytical Chemistry.1959; 31 (3):426–428.
22. Lowry OH, Rosenbrough NJ and Farr A. Randall RJ. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry.1951;193:265-275.
23. OlfaEllouze,SamiFattouch,FaouziMestiri,MohamedRadhouenAniba, Mohamed NejibMarzouki. Optimization of extracellular Xylanase production by *Sclerotiniasclerotiorum* S2 using

factorial design. Indian Journal of Biochemistry and Biophysics.2008;45:404-409.