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Statistical optimization of the production of a cellulase-free, thermo-alkali-stable, salt- and solvent-tolerant xylanase from *Bacillus halodurans* by solid state fermentation

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ABSTRACT

A cellulase-free, thermo-alkali-stable, salt- and solvent-tolerant xylanase (Bhxyl) was produced from mutant strain of a newly isolated Bacillus halodurans FNP 135. Bhxyl showed optimal activity at 75 ^{0}C and was stable at pH 6-10, displaying 60% activity even at pH 12. Its T½ was 45 min at 70 ^{0}C. Bhxyl retained 76% activity in 1M NaCl and was highly tolerant (> 85%) to organic solvents (50% v/v) and surfactants (1%). Thin layer chromatography analysis revealed that xylose was the sole end product of xylan hydrolysis by the crude enzyme. Bhxyl yield was enhanced by mutation and optimization of process parameters for wheat bran based solid state fermentation (SSF) using Plackett-Burman design and response surface methodology. Under optimal conditions viz. incubation time, 68.40 h; sodium carbonate, 2.31% (w/w) and wheat bran: moisture ratio, 1:0.8 (w/v) in tap water, a yield of 5056 ± 96 U/g wheat bran, a 16 fold increase over unoptimized conditions, was obtained. Cost effective enhanced production of a highly stable xylanase was achieved for its biotechnological applications in pulp and paper industry and biomass degradation.

Key words: Xylanase; Cellulase-free; Thermo-alkali-stable; Solvent-tolerant; *Bacillus halodurans.*

INTRODUCTION

Xylanases are wide-spread among fungi [1], actinomycetes [2], yeasts [3] and bacteria [4]. Currently, the biotechnological relevance of xylanases has expanded distinctly in paper manufacturing, animal feed processing and in the production of bioethanol [5]. Xylanases are of significant importance for use in the paper and pulp industry to diminish the utilization of chlorine chemicals and bring about improved pulp brightness. As industrial pulping takes place

at elevated temperate and alkaline pH, thermo-alkali-stability is an ideal characteristic of xylanases for efficient operation. To ensure nominal impairment to pulp fibers, xylanases should also be cellulase-free [6, 7].

Thermostable enzymes work at elevated temperature thereby decreasing the demand for adding more enzymes due to protein denaturation. Moreover, increased reaction rates, reduced viscosity and lower contaminant growth are additional advantages [8]. Industrial substrates are usually contaminated with considerable amount of solvents, salts and surfactants that affect the performance of most enzymes. Thus, enzymes that tolerate these conditions are of paramount importance for direct applications. To date, there are only few reports on solvent-, salt- and surfactant-tolerant xylanases [9, 10, 11].

Cost-effective production of xylanases from low-cost agro-industrial residues is vital for their extensive and economic industrial application. The level of microbial enzyme production is influenced by a variety of nutritional and physiological factors like supply of carbon and nitrogen, physical circumstances and chemical conditions [12]. To obtain the best outcome of a biotechnological process, it is imperative to consider the individual and combined influence of all involved parameters. In conventional optimization, one factor is varied keeping all other parameters constant. This method, while helpful in certain circumstances, usually fails to consider the combined effect of more than two factors. This limitation can be eliminated by factorial design optimization and response surface analysis. Plackett–Burman design is a powerful and efficient mathematical approach to screen and evaluate the important factors that influence the response without describing interaction among them [13]. Response surface methodology (RSM) is an assortment of mathematical and statistical techniques extensively used to find out the effects of numerous variables and to attain the best possible outcomes in diverse biotechnological processes [14].

Xylanases are produced by either solid state or submerged fermentation, but solid state fermentation (SSF) is recognized for its superior enzyme yield. SSF has become an attractive alternative process to produce microbial enzymes and metabolites due to its lower capital investment and lower operation cost [15]. Although some studies on purification and characterization of cellulase-free thermo-alkali-stable xylanases have been reported, systematic optimizations of their production conditions are still limited. In this study, the production of xylanase from a mutant of newly isolated *B. halodurans* FNP 135 was optimized in solid state fermentation with wheat bran using Placket-Burman design and response surface methodology.

MATERIALS AND METHODS

Organism identification

The organism was isolated from landfill soil in Chandigarh, India by growing on Horikoshi (HK) agar [16], supplemented with 0.5% birchwood xylan, pH 10.2 at 37 ^{0}C. Xylanase activity was detected by staining with Congo Red [17]. The organism was maintained on HK agar at 4 ^{0}C and subcultured every month.

Microscopic observation, cultural and biochemical characterization of the isolate were performed using standard procedures. Molecular characterization based on 16S rDNA sequence analysis

was also performed. Genomic DNA was prepared as described by Ausubel et al. [18]. The 16S rRNA gene was amplified by PCR using universal primers and the PCR products were sequenced directly. The sequence was further compared with sequences deposited in GenBank and analyzed by the BLAST program [19]. Multiple sequence alignment of sequences was performed using CLUSTAL_X (version 2.0) [20]. Aligned sequences were analyzed using the MEGA 4 software [21].

Solid state fermentation

Inoculum was prepared by transferring a single colony from a 24-h culture plate into 20-ml HK medium and incubating at 37 ^{0}C for 16 h at 160 rpm. Separately sterilized mineral salt solutions with variable composition (Table 1) or alkaline tap water (2.31% Na_2CO_3 (w/w)) were mixed with 0.5 ml inoculums, added to 5 g sterile wheat bran and incubated at 37 ^{0}C with intermittent shaking at ≥ 95% humidity.

Enzyme extraction

After incubation, the fermented substrate was suspended in 50 ml of 0.02% Tween 80, shaken at 160 rpm for 30 min and filtered through cheese cloth. The obtained solution was centrifuged at 12,000 x g for 15 min at 4 ^{0}C and the supernatant was used as crude enzyme.

Enzyme assays

The xylanase activity was determined by measuring the release of reducing sugars from 0.9 ml birchwood xylan (0.5% in 100 mM Tris.Cl buffer, pH 9) by 0.1 ml appropriately diluted enzyme at 75 ^{0}C for 5 min using the dinitrosalicylic acid method [22]. One unit of xylanase activity was the amount of enzyme required to release 1 µmol of xylose per min and was expressed as U/g dry substrate used. CMCase and FPase activities were determined according to the methods of Ghosh [23]. All experiments were carried out at least in triplicates and the results presented are mean values ± SD.

Mutation

The wild type strain was mutated using ethyl methane sulphonate (EMS) according to Miller [24]. The mutants were screened on modified HK agar supplemented with oat meal (20 g/l) instead of xylan, for economic reason, by growing them for 3 days at 37 ^{0}C. Mutants with bigger zones were grown in submerged culture [16] in HK medium and their activities compared quantitatively. The mutant with the highest activity was chosen for further work. The stability of the selected mutant was confirmed by repeated subculturing and xylanase activity measurement.

Effect of pH on activity and stability of xylanase

The optimum activity and stability were determined in the pH range of 4 to 12. After 1 h incubation, residual xylanase activity of the enzyme samples was determined.

Organic solvents, surfactants and salt tolerance of xylanase

The enzyme was incubated with solvents (50%), surfactants (1%) and NaCl (1 M) at 25 ^{0}C and 180 rpm for 6 h. Residual activity was measured under the standard assay conditions.

Effect of temperature on activity and stability of xylanase

The enzyme preparations were incubated at 60 ^{0}, 65 ^{0} and 70 ^{0}C at pH 9 for 3 h and the residual activity was determined.

Thin layer chromatography (TLC)

Birchwood xylan, 1.8 ml (1% w/v) and 0.2 ml of crude xylanase (5 U/ml) were incubated at 60 ^{0}C for 8 h. Samples were removed at different time intervals, centrifuged at 10,000 x g for 10 min and 5 µl of the supernatant was spotted on silica gel 60F_{254}. The solvent system was n-propanol: ethylacetate: water (18:1:8 v/v). Compounds were detected by spraying 10% sulfuric acid in ethanol followed by heating at 100 ^{0}C for 5 min. D-xylose was used as standard.

Selection of significant process parameters

A set of 12 experiments (Table 1) was carried out using the Plackett-Burman design of the Design expert (version 8.0.4) software (Stat-Ease Corporation, USA) for 11 components selected from literature as possible factors affecting xylanase production. Each variable was represented at two (upper and lower) levels of the range covered. From the regression analysis of the variables, the factors having significant effect on xylanase production were selected for further optimization.

Table 1. Parameters, experimental runs and responses of Placket-Burman design used for the selection of significant parameters

Run	Na_2CO_3 (%)	Moisture (Ratio)	$(NH_4)_2SO_4$ (g/l)	Urea (g/l)	$CaCl_2$ (g/l)	$FeSO_4$ (g/l)	$MgSO_4$ (g/l)	KH_2PO_4 (g/l)	K_2HPO_4 (g/l)	$CuSO_4$ (g/l)	Incubation Time (h)	Activity (U/g)
1	3.00	1.50	0.00	0.20	0.05	0.01	0.00	0.00	0.00	0.01	48.00	750±18
2	2.50	1.50	0.20	0.00	0.05	0.01	0.20	0.00	0.00	0.00	72.00	960±17
3	3.00	1.00	0.20	0.20	0.00	0.01	0.20	2.32	0.00	0.00	48.00	1527±27
4	2.50	1.50	0.00	0.20	0.05	0.00	0.20	2.32	7.54	0.00	48.00	580±22
5	2.50	1.00	0.20	0.00	0.05	0.01	0.00	2.32	7.54	0.01	48.00	1153±13
6	2.50	1.00	0.00	0.20	0.00	0.01	0.20	0.00	7.54	0.01	72.00	1333±22
7	3.00	1.00	0.00	0.00	0.05	0.00	0.20	2.32	0.00	0.01	72.00	1995±43
8	3.00	1.50	0.00	0.00	0.00	0.01	0.00	2.32	7.54	0.00	72.00	984±17
9	3.00	1.50	0.20	0.00	0.00	0.00	0.20	0.00	7.54	0.01	48.00	737±14
10	2.50	1.50	0.20	0.20	0.00	0.00	0.00	2.32	0.00	0.01	72.00	998±27
11	3.00	1.00	0.20	0.20	0.05	0.00	0.00	0.00	7.54	0.00	72.00	1887±55
12	2.50	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	48.00	856±20

Optimization using response surface methodology

A 2^3-factorial central composite design (CCD), with six axial points (a = 1.682) and six replications at the centre points (n_0 = 6) leading to a total number of 20 experiments was employed. Second degree polynomials, Eq. (1), which include all interaction terms were used to calculate the predicted response.

$$Y = \beta_0 + \Sigma \beta_i X_i + \Sigma \beta_{ii} Xi^2 + \Sigma \beta_{ij} X_i X_j \quad (1)$$

where Y represents response variable (xylanase U/g), β_0 is the interception coefficient, β_i, coefficient of the linear effect, β_{ii}, the coefficient of quadratic effect and β_{ij}, the coefficient of interaction effect. The significance of each coefficient was determined by student-t test and p values. The variability in the dependent variable was explained by R^2.

RESULTS AND DISCUSSION

Identification and mutation

The bacterial isolate was a Gram positive, endospore forming (terminal, slightly bulged, oval), motile and singly occurring big rod. It was tested negative for methyl red, Vogus Proskauer, citrate, gas and H_2S production, hydrolysis of esculin and urea. It hydrolyzed xylan, casein, gelatin, starch, tributyrine, Tween 40 and Tweeen 80, weakly reduced nitrate and was catalase and oxidase positive. It produced acid from dextrose and mannose but not from maltose, mannitol and xylose. It was identified as *B. halodurans* (99% similarity) based on BLASTn analysis of its 16S rRNA gene sequence (Fig. 1). The sequence was submitted to GenBank at NCBI (Accession no. HM 484173) and the culture was deposited in the Microbial Type Culture Collection and Gene Bank (MTCC) Chandigarh, India with accession no. MTTC 10957. The organism grew optimally at 37 ^{0}C and pH 11.5 in HK medium. No cellulase activity could be detected even in 10X concentrated culture supernatant (unpublished data).

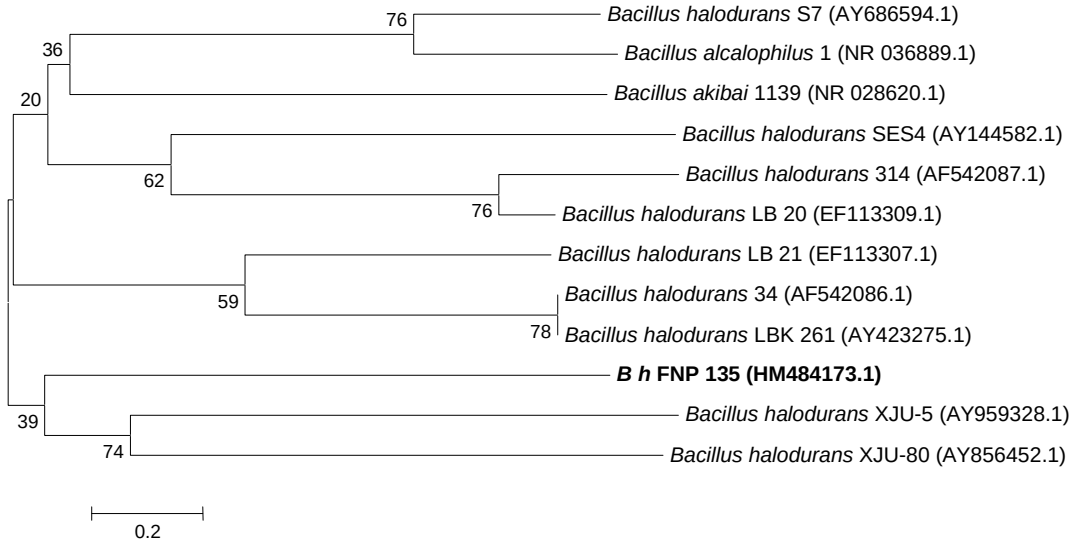


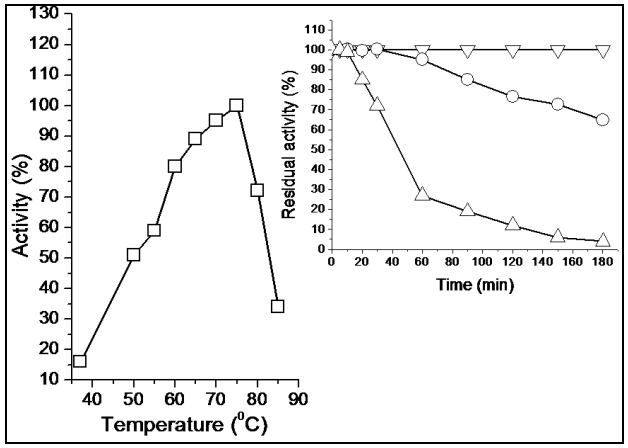
Fig. 1. Unrooted Neighbor joining Phylogenetic tree (Mega 4) based on 16S rDNA gene sequences of the isolate *B. halodurans* FNP 135 and closest species.

For mutant screening, expensive purified xylan was replaced with oatmeal that reduced the cost and avoided the resource intensive preparation of replica plates and staining procedures. Mutants could easily be identified by clear zones around their colonies. The selected mutant produced 228.6% (32 U/ml) xylanase compared to wild type strain (14 U/ml) in 0.5% birchwood xylan supplemented HK medium after 72 h incubation at 160 rpm and 37 ^{0}C. The mutant strain was used for further work.

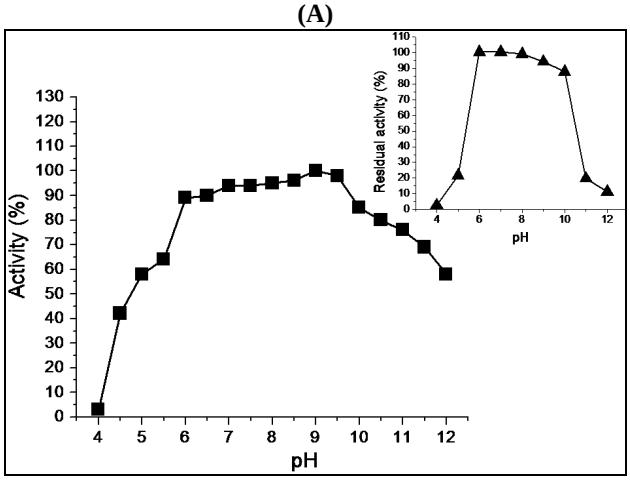
Effect of temperature and pH on Bhxyl activity and stability

The enzyme was optimally active at 75 ^{0}C (pH 9). At 60 ^{0}C and 65 ^{0}C the crude enzyme was 100% and 65% stable after incubation for 3h. T½ was 45 min at 70 ^{0}C and pH 9 (Fig. 2A). Bhxyl was active in a wide range of pH (6-12), optimally at 9-9.5 and retained 60% of its activity even at pH 12. It was completely stable from pH 6 to 8 and maintained 94% and 88% of its activity at pH 9 and 10 respectively, after incubation at 65 ^{0}C for 1 h (Fig. 2B). These properties showed

that Bhxyl was thermo-alkali-stable enzyme, which is desirable for application in the pulp and paper industry.



(A)



(B)

Fig. 2 (A). Effect of temperature on Bhxyl activity (□) and stability (inset) at pH 9.0. Stability was investigated by pre-incubating at 60 ^{0} (▽), 65 ^{0} (○) and 70 ^{0}C (△) for 3 h and measuring residual activity. 100% activity = 34 U/ml. (B). Effect of pH on Bhxyl activity (■) at 75 ^{0}C and stability (inset). Stability was studied by pre-incubating at pH 4-12 at 65 ^{0}C (▲) for 1h and measuring residual activity (Acetate buffer, pH 4-5.5; Phosphate buffer, pH 6-7.5; Tris.Cl buffer, pH 8-9; Glycine/NaOH buffer, pH 9.5-10.5; Phosphate/NaOH buffer, pH 11-11.5 and KCl/NaOH buffer, pH 12).

Organic solvents, surfactants and salt tolerance of the enzyme

Effect of organic solvents (50% v/v) with different log P values (a descriptor of hydrophobicity) on Bhxyl activity was studied. The activity of Bhxyl was enhanced slightly by acetone, benzene, hexane and isooctane; was not affected by dimethyl sulfoxide, petroleum ether, acetonitrile and dioxane; more than 90% activity was maintained in glycerol, ethanol and isopropanol but only 57 % in 10% ethyl acetate (Table 2). Stability of Bhxyl in organic solvents did not follow log P trend. The enzyme was highly tolerant to surfactants at 1% concentration. Non-ionic surfactants had negligible effect on the enzyme. The activity was slightly reduced by ionic surfactants. It was also tolerant to high concentration of NaCl. At 1 M, Bhxyl retained 76% activity. As many industrial processes are carried out in high concentrations of solvents, salts and surfactants, tolerance to these agents makes the enzyme more suitable and economical for application. For

instance, ethanol-tolerant xylanase is required for biofuel production [10], solvent- and salt-tolerant xylanases are applied for bioremediation of solvent-contaminated industrial wastewaters [25], solvent- and surfactant-tolerant xylanases are used in deinking of recycled paper [26] and solvent tolerance facilitates the selective precipitation, recovery and reuse of enzymes [27].

Table 2. Bhxyl stability in organic solvents, surfactants and NaCl. 100% relative activity was 34 U/ml.

Organic solvent (50 %)	Log *P*	Relative activity (%)
Glycerol	-2.55	95
Dimethyl Sulfoxide	-1.38	100
Dimethyl formamide	-1.04	30
Methanol	-0.76	86
Ethanol	-0.24	92
Acetone	-0.23	108
Acetonitrile	-0.15	97
Isopropanol	0.07	91
Formaldehyde	0.35	37
Ethyl acetate	0.71	0 (57*)
Dioxan	1.1	97
Benzene	2.0	112
Chloroform	2.0	88
Xylene	3.1	89
Hexane	3.6	127
Petroleum ether	4.0	99
Isooctane	4.5	114

Surfactant (1 %)	Relative activity (%)
CTAB	34
Polyethylene glycol	92
Tween 20	95
Tween 80	99
Triton X-100	99
Polyvinyl alcohol	103
NaCl (1M)	76

CTAB: Cetyltrimethylammonium Bromide

** relative activity at 10% solvent concentration*

Fig. 3. TLC analysis of the hydrolysis products released by Bhxyl from birchwood xylan.

Xylan hydrolysis

TLC analysis of the xylan hydrolysis end products showed that xylose was the only end product. As the reaction time was followed from 5 min to 8 h, the amount of xylose produced increased (Fig. 3), but no intermediate xylooligosaccharides could be detected indicating that endoxylanase

and β-xylosidase are involved [28]. In addition to providing fermentable sugar, the elimination of xylan and its oligomers to xylose was demonstrated to decrease their cellulase inhibitory effect in lignocelluloses' saccharification for biofuel production [29].

Selection of influential factors by Plackett-Burman design

Data obtained from the Placket-Burman design trials was statistically analyzed using the soft ware to evaluate and rank the 11 variables by their degree of impact on xylanase yield. The standard analysis of variance (ANOVA) results calculated from the experimental runs showed the significance of the model (0.0002) and of three parameters i.e. Sodium carbonate (0.0081), moisture level (0.0002) and incubation time (0.0021) with $p < 0.05$. The significant parameters were then optimized by response surface methodology. The other insignificant parameters were kept at their high levels as components of the moistening solution.

Optimization with RSM

The explanatory model obtained from the data was:

$$\text{Sqrt } Y = 62.76+7.12*A-8.75*B-2.61*C-3*A*B-1.65*A*C+2*B*C-8.15*A^2-5.15*B^2-9.40*C^2 \quad (2)$$

where Y is the response value (xylanase activity [U/g]) and A, B, and C are sodium carbonate (%), moisture level (v/w) and incubation time (h) respectively. Maximum activity (4163 U/g) was obtained at 2.5% Na_2CO_3, 0.8 moisture level and 60 h incubation (Table 2B).

Analysis of variance (ANOVA) of the model for the xylanase yield is shown in Table 4. All first order, second order and two level interactions were significant with 99.99% level of significance. The value of the correlation coefficient, R^2 (0.9971) showed that the regression model provides accurate description of the experimental data. A reasonable agreement between predicted (0.9912) and adjusted (0.9946) R^2 was also observed. The lack of fit F-value (0.29) was not significant relative to the pure error which is desirable. All these evaluations confirmed that the model can be used for the prediction of xylanase yield of the fermentation process within the given range of variables.

In order to determine the optimum conditions of xylanase production, a solution was given by the soft ware's numerical optimization option. The parameters moisture level, sodium carbonate and incubation time were adjusted to be in range while the xylanase yield was assumed to be maximum. The RSM model predicted that a medium containing sodium carbonate 2.31%, moisture level 0.8 (v/w), and incubation time 68.40 h gives maximum xylanase production of 4928 U/g of wheat bran used. Validation experiment was carried out under these conditions and 5009 ± 88 U/g of xylanase was obtained (Table 3C). The experimental value was found to be very close to the predicted value and hence, the model was successfully validated.

Table 3. (A). Experimental range and levels of independent test variables used in central composite rotary design. (B). Central composite rotary design matrix with experimental and predicted values of xylanase production. (C). Validation experiment result

A.

Independent variables	Level				
	-1.682	**-1**	**0**	**1**	**1.682**
Na_2CO_3 (%)	1.2	1.5	2.0	2.5	2.8
Moisture (v/w)	0.73	0.8	0.9	1.0	1.07
Incubation time (h)	52	60	72	84	92

B.

	Variables			Response		
Std run	**Na_2CO_3**	**Moisture**	**Incubation time**	**Actual**	**Predicted**	**Residual**
1	-1	-1	-1	1760±38	1806.95	-46.95
2	1	-1	-1	4163±75	4077.60	85.40
3	-1	1	-1	630±12	563.93	66.07
4	1	1	-1	1413±29	1354.08	58.92
5	-1	-1	1	1239±30	1268.68	-29.68
6	1	-1	1	2820±55	2856.82	-36.82
7	-1	1	1	800±13	856.16	-56.16
8	1	1	1	1040±15	963.80	76.20
9	-1.682	0	0	709±11	655.23	53.77
10	1.682	0	0	2560±37	2655.13	-95.13
11	0	-1.682	0	3887±76	3856.22	30.78
12	0	1.682	0	1147±17	1219.14	-72.14
13	0	0	-1.682	1651±25	1734.09	-83.09
14	0	0	1.682	995±20	953.27	41.73
15	0	0	0	4000±66	3939.32	60.68
16	0	0	0	3933±65	3939.32	-6.32
17	0	0	0	3900±58	3939.32	-39.32
18	0	0	0	3633±59	3939.32	-306.32
19	0	0	0	4120±74	3939.32	180.68
20	0	0	0	4057±70	3939.32	117.68

C.

	Variables			Response			
Std run	**Na_2CO_3 (%)**	**Moisture (ratio)**	**Incubation time (h)**	**Actual (U/g)**	**Predicted (U/g)**	**Residual**	**Error (%)**
1	2.31	1:0.8	68.40	5009±88	4928	81	1.64

Table 4. Analysis of variance (ANOVA) for the model developed for xylanase yield after fermentation

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	4248.08	9	472.01	387.24	< 0.0001	significant
A-Na_2CO_3	692.37	1	692.37	568.02	< 0.0001	
B-Moisture	1044.75	1	1044.75	857.11	< 0.0001	
C-Incubation time	92.87	1	92.87	76.19	< 0.0001	
AB	72.11	1	72.11	59.16	< 0.0001	
AC	21.75	1	21.75	17.84	0.0018	
BC	32.06	1	32.06	26.30	0.0004	
A^2	1043.37	1	1043.37	855.98	< 0.0001	
B^2	382.50	1	382.50	313.80	< 0.0001	
C^2	1273.67	1	1273.67	1044.91	< 0.0001	
Residual	12.19	10	1.22			
Lack of Fit	2.77	5	0.55	0.29	0.8976	not significant
Pure Error	9.42	5	1.88			
Cor Total	4260.27	19				

Further, the replacement of the mineral salt solution with tap water under the optimized conditions resulted in a comparable production (5056 ± 96 U/g) of the enzyme. This is important for easier production with reduced cost. A similar observation has been reported for another *Bacillus* sp. on xylanase production by SSF [30].

The interaction effect of two parameters on the response while the third variable was kept at its optimal level is represented in Fig. 3. Xylanase yield was maximum at 2.31% of sodium carbonate and then declined. Xylanase yield also increased when moisture content was increased up to 0.8 and weakened afterwards. Moisture content in SSF affects the physical properties of the solid substrate. A higher than optimum level causes decreased porosity and lower oxygen transfer [31] whereas, lower level leads to reduced solubility of the nutrients of the solid substrate [32]. The optimal enzyme production at high pH and reduced moisture content, at which most bacterial species fail to grow, has an advantage of preventing contamination. It is evident from the 3D graphs that xylanase yield increased up to 68 h incubation and declined thereafter which was probably because of the depletion of available nutrients for enzyme production and proteolysis of the produced xylanase [33].

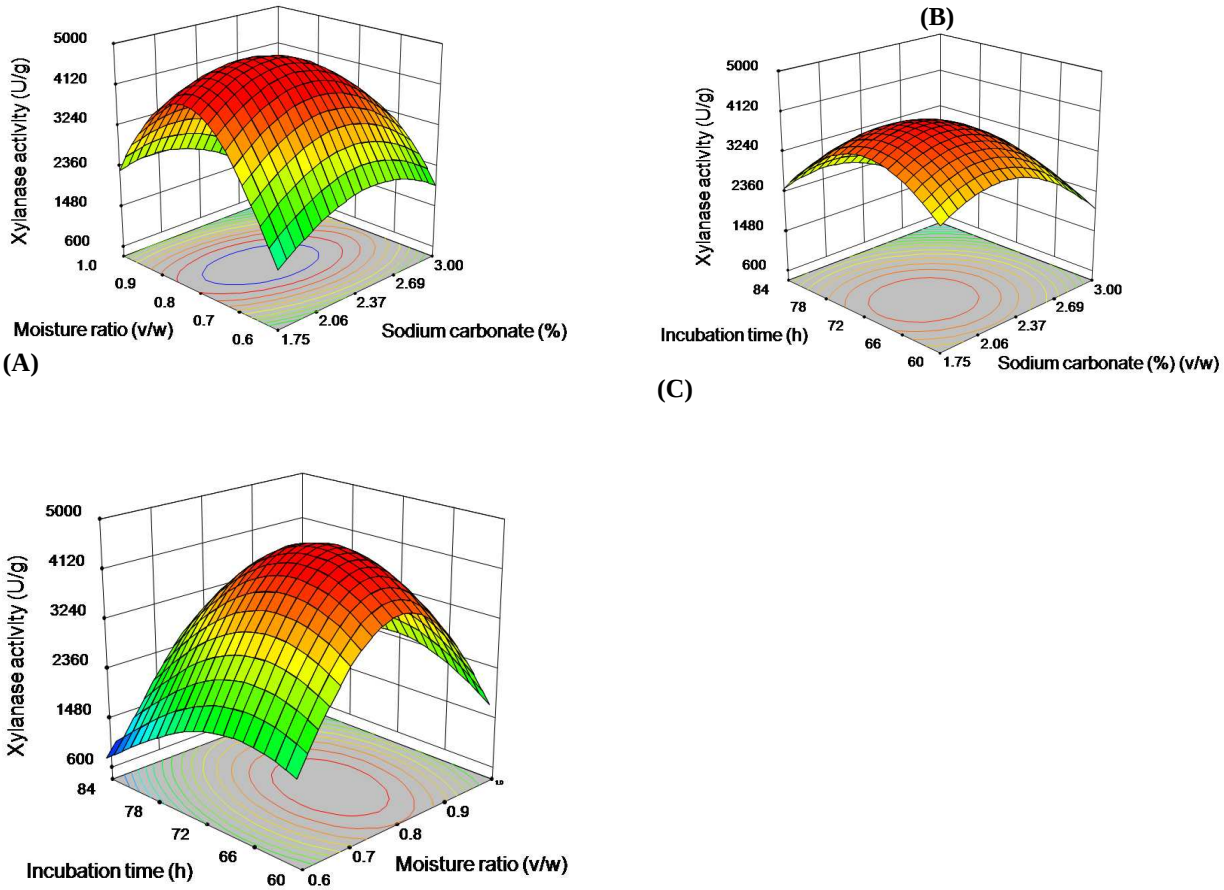


Fig. 3. 3D graph of the combined effects of sodium carbonate and moisture (A), moisture and incubation time (B) and sodium carbonate and incubation time (C) on Bhxyl production when the other factor was kept at optimal level.

The statistical optimization resulted in a 16 fold increase (5056 U/g) in xylanase production over unoptimized conditions (313 U/g) which is better than reported xylanase productions from *B. halodurans* strains to date i.e. 5.1 IU ml/1 in *B. halodurans* S7 [34], 40 IU/ml in *B. halodurans* TSEV1 using trypton and wheat bran as substrates [35] in submerged fermentations and 33.3 U/g in *B. halodurans* KR-1 [36] in solid state fermentation. Placket-Burman design and RSM have been successfully used to enhance xylanase yield by 2.19 fold in *Bacillus pumilus* ASH [37] and 2 fold in *Bacillus circulans* BL53 [14] over conventional methods in solid state fermentation with wheat bran and soybean residue respectively. Bhxyl was produced by solid state fermentation using abundantly available wheat bran, a universally suitable substrate with a considerable amount of soluble sugars like glucose, xylose, arabinose and galactose required for the initiation of growth [38], hemicelluloses (45%) as inducers and organic nitrogen sources (23%) for protein synthesis [39]. Wheat bran supports high levels of xylanase production in bacteria [40, 41].

CONCLUSION

A cellulase-free, thermo-alkali-stable, solvent-, salt- and surfactant-tolerant xylanase was produced from the newly isolated *B halodurans* FNP 135. Mutation and statistical optimization were successfully used to enhance its production to 5056 U/g dry substrate which is 16 fold increase over unoptimized conditions. The high yield of such a physico-chemically stable xylanase using alkaline tap-water moistened wheat bran provides a suitable means for its feasible industrial scale production and biotechnological applications.

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