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A MANNANASE FROM *BACILLUS NEALSONII* PN-11: STATISTICAL OPTIMIZATION OF PRODUCTION AND APPLICATION IN BIOBLEACHING OF PULP IN COMBINATION WITH XYLANASE

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ABSTRACT

β-Mannanases have number of industrial applications. One of the important application is in biobleaching of pulp, for which enzyme has to be thermo-alkali-stable and cellulase-free. In this work a new strain of *Bacillus nealsonii* PN-11 was isolated which produces a cellulase free, thermo-alkali-stable mannanase. The mannanase production from this strain has been optimized using Response surface methodology. On optimization enzyme yield increased by 17.2 fold. The enzyme was applied on softwood pulp to evaluate its bleaching potential individually and in combination with xylanase. PN-11 mannanase was able to improve the pulp properties when applied individually. Moreover, siginificant reduction in kappa number (32.23%), increase in roughness of pulp surface and enhancement of brightness (16.39%) as well as viscosity (3.20%) could be achieved when PN-11 mannanase was applied in combination with xylanase which makes it a potential candidate for application of pulp biobleaching at industrial scale.

KEYWORDS: Cellulase free mannanase, thermo-alkali-stable mannanase, *Bacillus nealsonii*, Response surface methodology, Pulp Biobleaching.

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INTRODUCTION

Hemicellulose is the second most abundant heteropolymer present in nature, usually associated with cellulose and lignin in plant cell walls[1]. The two most important and representative hemicelluloses are hetero-1, 4-β-D-xylans and hetero-1, 4-β-D-mannans[2,3]. Mannan is the predominant hemicellulosic polysaccharide in softwoods from gymnosperms. Major enzymes involved in the hydrolysis of linear mannans and glucomannans are 1, 4-β-D mannan mannohydrolases (called β-mannanases, EC 3.2.1.78), 1, 4-β-D-mannopyranoside hydrolases (called β-mannosidases, EC 3.2.1.25) and 1,4-β-D glucoside glucohydrolases (called β-glucosidases, EC 3.2.1.21). Out of these most important are β-mannanases attacking the internal glycosidic bonds of the mannan backbone chain, releasing short β-1, 4-manno-oligosaccharides[2,4]. Various mannanases from fungi, yeasts and bacteria as well as from germinating seeds of terrestrial plants have been reported[5,6,7,8,9,10,11]. Microbial mannanases are mainly extracellular and can act in a wide range of pH and temperature because of which they have found applications in industries like pulp and paper, pharmaceutical, food, coffee extraction, bioethanol production, textile, oil drilling and detergent. Thermo-alkali-stable β-mannanases have special importance with respect to their application in pulp biobleaching[12,13] which is carried out at very high temperature and pH. The majority of known β-mannanase cannot maintain catalyzing capacity and stability in such extreme environment. Moreover the enzymes to be employed in pulp industry needs to be cellulase-free[14,15,16]. The objective of the present study was to isolate cellulase-free, thermo-alkali-stable mannanase producing bacteria, optimize the conditions for its maximum enzyme yield using statistical designs of Plackett-Burman and Response Surface Methodology and to explore the application of this enzyme in biobleaching of softwood pulp individually or in combination with other hemicellulolytic enzymes like xylanase.

MATERIALS AND METHODS

Locust bean gum (LBG) were obtained from Sigma chemicals, USA. All other analytical media and reagents were purchased from SRL, Hi-Media, Qualigens, India. Unbleached softwood pulp used in this study was provided by Ballarpur Industries Limited (BILT), Yamunanagar, Haryana, India. Xylanase was obtained from alkalophilic *Bacillus* sp. NG-27 previously isolated in our laboratory and deposited at Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India (MTCC No. B0013)[14,15].

Screening and Isolation of mannanase producing bacteria

Soil samples were collected from the area where dry leaves and paper were decaying at forest area near Sukhna lake, Chandigarh, India and landfill areas of Haryana and Chandigarh, India. For enrichment, 1g of soil samples were added in Minimal Media (MM) containing LBG (0.5%)[17] and incubated at 37^0C for 96 h. After enrichment appropriate dilutions were made and plated on minimal media plates containing Locust bean gum (0.5%). The mannanase production was observed by visualization of zones around colonies (after staining with 0.2 % Congo red and then destaining by 1N NaCl).

Evaluation of the presence/absence of Carboxy Methyl Cellulase and Xylanase

All the positive isolates were grown on media containing 0.5% Carboxy Methyl Cellulose/avicell or 0.5% Xylan. The presence/absence of these enzymes was evaluated by zone of clearance around the colonies. (On staining with 0.2% Congo red and then destaining by 1N NaCl). The results were further confirmed by liquid assay (9, 24).

Mannanase production in liquid medium

20 ml broth of MM (pH 7.0) was taken in 100 ml flask. It was inoculated with 1% inoculum of log phase grown cells and incubated at 150 rpm/37°C for 24 h. The culture was centrifuged at 7826 x *g* for 10 min at 4^0C.

Mannanase activity was assayed in cell free supernatant.

Assay for Mannanase activity

Mannanase activity was assayed by measuring the amount of reducing sugars released by the enzyme using Dinitrosalicylic acid[19]. The mannanase assay mixture contained 0.5 ml of 1.0% (w/v) Locust bean gum (substrate), prepared in 100 mM Glycine-NaOH buffer, pH 8.8 and 0.5 ml of appropriately diluted enzyme. The reaction mixture was maintained at 65°C for 5 min and then 3 ml of DNSA reagent was added and boiled for 10 min. The optical density was taken at 560 nm. One unit of mannanase activity was defined as the amount of enzyme that produced 1µmol of reducing sugar as a D-mannose standard per minute by one ml of enzyme.

Temperature and pH stability

Temperature stability was determined at 60^0C and 65^0C and pH stability at 8.4 and 8.8 for different time interval up to 3 h.

Identification

The morphology of the isolate was studied by Gram staining and scanning electron microscope (SEM). Physiological and Biochemical characterization was done according to Bergey's Manual of Determinative Bacteriology. The 16S rDNA sequencing was done using forward primer (5'-AAGAGTTTGATCCTGGCTCAG-3') and reverse primer (5'-GGTTACCTTGTTACGACTT-3') and sequence was deposited in NCBI Genbank database under the Accession No JN624311.

Selection of significant process parameters

On the basis of results of one variable at a time studies (data not shown) and available literature, the variables that significantly influence the mannanase production were screened using Plackett Burman design of Design expert (version 8.0.4) software (Stat-Ease Corporation, USA). 11 independent medium compositions were evaluated at two levels (high and low). The significant variables were screened in 12 combinations in accordance with design matrix and the responses were measured. All experiments were carried out in triplicate and the average of the mannanase activity was taken as response. From the pareto chart, the factors showing highest positive effect were selected for optimization using central composited design of a response surface methodology.

Optimization using response surface methodology

Response surface methodology was used to further optimize the factor showing positive effect on mannanase production using central composite design. The behavior of the system was explained by the following quadratic equation.

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

Where Y represents response variable (mannanase Uml^{-1}), 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. A 2^3-factorial central composite design (CCD), with eight axial points ($\alpha = 2$) and six replications at the center points ($n_0 = 6$) leading to a total number of 30 experiments wherein the effect of each compound on mannanase production was taken as a response. Design Expert Version 8.0 (Stat-Ease Corporation, USA) was used for multiple regression analysis and to construct the plots of the obtained data[20]. The coded and uncoded values of the variables at various levels are given in Supplementary Table 2. The coded variables were Casein (A), LBG (B), Bactopeptone (C) and pH (D). These values were constructed into their actual values to find out the optimum range of the variables for the production of mannanase.

Biobleaching of softwood pulp

Enzyme pretreatments were carried out in 250 ml erlenmeyer flasks containing oven dried pulp (odp) at 5% consistency in a water bath set at 150 rpm. To optimize the parameters for biobleaching, pulp was treated with the different enzyme (manannase or xylanase) dose (0, 5,10,20,30,40,50,60,70,80 U/g odp [gram oven dried pulp]), reaction time (0, 30 min, 60 min, 90 min, 120 min, 150 min, 180 min), pH value (8.0,8.4,8.8) and temperature (50^0C, 55^0C, 60^0C, 65^0C). Enzyme treated pulp samples were filtered through muslin cloth and respective filtrates were studied for release of chromophores, hydrophobic compounds and release of reducing sugars[21,22]. For biobleaching, pulp was treated with mannanase or xylanase alone and in combination (simultaneously) under optimized conditions. After predetermined time intervals, reaction mixture was filtered and then treated pulp was washed with distilled water and dried at room temperature. These final bleached pulp samples were used for the determination of the physical and chemical properties. Kappa number (TAPPI Protocol, T-236 OM-85), brightness (T-452 OM-87), viscosity (T-230 OM-82) determined according to TAPPI method (Technical Association of Pulp and Paper Industry, USA).

UV- Visible-spectra of released colored compounds

To monitor the release of lignin, after enzyme treatment and washing, absorbance of collected filtrate was determined spectrophotometrically from λ 200 nm to λ 600 nm by using Hitachi UV-Visible spectrophotometer (UV-1900).

Scanning Electron Microscopy (SEM)

Samples of pulp fibers were processed for scanning electron microscopy. The fibers were washed thrice with deionized water and fixed with 2.5% glutaraldehyde solution prepared in phosphate buffer, pH 7.2, for 1h. Fibers were separated from glutaraldehyde and washed thrice with same buffer and were gradually dehydrated with acetone gradient between 30 and 90 % and finally suspended in 100 % acetone; small pieces of fibers were air dried and placed on the stubs, mounted with silver tape, and sputter coated with gold using fine coat (JEOL ion sputter, Model JFC-1100) and examined at 10 KV.

RESULTS

Screening and Isolation of Bacteria Producing Cellulase-free, Thermo-Alkali-Stable Mannanase

Soil samples were enriched using Locust bean gum (LBG) as a sole carbon source. On screening 20 bacterial colonies were found to produce mannanase. Out of these, 3 isolates were shortlisted on the basis of absence of Carboxy Methyl Cellulase enzyme (CMCase). Further selection was done on the basis of mannanase yield in liquid assay. Isolate No.11 was selected for further studies as it gave the maximum yield of cellulase-free mannanase and it was designated as PN-11. Temperature optima of PN-11 mannanase was 65^0C and pH optima was 8.8 (Fig 1A & Fig 1B). The enzyme was stable under conditions to be used for pulp biobleaching as it retained >72% and >52% activity at 60^0C and 65^0C respectively (after 3h) and for pH stability it was >70% and >50% stable at pH 8.4 and 8.8 respectively (after 3h) (Fig 2A & Fig 2B). The isolate PN-11 produced small amounts of xylanase in medium containing xylan. However, no expression of xylanase could be detected in the conditions used for the production of mannanase (MM+LBG 0.5%).

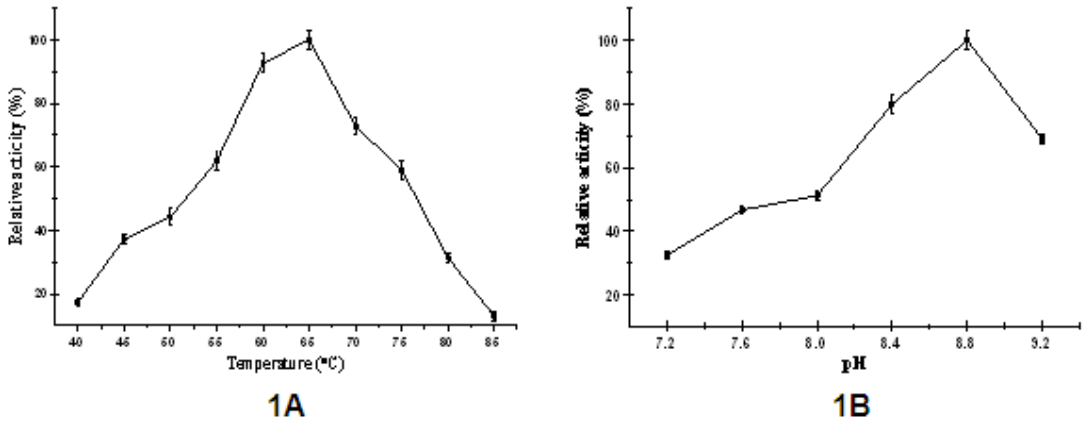


Figure 1
Effect of temperature and pH on activity of mannanase from PN-11
(A) temperature optima (B) pH optima

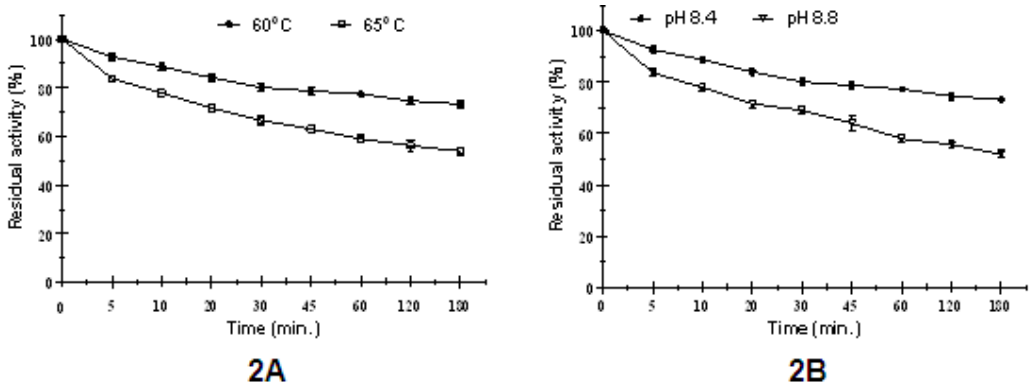
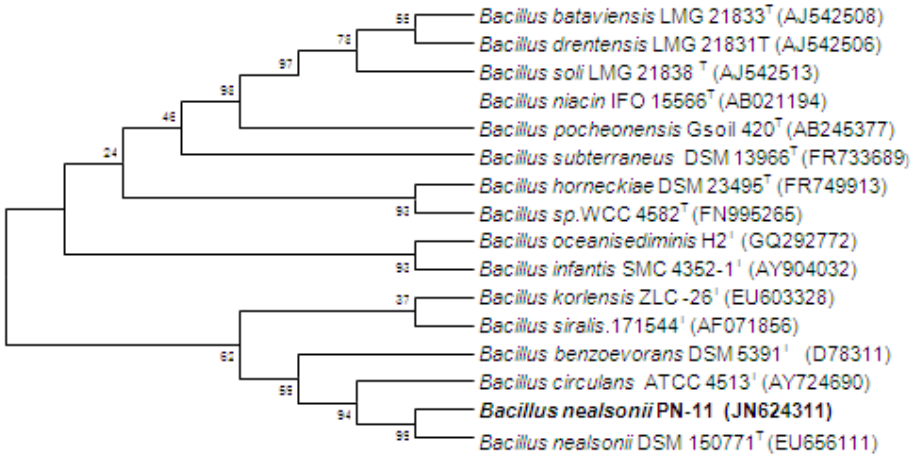


Figure 2
Temperature and pH stability of mannanase from PN-11
(A) temperature stability (B) pH stability

Identification of Mannanase Producer

The morphological and biochemical characterization of the strain was carried out according to Bergey's Manual of Systematic Bacteriology (9th Eds) and isolate PN-11 was found to be similar to that of genus *Bacillus* (Supplementary Table 1). For further identification, 16S rDNA gene sequencing was done. The 16s rDNA gene sequence (GenBank Accession No. JN624311) analysis revealed that the organism phylogenetically belonged to genus *Bacillus* and was closely related to the type strain *Bacillus nealsonii* (99.21 % similarity) (Supplementary Fig 1). The strain has been deposited in the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India (MTCC No. 11401).



Supplementary Figure 1

Unrooted neighbor-joining phylogenetic tree (Mega 4) based on 16S rDNA gene sequences of the isolate PN-11 and species of related genera. Bootstrap values (Expressed as percentages of 1,000 replications) >50% are given at nodes. The bar represents two substitutions per 100 nucleotides. GenBank accession numbers are shown in parentheses.

Supplementary Table 1

Morphological, Physiological, and biochemical characteristics of Bacillus nealsonii PN-11

(a) Microscopic characteristics	
Gram staining reaction	Gram-positive
Appearance	Rods
Spore formation	Sporulating
Arrangement	Placed singly (sometimes chains and bunches)
Capsule formation	Non-capsulated
Motility	Motile
Color	Whitish to milky in 18 h on MM LBG plate at 37°C
Size	2.5-3.5 mm in diameter after 40 h of growth
Shape & Texture	Irregular, rough and umbonate with undulate or lobate edges
Pigment formation	No (not even after 8 days at 30-37°C)
Aerobic/Anaerobic	Facultative anaerobic
Growth Temperature	25^0C-45^0C
Growth pH	2-10
Salinity	
(b) Biochemical characteristics	
Oxidase	-
Catalase	+
Lipase	+
Amylase	-
Gelatinase	-
Indole	+
Methyl-red	-
Voges-Proskauer	-
Citrate	-
Glucose	A
H/L	Motile
TSI	A/A
PPA	-
Urease	-
Mannitol	+
Mannose	+
Xylose	+

Sucrose	+
Nitrate	+
H_2S	-
Lysine	-
Ornithine	-
Arginine	-
Tryptophan	-
Growth on Blood agar	+
Growth on McConkey agar	-

Supplementary Table 2
Experimental range and levels of independent factors of CCD-RSM

Codes	Factors	Units	Levels				
			-2	-1	0	+1	+2
A	Casein	(%)	0.040	0.045	0.050	0.055	0.060
B	LBG	(%)	0.700	0.750	0.800	0.850	0.900
C	Bactopeptone	(%)	0.020	0.025	0.030	0.035	0.040
D	pH		7.000	7.500	8.000	8.500	9.000

Supplementary Table 3
Central composite rotary design matrix with experimental and predicted value of PN-11 mannanase production

Run	Factors				Mannanase Activity (Uml^{-1})		
	A	B	C	D	Actual	Predicted	Residual
1	-1	-1	-1	-1	70.22	71.09	-0.87
2	+1	-1	-1	-1	78.52	78.95	-0.43
3	+1	+1	-1	-1	68.73	68.33	0.40
4	+1	+1	-1	-1	65.08	64.19	0.89
5	-1	-1	+1	-1	62.33	62.06	0.27
6	+1	-1	+1	-1	59.92	59.78	-0.14
7	-1	+1	+1	-1	56.00	56.85	-0.85
8	+1	+1	+1	-1	42.15	42.56	0.41
9	-1	-1	-1	+1	31.50	30.83	0.67
10	+1	-1	-1	+1	40.70	30.82	0.88
11	-1	+1	-1	+1	52.27	52.38	-0.11
12	+1	+1	-1	+1	49.35	49.36	-0.11
13	-1	-1	+1	+1	50.45	51.31	-0.86
14	+1	-1	+1	+1	50.03	50.17	-0.14
15	-1	+1	+1	+1	71.10	70.41	0.69
16	+1	+1	+1	+1	58.15	57.25	0.90
17	-2	0	0	0	59.17	58.70	0.47
18	+2	0	0	0	52.27	52.38	-0.11
19	0	-2	0	0	49.35	49.36	0.14
20	0	+2	0	0	51.41	52.01	-0.60
21	0	0	-2	0	72.80	73.36	-0.58
22	0	0	+2	0	72.50	72.22	0.28
23	0	0	0	-2	61.00	60.43	-0.57
24	0	0	0	+2	34.00	34.86	-0.86
25	0	0	0	0	103.33	102.14	1.19
26	0	0	0	0	102.80	102.14	0.66
27	0	0	0	0	102.00	102.14	-0.14
28	0	0	0	0	101.00	102.14	-1.14
29	0	0	0	0	102.00	102.14	-0.14
30	0	0	0	0	101.70	101.14	-0.44

A: Casein, B: Locust Bean Gum, C: Bactopeptone, D: pH

Selection of influential factors by Plackett Burman design

The influence of eleven factors viz, incubation time, pH, temperature, inoculum %, inoculum age, locust bean gum, bactopeptone, ammonium sulphate, ammonium nitrate, sodium nitrate + meat extract and casein, for mannanase production by *Bacillus nealsonii* strain PN-11 was investigated in 12 runs using Plackett burman design. In 12 runs, variation ranging from 1.44 Uml^{-1} to 58.50

Uml^{-1} in the yield of mannanase was observed. This variation reflected the importance of optimization of medium components to attain higher yields. In pareto chart, five factors showing positive effects (pH, LBG, Inoculum age, Casein and Bactopeptone), out of these Casein, LBG, Bactopeptone and pH were selected for further optimization using Central composite design (CCD) (Fig 3).

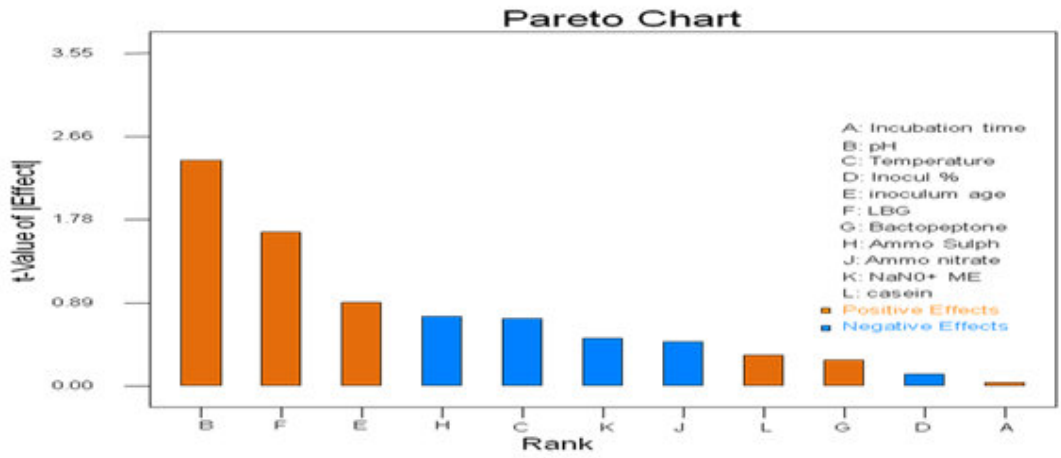


Figure 3
Pareto chart of eleven-factor affecting mannanase production

Optimization with Response Surface Methodology

A central composite design was employed to study the combined effect of variables, Casein (A), LBG (B), Bactopeptone (C) and pH (D). According to the design, 30 runs were performed and experimental as well as predicted responses were obtained (Supplementary Table 3). The relationships between factors were determined by applying a predictive quadratic polynomial equation obtained from 30 runs. The predicted and observed responses were analyzed by ANNOVA. The second order regression equation provided the levels of mannanase activity as the function of Casein, LBG, Bactopeptone and pH which can be presented in terms of coded factors as in the following equation:

$$Y(\text{response}) = +102.14 - 1.32 * A + 1.08 * B - 0.29 * C - 6.39 * D - 3.00 * A * B - 2.53 * A * C + 0.28 * A * D0.61 * B * C + 6.08 * B * D + 7.38 * C * D - 11.52 * A^2 - 13.07 * B^2 - 7.34 * C^2 - 13.62 * D^2 \quad (2)$$

where *Y* is the response value (mannanase activity [Uml^{-1}]) and *A*, *B*, *C* and *D* are Casein (%), LBG (%), Bactopeptone (%) and pH respectively. On Analysis of variance (ANOVA), a first order, second order and two level interactions were significant with 99.99% level of significance (Table 1). The value of the correlation coefficient, R^2 (0.9991) showed that the regression model provides an accurate description of the experimental data. A reasonable agreement between predicted (0.9959) and adjusted (0.9982) R^2 was also observed. From the Table 1, it can be seen that the factors with higher significant were A, B, D, AB, AC, BD, CD and squared terms of A^2, B^2, C^2 and D^2. The interaction terms AD and BC seem to be insignificant, which can be removed from the model without affecting the goodness of the model.

Table 1
Analysis of variance (ANOVA) for the model developed for mannanase yield after fermentation

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	13506.11	14	964.72	1180.43	<0.0001	significant
A	41.98	1	41.98	51.36	< 0.0001	
B	28.12	1	28.12	34.41	< 0.0001	
C	1.95	1	1.95	2.39	0.1433	
D	980.48	1	980.48	1199.71	< 0.0001	
AB	144.24	1	144.24	176.49	< 0.0001	
AC	102.82	1	102.82	125.81	< 0.0001	
AD	1.28	1	1.28	1.56	0.2305	
BC	6.03	1	6.03	7.37	0.0160	
BD	590.73	1	590.73	722.82	< 0.0001	
CD	871.14	1	871.14	1065.91	< 0.0001	
A^2	3640.85	1	3640.85	4454.91	< 0.0001	
B^2	4687.28	1	4687.28	5735.31	< 0.0001	
C^2	1476.22	1	1476.22	1806.29	< 0.0001	
D^2	5090.92	1	5090.92	6229.21	< 0.0001	
Residual	12.26	15	0.82			
Lack of Fit	8.87	10	0.89	1.31	0.4032	not significant
Pure Error	3.38	5	0.68			
Cor Total	13518.37	29				
Model fitting	C.V = 1.38	R-Sq = 99.91%		R-Sq (pred) = 99.59	R-Sq (adj) = 99.82%	

A: Casein, B: Locust Bean Gum, C: Bactopeptone, D: pH

The three dimensional (3D) response surface graphs of mannanase production based on the final model are depicted in Fig 4, which was generated for the pair-wise combination of the four factors while keeping the other one at its optimum levels. The response at the central point corresponds to a maximum degree of achievable mannanase activity for four factors. Almost all the interactions in the designed experiments produced a 'nearly spherical' variance function. All these evaluations confirmed that the model can be used for the prediction of mannanase yield within the given range of factors. The RSM model predicted that a medium containing casein 0.050(%), LBG 0.800(%), bactopeptone 0.030(%) and pH 8.0 should give maximum mannanase yield of 103.33 Uml^{-1}. Validation experiment under these conditions produced 104.0 Uml^{-1}. The experimental value was found to be very close to the predicted value and hence, the model was successfully validated. The alkaline mannanase production under unoptimized conditions was 6.0 Uml^{-1}, whereas with optimized medium the production was increased to 103.33 Uml^{-1} resulting in an approximately 17.2 fold increment in mannanase yield.

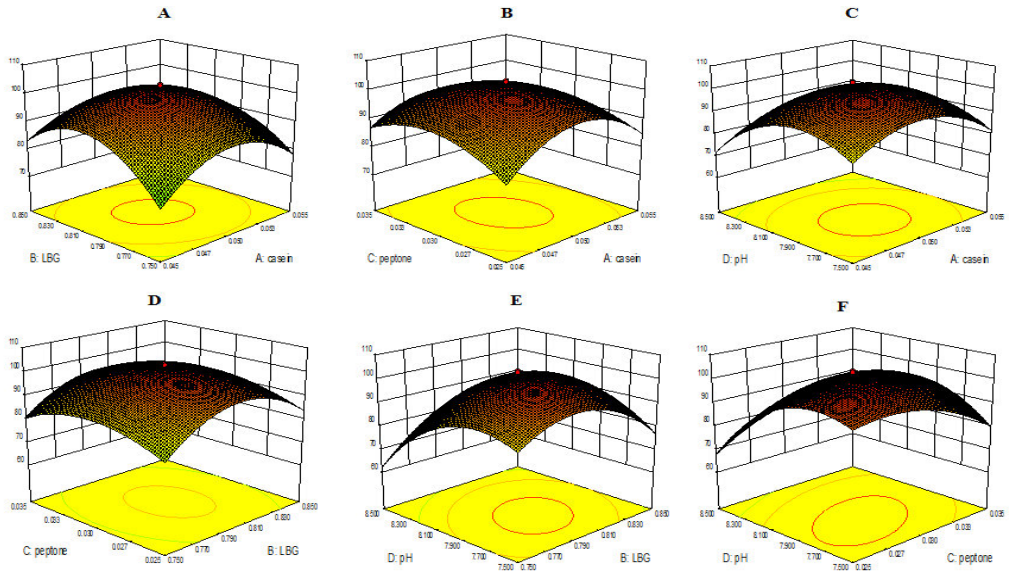
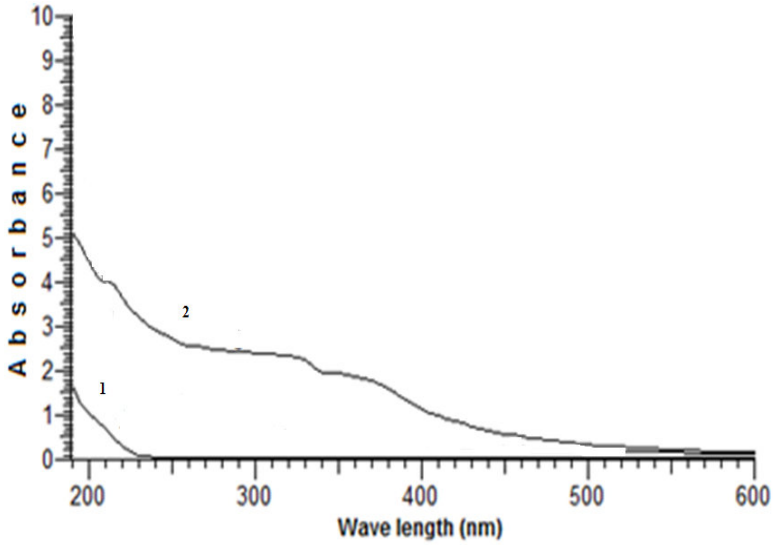


Figure 4
Three dimensional response surface plots for the effect of A) LBG and Casein, B) Bactopeptone and Casein, C) pH and Casein, D) Bactopeptone and LBG, E) pH and LBG and F) pH and Bactopeptone for the yield of PN-11 mannanase

Pulp biobleaching by PN-11 mannanase

For pulp biobleaching softwood pulp was treated with PN-11 mannanase alone and in combination with xylanase from *Bacillus* sp. NG-27 (reported earlier from our lab)[14,15] having activity in same range of temperature (70^0C) and pH (8.4) as that of PN-11 mannanase. On optimizing the conditions for pulp biobleaching temperature 60^0C, pH 8.4, and reaction time 1 h were found to be the optimal for both the enzymes. However optimum enzyme dose was 40 U/g odp [gram oven dried pulp] for PN-11 mannanase and 20 U/g odp for NG-27 xylanase respectively (unpublished data). When mannanase and xylanase were applied for biobleaching individually, under optimized conditions, 14.50% reduction of kappa number, 11.47% increase in brightness and 2.72% increase in viscosity was observed with mannanase and 22.34% reduction of kappa number, 14.75% increase in brightness and 2.40% increase in viscosity was observed with xylanase. When biobleaching were done simultaneously with mannanase and xylanase reduction in kappa number was significant (32.23%), more than when enzymes were applied individualy. Similarly significant increase of brightness (16.39%) and viscosity (3.20%) could be achieved (Table 2). The release of lignin from wood by the enzymatic treatment was confirmed by the increase in absorbance of the effluent at 200-600 nm (Supplementary Fig 2).



Supplementary Figure 2
UV-Visible spectra of the effluent of (1) untreated, (2) mannanase + xylanase treated softwood pulp

Table 2
Physicochemical properties of softwood pulp treated with PN-11 mannanase and xylanase

Treatment	Kappa number		Brightness		Viscosity	
	KN	% Reduction	ISO	% Increase		% Increase
Control	15.00	0	24.40	0	6.25	0
M	13.10	14.50	27.20	11.47	6.42	2.72
X	12.26	22.34	28.00	14.75	6.40	2.40
M + X*	10.30	32.22	28.40	16.39	6.45	3.20

M: Mannanase, X: Xylanase, M + X*: Mannanase + Xylanase (Simultaneous treatment)

Scanning electron micrograph of pulp fibre

Scanning electron micrographs of the untreated (Fig 5A), Mannanase +Xylanase treated (Fig 5B) softwood pulp clearly showed crack formation, increased roughness and flaking of fiber surfaces due to enzyme treatments. These changes are helpful in lowering down the concentration of active chemical ingredients to be used in the chemical bleaching process.

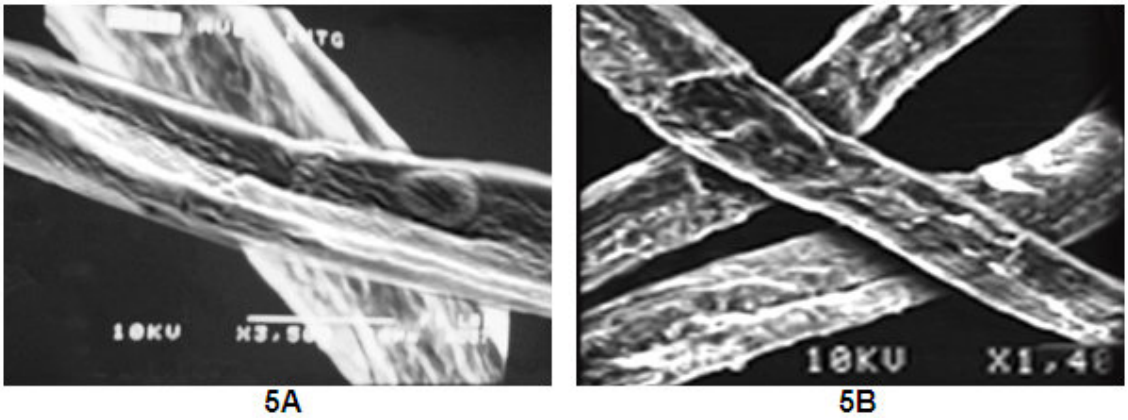


Figure 5
Scanning electron micrographs of (A) untreated, (B) Mannanase + Xylanase enzyme treated softwood pulp

DISCUSSION

Mannanases that randomly hydrolyse β-D-1,4 mannopyranoside linkages in β-1,4 mannans have applications in various fields, some important industrial applications of mannanases require the enzyme to be alkali-thermo-stable for example kraft pulping, detergent industry, hydraulic fracturing of oil well etc.[2,4]. A number of mannanases have been reported[2,4,23], some of these are alkali-stable but not thermostable[13,24,25,26] whereas others are thermostable but not alkalistable[27,29,29,30]. However for the above cited industrial applications both alkalistabilty and thermostability are required. To the best of our knowledge only reported mannanase which is alkali-thermo-stable is from alkalophilic *Bacillus* sp N16-5[31]. Moreover for the application of any enzyme in pulp industry it has to be cellulase free[14,15,16]. In the present study along with alkali-thermo-stablility, mannanase from *Bacillus nealsonii* PN-11 is also cellulase free. In addition, it has become a bottleneck problem for industrial application of extremozymes because the extremophiles directly isolated from nature have some shortcoming, for extremozyme production, such as longer generation time, lower biomass yield leading to lower productivity of the extremozymes, and the higher production costs[9,32,33]. The *Bacillus nealsonii* PN-11 is a mesophilie growing at a neutral pH but it produces a thermo-alkali-stable mannanase which provides an obvious advantage for the application of this enzyme at industrial level. All these properties together i.e. cellulase free, thermo-alkali-stable mannanase from mesophilic, neutrophilc *Bacillus nealsonii* PN-11 makes it highly suitable for application in pulp biobleaching. For industrial application enzyme yield is another important factor. The use of statistical models to optimize the enzyme yield has increased in present-day biotechnology. In present study significant improvement (17.2-fold) in the yield of alkaline mannanase from *Bacillus nealsonii* strain PN-11 has been achieved within a shorter cultivation period using statistical methods, which is much higher than other reports. A 2 fold increase in the mannanase yield has been reported from *Bacillus* sp. N16-5 and *Bacillus licheniformis* TJ-101[9,34]. Rashid et al[11] has reported 3 fold increase of enzyme yield from *Aspergillus terrus* SUK-1 and Mohamad et al[35] has reported 0.72 fold increase from *Aspergillus niger*.

There are only few reports on the application of mannanases in biobleaching of pulp but in all these cases all the required properties of the enzyme i.e. cellulase free, thermo-stability, alkali-stablility are not met[13,29,36]. Because PN-11 mannanase is cellulase-free, alkali-stable as well as thermo-stable therefore it is well suited for its application in pulp biobleaching. Moreover the application of mannanase in biobleaching can be of more use if it is applied in combination with xylanases[12,29,36,37,38]. Therefore the possible use of PN-11 mannanase in biobleaching was explored in combination with xylanase from alkalophlic *Bacillus* sp. NG-27 reported to have pulp biobleaching potential[14,15]. On optimization, biobleaching conditions (Temperature, pH, Incubation time) were found to be same for both the enzymes which further make their simultaneous application possible at industrial level. When applied individually both mannanase and xylanase could improve the pulp properties like reduction of kappa number, increase of brightness and viscosity. Moreover when they are applied in combination the additive effect of two was better than individual application of enzymes. Reduction in kappa number and increase in brightness is because of degradation of hemicellullolytic component and release of lignin from pulp. Increase in pulp viscosity to a smaller extent might have resulted from partial degradation of the low molecular weight hemicelluloses, leading to increase in cellulose fraction[39]. These results indicate that PN-11 mannanase can be a good candidate for pulp biobleaching alone as well as in combination with other hemcellulolytic enzymes.

CONCLUSION

In conclusion, the *Bacillus nealsonii* PN-11 is a mesophilie produces a cellulase free, thermo-alkali-stable mannanase which is

highly suitable for application in pulp biobleaching. A 17.2 fold increase in the yield of alkaline mannanase from *Bacillus nealsonii* strain PN-11 has been achieved within a shorter cultivation period using statistical methods. On application of PN-11 mannanase for pulp biobleaching individually and in combination with xylananse, significant reduction in kappa number, increase in brightness and viscosity was achieved. Therefore alkali-thermo-stable PN-11 mannanase can be a potential candidate for the development of feasible greener pulping industry.

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CONFLICTS OF INTEREST

None to declare.

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