Co-Production of Cellulase and Xylanase Enzymes By Thermophilic *Bacillus subtilis* 276NS

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Abstract: Co-production of thermostable extracellular cellulase and xylanase was investigated using bacterial soil isolate. To evaluate the effect of culture conditions on the c-oproduction of both enzymes by *Bacillus subtilis* 276NS (GenBank accession number JF801740), a Plackett-Burman fractional factorial design was applied. Among the tested variables, yeast extract, sucrose and incubation time were the most significant variables increased cellulase and xylanase productions. Both of xylan and CMC (Carboxymethyl Cellulose) induced the xylanse enzyme production. A near-optimum medium formulation was obtained which increased the cellulase and xylanase enzymes 5.7- and 1.08 – fold higher than the yield obtained with the basal medium, respectively. Thereafter, the response surface methodology was adopted to acquire the best process conditions among the selected variables (xylan, CMC and Yeast Extract (YE)) required for improving xylanase yield. The optimal combinations of the major medium constituents for xylanase production evaluated using non-linear optimization algorithm of EXCEL-solver, was as follows (g/L): D-sucrose, 10; xylan, 10.367, CMC, 10.535; $(NH4)_2$ SO_4, 1.0; YE, 1.71; Tween-80, 0.4 and $FeSO_4$, 0.25 mg/L, at pH 8.0, temperature 35°C and incubation time 24h under shaking. The predicted optimum thermostable xylanase activity was 360 U/ml, which was around 4-times the activity with the basal medium.

Keywords: Thermostable enzymes, Experimental design, Bacillus, Medium Optimization, Enzyme production.

1. INTRODUCTION

Cellulose is an unbranched glucose polymer, composed of anhydro-D-glucose units linked by 1,4-β-D-glucoside bonds, which can be hydrolyzed by cellulolytic enzymes [1-2]. Xylan is the main component of hemicellulose in the plant cell walls. Since, hemicellulose is the most abundant polysaccharide fibers in nature that is secondary to cellulose; it has a linear backbone comprised of β-1,4-linked D-xylopyranose residues, which, depending on the origin, may present ramifications containing mainly acetyl, arabinosyl and gluconosyl residues [3]. A cellulolytic enzyme system is a complex system of enzymes composed of endoglucanase (endo-1, 4 β-D-glucanase, EC 3.2.1.4), exo-glucanase (1,4 –β-D-glucan-cellobiohydrolase, EC 3.2.1.91), and β-glucosidase (β-D-glucoside glucanohydrolase, cellobiase, EC 3.2.1.21) that acts synergistically to degrade cellulosic substrate [4-5]. As well the xylanses (EC 3.2.1.18) are a complex system, it includes: xylanases (1, 4-β-D-xylan xylanohydrolase, EC 3.2.1.8) [6] and xylosidases (1,4 β-D-xylan xylohydrolase, EC 3.2.1.37) [7].

Cellulase and xylanse enzymes have been used in many applications including, energy generation, waste treatment, production of chemicals, clarification of juice, paper manufacture, animal feed and beverage industries [8-13].

Biomass resources, such as starchy and cellulosic materials of plant origin, are the most abundant renewable resources on earth. Cellulolytic enzymes are central to biomass processing for production of energy, chemicals and bioproducts [14-17]. High cost of these enzymes, however presents a significant barrier to commercialization of ethanol and chemicals. Due to the heterogeneity and complexity of lignocelluolytic biomass conversion requires multiple enzyme activities. An efficient and cost effective enzyme system should contain balanced activities of cellulases (both endo and exo-glucanase), β-glucosidase and xylanase, and such a system should also have high titer of these activities to offset the cost of ethanol production. Statistical optimization for enhancing the co-production of cellulase and xylanase enzymes has advantage of screening of many variables affecting the co-production through placket-Burman design. This process followed by optimization of the most significant variables to produce enzymes in a maximal titers. The mixed production of these enzymes allow the use of heterogenous products of agriculture wastes and agro-based industry [18]. Many microorganisms have been reported to produce cellulase or xylanase enzymes, though until now the majority of the work has focused on the use of fungal enzymes to saccharify or liquefy celluolytic materials into sugars. During recent years

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the enzyme working under harsh condition of temperature or pH has been investigated as a potential components in laundry detergents as well as in biomass conversion. Thermostable cellulases from Bacillus, Geobacillus and Archae have been highlighted and characterized by many authors [19-27]. Also, many xylanases have been reported in many literatures [28-31]. Experimental design is considered a valuable approach for optimization the production of different bio-products, algal growth and highlighted in many literatures [32-33].

In this paper we focused the attention to produce both enzymes simultaneously by a thermotolarant *Bacillus* sp. designated 276NS. The statistical experimental design strategies used was that of Plackett-Burman to determine which of the nutritional or environmental variables were the most significant on production of targeted enzymes. Sequentially, the optimum levels of the most significant identified variables were determined through application of response surface methodology.

2. MATERIAL AND METHODS

2.1. Sample Collection and Bacterial Isolation

Sandy soil samples were collected in summer from different location of Saudi Arabia (Al-Riyadh) and Egypt (Western- desert), transported to the laboratory, stored in a cold room then analyzed. Diluted LB (Luria Bertani) medium [tryptone, 10; YE, 5; Sodium chloride 5 (g/L)], at one-tenth strength was used for bacterial isolation and colony purification. The purified isolates were qualitatively tested for the co-production of cellulase and xylanase by streaking in a Petri-dish containing LB agar supplemented with CMC or/xylan substrate (0.5%). Plate screening method was followed to identify the positive hydrolyzing isolate to any of tested substrates [34], the method based on covering the plate with congo red solution (0.1%), incubated for 20 min, the dye removed then the color was fixed by adding 2M NaCl solution. The positive hydrolyzing isolates to any of the tested substrate showed a clear zone, whereas the rest of the plate stained with pink color.

2.2. Production Conditions

The bacteria able to hydrolyze the tested substrates were selected and allowed to grow in 50-ml of production medium dispensed in 250-ml Erlenmeyer flask and incubated at 50◦C for 24 h at 200 rpm. The used production medium has the following composition: (g/L) peptone, 10; yeast extract, 3.0; $K2HPO_4$, 10; $MgSO_4.7H2O$, 0.5; CMC/or xylan, 20, as described by Honglian [35]. Quantitative estimations for cellulase and xylanase enzymes were determined independently in the former medium after 24h incubation for each isolate under reaction conditions of temperature 50°C and pH 7.6.

2.3. DNA Isolation

An overnight culture of the target isolate grown at 50°C was used for the preparation of genomic DNA. The DNA was isolated using DNA isolation Kit (Promega, Fitchburg, Wisconsim, United States) according to the manufacturer's instructions.

2.4. Molecular Identification

The bacterium was identified by 16S rRNA gene sequencing using universal primers AGAGTTTGATCMTGGCTCAG (position 8 in the 16S rRNA gene according to *E. coli* numbering) and TACGGYACCTTGTTACG ACTT (position 1514 in the 16S rRNA gene according to *E. coli* numbering). The PCR mixture consisted of 25 pmol of each primer, 10 ng of chromosomal DNA, 200 mM dNTPs and 2.5 U of Taq polymerase in 50 µl of polymerase buffer (Fermentas, Germany). The PCR was carried out for 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. After completion, a fraction of the PCR mixture was examined using agarose gel electrophoresis [36] and the remnant mixture was purified using QIAquick PCR purification reagent (Qiagen, Hilden, Germany). The 16S rRNA gene fragment (1450 bp length) was sequenced in both direction and Blast program (www.ncbi.nlm.nih.gov/blast) was used to assess the DNA similarities. The sequence has been deposited in the GenBank under the accession number JF801740. Multiple sequence alignment and molecular phylogeny were performed using BioEdit7.0.5.3 (http://www.softpedia.com/get/Science-CAD/BioEdit.shtml) [37] and TreeViewX: (http://treeview-x.en.softonic.com/) [38].

2.5. Enzyme Assay

The cellulase and xylanase were measured by monitoring the reducing sugar concentration released as glucose by dinitrosalicylic acid (DNS) method [39]. A 0.5 ml of diluted enzyme sample in 50mM phosphate buffer pH 7.5 was mixed with 0.5 ml of 0.5% of CMC or Birchwood Xylane substrate and incubated at 50°C for

fifteen min. The reaction was then terminated by adding 1 ml of DNS and heating at 100°C for 10 min. The absorbance at 540nm was measured, where one unit of cellulase or xylanase was defined as the amount of enzyme producing 1 µg of reducing sugar equivalent to glucose per minute under standard test conditions.

2.6. Plackett-Burman Screening Design

A Plackett-Burman experimental design [40] was applied to investigate the significance of various medium composition on the co-production of the cellulase/xylanse enzymes. Fourteen variables were tested at two levels, -1 for the low and +1 for the high, based on a Plackett-Burman matrix design (Table 2), representing two level factorial design and allowing the investigation of *n-1* variables in at least *n*-experiments. In this study a design matrix with sixteen trials was used to study the selected fourteen variables. The Plackett-Burman experimental design was based on a first-order model:

$$Y = ßo + \sum ßiXi \quad (1)$$

where Y is the response (enzyme activity), ßo is the model intercept, ßi is the linear coefficient, and Xi is the level of the independent variable. This model does not describe any interaction among factors, and is used to screen and determine the important factors that influence the response. In the present work, 14 assigned variables were screened in 16 trail experimental designs.

2.7. Statistical Analysis of Data

The cellulase and xylanase enzyme(s) data were subjected to statistical analysis, where Essential Experimental Design free software [41] was used for the data analysis, determining the coefficients, and the polynomial model reduction. The factors with the highest *t*-value and a confidence level over 65% and 80% were considered to be highly significant for cellulase and xylanase enzymes production, respectively.

2.8. Box-Behnken Design

To describe the nature of the response surface in the experimental region and to identify the optimum conditions for enzyme production, a Box-Behnken design [42] was applied. Table **4** presents the design matrix, consisting of 13 trials to study the most significant variables affecting xylanase production. Each variable was studied in three levels, coded -1, 0, and +1 for low, middle, and high values, respectively. To predict the optimal point, a second order polynomial function was fitted to correlate the relationship between the independent variables and the response (xylanase activity), for three factors. The equation was:

$$Y= \beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{12}X_{12}+ \beta_{13}X_{13}+\beta_{23}X_{23}+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2 \quad (2)$$

where Y is the predicted response, β_0 is the model constant, X1, X2, and X3, are the independent variables, β_{12}, β_{13}, and β_{23} are the cross product coefficients, and β_{11}, β_{22}, and β_{33} are the quadratic coefficients. Microsoft Excel 97 was used for the regression analysis of the experimental data obtained. The quality of fit of the polynomial model equation was expressed by a coefficient of determination, R^2. The experiments were performed in triplicate and the mean values are given.

2.9. Statistical Analysis of Data

The enzyme activity data were subjected to multiple linear regression using Microsoft Excel 97 to estimate the *t*-values, *P*-values, and confidence levels expressing the *P*-values as a percentage. The optimal enzyme activity value was estimated using *Solver* function of the Microsoft Excel tools.

3. RESULTS AND DISCUSSION

3.1. Isolation, Screening and Identification

The screening program was performed for the isolation of thermostable cellulase xylanase enzyme

Table 1: Production of Cellulase and Xylanase Enzymes by Different Selected Bacterial Isolates

Isolate code	Cellulase activity (U/ml)	Xylanase activity (U/ml)
276 NS	36.0	89.7
183	34.8	86.0
187	34.6	88.0
124	36.0	59.2
212	24.0	82.0

producing bacteria. Three hundred isolates were obtained from different localities in Egypt and Saudi Arabia and tested for production of targeted enzymes. The isolate coded 276NS was found able to produce both cellulase and xylanase with a high potency in plate assay and production medium (Table **1**).

To investigate the phylogenetic affiliation of this isolate, the 16S rRNA gene was sequenced and deposited in GeneBank under the accession number JF801740. A phylogenetic tree was constructed using Clustal X 2.0.11 program (http://www.softpedia.com/get/Science-CAD/Clustal-X.shtml), and showed that the isolate 276NS is more related to *Bacillus subtilis* subsp. inaquosorum strain KTH-61 (ac: HM854250) with 99% identity.

3.2. Evaluation of Culture Conditions Affecting Co-production of Cellulase and Xylanase Enzymes by thermophilic *Bacillus subtilis* 276NS

Normally, there is no general defined medium for specific enzyme production by different microbial strains [43], as every microorganism has its own particular physico-chemical nutritional requirements for either growth or primary and secondary metabolite production. Thus for the simultaneous production of both thermostable cellluase and xylanase, a screening strategy for a large number of factors is required.

To evaluate the factors affecting the co-production of both enzymes by *Bacillus subtilis* 276NS, a Plackett-Burman statistical design was employed. The settings for the fourteen examined independent variables and experimental matrix were presented and carried out according to Table **2**, where the production of thermostable cellulase and xylanase enzymes was the measured response. A wide variation was found among the different trials as regards the cellulase production results (0-187 U/ml) and xylanase (0-165 U/ml), reflecting the importance of medium optimization to attain a high yield of target products. The main effects of the examined factors on celllulase and xylanase production were calculated and are presented graphically in Figure **1**. From an analysis of the regression coefficients and *t*-values for the 14-variables (Table **3**), yeast extract is the most significant

Table 2: Plackett–Burman Experimental Design for Evaluating Factors Influencing the Co-Production of Thermostable Cellulase and Xylanase Enzymes by *Bacillus subtilis* 276NS

Trial	Sucrose (g/l) (X1)	Xylan (g/l) (X2)	CMC (g/l) (X3)	Tween 80 (g/l) (X4)	$(NH4)_2SO4$ (g/l) (X5)	Yeast-Extract (g/l) (X6)	K_2HPO4 (g/l) (X7)	KH_2PO4 (g/l) (X8)	MgSO4.7H2O (g/l) (X9)	Triton-X100 (g/l) (X10)	FeSO4 (mg/l) (X11)	pH (X12)	Temperature (°C) (X13)	Incubation time (hr.) (X14)	Cellulase activity (U/ml)	Xylanase activity (U/ml)
1	1(10)	1(10)	-1 (0)	1(2.0)	-1(0.2)	-1(0.4)	-1(0.1)	1(0.5)	1(0.25)	1(2.0)	-1(0.5)	-1(6)	1(50)	-1(12)	0	0
2	-1 (2)	1(10)	1(10)	-1(0.4)	1(1.0)	1(2.0)	1(0.5)	-1(0.1)	1(0.25)	1(2.0)	-1(0.5)	-1(6)	-1(35)	1(24)	39	75
3	1(10)	-1 (0)	1(10)	1(2.0)	-1(0.2)	-1(0.4)	-1(0.1)	1(0.5)	-1(0.00)	1(2.0)	1(2.5)	-1(6)	-1(35)	-1(12)	0	53
4	-1 (2)	1(10)	-1 (0)	1(2.0)	1(1.0)	1(2.0)	1(0.5)	-1(0.1)	1(0.25)	-1(0.4)	1(2.5)	1(8)	-1(35)	-1(12)	71	71
5	-1 (2)	-1 (0)	1(10)	-1(0.4)	1(1.0)	1(2.0)	-1(0.1)	1(0.5)	-1(0.00)	1(2.0)	1(2.5)	1(8)	1(50)	-1(12)	30	32
6	-1 (2)	-1 (0)	-1 (0)	1(2.0)	-1(0.2)	-1(0.4)	1(0.5)	-1(0.1)	1(0.25)	-1(0.4)	-1(0.5)	1(8)	1(50)	1(24)	49	49
7	1(10)	-1 (0)	1 (0)	-1(0.4)	1(1.0)	1(2.0)	1(0.5)	1(0.5)	-1(0.00)	1(2.0)	1(2.5)	-1(6)	1(50)	1(24)	0	0
8	1(10)	1(10)	-1 (0)	-1(0.4)	-1(0.2)	-1(0.4)	-1(0.1)	1(0.5)	1(0.25)	-1(0.4)	-1(0.5)	1(8)	-1(35)	1(24)	186	165
9	1(10)	1(10)	1(10)	-1(0.4)	-1(0.2)	-1(0.4)	1(0.5)	-1(0.1)	1(0.25)	1(2.0)	1(2.5)	-1(6)	1(50)	-1(12)	0	31
10	-1 (2)	1(10)	1(10)	1(2.0)	-1(0.2)	-1(0.4)	-1(0.1)	1(0.5)	-1(0.00)	1(2.0)	-1(0.5)	1(8)	-1(35)	1(24)	0	57
11	1(10)	-1 (0)	1(10)	1(2.0)	1(1.0)	1(2.0)	-1(0.1)	-1(0.1)	1(0.25)	-1(0.4)	1(2.5)	-1(6)	1(50)	-1(12)	32	63
12	-1 (2)	1(10)	-1 (0)	1(2.0)	1(1.0)	1(2.0)	-1(0.1)	-1(0.1)	-1(0.00)	1(2.0)	1(2.5)	1(8)	-1(35)	1(24)	82	100
13	1(10)	-1 (0)	1(10)	-1(0.4)	1(1.0)	1(2.0)	1(0.5)	-1(0.1)	-1(0.00)	-1(0.4)	-1(0.5)	1(8)	1(50)	-1(12)	139	156
14	-1 (2)	1(10)	-1 (0)	1(2.0)	-1(0.2)	-1(0.4)	1(0.5)	1(0.5)	-1(0.00)	-1(0.4)	1(2.5)	-1(6)	1(60)	1(24)	77	76
15	1(10)	-1 (0)	1(10)	-1(0.4)	1(1.0)	1(2.0)	1(0.5)	1(0.5)	1(0.25)	-1(0.4)	-1(0.5)	1(8)	-1(35)	1(24)	187	154
16	-1 (2)	-1 (0)	-1 (0)	-1(0.4)	-1(0.2)	-1(0.4)	-1(0.1)	-1(0.1)	-1(0.00)	-1(0.4)	-1(0.5)	-1(6)	-1(35)	-1(12)	44	70

*Variable levels are presented between brackets as g/L or mg/L.

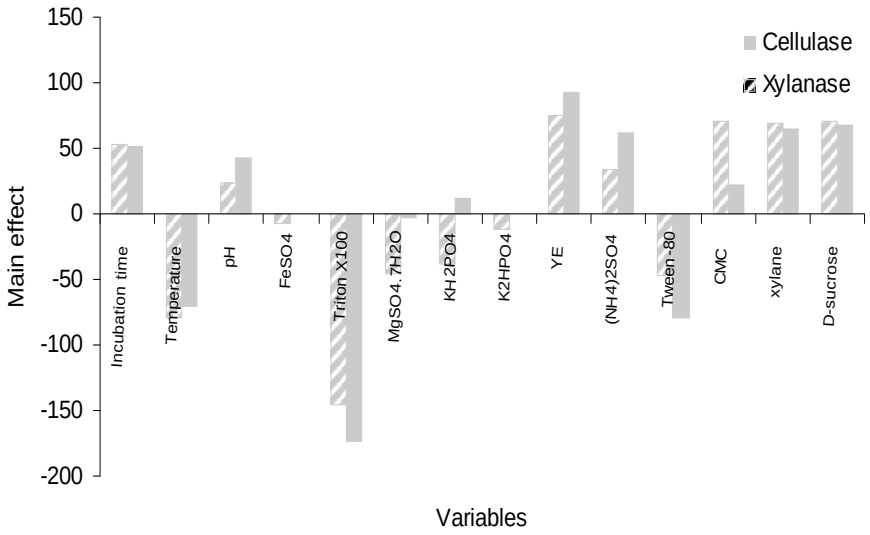

Figure 1: Effect of culture conditions on cellulase and xylanase enzymes produced by thermophilic *Bacillus subtilis* 276 NS based on Plackett-Burman design results.

Table 3: Statistical Analysis of Plackett-Burman Design Showing Coefficient Values, Plus *t* and *P*-Values, for each Variable

Variables	Cellulase (U/ml)				Xylanase (U/ml)			
	Coefficient	***t*-Statistic**	***P*-value**	**Confidence level %**	**Coefficient**	***t*-Statistic**	***P*-value**	**Confidence level %**
Intercept	36.55637	0.90946	0.530164	46.98363	67.05605	4.218063	0.148191	85.18089
X1	33.75185	1.214403	0.438553	56.14472	35.57829	3.236718	0.190764	80.92356
X2	32.44668	1.156900	0.453772	54.62285	34.91752	3.14792	0.195817	80.41834
X3	11.30450	0.442309	0.734886	26.51139	35.21009	3.483344	0.177975	82.20251
X4	-40.0212	-1.56590	0.361808	63.81925	-23,3420	-2.30923	0.260164	73.98358
X5	30.86089	1.100358	0.469604	53.03955	16.87839	1.521638	0.370138	62.98624
X6	46.00350	1.655221	0.345980	65.40197	37.35975	3.398785	0.182168	81.78324
X7	-0.28738	-0.01026	0.993467	0.653267	-5.67096	-0.51201	0.698745	30.12553
X8	5.657834	0.208888	0.868903	13.10969	-19.2503	-1.79703	0.323275	67.67247
X9	-1.16937	-0.04184	0.973380	2.661987	-22.9070	-2.07229	0.286222	71.37777
X10	-86.6922	-3.13749	0.196427	80.35730	-72.9990	-6.67997	0.094600	90.53997
X11	0.264497	0.009572	0.993906	0.609383	-3.91350	-0.35812	0.781075	21.89246
X12	21.36164	0.764298	0.584549	41.54511	11.54576	1.044492	0.486148	51.38518
X13	-35.3036	-1.30341	0.416622	58.33775	-39.8819	-3.72300	0.167054	83.29464
X14	25.70961	0.918045	0.527185	47.28149	26.36275	2.380203	0.253209	74.67906

variable increasing cellulase production, whereas sucrose, xylan and CMC were the most significant variables increasing xylanase production. Both Triton X-100, Tween- 80 and temperature are the most significant variables decreasing cellulase and xylanase enzymes production.

In the current experiments, variables with confidence levels greater than 65% were considered significant for cellulase, whereas those with confidence levels greater than 80% were considered significant for xylanase. Where, the quality of fit of the polynomial model equation was expressed by the coefficient of the determination of R2, which was 0.98 and 0.99 for the full model of cellulase and xylanase, respectively.

Therefore, when neglecting the insignificant terms with ($p> 0.5$) and ($p>0.2$) for cellulase and xylanase, respectively, the model equations for the enzyme yields could be written as:

$$Y_{cellulase}=36.55637+46.00350X_6 \quad (3)$$

$$Y_{xylanase}=67.05605+35.6X_1+35.0X_2+35.2X_3+37.4X_6 \quad (4)$$

where X1, X2, X3, and X6 are sucrose, xylan, CMC and YE, respectively.

One of the advantages of the Plackett-Burman is the ability to rank the effect of different variables on the measured response, independent on their nature (either nutritional or physical factor) or sign (whether it contributes positively or negatively). Figure **2**, shows the ranking of the factor estimates in a Pareto chart, which displays the magnitude of each factor estimate and is a convenient way to view the results of the Plackett-Burman design [44].

Thus from the confidence level of the variables, it was apparent that yeast extract was the most positive significant variable affecting cellulase enzyme, whereas sucrose, xylan and CMC were the most significant variables increasing xylanase production. Yeast extract is beneficial because of its organic nature, enriched with amino acids, activators and co-factors required for growth of the microorganism and enzyme production. This is in accordance with the results [45-46]. Among the carbon sources tested, sucrose, xylan and CMC all activated the xylanase production. This is attributed to the utilization of simple carbon sources such as sucrose for initiating the microbial growth. This is in accordance to [46-47]. Xylan is considered a biopolymer and reported as inducer for xylanase enzyme [48-49]. Also, CMC was reported as an inducer for cellulase enzyme [50], however in this study it enhanced the xylanase production by *Bacillus subtilis* 276NS. This could be related to saccharification of CMC into sugars by cellulase enzyme.

The present study investigated the significant factors affecting the co-production of thermostable celulase and xylanase enzymes in a batch fermentation by a thermotolarant *Bacillus subtilis* 276NS. The statistically designed experiment based on Plackett-Burman matrix allowed factors from different categories to be ranked to enable a better understanding of the effect of the medium. The Plackett-Burman design provides an effective and fast screening procedure and can mathematically compute the significance of a large number of factors in one experiment, thereby saving time and maintaining convincing information on each component. However, the priority of the screening program in the present study was not to examine the interaction between large numbers of variables, as only the most effective factors with positive significance needed to be selected for further optimization. Therefore, at this point, other suitable optimization statistical designs need to be applied to further optimize the significant variables determined in the present study to attain the maximum yield of each enzyme.

We concluded that the production of two enzymes are not concomitant by the studied isolate, but the

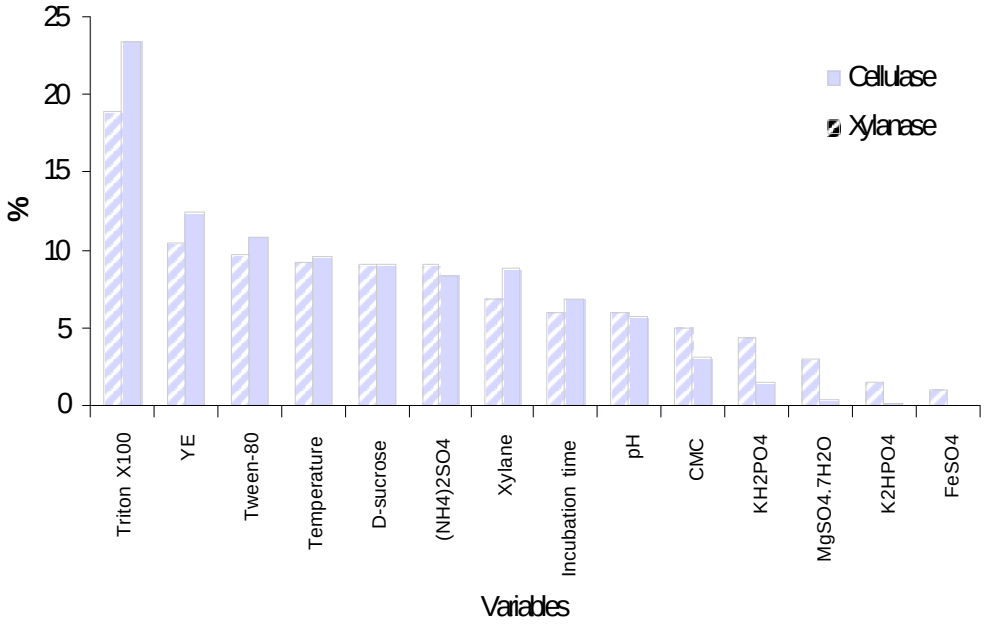

Figure 2: Pareto plot for Plackett-Burman parameter estimates of cellulase and xylanase enzymes produced by *Bacillus subtilis* 276 NS.

Table 4: Box-Behnken Factorial Design Representing Response of Thermostable Xylanase Enzyme Activity as Influenced by Xylane, CMC and Yeast Extract

Trial	Xylane (X1)	CMC (X2)	Y.E (X3)	Xylanase activity (U/ml)	
				Measured	Predicted
1	0 (1.0)	-1 (0.5)	-1 (0.1)	205	257.375
2	0 (1.0)	1 (1.5)	-1 (0.1)	156	248.375
3	0 (1.0)	-1 (0.5)	1 (0.3)	207	114.625
4	0 (1.0)	1 (1.5)	1 (0.3)	266	213.625
5	-1 (0.5)	-1 (0.5)	0 (0.2)	125	130.875
6	-1 (0.5)	1 (1.5)	0 (0.2)	207	172.875
7	1 (1.5)	-1 (0.5)	0 (0.2)	126	160.125
8	1 (1.5)	1 (1.5)	0 (0.2)	214	208.125
9	-1 (0.5)	0 (1.0)	-1 (0.1)	249	190.750
10	-1 (0.5)	0 (1.0).	1 (0.3)	19	105.500
11	1 (1.5)	0 (1.0)	-1 (0.1)	313	266.500
12	1 (1.5)	0 (1.0)	1 (0.3)	76	134.250
13	0 (1.0)	0 (1.0)	0 (0.2)	352	352.000

*Variable levels are presented between brackets expressed as g/L.

production of cellulase enzyme affects indirectly on xylanase production. Only, YE showed a high significant effect on cellulase production and the optimal value appeared at 0.8% and the maximum activity reached to 208U/ml/min. On the other hand, we noticed that xylan, CMC and YE showed a high significant effect on xylanase enzyme production.

Accordingly, based on Plackett-Burman results, a preliminary experiment was carried out using different formulations of trial number 7 which gave the highest yield of thermostable xylanase (165 U/ml) to find the most promising conditions (data not shown). A medium with the composition (g/L) of D-sucrose, 10; xylan, 10; CMC, 10; $(NH_4)_2\ SO_4$, 1.0; YE, 2.0; Tween-80, 0.4 and $FeSO_4$, 2.5 mg/L was found to be the most potent and gave 245 U/ml after 24h incubation time under shaking at 35°C and pH 8.0. Thus, it was used as basal medium for Box-Behnken design.

3.3. Response Surface Methodology for Optimization of Xylanase by *Bacillus* sp. NS276

The response surface methodology is widely applied to optimize the enzyme production and has been already reported in many studies [51-52]. Thus, to determine the optimum response region for thermostable xylanase enzyme production, the most significant variables (xylan, CMC and YE) were intensively studied at three levels: -1, 0, and +1. The 13 trial design matrix illustrating the Box-Behnken is represented in Table **4**, along with the experimental results of xylanase activity.

All the trials were performed in triplicates and the average observation was used. The results were presented in the form of surface plots (Figure **3**), The figure showed that when the xylan and CMC levels increased the xylanase level improved, especially at low level of yeast extract.

Thus to predict the optimal point within the experimental constrains, a second order polynomial function was fitted to the experimental results (non linear optimization algorithm) of xylanase activity.

$$Y=198+68.375X_1-42.875X_2-4.25X_3+23X_1X_2-25.75X_1X_3-12.75X_2X_3+29.25X_1^2-40.25X_2^2+112.5X_3^2 \quad (5)$$

Thermostable xylanase ativity, being a measure of fit for the model indicated that about 17% of the total variations were not explained by the enzyme activity. The optimal levels of the three components, as obtained from the maximum point of the polynomial model, were then estimated using the solver function of the Microsoft Excel tools and found to be (g/L): xylan, 10.367; CMC, 10.535 and YE, 1.71 with a predicted activity 360 U/ml. As such the value of the enzyme activity in the optimized medium was 4-folds the value under basal conditions, thereby this emphasizing the necessity and value of an optimization process.

The optimal conditions realized from the optimization experiment were verified experimentally and compared with the predicted optimum from the model. As a result, the estimated thermostable xylanase activity was 368 U/ml, where the predicted

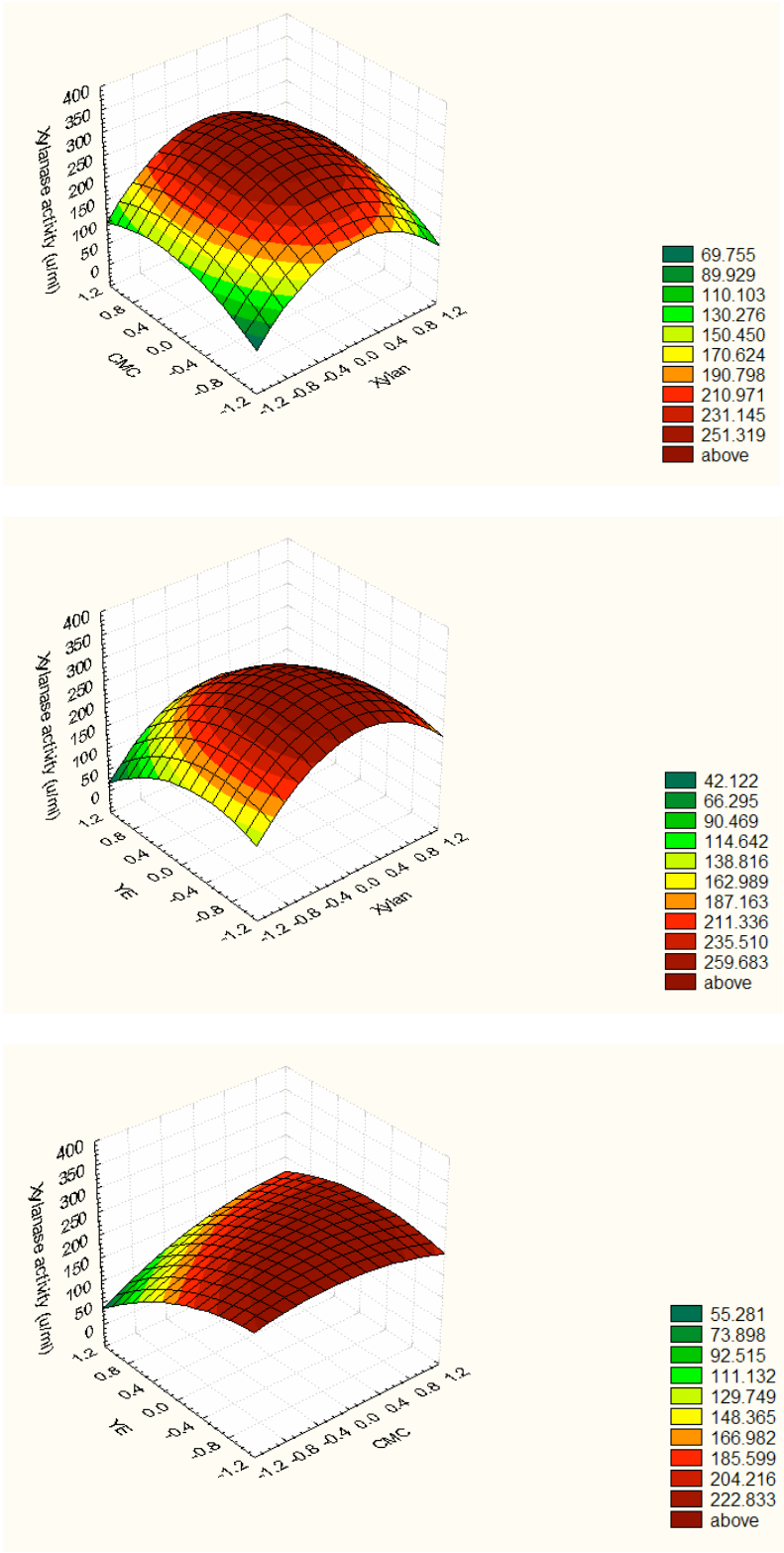

Figure 3: Three dimensional response surfaces representing thermostable xylanase enzyme yield (U/ml) from *Bacillus subtilis* 276NS as affected by culture conditions.

value from the polynomial model was 360 U/ml. Therefore, this high degree of accuracy confirmed the validity of the model under the following optimal conditions (g/L): D-sucrose, 10; xylan, 10.367, CMC, 10.535; $(NH_4)_2 SO_4$, 1.0; YE, 1.71; Tween-80, 0.4 and $FeSO_4$, 2.5 mg/L at pH 8.0 with a cultivation temperature at 35°C and incubation time 24h under shaking.

4. CONCLUSION

The present study investigated the factors affecting the co-production of thermostable cellulase and xylanase enzymes by a thermotolarant *Bacillus* sp. 276NS. The variables are highlighted, where it was found that yeast extract was the most significant factor affecting cellulase production, whereas xylan, CMC and YE were the most significant factors affecting xylanase production. The production of the two enzymes was not concomitant but, the production of cellulase enzymes and its saccharification of CMC plays an important and indirect role in production of xylanase enzyme. Therefore, we tried to make further optimization for the production of xylanase enzyme using response surface methodology and succeeded in increasing the yield 4-folds compared to the basal medium. Also, the yield of cellulase enzyme was improved and reached 5 –folds through early primary screening experiment..

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