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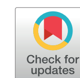
Research article

Bleach enhancement of mixed wood pulp by mixture of thermo-alkali-stable xylanase and mannanase derived through co-culturing of Alkalophilic *Bacillus* sp. NG-27 and *Bacillus nealsonii* PN-11



Steffy Angural, Indu Bala, Aditya Kumar, Deepak Kumar, Sunena Jassal, Naveen Gupta *

Department of Microbiology, Panjab University, Chandigarh, India

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ABSTRACT

The Application of a combination of enzymes is the best alternative to reduce the use of chemicals in the paper industry. *Bacillus* sp. NG-27 and *Bacillus nealsonii* PN-11 are known to produce thermoalkali stable xylanse (X) and mannanase (M) respectively having potential for pulp biobleaching. The Present study, reports the production of a mixture of X + M by co-culturing of strains in SSF and standardizing its application for pulp biobleaching. Production of enzymes by co-cultivation in SSF was optimized by statistical methods. Substantial increase in the yield of enzymes; 3.61 fold of xylanase and 37.71 fold of mannanase was achieved. Application of enzyme cocktail for pulp biobleaching resulted in a 45.64% reduction of kappa number with 55 IU g^{-1}odp of enzyme dose (xylanase:mannanase; 3:1) at pH 8.0 in 1h at 65 °C along with significant increase in brightness (11%) and whiteness (75%). The Same quality of paper as made up from chemical treated pulp can be made from enzyme-treated pulp with 30% less use of chlorine. Structural analysis of enzyme-treated pulp showed dissolution of hemicellulose as indicated by pores, cracks and increased roughness all over the surface. Cocktail of X + M produced economically in a single fermentation having all the requisite characteristics for pulp biobleaching is a highly suitable candidate for application in the pulp and paper industry.

1. Introduction

In the process of papermaking, chlorinated organic substances released in the effluent are known to be toxic and harmful [1, 2]. Although a lot of research is being done to standardize the treatment of these toxic effluents [3, 4, 5, 6] but devising processes with reduced use of chemicals is the best alternative to reduce pollution and making the process energy efficient [7, 8, 9]. Biotechnology has come a long way in developing such environment-friendly processes [10, 11, 12]. Bioprocessing of pulp using microbial enzymes is one of the most suitable alternatives [1].

Hemicellulolytic enzymes such as xylanases and mannanases are known to dissociate the chromophores bound to carbohydrates and depolymerise the hemicellulosic components present on the surface of pulp fiber [13, 14] which increases the access of bleaching chemicals to the lignin by opening the pulp structure [15, 16]. Therefore, the enzyme assisted bleaching of pulp leads to less use of chemicals [17].

For the application of enzymes in pulp biobleaching; they should be active/stable under extreme conditions (high temperature and alkaline pH) [18, 19] and free from any cellulolytic activity [20, 21]. Most importantly for effective biobleaching, removal of lignin as well as hemicelluloses present in wood is required, therefore no single enzyme is sufficient, rather a cocktail of enzymes is required [17, 18].

A cocktail of enzymes for pulp biobleaching can be prepared by using different strategies (i) preparing a mixture of enzymes by growing different microorganisms separately [22] however; it makes the process economically unviable (ii) isolating a microorganism producing a cocktail of enzymes; some reports of the cocktail of enzymes of fungal origin are there [23, 24] but due to their less stability at high temperature and pH they are not suitable for their application in pulp and paper industry (iii) co-culturing of microorganisms producing different enzymes is the most suitable methods. There is no report on the production of a mixture of thermo-alkai stable hemicellulolytic enzymes such as xylanase and mannanase by this strategy.

Alkalophilic *Bacillus* sp. NG-27 [25] and *Bacillus nealsonii* PN-11 [26] are known to produce novel extracellular thermo-alkali stable xylanase (Temp/pH optima 70ºC/8.4) and mannanase (Temp/pH optima 65ºC/8.8) respectively. The enzymes are known to have high potential

* Corresponding author.
E-mail address: ng.puchd@gmail.com (N. Gupta).

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for pulp biobleaching [25, 26]. The Present study was undertaken to produce a mixture of these enzymes by co culturing in solid-state fermentation and standardize the conditions for their application in pulp biobleaching.

2. Material and methods

2.1. Organisms

Alkalophilic *Bacillus* sp. NG-27 and *Bacillus nealsonii* PN-11 were previously isolated in our laboratory and are known to produce thermoalkali stable xylanase [25] and mannanase [26] respectively having application in pulp biobleaching and other industrially important processes. The strains have been deposited in Microbial Type Culture Collection and Gene Bank (MTCC) India with Accession Numbers:

Alkalophilic *Bacillus* sp. NG-27: MTCC B0013.

Bacillus *nealsonii* PN-11: MTCC 11401.

2.2. Substrate and chemicals

Locust bean gum and birchwood xylan were purchased from Sigma Aldrich, USA. Wheat bran, copra meal, saw dusts were taken from the local market, Chandigarh, India. All other chemicals were of analytical grades.

2.3. Pulp

Mixed wood pulp was obtained from Ballarpur Industries Limited (BILT), Yamunanagar, Haryana, India.

2.4. Hyperproduction of xylanase and mannanase

2.4.1. Production of xylanase and mannanase by solid-state by co-cultivation

To check the production of enzyme by co-cultivation in solid state fermentation, 5g of wheat bran with moisture ratio 1:1 was used as a substrate in 250 ml Erlenmeyer flask, inoculated with 0.5% overnight grown culture (0.25% *Bacillus* sp. NG-27 and 0.25% *Bacillus nealsonii* PN-11) and incubated at 37 °C for 120h. After incubation crude enzymes was extracted in Tris-HCl buffer (0.1 M, pH 8.0) and the supernatant was assayed for xylanase and mannanase activity.

2.4.2. Optimization of enzymes production by co-cultivation using OVAT and RSM

Various physiochemical factors viz. substrate, time, temperature, moisture ratio and inoculum size were optimized initially by conventional one variable at a time (OVAT) method. Further optimization of the significant factors was done by studying their interactive effect by employing statistical methods. The variables (Incubation temperature, moisture ratio, inoculums size and incubation time) were optimized using central composite design (CCD). The minimum and maximum range of the selected variables i.e. incubation temperature (30-37 °C), moisture ratio (1:2–1:4), inoculum size (0.5–1%) and incubation time (72–120h) were used in 30 combinations (Table S1). The RSM model was validated further for predicted versus actual responses.

2.5. Enzymatic treatment of pulp with a mixture of xylanase (X) and mannanase (M)

2.5.1. Pretreatment of pulp

Mixed wood pulp was washed with water until clear water eluted from the pulp and the pH of filtrate turned neutral. The washed pulp was then dried at 55 °C overnight and was designated as oven dried pulp (odp).

2.5.2. Treatment of pulp with enzymes mixture

1g of pulp was put in a 250 ml Erlenmeyer flask and 20 ml of suitability diluted enzymes mixture [Ug^{-1}(odp)] was added. The reaction mixture was incubated at the required temperature in a water bath shaker. After enzymatic treatment pulp was washed with an excess of water and filtered. In the control reaction enzyme was replaced with buffer. The Measurement of kappa number was done by TAPPI Protocol (T-236 OM-85). All the reactions were done in triplicate and the results were taken as average.

2.5.3. Optimization of pulp biobleaching by response surface methodology (RSM)

The biobleaching was statistically optimized using response surface methodology (RSM). Central composite design (CCD) at α value ±2 was employed using Design-Expert software to further optimize the levels of significant variables. The minimum and maximum range of the selected variables i.e. Enzyme dose; 40–70 IU $godp^{-1}$ (X:M; 3:1), time (30–90min.), pH (7–9) and temperature (55-75 °C) was used in 30 combinations (Table S2). The RSM model was validated further for predicted versus actual responses. Percentage reduction in kappa number was calculated relative to the control pulp. The data were analyzed by the software to generate 3D plots.

2.5.4. Chemical bleaching of pulp and its analysis

Enzymatically treated pulp was subjected to chemical bleaching by using standard methods of chlorination and alkali-extraction [27] using varying amounts of chlorine. Pulp without enzymatic treatment was taken as control. Standard methods of TAPPI (Technical Associations of Pulp and Paper Industry) were used to analyze the brightness (T-452 OM-87) and whiteness (T-1216 OM).

2.6. Analysis of enzymatically treated pulp

Pulp was treated with a mixture of X + M under standardized conditions and was characterized with respect to scanning electron microscopy (SEM) analysis and Fourier transformed infrared (FTIR) spectroscopy.

2.6.1. Scanning electron microscopy of pulp fibers

Scanning electron microscopy was done with control and enzyme-treated pulp. Small pieces of fibers were placed on the stubs after air drying, mounted with silver tape, and sputter-coated with gold using fine coat (JEOL ion sputter, Model JFC-1100) and examined under a scanning electron microscope.

2.6.2. Fourier transformed infrared spectroscopy (FTIR)

The control and enzyme-treated pulp samples were embedded in KBr disk with Perkin Elmer-hydraulic press and subjected to Fourier-transformed infrared spectra (FTIR) with a Perkin Elmer-spectrum 400 FT-IR/FT-FIR spectrometer at room temperature. Peaks were assigned based on known the FTIR database for lignin [28].

3. Results and discussion

Alkalophilic *Bacillus* sp. NG-27 and *Bacillus nealsonii* PN-11 are known to produce novel extracellular thermo-alkali stable xylanase (X) and mannanase (M) respectively [25, 26] having application in the pulp and paper industry [22, 26]. Co-culturing is the most viable method for the production of a cocktail of enzymes if they are produced by different microbial strains [18, 29]. Moreover, solid-state fermentation (SSF) is more useful for co-culturing because of better colonization of organisms due to symbiotic interactions [30, 31]. For the production of a mixture of enzymes (X + M) strains were grown in co-culture using SSF with wheat bran as substrate at 37 °C. Under unoptimized conditions 43.1 IUg^{-1} of

Table 1. Optimization of enzymes (X + M) production by classical OVAT method.

	Unit	Range	Optimum	Xylanase Activity (IUg^{-1})	Mannanase Activity (IUg^{-1})
*Initial Unoptimized conditions	-	-	-	43.1 ± 0.3	1.4 ± 0.65
Type of substrate	G	Wheat bran Copra meal Saw dust Pulp Wheat bran + Copra meal (In the ratio of 1:1)	Wheat bran + Copra meal	48.8 ± 0.54	13.8 ± 0.14
Incubation time	h	24–120	120	76 ± 0.42	19.1.9 ± 0.72
Incubation Temperature	°C	25–45	37	78.6 ± 0.64	20.9 ± 0.24
Moisture ratio	%	1:1–1:4	1:2	88 ± 0.42	23.8 ± 0.72
Inoculum size; 1:1 *Bacillus* sp. NG-27 + *Bacillus nealsonii* PN11	%	0.5–2.0	1.0	98.0 ± 0.52	28.8 ± 0.25

* Initial Unoptimized conditions: Incubation time: 120h; temperature: 37 °C; Moisture ratio- 1:1; inoculums size 0.5%.

Table 2. Central composite design matrix with experimental and predicted values of xylanase and mannanase yield.

Factor % (W/V)					Xylanase activity IUg^{-1}			Mannanase activity IUg^{-1}		
Run	A: Incubation temperature	B: Moisture Ratio	C: Inoculum size	D: Incubation time	Actual	Predicted	Residual	Actual	Predicted	Residual
1	33.5	5	0.75	96	16	23.56 ± 0.62	-7.56	10.24	12.26 ± 1.76	-2.02
2	37	4	1	72	39	28.69 ± 1.25	10.31	16.8	18.22 ± 1.45	-1.42
3	30	4	1	72	30.5	29.50 ± 0.83	1.0000	11.2	6.88 ± 1.25	4.32
4	33.5	3	0.75	96	99	109.30 ± 0.25	-10.30	40.4	41.00 ± 0.65	-0.6000
5	33.5	3	0.75	96	156	109.30 ± 1.43	46.70	52.8	41.00 ± 0.99	11.80
6	37	2	0.5	72	151	149.60 ± 1.61	1.40	12.4	12.15 ± 1.45	0.2517
7	33.5	3	0.75	96	102	109.30 ± 1.5	-7.30	41.2	41.00 ± 2.1±	0.2000
8	30	4	1	120	150	149.52 ± 0.94	0.4792	31.6	31.09 ± 1.89	0.5117
9	30	4	0.5	120	95	81.00 ± 0.76	14.00	32	29.25 ± 1.47	2.75
10	37	2	1	120	141	145.27 ± 1.42	-4.27	53.8	52.85 ± 2.45	0.9517
11	30	2	1	120	144	132.33 ± 1.65	11.67	49.2	48.41 ± 2.21	0.7933
12	33.5	3	0.25	96	80	85.48v1.43	-5.48	16	18.27 ± 1.35	-2.27
13	33.5	3	0.75	96	90	109.30 ± 1.89	-19.30	41.2	41.00 ± 0.89	0.2000
14	33.5	3	0.75	96	102	109.30 ± 1.34	-7.30	44	41.00 ± 0.65	3.00
15	40.5	3	0.75	96	110	108.31 ± 1.25	1.69	36	34.21 ± 1.45	1.79
16	33.5	3	0.75	144	154	157.65 ± 0.65	-3.65	46.4	47.27 ± 1.78	-0.8717
17	37	2	0.5	120	108	98.50 ± 0.76	9.50	19.8	21.31 ± 1.1	-1.51
18	30	2	0.5	120	85	93.44 ± 1.25	-8.44	26.4	24.22 ± 1.45	2.18
19	37	4	0.5	120	44	49.94 ± 2.1	-5.94	29	29.29 ± 2.4	-0.2883
20	30	2	0.5	72	130	122.17 ± 2.2	7.83	14	11.11 ± 1.24	2.89
21	33.5	3	1.25	96	77	83.90 ± 1.54	-6.90	36.4	37.71 ± 1.46	-1.31
22	37	4	0.5	72	68	69.17 ± 1.54	-1.17	25	22.98 ± 1.67	2.02
23	33.5	1	0.75	96	82	86.81 ± 1.98	-4.81	17.2	18.75 ± 0.97	-1.55
24	37	2	1	72	76	79.50 ± 2.2	-3.50	29.8	29.74 ± 1.45	0.0600
25	33.5	3	0.75	96	106.8	109.30 ± 1.25	-2.50	26.4	41.00 ± 1.76	-14.60
26	30	2	1	72	52	44.19 ± 1.87	7.81	22.4	21.35 ± 1.24	1.05
27	37	4	1	120	129	126.33 ± 1.54	2.67	38.4	38.48 ± 1.5	-0.0800
28	33.5	3	0.75	48	80	88.73 ± 2.23	-8.73	11.2	13.91 ± 2.4	-2.71
29	30	4	0.5	72	84	77.85 ± 1.43	6.15	18.8	18.99 ± 1.43	-0.1883
30	26.5	3	0.75	96	90	104.06 ± 2.71	-14.06	20.4	25.77 ± 1.94	-5.37

Values represent mean ± Standard deviation (n = 3).

xylanase and 1.4 IUg^{-1} of mannanase yield were obtained in crude enzyme extract.

3.1. Hyperproduction of enzymes

For the hyperproduction of X + M optimization was done by one variable at a time (OVAT) and by statistical methods.

3.1.1. Optimization of physicochemical factors by one variable at a time (OVAT) method

In solid-state fermentation, parameters that which are significantly known to influence enzyme production are substrate matrix, incubation time, temperature, inoculums size and moisture ratio [32, 33, 34]. Using the conventional 'one variable at a time' (OVAT) approach, the involvement of various cultural/nutritional factors were optimized by

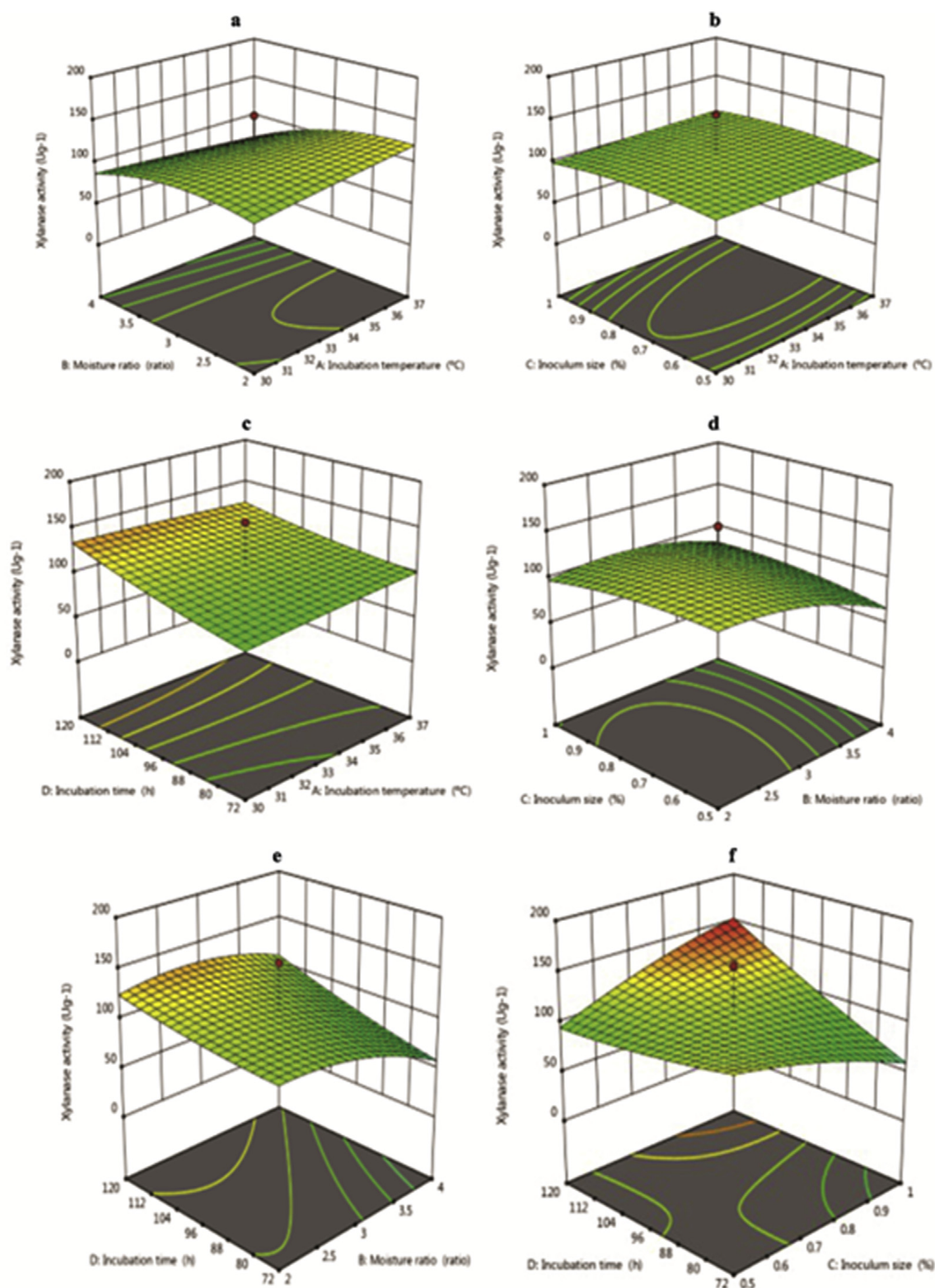

Figure 1. Three dimensional response surface plots showing the yield of xylanase [a] moisture ratio and incubation temperature; [b] inoculum size and incubation temperature [c] incubation time and incubation temperature; [d] inoculum size and moisture ratio; [e] incubation time and moisture ratio; [f] incubation time and inoculums size.

changing one parameter at a time while keeping all other factors constant. This approach is simple to execute and helps in the selection of significant parameters influencing enzyme yield [35, 36]. Maximum enzyme yield (X + M) was achieved when a mixture of wheat bran and copra meal was used as substrate (In the ratio of 1:1). Use of various substrates such as wheat bran, rice bran and other agricultural waste residues have been reported for the production of xylanase/s [14, 37] among which wheat bran is most widely used [18, 22, 32]. Because of the higher content of mannan in it copra meal is a known substrate for the production of manannases [38, 39,40]. After initial optimization by OVAT 2.27 and 20.5 fold yield increase was achieved for xylanase and manannase respectively with incubation time 120h, temperature 37 °C, moisture ratio 1:2, inoculums size 1.0% (Table 1).

3.1.2. Optimization of enzyme (X + M) production by statistical methods

The conventional (OVAT) method has one limitation that it is not able to identify the combined or interactive effect of different parameters [41]. A statistical experimental design such a as central composite

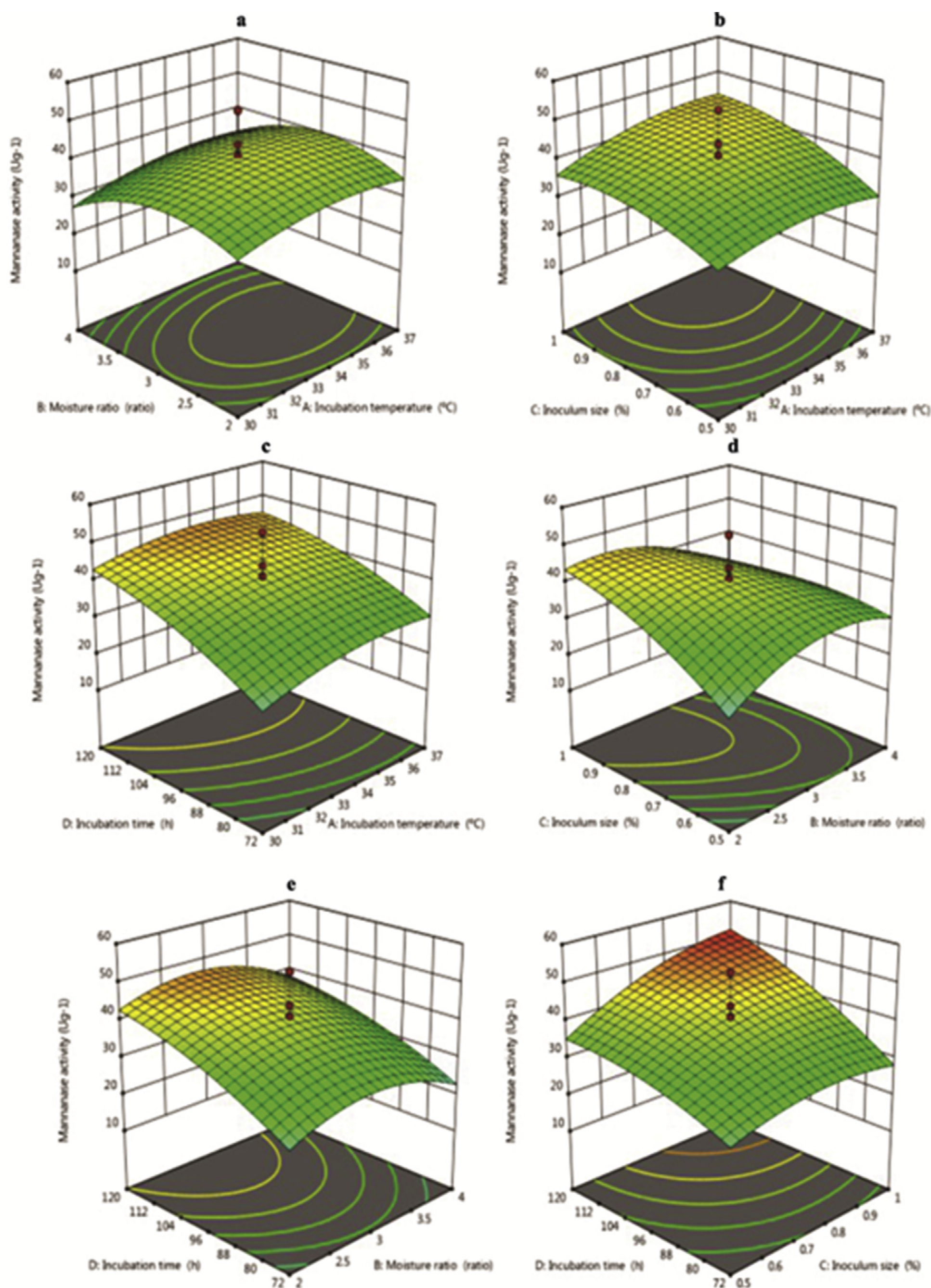

Figure 2. Three dimensional response surface plots showing the yield of mannanase [a] moisture ratio and incubation temperature; [b] inoculum size and incubation temperature [c] incubation time and incubation temperature; [d] inoculum size and moisture ratio; [e] incubation time and moisture ratio; [f] incubation time and inoculums size.

experimental design provides information taking care of interactions between various factors with minimum numbers of experiments [42].

To see the interactive effect of various parameters on enzyme (X + M) production a central composite design with 30 runs was applied and responses were observed in terms of yield of xylanase and manannase (Table2).

Multiple regressions were applied to construct predictive quadratic polynomial equations (R1, R2) to get the correlation between parameters and responses. ANNOVA was used to analyze predicted and observed responses (Supplementary Table S3 and S4).

$$R1 = +109.30 + 1.06*A-15.81*0.3958*C+17.23*D-9.03*AB+1.97*AC-5.59*AD+7.41*BC+7.97*BD+29.22*CD- 0.7781*A^2-13.53*B^2-6.15C^2 + 3.47*D^2 \quad \text{Eq. 1}$$

$$R2 = +41.00 + 2.11*A-1.62*B+4.86*C+8.34*D+0.7375*AB+1.84*AC-0.9875*AD-5.59*BC-0.7125*BD+3.49*CD- 2.75*A^2-6.37*B^2-3.25*C^2-2.60*D^2 \quad \text{Eq. 2}$$

R1 is the response 1 (Xylanase yield, IUg^{-1}) and R2 is response 2 (Mannanase yield, IUg^{-1}). A, B, C and D were the coded values of incubation temperature, moisture ratio, inoculums size and incubation time respectively.

p values < 0.0001 indicated that the linear, interactive, and squared terms significantly influenced both the responses. The p values for the lack of fit were 0.97 and 0.99 for xylanase and mannanase yield respectively, which indicated that this model adequately fitted into the

Table 3. Central composite design matrix with actual and predictive values of reduction in kappa number.

Run	Enzymes Dose (IU godp^{-1}) (xylanase:mannanase) (3:1)	Time (h)	pH	Temp. (°C)	Kappa number reduction (%)	Predicted value	Residual Activity
1	25	60	8	65	9.12 ± 0.56	9.73	-0.6075
2	40	90	7	55	44.52 ± 1.21	39.82	4.70
3	55	60	8	65	39.59 ± 0.95	35.93	3.66
4	70	30	7	55	44.47 ± 0.58	44.46	0.0125
5	40	30	9	75	7.12 ± 1.1	4.86	2.26
6	55	60	6	65	38.35 ± 0.94	37.01	1.34
7	55	60	8	45	44.53 ± 1.25	43.99	0.5408
8	55	120	8	65	15.63 ± 1.64	19.11	-3.48
9	70	30	9	75	22.76 ± 0.89	23.75	-0.9875
10	55	60	8	65	36.11 ± 0.45	35.93	0.1783
11	70	90	9	75	7.12 ± 0.98	4.83	2.29
12	70	30	7	75	43.59 ± 0.48	40.66	2.93
13	70	90	7	55	20.52 ± 0.74	23.81	-3.29
14	40	30	7	75	9.9 ± 1.2	11.66	-1.76
15	40	30	9	55	20.54 ± 1.45	17.91	2.63
16	70	90	7	75	19.1 ± 0.65	18.01	1.09
17	55	60	8	65	45.64 ± 0.45	35.93	9.71
18	85	60	8	65	10.52 ± 0.23	12.61	-2.09
19	55	60	8	65	38.6 ± 0.37	35.93	2.67
20	55	60	8	65	30.52 ± 1.35	35.93	-5.41
21	55	0	8	65	23.9 ± 1.49	23.12	0.7825
22	40	90	9	55	32.6 ± 0.44	36.55	-3.95
23	70	90	9	55	15.9 ± 1.12	10.43	5.47
24	55	60	10	65	12.8 ± 0.89	16.83	-4.03
25	70	30	9	55	26.7 ± 0.94	27.35	-0.6517
26	40	30	7	55	21.6 ± 0.85	24.91	-3.31
27	40	90	7	75	24.2 ± 0.69	24.57	-0.3683
28	40	90	9	75	25.2 ± 0.45	21.50	3.70
29	55	60	8	85	21.9 ± 0.57	25.14	-3.24
30	55	60	8	65	25.13 ± 1.19	35.93	-10.80

Values represent mean ± Standard deviation (n = 3).

data. The determination coefficients $R1^2$ 0.9015 and $R2^2$ 0.9074 indicated perfect coherence between predicted and experimental values. The values of adjusted $R1^2$ and $R2^2$ suggested that the variation in the responses was attributed to the independent variables and only 0.23% and 0.14% of the total variations could not be explained by the models. The three dimensional (3D) response surface graphs of xylanase and mannanase (Figures 1 and 2) depicted that all the interactions in the designed experiments produced a 'nearly spherical' variance function.

All these results confirmed that the models can be used for the prediction of the maximum yield of X + M. The Maximum yield of xylanase (156.0 IUg^{-1}) was obtained in run 5 at 33.5 °C, 1:3 moisture ratios, 0.75% inoculums and 96h incubation which was at the central value of the model hence the model was internally validated. The Maximum yield of mannanase (53.80 IUg^{-1}) was in run 10 at 37 °C, 1:2 moisture ratios, 1.0% inoculum and 120h incubation, which was not at the central value. The Validation experiment gave maximum yield of mannanase (54.84 IUg^{-1}) at 37 °C temperature, 1:2 moisture ratios, 1.0% inoculums, and 120h of incubation. As the aim of the study was to have the co-production of xylanase and mannanase in high yields, therefore standard run 5 (33.5 °C incubation temperature, 1:3 moisture ratio, 0.75% inoculum size and 96h incubation time) was selected as it gave the maximum xylanase yield and mannanase yield of 52.8 IUg^{-1} (which was comparable to its maximum yield of 54.84 IUg^{-1}). Optimization resulted in an approximately 3.61 fold increase in the yield of xylanase and a 37.71 fold increase in the yield of mannanase.

There are number of reports on the production of enzymes by co-culturing from fungi as well as bacteria. *Bacillus nealsonii* PN-11 has been used for the co-production of mannanase and protease in SSF [43]. However, there is no report on the production of mixture of xylanase and mannanase by co-culturing.

The ratio of xylanase and mannanase in final enzyme preparation was 3:1; which was suitable for pulp biobleaching as xylan content is often higher in wood than other components of hemicellulse such as mannan. Moreover enzyme preparation was having no cellulolytic activity which is a prerequisite for the application of an any enzyme in the process of papermaking [14].

3.2. Application of a mixture of xylanase and manannase for pulp biobleaching

Wood from which the pulp is made for papermaking contains various types of hemicelluloses most abundant of which are xylan and manann [27]. Therefore to have an effective removal of hemicelluloses a cocktail of xylanase/s and manasses/s is required [13]. As the mixture of xylanase and mananase produced in this study was cellulose free and both the enzymes were active in the temperature range of 60–70 °C and pH 7–9, therefore it was ideal for its application for biobleaching of pulp.

3.2.1. Optimization of the process for pretreatment of pulp with a mixture of X + M

Enzymatic biobleaching of pulp is affected by various parameters such as enzyme doze, incubation time, pH and incubation temperature [26]. To see the interactive effect of these parameters and develop an optimal process for pretreatment of pulp with a concoction of X + M a central composite design was formulated and biobleaching was measured by taking a reduction in kappa number as a response (Table 3).

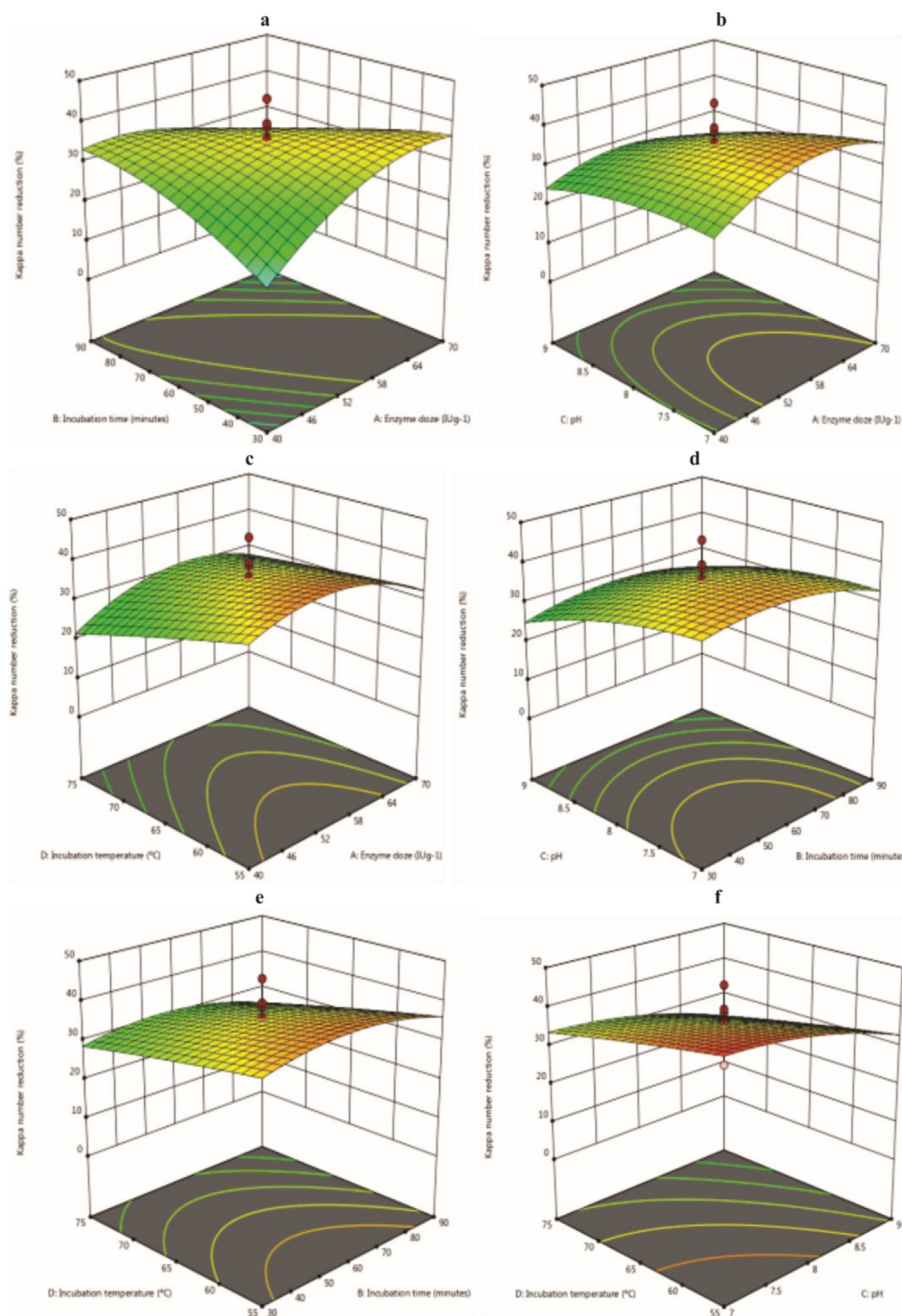

Figure 3. Three dimensional response surface plots showing the reduction in kappa no. [a] incubation time and enzyme doze; [b] pH and enzyme doze [c] incubation temperature and enzyme doze; [d] pH and incubation time; [e] incubation time and incubation temperature; [f] incubation temperature and pH.

The predicted and observed responses were analyzed by ANNOVA (Table S5) which indicated that the model to be significant. Correlation between responses and parameters was studied by applying a predictive quadratic polynomial equation (Eq. 3).

$$R\text{ (kappa number reduction)} = +35.93 + 0.7200^{*}A - 1.00^{*}B - 5.04^{*}C - 4.71^{*}D - 8.89^{*}AB - 2.53^{*}AC + 2.36^{*}AD + 0.9325^{*}BC + 0.0488^{*}CD - 6.19^{*}A^2 - 3.70^{*}B^2 - 2.25^{*}C^2 - 0.3423^{*}D^2 \quad \text{Eq. 3}$$

Where, R is the response (kappa number reduction) and A, B, C and D were the coded values of enzymes doze, time, pH and temperature respectively.

Graphs of response formed by pair wise combination of the two factors while keeping the other one at its optimum level confirmed a positive interaction Figure 3. Results indicated that the model can be used for maximum kappa no. reduction. Almost all the interactions in the designed experiments produced a 'nearly spherical' variance function.

Maximum reduction in kappa number (45.64%) was obtained with 55 IU godp-1 enzyme doze, 1h incubation time, 8 pH and 65 °C these conditions were at the central value of the model hence model was internally validated. In X + M treated pulp brightness and whiteness also increased by 6.0% and 1.48% respectively (Table 4A). Chauhan et al. [26, 44] has reported a 32.22% reduction in kappa number with the same

Table 4. Effect of enzyme treatment (X + M) on pulp and reduction of chlorine consumption in chemical based biobleaching.

A
Effect of enzymatic treatment

Parameters	U*	X + M#	% improvement	
Enzyme treatment stage				
Kappa no.	15.2 ± 0.32	8.2 ± 0.20	**46.05↓**	
Brightness (%)	28.60 ± 0.25	30.31 ± 0.24	**6.0↑**	
Whiteness (%)	-41.25 ± 0.40	-39.72 ± 0.38	**3.70↑**	
B **Reduction in chlorine consumption**				
	U*	**X + M#**	**X + M#**	**X + M#**
Chlorine charged	100%	100%	80%	60%
C-stage				
Brightness (%)	46.78 ± 0.39	49.54 ± 0.15	47.25 ± 0.16	44.42 ± 0.33
Whiteness (%)	-18.81 ± 0.34	-14.62 ± 0.27	-16.10 ± 0.18	-19.36 ± 0.16
Alkali extraction and EP-Stage				
Brightness (%)	**53.80 ± 0.27**	**59.73 ± 0.25(11%↑)**	**57.20 ± 0.03**	**52.25 ± 0.32**
Whiteness (%)	**14.43 ± 0.33**	**25.27 ± 0.40(75.12%↑)**	**21.66 ± 0.22**	**13.36 ± 0.16**

Particular parts of the table were made bold to highlights the important result.
* U = Untreated pulp.
# X+M = Xylanase+Mannanase.

enzymes as used in the present study but the enzymes were produced individually and enzymes doze used was also much higher. Biobleaching using xylanase and mannanase from *Streptomyces galbus* NR has also been reported by Kansoh et al. [45] but the required treatment time and enzyme dose was much higher. A mixture of xylanase from *Bacillus halodurans* FNP 135 and mannanase from *Bacillus* sp. were able to give only a 26.7% reduction in kappa number [37]. Clarke et al. [46] has also reported the use of a mixture of xylanase and mannanase/s but the enzymes were produced by growing the organisms individually and for pulp biobleaching enzymes were added in a sequential manner. Gupta et al. [18] and Angural et al. [47, 48] have also shown the pulp biobleaching using a mixture of lignolytic and hemicellulolytic enzymes.

3.2.2. Reduction in chlorine consumption in bleaching of enzyme (X + M) pulp

Chemical-based bleaching of enzymatically (X + M) treated pulp lead to a significant increase in brightness (11%) and whiteness (75.12%) (Table 4B); which might be due to the effective removal of already loosened hemicellulosic and lignin component of the pulp due to the action of enzymes. Similar results have been shown by Gupta et al. [18] with laccase and xylanse but enzyme treatment was given in a sequential manner [E-C-EP].

When enzyme-treated pulp was subjected to decreased amounts of chlorine; it was observed that the same level of brightness and whiteness as that of untreated pulp (with 100% of a chlorine treatment) can be achieved with about 70% of chlorine dose. Therefore about 30% of less chlorine can be applied to the pulp treated with an enzyme mixture of X + M to achieve the same quality of paper like that of untreated pulp (Table 4).

3.3. Structural analysis of enzymatic (X + M) treated pulp

Enzymatic treatment of pulp leads to various changes in the pulp structure [22] to analyze various structural changes in the pulp treated with a mixture of X + M it was analyzed by scanning electron microscopy (SEM) and Fourier transformed infrared (FTIR) spectroscopy.

3.3.1. Analysis of enzymatic treated pulp using SEM

On analysis by SEM enzymatic treated pulp showed pores, cracks and increased roughness all over the surface due to fiber explosion (Figure 4). These changes are known to be helpful in the removal of the

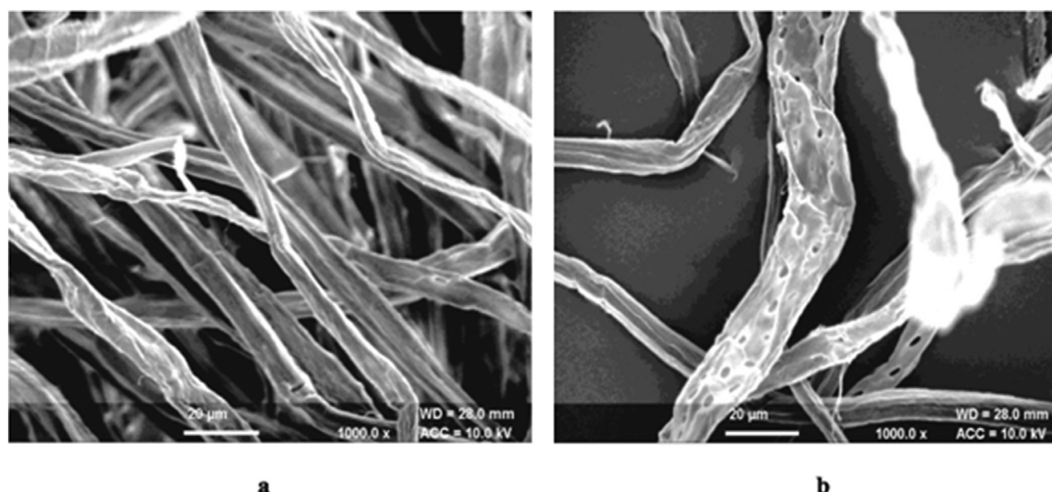

Figure 4. Scanning electron micrographs of mixedwood pulp (A) untreated, (B) xylanse and mannanase treated.

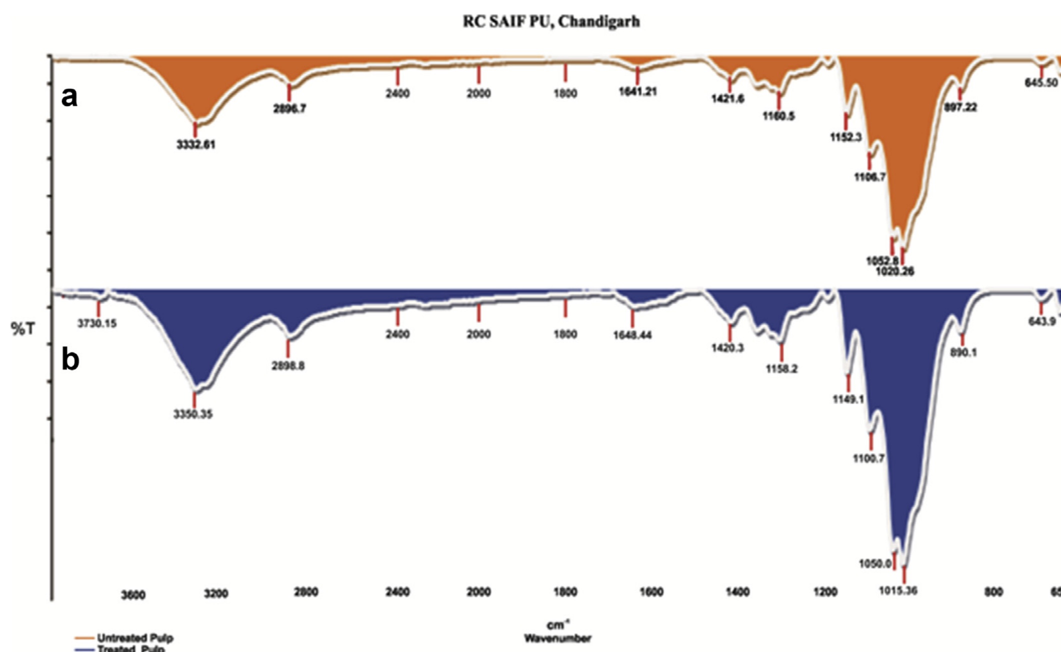

Figure 5. FTIR analysis of pulp fibres. a) Control Pulp (Without enzymatic treatment) b) Pulp treated with concoction of xylanase and mannanase.

hemicelluloses, lignin with fewer concentrations of chlorine in the chemical bleaching process [18].

3.3.2. Analysis of enzymatically treated pulp using FTIR

The changes in the chemical groups of pulp with enzymatic treatment were determined by doing FTIR and comparing the spectra with untreated pulp (Figure 5) and analyzing the changes with respect to the standard positions of various bonds (Table S6).

Some shift with an increase in the bandwidth as well as intensity was observed in the peaks around 3300-3200 cm^{-1} and 2900-2800 cm^{-1} which can be attributed to the N–H stretching of amide bonds in cellulose and stretching of C–H bonds of lignocellulosic components respectively. There was no change in the peaks around 2400, 2000 and 1800 cm^{-1} which might be due to presence of high energy triple bonds C≡C, C≡N in this region. Band shift as well some increase in the intensities were also observed at 1600 cm^{-1} which are known to be because of vibrations of the aromatic and carbonyl groups of lignin. Similar changes were seen in the various peaks between 1400-1100 cm^{-1} due to changes in bonds such as C=O, C–O in lignin and xylan. A significantly increased intensity of peak was there in the spectra around 1050–1000 indicating the changes in C–C, C–OH, C–H ring and side groups. Similarly some changes were noticed in other regions which indicated that enzymatic treatment leads to the structural changes of the pulp.

4. Conclusion

Developing a process using microbial enzymes for pulp bio bleaching is one of the most viable alternatives to make the process of paper making greener. The present study reports the economical production of a mixture of xylanase and manannase by co-culturing of alkalophilic *Bacillus* sp. NG-27 and *Bacillus nealsonii* PN-11 in SSF. Conditions were standardized to have the high yields of both the enzymes in a single fermentation. Optimization resulted in a-many fold increase in the production of enzymes. Analysis of pulp treated with enzymatic mixture denoted effective degradation of hemicelluloses in terms of high reduction of kappa number and enhancement of paper quality such as brightness and whiteness. Moreover, enzyme treatment led to about 30% less use of chlorine in subsequent chemical-based bleaching. SEM and FTIR showed the structural changes which are known to be helpful in the removal of the hemicelluloses and lignin with fewer concentrations of chlorine in the chemical bleaching process. As the concoction of hemicellulolytic enzymes produced in the present study could be produced in an economical manner and both the enzymes are having all the desired characteristics for pulp bio bleaching, therefore, it is a highly suitable candidate for application in the paper mill to reduce the use of chemicals and develop an environment-friendly process, however, studies at industrial scale are required.

Declaration

Author contribution statement

Steffy Angural: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Indu Bala: Performed the experiments.

Aditya Kumar: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Deepak Kumar: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Sunena Jassal: Analyzed and interpreted the data.

Naveen Gupta: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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