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Research article

Statistical optimization of thermo-alkali stable xylanase production from *Bacillus tequilensis* strain ARMATI

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ABSTRACT

Background: Xylanase from bacteria finds use in prebleaching process and bioconversion of lignocelluloses into feedstocks. The xylanolytic enzyme brings about the hydrolysis of complex biomolecules into simple monomer units. This study aims to optimize the cellulase-free xylanase production and cell biomass of *Bacillus tequilensis* strain ARMATI using response surface methodology (RSM).

Results: Statistical screening of medium constituents and the physical factors affecting xylanase and biomass yield of the isolate were optimized by RSM using central composite design at N = 30, namely 30 experimental runs with 4 independent variables. The central composite design showed 3.7 fold and 1.5 fold increased xylanase production and biomass yield of the isolate respectively compared to 'one factor at a time approach', in the presence of the basal medium containing birchwood xylan (1.5% w/v) and yeast extract (1% w/v), incubated at 40°C for 24 h. Analysis of variance (ANOVA) revealed high coefficient of determination (R^2) of 0.9978 and 0.9906 for the respective responses at significant level ($p < 0.05$). The crude xylanase obtained from the isolate showed stability at high temperature (60°C) and alkaline condition (pH 9) up to 4 h of incubation.

Conclusions: The cellulase-free xylanase showed an alkali-tolerant and thermo-stable property with potentially applicable nature at industrial scale. This statistical approach established a major contribution in enzyme production from the isolate by optimizing independent factors and represents a first reference on the enhanced production of thermo-alkali stable cellulase-free xylanase from *B. tequilensis*.

1. Introduction

Xylan is a polymer of xylose molecules and a major component of hemicelluloses in plant, which plays a major role to hold the cell walls together. It is one of the most abundant renewable polysaccharides in nature after cellulose that constitutes 35–40% of the total dry weight of higher plant [1,2]. Xylan contains β-1,4-linked xylose residues in the backbone. Short side chains of o-acetyl, α-L-arabinofuranosyl, D-α-glucuronic and phenolic acid residues are attached to it [3]. Complete hydrolysis of xylan requires different xylanolytic enzymes such as endoxylanase, β-xylosidase, α-arabinofuranosidase, acetyl esterase, and α-glucoronidase. Among them, endo-β-1,4-xylanase (EC 3.2.1.8) plays an important role to catalyze the hydrolysis of long-chain xylan into short oxylo oligosaccharides [4,5]. Xylanases are hemicellulases produced by microorganisms to break down hemicelluloses and xylan, and release xylose. The main hydrolysis products are β-D-xylopyranosyl oligomer but later, mono-, di- and trisaccharides of β-D-xylopyranosyl are also produced [6].

Bacteria are major attractive producer of high levels of extracellular xylanase [7,8]. In the present scenario, large-scale cultivation of fungi and actinomycetes is very difficult because of their slow generation time, coproduction of highly viscous polymers, and poor oxygen transfer [9]. To overcome these problems, researchers have concentrated on the large-scale production of xylanase from bacteria. The genus *Bacillus* has been studied more extensively among bacterial xylanases. *Bacillus* species are industrially important bacteria due to their rapid growth rate and for their capacity to secrete important extracellular enzymes and proteins in the medium. Currently, cellulase-free xylanases are playing the most important role in paper and pulp industries (prebleaching of kraft pulp) in order to reduce the use of toxic chlorine chemicals [10] and the enzymes are also found to be effective in saccharification process [11].

The commercial application of cellulase-free xylanase in various industrial processes is very limited due to partial hydrolysis of substrate, thermal and pH instability of enzyme, and time dependent enzyme production. The maximum production of xylanase with cost effective way in less time period can be achieved by employing

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alternate strategies of culture medium optimization. Therefore, an intensive investigation is necessary to scale up the enzyme production from new bacterial isolates under submerged fermentation (SmF) by various optimization means. Fermentation medium optimization plays a critical role in enhancing the production yields of the industrially important enzyme. Optimization for enhanced production of enzyme depends upon medium components like carbon source, nitrogen sources, pH, temperature, agitation and incubation time. The optimization of the fermentation medium by one factor at a time (OFAT) is not only a time consuming process but also may lead to inaccurate results and conclusions. This classical method of optimization is unable to detect the interaction between two factors responsible for enhanced enzyme production. Response surface methodology (RSM) is an alternate statistical strategy to improve enzyme yield by designing minimum number of experiments for large number of factors [12]. RSM explains the combined effects of all the independent variables in a fermentation process and explores an approximate interaction between a response variable and a set of design independent variables. Therefore, keeping in view the importance of statistical approach, an attempt has been made to enhance the production of cellulase-free xylanase by RSM using central composite design (CCD) with four independent variables from poultry farm isolate, *Bacillus tequilensis* strain ARMATI in SmF.

2. Materials and methods

2.1. Sample collection

Feces soil samples were collected from poultry farm of Guduvanchery, Tamil Nadu, India. The samples were kept in sterilized polythene bags and stored at 4°C in laboratory for further experimental analysis.

2.2. Screening of hyperxylanolytic bacterial strain

Xylan agar media (in g/L: polypeptone — 10.0; yeast extract — 5.0; KH_2PO_4- 1.0; $MgSO_4.7H_2O$ — 0.2; Na_2CO_3 — 10.0; Xylan — 5.0; NaCl — 3.0; agar — 20.0; and pH — 7.0) were prepared and transferred to the sterile petri plates aseptically. The serially diluted soil samples were spread onto the cooled agar media under aseptic conditions. The plates were incubated overnight at 37°C for the appearance of xylanolytic bacterial colonies. After overnight incubation, xylanase producing bacterial colonies were selected by staining the plates with Congo Red (1%) for 5 min followed by destaining with 1 M NaCl for 10–15 min. The selected cultures were purified and maintained as glycerol stock. Isolated pure cultures were screened on the basis of their extracellular xylanase production efficiency. Hyperxylanase producing isolate was selected for its characterization and cellulase-free xylanase production ability.

2.3. Molecular characterization and identification of hyperxylanolytic bacterial strain

Genomic DNA of the strain was isolated and amplification of the 16S ribosomal RNA was done using the thermal cycler (Eppendorf Gradient) with *Taq* DNA polymerase and primers. The conditions for thermal cycling were as follows: denaturation of the target DNA at 94°C for 4 min followed by 30 cycles at 94°C for 1 min, primer annealing at 52°C for 1 min and primer extension at 72°C for 1 min. At the end of the cycling, the reaction mixture was held at 72°C for 10 min and then cooled to 4°C. PCR amplification was detected by agarose gel electrophoresis and visualized by gel doc. The amplicon was purified and the same was sequenced using an automated sequencer (Genetic Analyzer 3130, Applied Biosystems, and USA). The sequence was compared for similarity search with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST. The sequence was submitted to GenBank and the accession number KC424491 was assigned for the isolate. Phylogenetic relationship of the isolate with other *Bacillus* species were inferred from phylogenetic comparison of the 16S rRNA sequences using the Neighbor-Joining (NJ) algorithm in Molecular Evolution Genetic Analysis (MEGA) software version 4.0 [13].

2.4. Extracellular xylanase production and extraction

Five hundred microliter of overnight grown bacterial inoculum was inoculated into 50 mL of fermentation medium. The fermentation medium consisted of (g/L): polypeptone — 5.0; yeast extract — 5.0; KH_2PO_4 — 1.0; $MgSO_4 \cdot 7H_2O$ — 0.2; Na_2CO_3 — 10.0; Xylan birchwood — 10.0; NaCl — 3.0; and pH — 7.0. The flask was kept for incubation in rotatory shaker at 37°C. After overnight incubation the broth culture was centrifuged at 8000 g for 10 min. The supernatant was collected and the quantitative assay for xylanase production was performed according to standard procedure.

2.5. Estimation of cellulase-free xylanase activity

Xylanase activity was measured according to Bailey et al. [14] with some modifications. The supernatant obtained from overnight bacterial broth culture was used as crude enzyme. One milliliter of enzyme solution was added to the test tube containing 1 mL of 1% solubilized birchwood xylan solution. The reaction mixture was incubated at 50°C in water bath for 10 min. The reaction was stopped by adding 1 mL of dinitrosalicylic acid (DNSA) into it. The tubes were incubated in boiling water bath for 5 min. After cooling, the solution was centrifuged at 8000 g for 5 min, and the supernatant was analyzed for absorbance at 540 nm. A reagent blank and an enzymatic blank were prepared under the same condition. D-xylose (100–1000 μg/mL) was used as the standard. One unit (IU) of xylanase activity was defined as the amount of enzyme that liberates 1 μmol of reducing sugars equivalent to xylose per minute under the assay conditions described.

Cellulase activity was determined by similar method using 1% (w/v) carboxy methyl cellulose (CMC) as substrate and glucose as standard. This assay was performed to adjudge the cellulase free nature of the crude xylanase produced from the bacteria. The cellulase-free xylanase will provide additional benefit during optimization process.

2.6. Estimation of protein

Estimation of total extracellular protein was performed through Bradford test (Bradford, 1976). Bovine serum albumin (BSA) was used as standard. Protein content per milliliter of test samples was determined against the standard curve.

2.7. Biomass estimation

Two milliliter sample was collected in a pre-weighed Eppendorf tube and centrifuged at 8000 rpm for 10 min. Supernatant was discarded and the pellet was washed thrice with sterile distilled water, followed by drying the pellets at 95°C till attaining constant weight and expressed in dry cell weight (mg/mL).

2.8. Optimization of process parameters for xylanase production and biomass yield

The effect of different parameters such as pH (5–10), incubation temperature (30°C–50°C), agitation speed (120–160 rpm), incubation time (12–96 h), carbon sources (glucose, birchwood xylan, xylose, sucrose, lactose and mannose) and nitrogen sources (peptone, yeast extract, beef extract, tryptone, KNO_3, ammonium sulfate and ammonium chloride) were studied using the 'one factor at a time

approach' (keeping the rest factor constant) in order to estimate xylanase activity and biomass yield under submerged fermentation. A total of four independent variables were selected for further optimization process by RSM using CCD based on previous experimental results.

2.9. Effect of temperature and pH on the enzyme stability

The temperature stability of the enzyme was studied using crude xylanase. To evaluate thermal stability, the enzyme solution was incubated at temperatures of 30–70°C for up to 4 h. The residual enzyme activity was recorded at 1 h interval during 4 h incubation. The enzyme activity was determined as described earlier.

pH stability was measured by incubating the enzyme at pH 5 to 10 in different buffers (0.1 M) such as Sodium phosphate (pH 5.0 to 7.0), Tris–HCl (pH 8.0, 9.0) and carbonate–bicarbonate (pH 10.0). To evaluate the stability of the enzyme at each pH, the crude enzyme was incubated into the respective buffer over a pH range of 5.0–10.0 for up to 4 h at optimum temperature. The residual enzyme activity was determined at 1 h interval during 4 h incubation. The enzyme activity was determined as described earlier.

2.10. Response surface methodology

RSM was used to optimize the fermentation parameters for enhancing extracellular xylanase production and biomass yield of the strain. A central composite design [2] was employed for the optimization process. The independent variables selected for this study were birchwood xylan, yeast extract, incubation temperature and incubation time. Each independent variable in the design matrix was studied at five different levels ($-\alpha$, -1, 0, $+1$, $+\alpha$) as shown in Table 1. The design involved 6 center points with an alpha value being ± 2. The five coded levels of alpha, studied in the present study were -2, -1, 0, $+1$ and $+2$. According to the present experimental design, the total number of treatment combinations is $2^k + 2\,k + n$, where 'k' is the number of independent variables and 'n' is the number of repetition of experiments at the central point. The experimental design consisted of 30 experiments of four variables in order to optimize the xylanase production. All variables were set at a central coded value of zero. The experimental plan of independent variables with respect to their values in actual and coded form is shown in Table 2. All the experimental runs were performed in triplicate and average xylanase activity (Y) and biomass production (Z) were taken as dependent variable. The effects of variables to the response were analyzed by using a second-order polynomial equation [Equation 1]:

$$Y, Z = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD \quad \text{[Equation 1]}$$

where Y, Z = response variable; β_0 = intercept; β_1, β_2, β_3 and β_4 = linear coefficients; β_{11}, β_{22}, β_{33} and β_{44} = squared coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34} = interaction coefficients; A, B, C, D, A^2, B^2, C^2, D^2, AB, AC, AD, BC, BD and CD = levels of independent variables.

Table 1
Experimental range, level and code of independent variables for CCD design.

Variables	Code	Range and levels				
		$-\alpha$	-1	0	$+1$	$+\alpha$
Birchwood xylan (%)	A	0.5	1	1.5	2	2.5
Yeast extract (%)	B	0.3	0.5	1	1.5	2
Temperature (°C)	C	30	35	40	45	50
Incubation period (h)	D	12	18	24	36	48

The goodness of fit of the polynomial equation was expressed by coefficient of determination R^2 and its statistical significance level was checked by *F* test. The desirability was kept at maximum. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination R^2. Each experimental design was carried out in triplicate, and the mean values were given.

2.11. Validation of model

The validation of the design was validated by running the experiment based on the optimum values for variables given by response optimization using CCD to confirm the experimental value and predicted value of xylanase production and biomass yield.

2.12. Software used

The independent variables of the experimental design were optimized and interpreted using Design Expert Version 10.0.0 (Stat-Ease Inc., Minneapolis, Minnesota, USA) statistical software. ANOVA was used to validate statistical parameters.

3. Results

3.1. Identification of bacteria

Out of ten isolates, one isolate showed maximum production of xylanase on xylan agar plate. An amplicon of 740 bp size (Figure not shown) was observed through PCR amplification and sequencing. The sequence was subjected to a multiple sequence alignment using the BLAST program of NCBI. The 16S rRNA sequence showed a homology of 100% with *B. tequilensis*. The sequence was deposited in GenBank, maintained by NCBI, USA (Accession No: KC424491) and the organism was identified as *B. tequilensis* strain ARMATI (Fig. 1). The phylogenetic tree (Fig. 2) was constructed using bioinformatics software MEGA 4.0.

3.2. Optimization of process parameters

Among various dependent and independent variables tested for xylanase and biomass production through OFAT method, birchwood xylan (1% w/v), yeast extract (0.5% w/v), incubation period (24 h) and incubation temperature (35°C) were found to be potential factors in order to increase xylanase and biomass production in strain ARMATI. There was no trace of cellulase production during the submerged fermentation process. It indicates the cellulase-free xylanase production from strain ARMATI.

3.3. Effect of pH and temperature on xylanase activity and stability

The xylanase produced by strain ARMATI was assessed at different pH and temperature ranges. The isolate showed maximum xylanase activity at pH 7 (Fig. 3a). There was a significant reduction in the xylanase activity at acidic and alkaline medium. The enzyme was found to be stable at a wide range of pH from 5.0 to 9.0. The strain was found stable with 66% of activity up to pH 9.0 for 4 h of incubation (Fig. 3b). On the other hand, the strain showed maximum production of xylanase at 35°C (Fig. 4a). The enzyme activity was found to be reduced at high temperature. Crude xylanase obtained from strain ARMATI showed stability till 60°C for 4 h of incubation and retained up to 51% of the activity. The enzyme stability was reduced significantly above 60°C (Fig. 4b).

3.4. Optimization using response surface methodology

The independent factors with their respective high and low concentrations used in optimization process are represented in Table 1. The central composite design matrix of the independent

Table 2
Central composite design along with experimental and predicted values of dependent variables.

					Xylanase activity (IU/mL)		Protein (mg/mL)		Biomass (mg/mL)	
Run Order	A	B	C	D	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	0	0	0	0	85.52	85.76	1.23	1.50	4.10	4.18
2	+1	+1	-1	-1	70.02	69.99	1.13	1.10	2.24	2.17
3	-α	0	0	0	78.13	77.56	1.27	1.20	1.92	1.78
4	0	+α	0	0	82.14	81.55	1.38	1.50	2.87	2.83
5	+1	-1	-1	+1	78.14	77.63	1.35	1.30	1.76	1.81
6	-1	+1	+1	+1	72.01	72.66	1.11	1.12	2.10	2.23
7	-1	-1	-1	+1	71.16	71.87	1.20	1.17	1.45	1.56
8	0	0	0	-α	48.65	48.38	0.86	0.79	1.11	1.12
9	0	0	+α	0	62.18	62.12	0.98	0.91	1.88	1.90
10	0	0	0	+α	63.43	63.12	0.99	1.03	2.10	1.95
11	+1	+1	+1	-1	64.44	64.84	0.94	0.99	1.72	1.83
12	-1	-1	-1	-1	61.02	60.88	0.92	0.93	1.22	1.24
13	-1	-1	+1	-1	58.12	58.61	0.91	0.93	1.42	1.54
14	+1	-1	-1	-1	65.04	65.50	0.99	1.01	1.38	1.47
15	+α	0	0	0	78.14	78.13	1.12	1.15	2.14	2.14
16	+1	-1	+1	+1	77.10	77.71	1.23	1.20	2.21	2.32
17	0	0	0	0	86.62	85.76	1.55	1.50	4.32	4.18
18	0	-α	0	0	78.02	78.03	1.25	1.27	2.10	2.0
19	0	0	0	0	85.32	85.76	1.60	1.50	4.12	4.18
20	+1	+1	+1	+1	69.0	68.60	0.97	0.99	2.45	2.35
21	-1	-1	+1	+1	74.13	73.62	1.12	1.17	2.11	2.10
22	-1	+1	+1	-1	70.06	70.04	1.10	1.17	1.86	1.73
23	-1	+1	-1	-1	73.0	73.50	1.14	1.19	1.92	2.03
24	0	0	-α	0	66.04	65.51	0.98	1.02	1.86	1.70
25	+1	-1	+1	-1	62.12	61.56	0.89	0.91	1.88	1.74
26	-1	+1	-1	+1	72.07	72.10	1.13	1.24	2.23	2.29
27	+1	+1	-1	+1	69.10	69.72	1.10	1.10	2.34	2.44
28	0	0	0	0	85.52	85.76	1.54	1.50	4.16	4.18
29	0	0	0	0	86.04	85.76	1.59	1.50	4.18	4.18
30	0	0	0	0	85.52	85.76	1.51	1.50	4.18	4.18

variables in coded units along with predicted and experimental values of response is given in Table 2.

The production of xylanase was predicted by [Equation 2]:

$$Y\,(IU/mL) = 85.78 + 0.14A + 0.88B - 0.85C + 3.69D - 2.03AB - 0.42AC + 0.28AD - 0.3BC - 3.1BD + 1.01CD - 1.98A^2 - 1.49B^2 - 5.49C^2 - 7.5D^2 \quad \text{[Equation 2]}$$

The biomass yield (Z) was predicted by [Equation 3]:

$$Z(mg/mL) = 4.18 + 0.088A + 0.21 + 0.052C + 0.21D - 0.024AB - 8.125E{-}003AC + 4.375E{-}003AD - 0.15BC - 0.016BD + 0.061CD - 0.55A^2 - 0.44B^2 - 0.59C^2 - 0.66D^2 \quad \text{[Equation 3]}$$

The highest xylanase activity was obtained from Run No. 17 (Supplementary Fig. 1), which consisted of birchwood xylan (1.5% w/v), yeast extract (1% w/v), incubation temperature (40°C) and time period (24 h). The biomass value of the strain using RSM optimization was enhanced 1.5 fold compared to one factor at a time approach.

ACATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGG
GTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCA
TGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTG
AGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGA
CACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAA
CGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAA
TAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAC
GTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTG
AAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAA
TTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTG
TAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGG

Fig. 1. 16S rRNA sequence (740 bp) of *Bacillus tequilensis* strain ARMATI.

Analysis of variance (ANOVA) for xylanase quadric model is given in Table 3. Model terms having p value <0.05 were considered significant. The model F value of 492.88 implies the model is significant. There is only 0.01% chance that a large "Model F value" could occur due to noise. Values of "Prob > F" < 0.05 indicate model terms are significant. In this case B, C, D, AB, AC, BD, CD, A^2, B^2, C^2 and D^2 are significant model terms (Table 3). The "Lack of Fit F-value" of 1.98 implies that the Lack of Fit is not significant relative to the pure error. Non-significant lack of fit is good for the model to fit. The multiple correlation coefficient (R^2) closer to 1 denotes better correlation between experimental and predicted values and indicates the model accurate with better response. In the present study, a low CV (0.86%) denotes that the experiments performed are highly reliable and precise. The "Predicted R^2" of 0.9978 is in reasonable agreement with the "Adj R^2" of 0.9958. "Adeq Precision" ratio of 84.543 indicates an adequate signal due to the value greater than 4. This model can be used to navigate the design space.

ANOVA for biomass quadric model is given in Table 4. The model F value of 112.51 implies the model is significant. There is only 0.01% chance that a large "Model F value" could occur due to noise. In this case A, B, D, BC, A^2, B^2, C^2 and D^2 are significant model terms. The "Lack of Fit F-value" of 3.93 implies that the Lack of Fit is not significant relative to the pure error. The multiple correlation coefficient (0.9906) closer to 1 denotes better correlation between experimental and predicted values and indicates that model accurate with better response. In the present study, a low CV (5.59%) denotes that the experiments performed are highly reliable and precise. The "Predicted R^2" of 0.9503 is in reasonable agreement with the "Adj R^2" of 0.9818. "Adeq Precision" ratio of 32.516 indicates an adequate signal due to the value greater than 4.

Distribution of experimental and predicted values for xylanase activity was shown by Parity plot (Fig. 5) where data points are localized close to the diagonal line, suggesting that the model is accurate and satisfactory.

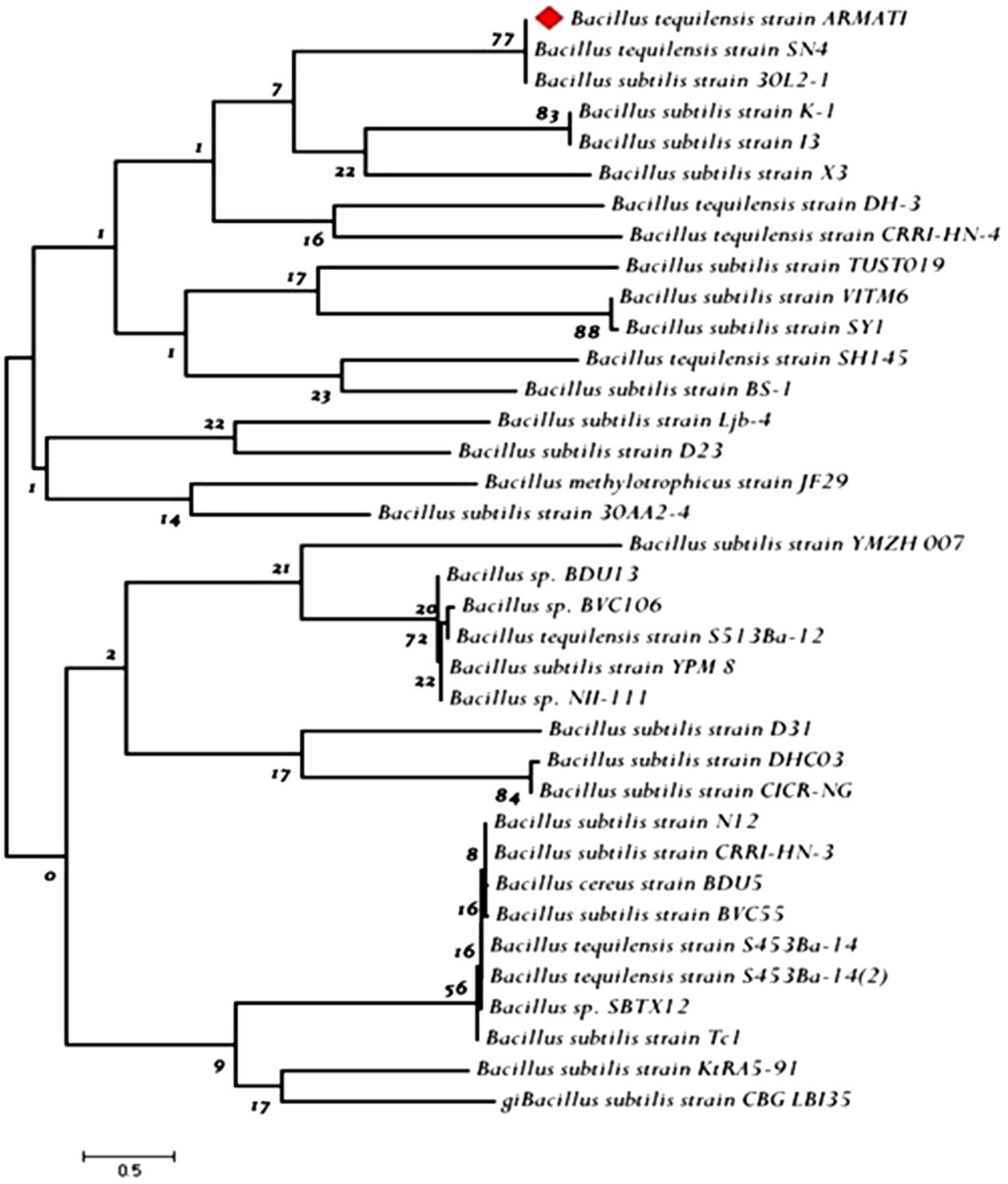

Fig. 2. Neighbor joining tree constructed showing relationship of *Bacillus tequilensis* strain ARMATI among the genus *Bacillus* based on 16S rRNA gene sequences.

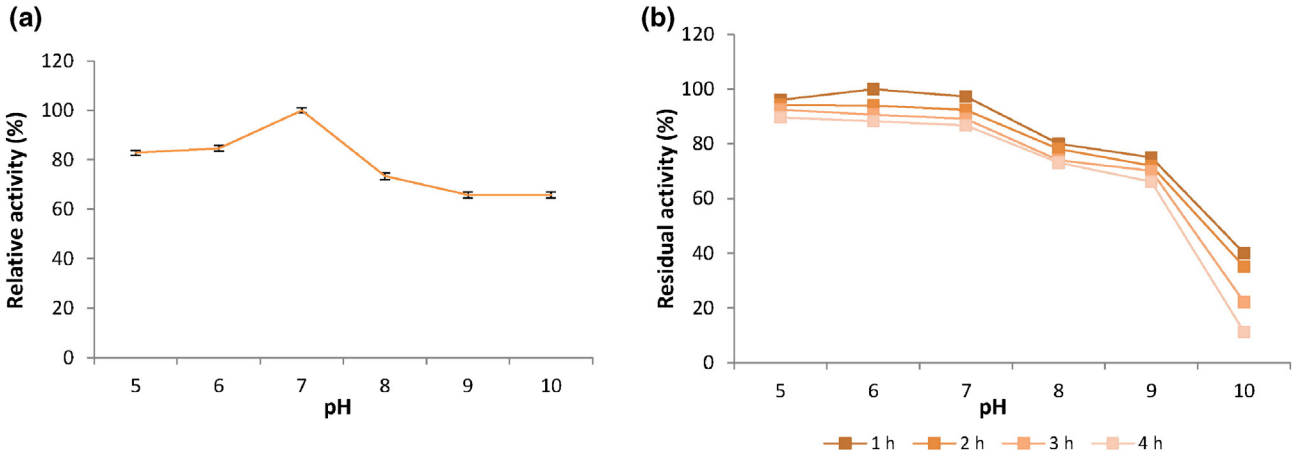

Fig. 3. Effect of pH on xylanase activity (a) and stability (b).

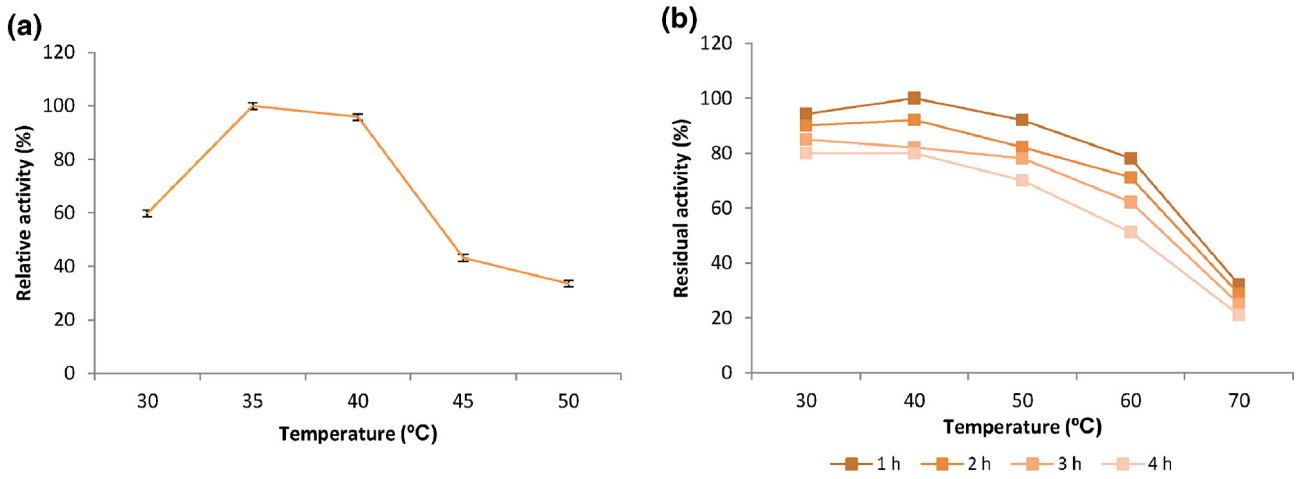

Fig. 4. Effect of temperature on xylanase activity (a) and stability (b).

Fig. 6 depicts that xylan birchwood (A/X1) and yeast extract (B/X2) at their low level showed significant effect on the production of xylanase. Maximum xylanase activity was observed with lower to middle level of birchwood xylan concentration (0.5–1.5% w/v) and middle to high level of yeast extract concentration (1–2% w/v). Interaction between birchwood xylan concentration (A/X1) and incubation temperature (C/X3) on xylanase activity is shown in Fig. 7 Both the variables at their respective middle levels showed increment in xylanase production. Fig. 8 depicts the significant reduction in the xylanase activity at the lower and higher concentrations of birchwood xylan concentration (A/X1) and incubation time (D/X4). Fig. 9 represents that yeast extract concentration (B/X2) and incubation temperature (C/X3) at their respective middle to slightly higher levels showed increased xylanase production. Fig. 10 depicts that the enzyme production was increased at lower levels of yeast extract concentration (0.3–0.5%) and higher levels of incubation time (24–48 h). Fig. 11 depicts that an enhancement in the xylanase production was obtained at middle level of incubation temperature (40°C), while lower to middle level of incubation time (18–24 h) enhanced the response significantly. The normality assumption v_s internally studentized residuals were found to be satisfactory due to the plot obtained in straight line (Supplementary Fig. 2).

3.5. Validation of model

The central point representing the maximum xylanase production had the following values — birchwood xylan 1.5% (w/v), yeast extract 1% (w/v), incubation temperature 40°C and incubation period 24 h. The validation of the optimization was carried out under above mentioned optimized condition of the variables. It showed the dependent response of 86.62 IU/mL which is very much close to the predicted response. The RSM optimization showed 44% enhancement in the response, compared to the lowest response of 48.65 IU/mL. The optimized conditions revealed the responses values close to the predicted one, indicating the validation strategy towards biomass yield and protein production. The reasonable accepted values of R^2 define the true behavior of the statistical system that can be used for interpolation in the experimental domain. A comparison of xylanase production from the isolate of the present study revealed that the

Table 3
ANOVA for xylanase activity as a function of independent variables.

Source	Sum of Squares	df	Mean Square	F Value	p-Value Prob > F
Model	2697.09	14	192.65	492.88	<0.0001 significant
A-A	0.48	1	0.48	1.24	0.2831
B-B	18.57	1	18.57	47.50	<0.0001
C-C	17.22	1	17.22	44.06	<0.0001
D-D	325.98	1	325.98	833.98	<0.0001
AB	66.22	1	66.22	169.42	<0.0001
AC	2.81	1	2.81	7.20	0.0170
AD	1.29	1	1.29	3.31	0.0889
BC	1.43	1	1.43	3.67	0.0747
BD	153.47	1	153.57	392.91	<0.0001
CD	16.22	1	16.22	41.50	<0.0001
A^2	107.23	1	107.23	274.33	<0.0001
B^2	60.97	1	60.97	155.99	<0.0001
C^2	825.48	1	825.48	2111.92	<0.0001
D^2	1543.24	1	1543.24	3948.28	<0.0001
Residual	5.86	15	0.39		
Lack of Fit	4.68	10	0.47	1.98	0.2343 not significant
Pure error	1.18	5	0.24		
Cor total	2702.95	29			

R^2: 0.9978, adj R^2: 0.9958, predicted R^2: 0.9894; CV: 0.86%, adeq precision: 84.543; df = degree of freedom; Highly significant, $p \leq 0.0001$; Significant, $p \leq 0.05$; non-significant, $p > 0.05$.

Table 4
ANOVA for biomass as a function of independent variables.

Source	Sum of Squares	df	Mean square	F value	p-Value Prob > F
Model	27.84	14	1.99	112.51	<0.0001 significant
A-A	0.19	1	0.19	10.49	0.0055
B-B	1.03	1	1.03	58.22	<0.0001
C-C	0.065	1	0.065	3.68	0.0742
D-D	1.04	1	1.04	58.69	<0.0001
AB	9.506E-003	1	9.506E-003	0.54	0.4747
AC	1.056E-003	1	1.056E-003	0.060	0.8102
AD	3.063E-004	1	3.063E-004	0.017	0.8970
BC	0.36	1	0.36	20.53	0.0004
BD	3.906E-003	1	3.906E-003	0.22	0.6451
CD	0.059	1	0.059	3.33	0.0882
A^2	8.42	1	8.42	476.30	<0.0001
B^2	5.32	1	5.32	300.80	<0.0001
C^2	9.68	1	9.68	547.55	<0.0001
D^2	11.96	1	11.96	676.49	<0.0001
Residual	0.27	15	0.018		
Lack of Fit	0.24	10	0.024	3.93	0.0721 not significant
Pure Error	0.030	5	5.987E-003		
Cor Total	28.11	29			

R^2: 0.9906, adj R^2: 0.9818, predicted R^2: 0.9503; CV: 5.59%, adeq precision: 32.516; df = degree of freedom; Highly significant, $p \leq 0.0001$; Significant, $p \leq 0.05$; Non- significant, $p > 0.05$.

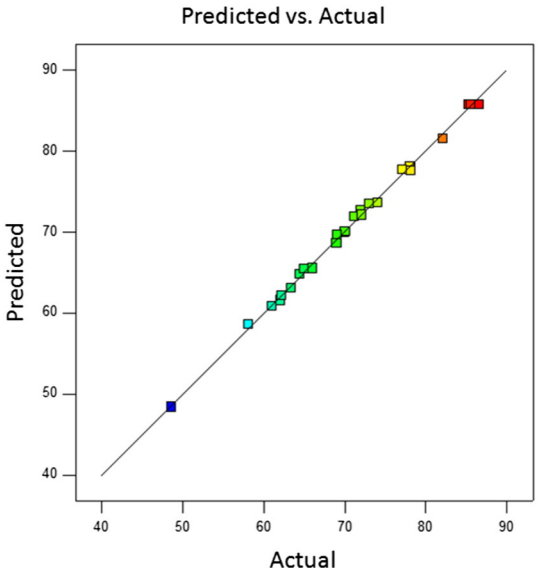

Fig. 5. Actual values vs predicted values for xylanase activity residing close to the diagonal line.

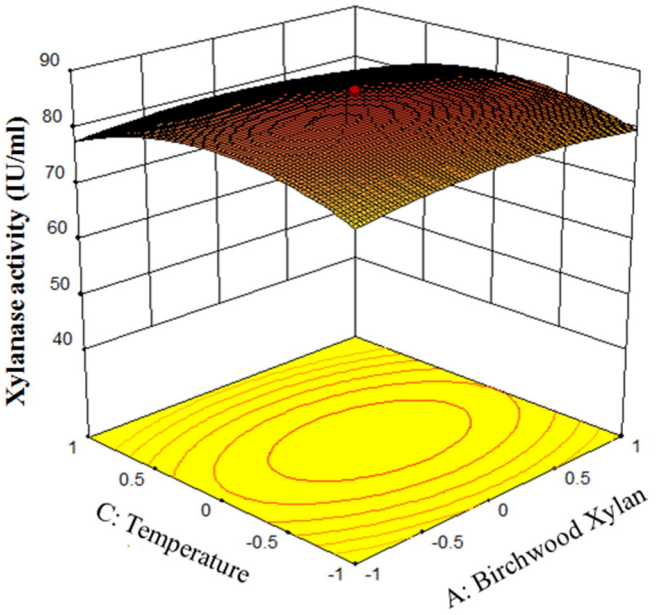

Fig. 7. 3D response plot showing interaction between birchwood xylan and temperature.

enzyme titer value was higher than the other *B. tequilensis* strains but the activity of strain ARMATI varied substantially from other reported *Bacillus* sp. (Table 5).

4. Discussion

Xylanase is an important group of enzymes that depolymerize the xylan molecules into monomers. Xylanolytic enzymes are receiving increasing attention because of their potential application in pulp bleaching [15,16], resulting in reduced use of toxic chemicals by avoiding chlorine. There is a constant search of bacteria producing temperature and alkali-stable cellulase-free xylanase due to their vast industrial application. In that case *B. tequilensis* strain ARMATI was found to be a potential bacterial strain with significant xylanolytic activity, temperature stability and alkali tolerant ability. The preliminary study of this research was focused to identify the potential variables enhancing xylanase production in strain ARMATI using 'one factor at one time' approach by optimizing various medium components and physical parameters. Birchwood xylan, yeast extract, incubation temperature and incubation period were found to be potential independent variables influencing the xylanase production.

Xylanase activity was moderately affected by change in pH of the production medium. pH played an essential role in the secretion and production of xylanase by isolate. In the present study strain ARMATI showed maximum xylanase activity at neutral pH. The result of the present context was in good agreement with the findings of Sanghi et al. [17] and Ammoneh et al. [18] who demonstrated the neutral pH of the medium as influencing factor for maximum xylanase production from *Bacillus* sp. pH played an important role in the production of extracellular xylanase by the isolate due to the reason that substrate binding and catalysis are often dependent on charge distribution on both substrate and in particular enzyme molecules.

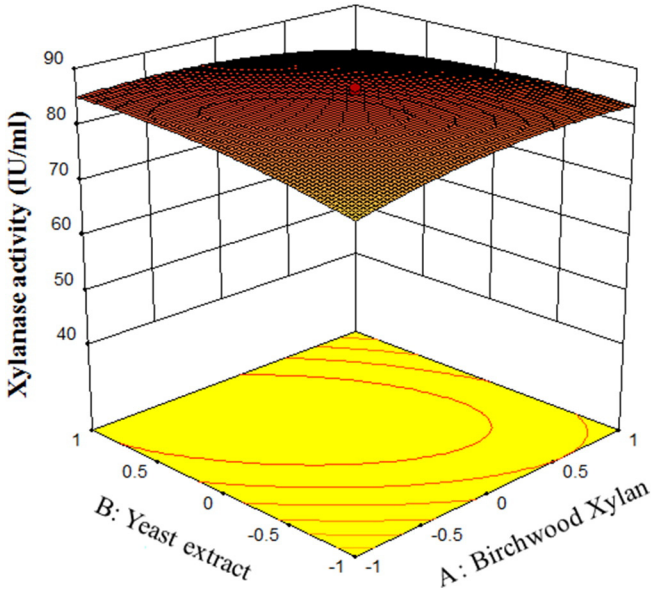

Fig. 6. Response surface plot showing the interaction between birchwood xylan and yeast extract.

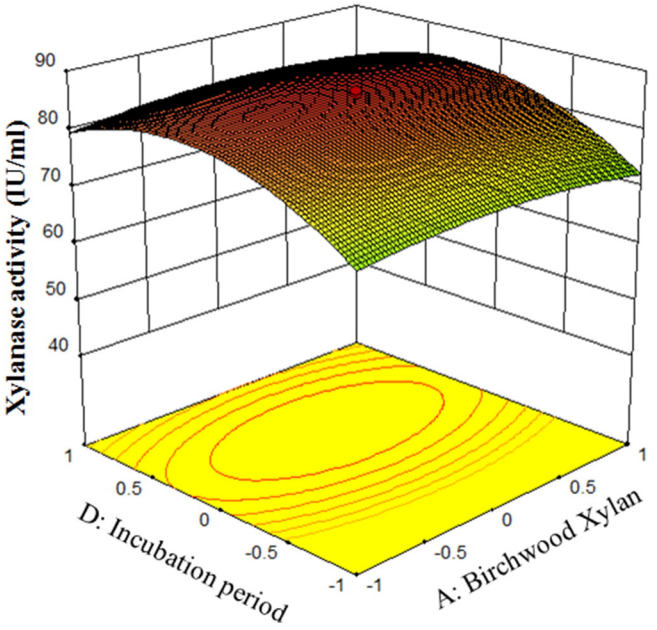

Fig. 8. Response surface graph showing interaction between birchwood xylan and incubation period.

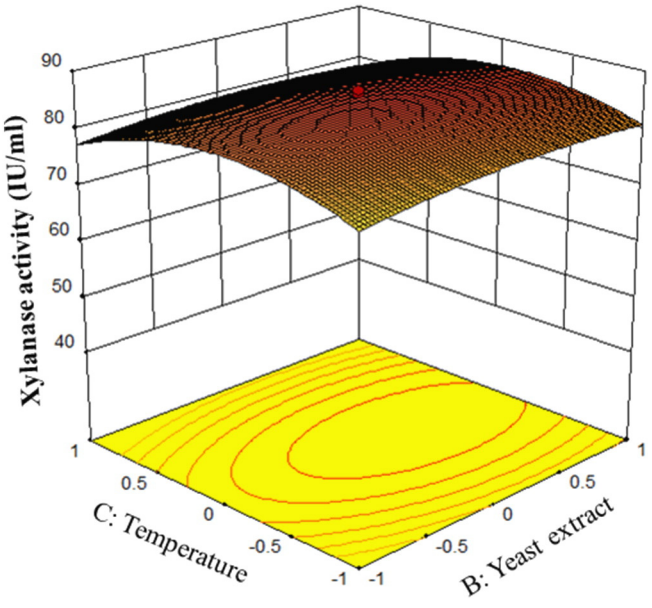

Fig. 9. Response surface graph showing interaction between yeast extract and incubation temperature.

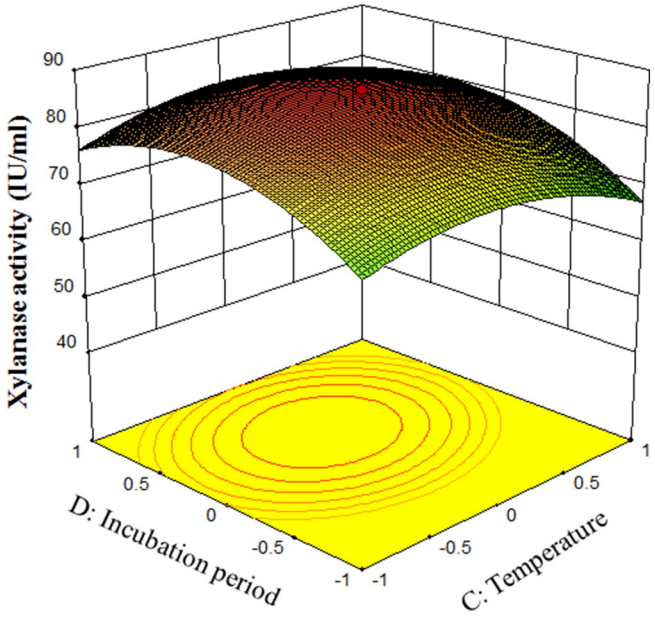

Fig. 11. Response surface plot showing interaction between temperature and incubation period.

The most important characteristic of novel isolate of the present investigation was stability at alkaline pH. The crude xylanase obtained from strain ARMATI was stable at a wide range of pH from 5.0 to 9.0. The outcome of the present study is more or less similar to the finding of Bai et al. [19] and Ko et al. [20] who demonstrated stability of xylanase at a wide range of pH 5.5 to 9.5. On the other hand the present investigation was in good agreement with the finding of Patthra et al. [21] who found that microorganisms produce maximum xylanase at neutral pH but the enzyme stability occurred in alkaline conditions. Charged amino acid residues result the enzymes to be stable at alkaline pH. Strain isolated in the present context could be a good source for biotechnological applications because of the enzyme stability at alkaline pH.

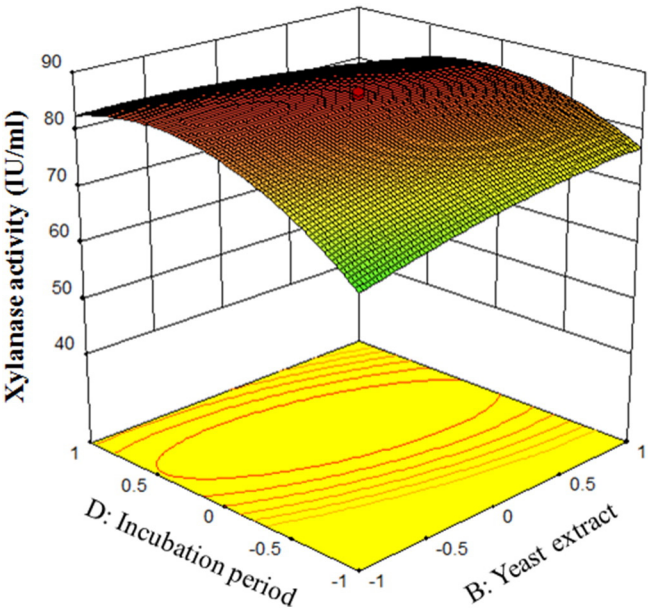

Fig. 10. 3D surface response showing interaction between yeast extract and incubation period.

The fermentation temperature had profound effect on the production of xylanase by bacterial isolate. The finding of this present study is more or less similar to the reports of Battan et al. [22] who demonstrated that optimum xylanase production by *Bacillus* sp. was obtained at 37°C. The variations between our finding and the previous report may be because of the source of the isolation and type of bacterial strain. Xylanases collide and bind to the substrate at the active site in order to convert xylan into product. Change in the temperature of a system will increase or decrease the number of collisions of enzyme and substrate per unit time resulting change in the reaction. Thermal denaturation of the protein and enzymes occurs for the mesophilic bacteria at high temperature. Thus, high temperature may cause the rate of an enzyme catalyzed reaction to decrease due to the denaturation and inactivation of substrates or enzymes.

In the present investigation crude xylanase obtained from strain ARMATI was more stable at 60°C for 4 h of incubation. Our study favors the finding of Kamble and Jadhav [23] who demonstrated that xylanase obtained from *Cellulosimicrobium* sp. MTTCC 1065 was more stable up to 4 h of incubation. Xylanases exhibiting almost similar temperature optima have been reported by other researchers from several *Bacillus* sp. [24,25]. Mesophilic bacteria are the potential producer of thermostable xylanases [8]. Results of the present study concluded that the crude xylanase from the isolate is temperature stable. Thus, enzyme stability at higher temperatures would be important criteria for several industrial applications.

The bacterial isolate showed enhancement in the enzyme production when grown in the media supplemented with xylan birchwood (1% w/v) and yeast extract (0.5% w/v) as sole carbon and nitrogen source respectively using OFAT method. The results totally agree with the finding of Kamble and Jadhav [23] and Sugumaran et al. [26] who demonstrated maximum xylanase production by the bacterium in the presence of birchwood xylan and yeast extract respectively. In the present investigation, xylan birchwood acts as stimulator in the production medium in order to enhance extracellular xylanase production. This might be due to the reason that xylan is metabolized slowly by the bacteria as a complex carbon source and accumulates maximum enzyme in the fermentation medium. Yeast extract plays an important role in xylanase production due to the presence of essential elements and growth factors.

Table 5
Comparison of xylanase production by *Bacillus* sp.

Organism	Method	Carbon source	Xylanase activity (IU/mL)	References
B. tequilensis strain ARMATI	CCD, RSM	Birchwood xylan	86.62	Present investigation
B. tequilensis SH0	OFAT	Wheat bran	41.3	Kumar et al. [29]
B. tequilensis	Non-statistical	Wheat bran	74.23	Rathee et al. [30]
Bacillus sp.	Non-statistical	Wheat bran	3.5	Azeri et al. [31]
Bacillus sp.	Non-statistical	Birchwood xylan	49.0	Yang et al. [32]
B. subtilis	Non-statistical	Oat spelt xylan	18.0	Sa-Pereira et al. [33]
B. subtilis	Non-statistical	Oat spelt xylan	128.0	Annamalai et al. [5]
B. pumilus B20	Factorial design	Wheat bran	313.0	Geetha and Gunasekaran [34]
B. subtilis ASH	Non-statistical	Wheat bran	410.0	Sanghi et al. [35]
B. subtilis	Non-statistical	Pineapple peel	18.87	Ling Ho and Heng [36]
B. subtilis	Non-statistical	Wheat bran	22.07	Ho [37]
Bacillus sp.	Non-statistical	Wheat bran	16.13	Gupta and Kar [38]
Bacillus sp.	CCD, RSM	Wheat bran	205.3	Shanthi and Roymon [39]
B. pumilus	CCD, RSM	Corn husk	2.5	Ayishal Begam et al. [40]
B. subtilis	Non-statistical	Sugarcane bagasse	439.5	Irfan et al. [4]
B. weihenstephanesis strain ANR1	Non-statistical	Watermelon rind	426.0	Harris and Ramalingam [41]
B. amyloliquefaciens XR44A	Non-statistical	Birchwood xylan	10.5	Amore et al. [42]
B. pumilus SV-205	Non-statistical	Wheat bran	7382.7	Nagar et al. [43]

Time period plays a critical role in the enzyme activity. The isolate showed maximum xylanase production after 24 h of incubation. After 24 h, the xylanase activity was drastically decreased till 96th h in a constant manner. A decline in xylanase activity afterwards was probably due to depletion of nutrients in the medium. This unfavorable condition for the bacteria results in reduction of enzyme activity. The present study results were more or less similar to Khodayari et al. [27] who observed significant xylanase production for *Bacillus* sp. at the end of 24 h. Findings of the current investigation concluded that the time course of enzyme activity varies with the source of isolation, types and genetic makeup of strains, and their cultivation conditions.

The results obtained from the strain ARMATI using 'one factor at a time' (OFAT) approach was not satisfactory due to the less enzyme productivity and biomass yield of strain ARMATI. The classical method of optimization *i.e.* OFAT is not only a time consuming method but also a tedious process with unreliable results. Nowadays statistical design is a growing approach in order to optimize medium components and physical parameters for enhancing the enzyme production. Previous reports had shown xylanase production from microbial sources using statistical method [1,28]. In the present investigation, cellulase-free xylanase production from strain ARMATI was enhanced 3.7 fold compared to the OFAT method by response surface optimization using CCD. Coman and Bahrin [2] also reported the 3 fold enhancement in the xylanase production from microbial source using central composite design. On the other hand Walia et al. [1] and Thomas et al. [28] showed 3.1 fold and 5 fold increase of xylanase production respectively after RSM optimization.

The statistical approach applied to the optimization of cellulase-free xylanase production and biomass yield from the strain showed the effect of four independent variables at five different levels. The results suggested a close agreement between the observed and predicted values of both the responses, which showed the accuracy of RSM in order to optimize the parameters for enhanced enzyme production. The ANOVA (*F* test) showed the model adjustment with the observed responses. A lower value of CV calculated from both the responses indicated a better precision and reliability of the model that was further checked by the correlation coefficient (R) and determination coefficient (R^2). Later the normality assumption v_s internally studentized residual plot existing in straight line showed the better accuracy of the model. Perturbation graph for biomass production from strain ARMATI established an interactive effect of variables on the response. In the present context the statistical optimization had been applied efficiently to submerged fermentation that had overcome the limitations of one factor at a time approach.

The interaction between the independent variables such as birchwood xylan and yeast extract, birchwood xylan and temperature, birchwood xylan and incubation time, yeast extract and temperature, yeast extract and incubation period, and temperature and incubation period showed significant effect on both the responses. The enhanced production of both the responses showed mutual interconnection among the variables at their respective middle values. The experimental data obtained after statistical optimization had resulted in increased enzyme production by SmF. In spite of the wide application of SmF for enzyme production using bacteria, the present investigation showed that *B. tequilensis* strain ARMATI can be used for the enhanced production of cellulase-free xylanase using birchwood xylan (1.5% w/v) and yeast extract (1% w/v) within a shorter period of incubation (24 h) at 40°C.

In a nutshell, strain ARMATI is a potential bacterium that can be utilized at commercial scale in biobleaching process and lignocellulose bioconversion system because of long term thermostable and alkali-tolerant nature of crude xylanase. The RSM applied to the optimization and enhancement of xylanase production in strain ARMATI is a reliable and precise tool that explores an approximate functional relationship between a response independent variable and a set of design variables.

5. Conclusion

Response surface methodology using central composite design appeared to be a powerful statistical tool for the enhanced production of xylanase and biomass yield in *Bacillus tequilensis* strain ARMATI during SmF. Maximal production of enzyme was obtained with the fermentation conditions of birchwood xylan (1.5% w/v), yeast extract (1% w/v), incubation temperature (40°C) and incubation period (24 h). Under optimal conditions of independent variables, the experimental responses showed close agreement with predicted responses, confirming the validity of model. The optimized model through CCD showed 3.7 fold enhancements in xylanase production compared to OFAT approach. Further, the crude enzyme showed stability at a wide range of temperature and pH with 51% and 66% of residual activity respectively. These findings suggest that the cellulase-free xylanase obtained from strain ARMATI has properties suitable for industrial application especially in bleaching processes. Further study is in progress to scale up enzyme production in a batch fermenter using CCD optimized conditions.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ejbt.2016.04.002.

Conflict of interest

None declared.

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References

[1] Walia A, Mehta P, Guleria S, Shirkot CK. Improvement for enhanced xylanase production by *Cellulosimicrobium cellulans* CKMX1 using central composite design of response surface methodology. 3 Biotech 2015;5:1053–66. http://dx.doi.org/10.1007/s13205-015-0309-2.
[2] Coman G, Bahrim G. Optimization of xylanase production by *Streptomyces* sp. P12-137 using response surface methodology and central composite design. Ann Microbiol 2011;61:773–9. http://dx.doi.org/10.1007/s13213-010-0195-0.
[3] Coughlan MP, Hazlewood G. β-1,4-D-xylan-degrading enzyme systems: Biochemistry, molecular biology and applications. Biotechnol Appl Biochem 1993;17:259–89.
[4] Irfan M, Nadeem M, Quratualain S, Baig S. Effect of medium composition on xylanase production by *Bacillus subtilis* using various agricultural wastes. Am Eurasian J Agric Environ Sci 2012;12:561–5.
[5] Annamalai N, Thavasi R, Jayalakshmi S, Balasubramanian T. Thermostable and alkaline tolerant xylanase production by *Bacillus subtilis* isolated from marine environment. Indian J Biotechnol 2009;8:291–7.
[6] Kuhad RC, Singh A, Eriksson KEL. Microorganisms and enzymes involved in the degradation of plant fiber cell wall. Adv Biochem Eng Biotechnol 1997;57:47–125. http://dx.doi.org/10.1007/BFb0102072.
[7] Aarti C, Arasu MV, Agastian P. Lignin degradation: A microbial approach. S Indian J Biol Sci 2015;1:119–27.
[8] Nagar S, Jain RK, Thakur VV, Gupta VK. Biotechnological application of cellulase poor and alkali stable xylanase by *Bacillus pumilus* SV-85S in biobleaching. 3 Biotech 2013;3:277–85.
[9] Margaritatis A, Zajic JE. Mixing, mass transfer and scale up of polysaccharide fermentations. Biotechnol Bioeng 1978;20:939–1001. http://dx.doi.org/10.1002/bit.260200702.
[10] Mittal A, Nagar S, Gupta VK. Production and purification of high levels of cellulase-free bacterial xylanase by *Bacillus* sp. SV-34S using agro-residue. Ann Microbiol 2013;63:1157–67. http://dx.doi.org/10.1007/s13213-012-0574-9.
[11] Kumar L, Kumar D, Nagar S, Gupta R, Garg N, Kuhad RC, et al. Modulation of xylanase production from alkaliphilic *Bacillus pumilus* VLK-1 through process optimization and temperature shift operation. 3 Biotech 2014;4:345–56.
[12] Bocchini DA, Alves-Prado HF, Baida LC, Roberto IC, Gomes E, Da Silva R. Optimization of xylanase production by *Bacillus circulans* D1 in submerged fermentation using response surface methodology. Process Biochem 2002;38:727–31. http://dx.doi.org/10.1016/S0032-9592(02)00207-8.
[13] Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 2007;24:1596–9. http://dx.doi.org/10.1093/molbev/msm092.
[14] Bailey MJ, Biely P, Poutanen K. Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol 1992;23:257–70. http://dx.doi.org/10.1016/0168-1656(92)90074-J.
[15] Nagar S, Mittal A, Kumar D, Kumar L, Kuhad RC, Gupta VK. Hyper production of alkali stable xylanase in lesser duration by *Bacillus pumilus* SV-85S using wheat bran under solid state fermentation. New Biotechnol 2011;28:581–7. http://dx.doi.org/10.1016/j.nbt.2010.12.004.
[16] Singh V, Pandey VC, Agrawal S. Potential of *Laceyella sacchari*s train B42 crude xylanase in biobleaching of kraft pulp. Afr J Biotechnol 2013;12:570–9.
[17] Sanghi A, Garg AN, Gupta VK, Mittal A, Kuhad RC. One-step purification and characterization of cellulase-free xylanase produced by alkalophilic *Bacillus subtilis* ash. Braz J Microbiol 2009;41:467–76. http://dx.doi.org/10.1590/S1517-83822010000200029.
[18] Ammoneh H, Harba M, Akeed Y, Al-Halabi M, Bakri Y. Isolation and identification of local *Bacillus* isolates for xylanase biosynthesis. Iran J Microbiol 2014;6:127–32.
[19] Bai Y, Wang J, Zhang Z, Yang P, Shi P, Luo H, et al. A new xylanase from thermoacidophilic *Alicyclobacillus* sp. A4 with broadrange pH activity and pH stability. J Ind Microbiol Biotechnol 2010;37:187–94. http://dx.doi.org/10.1007/s10295-009-0662-4.
[20] Ko CH, Tsaia CH, Tu J, Lee HY, Kua LT, Kuod PA, et al. Molecular cloning and characterization of a novel thermostable xylanase from *Paenibacillus campinasensis* BL11. Process Biochem 2010;4:1638–44. http://dx.doi.org/10.1016/j.procbio.2010.06.015.
[21] Pason P, Kosugi A, Waeonukulet R, Tachaapaikoon C, Ratanakhanokchai K, Arai T, et al. Purification and characterization of a multienzyme complex produced by *Paenibacillus curdlanolyticus* B-6. Appl Microbiol Biotechnol 2010;85:573–80. http://dx.doi.org/10.1007/s00253-009-2117-2.
[22] Battan B, Sharma J, Kuhad RC. High-level xylanase production by alkaliphilic *Bacillus pumilus* ASH under solid-state fermentation. World J Microbiol Biotechnol 2006;22:1281–7. http://dx.doi.org/10.1007/s11274-006-9173-x.
[23] Kamble RD, Jadhav AR. Production, purification and characterisation of alkali stable xylanase from *Cellulosimicrobium* sp. MTCC 10645. Asian Pac J Trop Biomed 2012;2:1790–7. http://dx.doi.org/10.1016/S2221-1691(12)60496-1.
[24] Blanco A, Vidal T, Colom J, Pastor FIJ. Purification and properties of xylanase-A from alkali-tolerant *Bacillus* sp. strain BP-23. Appl Environ Microbiol 1995;61:4468–70.
[25] Archana A, Satyanarayana T. Xylanase production by thermophilic *Bacillus licheniformis* A99 in solid-state fermentation. Enzyme Microb Technol 1997;21:12–7. http://dx.doi.org/10.1016/S0141-0229(96)00207-4.
[26] Sugumaran KR, Kumar BK, Mahalakshmi M, Ponnusami V. Cassava baggase — Low cost substrate for thermo-tolerant xylanase production using *Bacillus subtilis*. Int J ChemTech Res 2013;5:394–400.
[27] Khodayari F, Cebeci Z, Ozcan BD. Optimization of xylanase and α-amylase production by alkaline and thermophilic *Bacillus* isolate KH-13. J Entomol Zool Stud 2014;2:295–303.
[28] Thomas L, Sindhu R, Binod P, Pandey A. Production of a cellulase-free alkaline xylanase from *Bacillus pumilus* MTCC 5015 by submerged fermentation and its application in biobleaching. Ind J Exp Biol 2015;53:356–63.
[29] Kumar S, Sharma N, Pathania S. Purification, characterization and gene encoding of xylanase produced from *Bacillus tequilensis* SH0 isolated from compost using low cost wheat bran as substrate. Global J Biol Agric Health Sci 2014;3:1–15.
[30] Rathee P, Dahiya D, Battan B. Xylanase production under submerged fermentation by isolated *Bacillus tequilensis* strain and its potential application in animal feed. Int J Appl Phys BioChem Res 2014;2:1–8.
[31] Azeri C, Tamer AU, Oskay M. Thermoactive cellulase-free xylanase production from alkaliphilic *Bacillus* strains using various agro-residues and their potential in bioleaching of kraft pulp. Afr J Biotechnol 2010;9:63–72.
[32] Yang VW, Zhuang Z, Elegir G, Jeffries TW. Alkaline-active xylanase produced by an alkaliphilic *Bacillus* sp. isolated from kraft pulp. J Ind Microbiol 1995;15:434–41. http://dx.doi.org/10.1007/BF01569971.
[33] Sá-Pereira P, Mesquita A, Duarte JC, Barros MRA, Costa-Ferreira M. Rapid production of thermostable cellulose-free xylanase by a strain of *Bacillus subtilis* and its properties. Enz Microb Technol 2002;30:924–33. http://dx.doi.org/10.1016/S0141-0229(02)00034-0.
[34] Geetha K, Gunasekaran P. Optimization of nutrient medium containing agricultural waste for xylanase production by *Bacillus pumilus* B20. Biotechnol Bioprocess Eng 2010;15:882–9. http://dx.doi.org/10.1007/s12257-009-3094-0.
[35] Sanghi A, Garg N, Kuhar K, Kuhad RC, Gupta VK. Enhanced production of cellulase-free xylanase by alkalophilic *Bacillus subtilis* ASH and its application in biobleaching of kraft pulp. BioResources 2009;4:1109–29.
[36] Ling Ho H, Heng KL. Xylanase production by *Bacillus subtilis* in cost-effective medium using Soybean hull as part of medium composition under submerged fermentation (SmF) and solid state fermentation (SSF). J Biodivers Bioproc Dev 2015;2:143. http://dx.doi.org/10.4172/2376-0214.1000143.
[37] Ho HL. Xylanase production by *Bacillus subtilis* using carbon source of inexpensive agricultural wastes in two different approaches of submerged fermentation (SmF) and solid state fermentation (SSF). J Food Process Technol 2015;6:437. http://dx.doi.org/10.4172/2157-7110.1000437.
[38] Gupta U, Kar R. Xylanase production by a thermo-tolerant *Bacillus* species under solid-state and submerged fermentation. Braz Arch Biol Technol 2009;52:1363–71. http://dx.doi.org/10.1590/S1516-89132009000600007.
[39] Shanthi V, Roymon MG. Response surface optimization of xylanase production by indigenous thermoalkalophillic *Bacillus* sp. Afr J Microbiol Res 2015;9:1699–705. http://dx.doi.org/10.5897/AJMR2015.7557.
[40] Ayishal Begam M, Annu A, Shameera Banu S, Vishnu Priya D. Comparison and optimization of thermostable xylanase production by *Bacillus pumilus* and *Bacillus cereus* using corn husk. Int Adv Res J Sci Eng Technol 2015;2:30–5. http://dx.doi.org/10.17148/IARJSET.2015.2907.
[41] Harris ADS, Ramalingam C. Production of xylanase from watermelon rind by *Bacillus weihenstephanesis* strain ANR1. Int J ChemTech Res 2015;8:1–5.
[42] Amore A, Parameswaran B, Kumar R, Birolo L, Vinciguerra R, Marcolongo L, et al. Application of a new xylanase activity from *Bacillus amyloliquefaciens* XR44A in brewer's spent grain saccharification. J Chem Technol Biotechnol 2015;90:573–81. http://dx.doi.org/10.1002/jctb.4589.
[43] Nagar S, Mittal A, Kumar D, Gupta VK. Production of alkali tolerant cellulase free xylanase in high levels by *Bacillus pumilus* SV-205. Int J Biol Macromol 2012;50:412–20. http://dx.doi.org/10.1016/j.ijbiomac.2011.12.026.