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ORIGINAL ARTICLE

Statistical optimization of alkaline xylanase production from *Streptomyces violaceoruber* under submerged fermentation using response surface methodology

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Abstract Response surface methodology employing central composite design (CCD) was used to optimize fermentation medium for the production of cellulase-free, alkaline xylanase from *Streptomyces violaceoruber* under submerged fermentation. The design was employed by selecting wheat bran, peptone, beef extract, incubation time and agitation as model factors. A second-order quadratic model and response surface method showed that the optimum conditions for xylanase production (wheat bran 3.5 % (w/v), peptone 0.8 % (w/v), beef extract 0.8 % (w/v), incubation time 36 h and agitation 250 rpm) results in 3.0-fold improvement in alkaline xylanase production (1500.0 $IUml^{-1}$) as compared to initial level (500.0 $IUml^{-1}$) after 36 h of fermentation, whereas its value predicted by the quadratic model was 1347 $IUml^{-1}$. Analysis of variance (ANOVA) showed a high coefficient of determination (R^2) value of 0.9718, ensuring a satisfactory adjustment of the quadratic model with the experimental data.

The economical and cellulase-free nature of xylanase would enhance its applicability in pulp and paper industry.

Keywords Alkaline · CCD · RSM · *Streptomyces violaceoruber* · Xylanase

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Introduction

Xylan is the main hemicellulosic polysaccharide found in plant cell walls and is composed of a backbone chain of 1→4 linked β-xylosyl residues and short side chains of arabinosyl, glucoronosyl and acetyl residues. Xylanase (endo-β-1,4-xylanase) and β-xylosidase (β-D-xyloside xylohydrolase) the main constituents of the xylanolytic enzyme system, convert xylan into a more readily fermentable form[1–3]. In recent years, interest in xylanases has increased markedly due to their usage in the pulp and paper industry[1,4–7]. Other potential applications include the clarification of fruit juices and wine, the extraction of plant oil, coffee and starch, the production of oligosaccharides and improvement of the nutritional value of animal feed[2,8–10].

It is well documented that extra cellular xylanase production by microbes is greatly influenced by media components, especially carbon and nitrogen sources, minerals and physical factors such as pH, temperature, agitation, dissolved oxygen and inoculum density[1]. In order to obtain optimum yield of an enzyme, development of a suitable medium and cultural conditions is obligatory. Statistical optimization not only allows quick screening of a large experimental domain, but also reflects the role of each of the components. Response Surface Methodology (RSM), a collection of mathematical and statistical techniques for building empirical models[11], is gaining importance for optimizing conditions for the production of industrially important products such as chemicals and enzymes[12–15] and for studying enzyme kinetics[16]. Here the term ‘‘optimum conditions’’ means the operating conditions for maximizing the production of enzymes. The optimum result is called the stationary point of minimum response or a saddle point[17].

The optimal design of the culture medium is a very important aspect in the development of fermentation processes. Optimization studies help in understanding the interactions among the nutrients at varying concentrations and in calculating the optimal concentration of each nutrient for a given target, i.e., maximal enzyme production in less time and at lower cost. The determination of an optimized factor level by standard univariate techniques is not always feasible for enzyme production. Multivariate experiments are designed to reduce the number of experiments necessary in the optimization process and to produce more precise results than those obtainable by univariate strategies[18]. This investigation deals with the optimization of the most important fermentation variables using RSM for the production of alkaline xylanase from *Streptomyces violaceoruber*.

Material and Methods

Microorganism

Streptomyces violaceoruber was isolated from effluent of pulp and paper industry and maintained on Horikoshi agar medium[19] (pH 8.5) containing (% w/v): glucose, 0.5; peptone, 0.5; yeast extract, 0.5; KH_2PO_4, 0.1; $MgSO_4.7H_2O$, 0.01; agar, 20.0.

16S rDNA sequencing

The actinomycete isolate was identified by phylogenetic analysis of a partial 16S rDNA sequence according to TREEVIEW[20]. A 500-bp 16S rDNA sequence was amplified with Microseq 500 16S rDNA PCR module (PE Applied Biosystems). The reaction mixture (50 µl) contained 25 µl of diluted genomic DNA and 25 µl of the ready reaction mixture. Cycling conditions for the amplification reaction were initial denaturation at 95°C for 10 min, 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s (rapid thermal ramp of 1°C/s between steps), final extension at 72°C for 10 min, and lastly a 4°C soak. The PCR products were purified with a Microcon PCR centrifugal filter device (Millipore Corp. Bedford, Mass.) according to the manufacturer's protocol. The purified DNA was recovered in 25 µl of deionized water. The amplified 16S rDNA was subjected to cycle sequencing with the Microseq 500 16S rDNA-sequencing module. The reaction mixture (20 µl) contained 3 µl of purified PCR product, 4 µl of deionized water, and 13 µl of sequencing reaction mixture (forward and reverse sequencing mixture in separate reactions). The cycling conditions were 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min (rapid thermal ramp of 1°C/s between steps), followed by a 4°C soak. The cycle-sequenced DNA was precipitated with ethanol (95%) and 3 M sodium acetate (pH 4.6) and finally analyzed with an ABI Prism 310 genetic analyzer (PE Applied Biosystems).

Xylanase production

Each 250 ml Erlenmeyer flask containing 50 ml of Horikoshi medium containing (% w/v): wheat bran, 2.5, peptone, 0.6, beef extract, 0.6, KH_2PO_4, 0.1, $MgSO_4.7H_2O$, 0.01; pH 8.5 was inoculated with 1% (v/v) of 36 h old seed culture of *Streptomyces violaceoruber* and incubated at 37°C for 54 h under shaking (200 rpm). The cell free supernatant obtained by centrifugation at 10,000 x g, for 20 min. at 4°C was assayed for xylanase activity.

Enzyme assay

The xylanase activity was determined by measuring the release of reducing sugars from birch wood xylan (1% w/v) using dinitrosalicylic acid method[21]. One unit of xylanase was defined as amount of enzyme required to release one µmol of xylose from birch wood xylan in one min under the assay conditions (60°C, 200mM glycine-NaOH buffer, pH 9.0).

Response surface methodology

The conventional one-factor-at-a-time method was used to select the effective factors and the initial test range of each of five variables: wheat bran (A), peptone (B), agitation (C), incubation period (D) and beef extract (E). Taking these factors into consideration, a response surface methodology using central composite design was adopted for improving xylanase production from *Streptomyces violaceoruber*. The statistical software package 'Design-Expert® 6.0', Stat-Ease, Inc., Minneapolis, USA was used to analyze the experimental design. A 2^5 factorial central composite experimental design, with five factors and eight replicates at the centre point, leading to a set of 50 experiments, was used to optimize the production of an alkaline xylanase from *Streptomyces violaceoruber*. All the variables were taken at a central coded value considered as zero. The minimum and maximum ranges of variables investigated, and the full experimental plan with respect to their values in actual and coded form, are listed in Table 2. The average maximum xylanase activity was taken as the dependent variable or response (Y). Regression analysis was performed on the data obtained.

Table 1 Characteristics of *Streptomyces violaceoruber.*

Characteristics	Results
Gram Character	+
Morphology	Filamentous, Rod shape
Substrate mycelium	Yellow
Aerial mycelium	White
Spore mass	Grey color
Metabolism	Aerobic
Catalase	+
Oxidase	+
Melanin pigment	+
Nitrate reduction	–
Phenylalanine deaminase	–
Indole formation	–
Methyl red	–
Voges-Prauskaur	–
Growth temperature	25–55°C
Growth pH	3.0–10.0
Growth in NaCl	0–2.0% (w/v)
Hydro Hydrolysis profile	
Xylan, Tween-40, Tween-80, tributyrin, starch, casein, phytic acid	+
Urea, pectin, tannic acid, poly R-478, Avicel cellulose, chitin, mannan	–
Utilization of carbon sources	
Lactamide, α-hydroxybutyric acid, β-hydroxybutyric acid, α-methyl-D-mannoside, α-methylgalactoside, β-methyl- galactoside, 3-methylglucose, γ-hydroxybutyric acid, p-hydroxy phenylacetic acid, α-ketovaleric acid, glucose-1-phosphate‡, fructose-6-phosphate‡, arabinose*, glucose, cellobiose*, glycerol†, maltose, adenosine-5'-monophosphate‡, lactose†, ribose, xylose, mannose, sucrose, fructose, α-ketoglutaric acid, uridine‡, xylitol, trehalose, melezitose*, palatinose, psicose, salicum, sedoheptulosan, inositol, inosine, lactulose, stachyose, tagatose, esculin, galactose, arbitol, arbutin, gluconic acid	+
Citrate, dulcitol, alginate, inulin, raffinose, rhamnose, adonitol, mellibiose, dextrin, mannitol, sorbitol, malonate, maltotriose, succinic acid, L-alaninamide, 2,3-butanediol, α-methyl-D-glucoside, β-methyl-D-glucoside, glucose-6-phosphate, D-L-α-glycerolphosphate, thymidine-5'-monophosphate, uridine-5'-monophosphate, thymidine, adenosine	
Utilization of nitrogen sources	
Valine, phenylalanine, tryptophan, arginine, isoleucine, L-alanine†, L-alanyl-glycine, L-asparagine, L-glutamic acid, glycyl-L-glutamic acid, L-pyroglutamic acid, L-serine, putrescin	+
L-alaninamide, D-alanine, proline	–

*96 h of incubation; ‡120 h of incubation; †240 h of incubation

Table 2 Experimental range and levels of five independent variables studied using CCD in terms of actual and coded factors.

Variable	Coded level of variable				
	– α	– α	0	+1	+α
Wheat bran (%w/v)	0.5	1.5	2.5	3.5	4.5
Peptone (%w/v)	0.2	0.4	0.6	0.8	1.0
Agitation (rpm)	100	150	200	250	300
Incubation period (h)	18	36	54	72	90
Beef extract (%w/v)	0.2	0.4	0.6	0.8	1.0

The results of a CCD were used to fit a second-order polynomial equation represented as:

$$Y=\beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_4D + \beta_5E + \beta_{12}AB + \beta_{13}AC + \beta_{14}AD + \beta_{15}AE + \beta_{23}BC + \beta_{24}BD + \beta_{25}BE + \beta_{34}CD + \beta_{35}CE + \beta_{45}DE .$$

Where: Y = Predicted response, β_0 = Regression coefficient, $\beta_1\beta_2\beta_3\beta_4\beta_5$ = Linear coefficients

$\beta_{12}\beta_{13}\beta_{14}\beta_{15}\beta_{23}\beta_{24}\beta_{25}\beta_{34}\beta_{35}\beta_{45}$ = Interaction coefficients.

Analysis of variance (ANOVA) was performed. The proportion of variance explained by the polynomial models obtained was given by the multiple coefficient of determination, R^2. In order to confirm the maximum xylanase production predicted by the model, a new set of production was performed using the optimal conditions as indicated.

Results and Discussion

The morphological characters of actinomycete isolate included the presence of highly differentiated branched mycelia with spores (warty ornamentation) bearing aerial hyphae. The isolate showed Gram positive and acid-fast reaction. The physiological and biochemical features of actinomycete included the ability to grow in a broad range of pH (3.0–10.0) and temperature (25–55°C) with a low salt tolerance level (0–2% w/v), ability to metabolize a wide range of diverse carbohydrates and organic compounds and produce respiratory enzymes (catalase and oxidase). It did not exhibit indole formation and was negative for methyl red, Voges-Proskauer and citrate utilisation tests (Table 1). The 16S rDNA gene sequence showed that the actinomycete isolate exhibited 91% homology with the gene sequence of actinomycete belonging to genus-*Streptomyces* and specie-*violaceoruber* (Accession No AF 434717).

The results of CCD experiments for studying the effect of five independent variables are presented along with the mean predicted and observed responses in Table 3. The regression equations obtained after the ANOVA gave the level of xylanase production as a function of the initial values of wheat bran, peptone, agitation, incubation period and beef extract. The final response equation that represented a suitable model for xylanase production is given below:

$$Y= 13700+2487A + 306.58B + 1105.17C - 1201.25D - 1331.87A^2 - 2190.25B^2 - 1695.75C^2 - 2613.13D^2 + 462.75AB - 32.38AC - 867.37AD - 333.63BC - 81.88BD - 308.50CD$$

where, Y enzyme production, A wheat bran, B peptone, C agitation, D incubation period and E beef extract.

The regression equation indicated that coefficient of determination (R^2) was 0.9718 (a value of $R^2 > 0.75$ indicates the aptness of the model) for xylanase production and thus the model could explain more than 97.18% of variability in the response (Table 4). The R^2 value is always between 0 and 1. The closer the R^2 is to 1.0, the stronger the model and the better it predicts[22] Moreover, R^2 is in reasonable agreement with adjusted R^2 of 0.9524. The R^2 value provided a measure of variability in the observed response values, which could be explained by the experimental factors and their interactions. The adjusted R^2 corrects the R^2 value for the sample size and number of terms in the model. If there are many terms in the model and the sample size is not very large, the adjusted R^2 may be noticeably smaller than predicted R^2. The purpose of statistical analysis is to determine the experimental factors, which generate signals that are large in comparison to noise. The adequate precision measuring the signal to noise ratio was found to be 23.645 (Table 4). A signal to noise ratio greater than 4 is desirable. The model is thus fit and could be used to navigate the design space. The predicted sum of squares (PRESS), which is a measure of how a particular model fits each point in design, was 8.328E+005. The computed *F*-value (49.97), which is the ratio of mean square due to regression to the mean square due to error and indicates the influence (significant or not) of each controlled factor on tested model was significant at high confidence level. The low probability p-value (<0.05) indicated that model terms are significant. For xylanase production, A, C, D, E, A^2, B^2, C^2, D^2, E^2, AC, AD and BE are significant model terms.

The three dimensional response surfaces were plotted to study the interaction among the various factors selected and to determine the optimum concentration for attaining maximum xylanase production. The plots were generated by plotting the response using the z-axis against two independent variables while keeping the other independent variables at their O-level. The coordinates of the central point within the highest contour levels in each of the figures correspond to the optimum concentrations of the respective components.

Figs. 1 & 2 show the response for the interaction of wheat bran (A) with incubation period (D) (Fig. 1) and agitation (C) (Fig. 2). From Figs. 1 & 2, it is clear that xylanase yield increased upon increasing the concentration of wheat bran (up to 3.5%w/v) and decreasing the incubation period to 36 h (1500.0 $IUml^{-1}$). High levels of xylanase production with increase in wheat bran concentration could be due to the fact that wheat bran is a nutrient reservoir for xylanolytic microorganisms since it contains a blend of complex hemicellulose (36%) and substituted insoluble xylan, in addition to 64% digestible nitrogen. The overproduction of xylanase

Table 3 Results of CCD using five independent variables and eight centre points showing observed and predicted response.

Standard order	Wheat barn (%w/v) (A)	Peptone (%w/v) (B)	Agitation (rpm) (C)	Incubation Period (h) (D)	Beef extract (%w/v) (E)	Actual value ($IUml^{-1}$)	Predicted value ($IUml^{-1}$)
1	1.5	0.4	150.0	36.0	0.4	407.0	336.0
2	3.0	0.4	150.0	36.0	0.4	847.0	789.0
3	1.5	0.8	150.0	36.0	0.4	265.0	202.0
4	3.5	0.8	150.0	36.0	0.4	569.0	616.0
5	1.5	0.4	250.0	36.0	0.4	400.0	444.0
6	3.5	0.4	250.0	36.0	0.4	1100.0	1102.0
7	1.5	0.8	250.0	36.0	0.4	354.0	428.0
8	3.5	0.8	250.0	36.0	0.4	1100.0	1047.0
9	1.5	0.4	150.0	72.0	0.4	325.0	417.0
10	3.5	0.4	150.0	72.0	0.4	365.0	446.0
11	1.5	0.8	150.0	72.0	0.4	254.0	341.0
12	3.5	0.8	150.0	72.0	0.4	365.0	330.0
13	1.5	0.4	250.0	72.0	0.4	474.0	404.0
14	3.5	0.4	250.0	72.0	0.4	657.0	639.0
15	1.5	0.8	250.0	72.0	0.4	482.0	447.0
16	3.5	0.8	250.0	72.0	0.4	600.0	641.0
17	1.5	0.4	150.0	36.0	0.8	235.0	301.0
18	3.5	0.4	150.0	36.0	0.8	839.0	879.0
19	1.5	0.8	150.0	36.0	0.8	423.0	354.0
20	3.5	0.8	150.0	36.0	0.8	789.0	892.0
21	1.5	0.4	250.0	36.0	0.8	532.0	434.0
22	3.5	0.4	250.0	36.0	0.8	1223.0	1216.0
23	1.5	0.8	250.0	36.0	0.8	512.0	604.0
24	3.5	0.8	250.0	36.0	0.8	1500.0	1347.0
25	1.5	0.4	150.0	72.0	0.8	321.0	277.0
26	3.5	0.4	150.0	72.0	0.8	458.0	429.0
27	1.5	0.8	150.0	72.0	0.8	254.0	388.0
28	3.5	0.8	150.0	72.0	0.8	569.0	500.0
29	1.5	0.4	250.0	72.0	0.8	154.0	290.0
30	3.5	0.4	250.0	72.0	0.8	654.0	647.0
31	1.5	0.8	250.0	72.0	0.8	623.0	518.0
32	3.5	0.8	250.0	72.0	0.8	654.0	835.0
33	0.1	0.6	200.0	54.0	0.6	290.0	245.0
34	4.8	0.6	200.0	54.0	0.6	1160.0	1161.0
35	2.5	0.1	200.0	54.0	0.6	375.0	377.0
36	2.5	1.1	200.0	54.0	0.6	487.0	442.0
37	2.5	0.6	81.0	54.0	0.6	404.0	344.0
38	2.5	0.6	319.0	54.0	0.6	854.0	871.0
39	2.5	0.6	200.0	11.0	0.6	425.0	496.0
40	2.5	0.6	200.0	97.0	0.1	100.0	114.0
41	2.5	0.6	200.0	54.0	1.1	326.0	326.0
42	2.5	0.6	200.0	54.0	0.6	560.0	516.0
43	2.5	0.6	200.0	54.0	0.6	1370.0	136.0

Table 3 (*Continued*)

Standard order	Wheat barn (%w/v) (A)	Peptone (%w/v) (B)	Agitation (rpm) (C)	Incubation Period (h) (D)	Beef extract (%w/v) (E)	Actual value ($IUml^{-1}$)	Predicted value ($IUml^{-1}$)
44	2.5	0.6	200.0	54.0	0.6	1370.0	1366.0
45	2.5	0.6	200.0	54.0	0.6	1370.0	1366.0
46	2.5	0.6	200.0	54.0	0.6	1370.0	1366.0
47	2.5	0.6	200.0	54.0	0.6	1370.0	1366.0
48	2.5	0.6	200.0	54.0	0.6	1370.0	1366.0
49	2.5	0.6	200.0	54.0	0.6	1370.0	1366.0
50	2.5	0.6	200.0	54.0	0.6	1370.0	1366.0

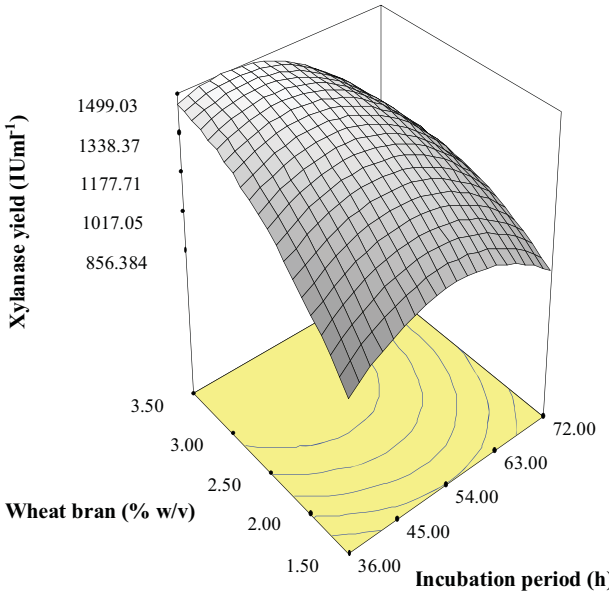

Fig. 1 Response surface plot showing the effect of incubation period and wheat bran on xylanase production.

Table 4 Analysis of variance (ANOVA) for response surface quadratic model for xylanase production.

Term	Xylanase yield
F- value*	49.97
P>F**	0.0500
Mean	684.90
R^2	0.9718
Adj R^2	0.9524
Pred R^2	0.9004
Coefficient of variance	13.16
Adequate precision	23.645
PRESS	8.328E+005

** Value of "P>F" less than 0.0500 indicate model terms are significant.

* The model F-value of 49.97 for xylanase yield implies that the model is significant. There is only 0.01% chance that a "model F-value" this large could occur due to noise.

may also be attributed to the better expression of multiple isoforms on wheat bran, as has been reported previously[23]. For enzyme production no pretreatment was given to the lignocellulosics (wheat bran), which has been often cited in literature[24]. However, decline in enzyme production at higher wheat bran levels (4.5 % w/v) could be due to the formation of a thick suspension and improper mixing of the substrate in shake flasks[25–26]. Reduced synthesis of enzyme in high concentration of xylan containing lignocellulosic material could also be due to the accumulation of more reducing sugars[27].

The yield of xylanase also increased on increasing the agitation up to 250 rpm. Mechanical agitation is known to be a crucial factor in fermentation processes because of its effectiveness in mixing the contents of the medium, uniform air distribution and prevention of cell clumping[28]. There was a significant reduction in xylanase production by *Streptomyces violaceoruber* at higher working volumes

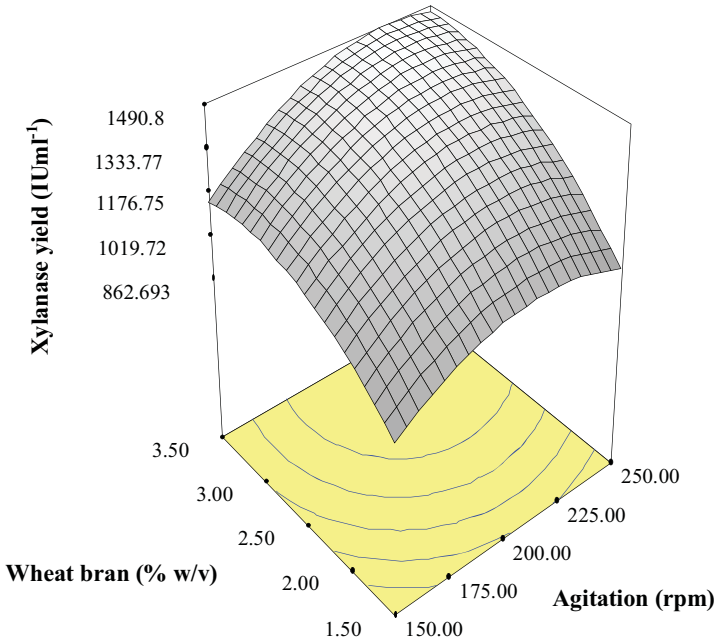

Fig. 2 Response surface plot showing the effect of agitation and wheat bran on xylanase production.

Table 5 Validation of response surface quadratic model for xylanase production.

Experiment No	Wheat bran (% w/v)	Peptone (% w/v)	Agitation (rpm)	Incubation period (h)	Beef extract (% w/v)	Predicted value ($IUml^{-1}$)	Experimental value ($IUml^{-1}$)
1	2.0	0.6	224.0	54.0	0.6	1249.0	1272.0
2	3.0	0.8	235.0	54.0	0.6	1382.0	1396.0
3	3.3	0.8	200.0	45.0	0.6	1364.0	1372.0
4	3.3	0.6	200.0	54.0	0.6	1442.0	1452.0
5	3.4	0.6	200.0	36.0	0.6	1543.0	1472.0
6	3.5	0.6	200.0	40.0	0.7	1503.0	1672.0
7	3.4	0.6	232.0	36.0	0.6	1531.0	1675.0
8	2.7	0.4	232.0	36.0	0.5	1154.0	1160.0
9	3.3	0.4	232.0	42.0	0.6	1406.0	1430.0
10	3.3	0.4	232.0	36.0	0.6	1384.0	1384.0

(above 250 rpm). This may be due to the mechanical shearing of mycelium by high agitation. A similar observation has also been made earlier where shearing forces originating from agitation caused disruption of fungal biomass that resulted in a decreased yield of xylanase[29]. The shear stress sensitivity of mycelium has also been reported in the case of xylanase production by *Thermactinomyces thalophile* subgroup C[30]. Furthermore, the shearing of mycelium at high agitation rates also release intracellular proteins in broth, which increase foam generation during the fermentation process and thus reduce xylanase yield by affecting the oxygen transfer ratios.

To validate and confirm these predictions, 10 experiments were designed with random levels of nutrients. The model was successfully validated as the values predicted by the model were in good agreement with the results obtained on validation for different levels of wheat bran, peptone, beef extract, incubation period and agitation (Table 5).

Over the last few decades, even though several papers regarding optimization of xylanase production have been reported, little information about the optimization of this enzyme production using *Streptomyces* sp. by submerged fermentation is available in the scientific literature. The results of CCRD for xylanase production from *Aspergillus fischeri* under SSF indicate the significance of $NaNO_2$ on production of xylanase[31]. The response surface showed that the interaction of different variables tested in this

investigation was negligible. In this study, a reduced level of protease activity was noticed in the optimized medium compared to the medium before optimization and this reduction was directly responsible for retention of xylanase activity in the optimized medium. A response surface method with three-factor-three-level design has been used to optimize the medium components and its pH for maximum xylanase production by *Bacillus circulans* D1 in submerged fermentation (SmF), which resulted in a maximum production of 22.45 Uml^{-132}. Similarly, xylanase production by *Schizophyllum commune* and *Thermomyces lanuginosus* has been maximized by CCRD method, and the maximum xylanase yields were 5.74 Uml^{-1} and 2.7 Uml^{-1} respectively in SmF[33–34].

Conclusion

Xylanase production from *Streptomyces violaceoruber* could be improved by controlling various physical and nutritional factors, and a statistical approach has proved to be a useful and powerful tool for rapid identification of the signal parameters and development of optimal culture conditions with a minimum number of experimental trials.

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