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Original Article

Effect of alkali treatment (NaOH) of eucalyptus leaves for hypercellulase production by *Bacillus subtilis* through submerge fermentation

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Abstract
Eucalyptus leaves were pretreated through Box-Bhenken design of response surface methodology using three variables with three levels such as NaOH concentration (0.6, 0.8, 1.0%), substrate concentration (5, 10, 15%) and residence time (4, 6, 8h) with and without steam (autoclaving at 121^{o}C for 15min and 15psi). After pretreatment, cellulase production was conducted in 250ml Erlenmeyer flask at 50 ºC, pH 5 with shaking speed of 120 rpm using 2% pretreated eucalyptus leaves as carbon source and 2% v/v inoculum size in submerged fermentation for 24h. Results showed that among these two treatments, only chemical treated substrates gave better enzyme yield as compared to chemical followed by steam. The highest FPase (2.526 IU/ml/min) activity was found at pretreatment conditions of 0.6% NaOH conc., 10% biomass loading and 4h reaction time while CMCase (2.803 IU/ml/min) at 1% NaOH conc., 15% biomass loading and 6h of reaction time. The proposed model was found highly significant as revealed by their *F*-values, *P*-values and R^2 values. Results suggest the efficiency of *Bacillus subtilis* K-18 for cellulase production from eucalyptus leaves and it would be fruitful for future industrial exploitation in all perspectives.
Keywords: Alkali pretreatment, Eucalyptus, RSM, cellulase, *Bacillus* sp.

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INTRODUCTION

By the recognition of the effect of fossil fuel on environment which adds to the global warming further enforce the necessity of the biofuel (Mclntosh *et al*., 2016). From the last decade research efforts increased for the second-generation biofuel production from lignocellulosic material rather than the edible crops (Pinkert *et al*., 2009; Mussatto *et al*., 2010; Lienqueo *et al*., 2016). Globally the use and production of ethyl alcohol has been encouraged as biofuel is comprises of 35% oxygen which reduce the emanation of particulate matter and NO_X from fuel incineration (Ruangmee *et al*., 2013).

The production of glucose or alcohol or biofuel or chemical initiatives from organic wastes is on its way (El-Shistawy *et al*., 2015). Crops with the characteristics of short yield rotation, least compost contribution and quick growth while plants like pine and spruce, eucalyptus and poplar, miscanthus and switch grass from soft wood, hard wood and grasses respectively have been stipulated as preferential biofuel crops and plants species (Li *et al.,* 2015). Eucalyptus leaves are the latent auspicious cradle for biofuel production because of its cosmopolitan distribution, good growth rates and high ingathering (Mclntosh *et al.,* 2016). Eucalyptus leaves are the intriguing substrate attributable to its surpassing sugar level compared to pentose sugars (Fernandes *et al.,* 2016).

Major composition of lignocellulosic biomass is cellulose, hemicellulose and lignin (Xu *et al.,* 2013). Second bountiful ingredient of lignocellulosic biomass is hemicellulose comprises of polysaccharide with diverse five and six carbon monosaccharide (Rubin 2008; Xu *et al.,* 2013) while macromolecule is lignin, possess several active sites swaying its responsiveness (Pandey and Kim, 2011;

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Norambuena *et al*., 2016). Lignocellulosic biomass has intractability due to more crystallinity and composite association of lignin and hemicellulose (Himmel *et al.,* 2007; Zaho *et al.,* 2012; Kundu *et al.,* 2016). Pretreatment technique either of thermochemical or physiological or any-other are necessary for the fractionation of lignocellulosic biomass will promote enzymatic hydrolysis followed by saccharification for bioethanol production (Alvira *et al.,* 2010; Akhtar *et al.,* 2016; Mclntosh *et al*., 2016). Cellulases are specified enzymes used for the β-1-4 glycosidic bond cleavage of glucane (Olofsson *et al.,* 2008). Cellulases enzymes are a set of three considerable enzyme categories as endo [1-4]-β-D-glucanase, exo [1-4]-β-D-glucanase and β-D-glucosidases being designed to transform the accessible cellulosic contents to fermentable sugars (Azadian *et al*., 2016; Chiarello *et al*., 2016).

Distinct cell molders as bacteria, fungi and yeasts yields cellulases which could flourish on low-cost media proven a reasonable source for the production of fermentable sugars (El-Shistawy *et al*., 2015). Bacterial species of *Clostridium, Bacillus, Acetovibrio, Bacterioides, Microbispora,* yield cellulases. Among *Bacillus* strains *B. carboniphilus, B. sphaericus, B. subtilus, B.* sp. L1 revealed the cellulytic activity (Sun and Cheng., 2002; Azadian *et al.,* 2016). Fungi belonging to *P. Chrisosoprium, Aspergillus, Trichoderma* and *Shizophyllum* produce cellulases. Among all these *Trichoderma* effectively used for cellulose production from last few decades because of its high cultivation and inducible property but it gave optimum saccharification at 50 ^{o}C (Sun and Cheng., 2002; El-Shistawy *et al.,* 2015; Kazeem *et al.,* 2016). Among actinomycetes *C. fimi, C. uda, S. lividans, T. curvata* and *C. bioazotea* reported for cellulolytic activity (Kuhad *et al.,* 2011). From last three decades cellulases are commercially available and exploited equally at industrial and speculative research (Kuhad *et al.,* 2011).

Response surface methodology is mathematical and statistical framework, useful designed for collective consequences of various variants to search the best situation for multivariable scheme (Ruangmee *et al*., 2013; Kim *et al.,* 2016). Box- Behenken Design (BBD) for three statistic at three levels was designated (Li *et al.,* 2007; Ahmed *et al*., 2012). Current study involves the analysis of cellulolytic activity from *Bacillus subtilis* K-18 using alkaline (NaOH) pretreated Eucalyptus leaves.

MATERIAL AND METHODS

Biomass Preparation

Eucalyptus leaves were collected from eastern garden of main library Quaid-e-Azam campus, Lahore, Punjab Pakistan, geographical coordinates are 31° 30' 15" North, 74° 18' 23" East. Collected leaves were washed to remove dust and then sundried followed by oven drying at 70 ^{o}C for overnight. The dried eucalyptus leaves were chopped and finally ground to powder form (approximately 2 mm) and used for pretreatment process.

Pretreatment Experiments

Pretreatment of eucalyptus leaves was done as described in our earlier reports (Irfan *et al.,* 2010).

Enzyme production

Enzyme production was done in 250 ml Erlenmeyer flask containing 25ml of fermentation medium (2% pretreated substrate and 1% yeast extract with initial medium pH 5) was autoclaved at 121^{o}C, for 15 minutes and 15 Psi pressure. After sterilization, the flasks were allowed to cool at room temperature and 2% (v/v) of the vegetative cell culture was transferred aseptically to each of the fermentation flasks. After inoculation, the flasks were incubated at 50 ^{o}C with agitation speed of 120 rpm for 24h of fermentation period. After completion of the fermentation period, the fermented broth was filtered through muslin cloth followed by centrifugation (Sigma 2-16 PK) for 10 minutes at 10,000xg and 4^{o}C for the removal of cell mass and unwanted particles. The clear filtrate obtained after centrifugation was used as a crude source of enzyme. Triplicate readings were taken for each of the experiment.

Analytical methods

The CMCase and FPase activities were determined as described by Arooj *et al.,* (2017).

Design of Experiment

A three variable Box-Behnken design for response surface methodology was used to study the combined effect of NaOH concentration, substrate loading and time on cellulase production over three levels. The coded and actual values of Box-Behnken design are shown in Table I. The Box-Behnken design is appropriate for examination of quadratic

response surfaces and creates a second-degree polynomial model, which in turn is used in improving a process using a little number of experimental runs. Three factor Box-Behnken design with experimental as well as predicted responses of dependent variable (acid pretreatment) in which total sugar and total phenolic concentration with observed, predicted as well as residual values shown in Table II. The 13 experimental runs were randomized to exploit the effects of unsolved variability in the observed responses due to extraneous factors. The levels of the independent variables as shown in Table I were selected based on initial experiments. The relation between the coded values and actual values are described as follows:

$$x_i = \frac{X_i - X_o}{\Delta X_i}$$

Where *xi* and *Xi* are the coded and actual values of the independent variable respectively. *Xo* is the actual value of the independent variable at the center point, and Δ*Xi*is the step change of *Xi*. A second degree polynomial was fitted to the experimental data using the statistical package software Minitab v. 17.0 to estimate the response of the dependent variable and predict the optimal point. The second degree polynomial was expressed as follows:

$$Y=b_0+b_1X_l+b_2X_2+b_3X_3+b_{12}X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{11}X^2{}_1+b_{22}X_2{}^2+b_{33}X^2{}_3$$

Where Y is predicted response, X_1, $X_2$and $X_3$are independent variables, b_0 is offset term, b_1, b_2,$b_3$are linear effects, b11, b22, b13are interaction terms.

RESULT AND DISCUSSION

In the current study CMCase and FPase yield were analyzed from alkaline and steam alkaline pretreated eucalyptus leaves as substrate by *Bacillus subtilis* K-18 at 50 ºC, pH 5 through submerged fermentation. Prior to enzymatic analysis eucalyptus leaves were submitted to alkaline pretreatment with NaOH concentrations (0.65, 0.8, 1%), biomass loading (5, 10, 15g) and reaction time (4, 6, 8h) and for steam alkaline pretreatment were being autoclaved at 121 ºC at 15 Psi in accordance to Box Behnken design to analyze the optimal conditions for cellulase production. After pretreatment, the substrate was dried and used for cellulase production in submerged fermentation. Experiments were conducted in triplicates and the response obtained was calculated through second order polynomial regression equations (eq 3-6). Experimental and predicted values of CMCase and FPase were mentioned in Table II and III using Box-Bhenken design. Highest FPase (2.527 IU/ml/min) production was observed in alkali treated eucalyptus leaves using pretreatment conditions of 0.6% NaOH conc., 10% substrate concentration and 4h residence time. The highest CMCase (2.803 IU/ml/min) production was noted in alkali treated eucalyptus leaves with pretreatment conditions of 1% NaOH concentration, 15% substrate concentration and 6h residence time.

Regression Equation in coded units for CMCase and FPase alkaline treated eucalyptus leaves

$$\text{CMCase (IU/ml/min)} = 8.06 - 14.59\,X_1 - 0.065\,X_2 + 0.039\,X_3 + 5.26\,X_1^2 - 0.00674\,X_2^2 - 0.0607\,X_3^2 + 0.2403\,X_1{*}X_2 + 0.581\,X_1{*}X_3 + 0.01400\,X_2{*}X_3 \quad \textbf{Eq. (3)}$$

$$\text{FPase (IU/ml/min)} = 6.428 - 9.465\,X_1 + 0.1914\,X_2 - 0.5214\,X_3 + 4.170\,X_1^2 - 0.008365\,X_2^2 + 0.00202\,X_3^2 - 0.0242\,X_1{*}X_2 + 0.4917X_1{*}X_3 + 0.00499\,X_2{*}X_3 \quad \textbf{Eq. (4)}$$

Regression Equation in coded units for CMCase and FPase steam alkaline treated eucalyptus leaves

$$\text{CMCase (IU/ml/min)} = 6.894 - 8.58\,X_1 - 0.0553\,X_2 - 0.6547\,X_3 + 3.610\,X_1^2 + 0.00347\,X_2^2 + 0.02761\,X_3^2 + 0.0038\,X_1{*}X_2 + 0.3388\,X_1{*}X_3 + 0.00264\,X_2{*}X_3 \quad \textbf{Eq. (5)}$$

$$\text{FPase (IU/ml/min)} = 1.984 - 1.63X_1 + 0.2030\,X_2 - 0.141X_3 + 0.993\,X_1^2 - 0.0022\,X_2^2 - 0.0088\,X_3^2 - 0.1814\,X_1{*}X_2 + 0.2217X_1{*}X_3 + 0.00456\,X_2{*}X_3 \quad \textbf{Eq. (6)}$$

For the assesment of significant level of second degree polynomial equation for cellulase production was performed by the implementation of analysis of variance (ANOVA). Table IV-V represents the experimental results which demonstrate the

model's effect on CMCase and FPase yeild by alkaline and steam alkaline pretreatment. For alkaline pretreatment computed Fishers F-value for CMCase was 7.15 with P-value 0.022 whereas for FPase F-value was128.54 with corresponding P-value 0.000 shows the models fitness. Model's significant level for steam alkaline pretreatment is also high-pitched as for CMCase models F-value was 29.08 with P-value 0.001 and for FPase F-value was 13.76 followed by 0.005 P-value.

Table I: Three stages and series of BBD factorial design of coded and actual variables

Independent variables	Symbols	Coded and actual values		
		-1	0	+1
NaOH concentration (%)	X_1	0.6	0.8	1
Substrate concentration (%)	X_2	5	10	15
Time (Hours)	X_3	4	6	8

Table II: Cellulase production by alkaline treated eucalyptus leaves using Box-Behnken design.

Run #	X_1	X_2	X_3	CMCase activity (IU/ml/min)			FPase activity (IU/ml/min)		
				Observed	Predicted	Residual	Observed	Predicted	Residual
1	0.8	10	6	2.028056	2.028056	-0.00000	2.011852	2.011852	-0.000000
2	1.0	10	8	1.804556	2.002380	-0.19782	2.239852	2.258574	-0.018722
3	1.0	15	6	2.803241	2.634499	0.168742	2.092593	2.101741	-0.009148
4	1.0	10	4	1.790759	1.874562	-0.08380	2.087556	2.080722	0.006833
5	1.0	5	6	1.503796	1.390911	0.112885	1.824148	1.803111	0.021037
6	0.6	15	6	2.155648	2.268534	-0.11288	2.163259	2.184296	-0.021037
7	0.8	5	4	1.514833	1.543916	-0.02908	1.767111	1.794981	-0.027870
8	0.6	10	8	1.736287	1.652484	0.083803	1.892593	1.899426	-0.006833
9	0.8	15	8	1.999083	1.970001	0.029082	1.954370	1.926500	0.027870
10	0.6	10	4	2.651741	2.453917	0.197824	2.526963	2.508241	0.018722
11	0.6	5	6	1.817593	1.986334	-0.16874	1.798074	1.788926	0.009148
12	0.8	5	8	1.012028	0.927089	0.084939	1.477333	1.479648	-0.002315
13	0.8	15	4	1.941852	2.026791	-0.08493	2.044444	2.042130	0.002315

Table III: Cellulase production by steam alkaline treated eucalyptus leaves using Box-Behnken design.

Run #	X_1	X_2	X_3	CMCase activity (IU/ml/min)			FPase activity (IU/ml/min)		
				Observed	Predicted	Residual	Observed	Predicted	Residual
1	0.8	10	6	1.012648	1.012648	-0.00000	1.841778	1.841778	0.000000
2	1.0	10	8	1.151852	1.203899	-0.05204	1.704296	1.782056	-0.07775
3	1.0	15	6	1.299630	1.264663	0.034966	1.783259	1.736778	0.046481
4	1.0	10	4	1.020926	1.036545	-0.01561	1.686074	1.699833	-0.01375
5	1.0	5	6	0.960935	0.928235	0.032700	1.746963	1.701926	0.045037
6	0.6	15	6	1.518972	1.551672	-0.03270	2.264889	2.309926	-0.04503
7	0.8	5	4	1.106463	1.123544	-0.01708	1.612593	1.643870	-0.03127
8	0.6	10	8	1.243046	1.227427	0.015619	1.828889	1.815130	0.013759
9	0.8	15	8	1.365833	1.348752	0.017081	1.977630	1.946352	0.031278
10	0.6	10	4	1.654176	1.602129	0.052047	2.165333	2.087574	0.077759
11	0.6	5	6	1.195370	1.230337	-0.03496	1.503111	1.549593	-0.04648
12	0.8	5	8	0.986435	0.967088	0.019347	1.490222	1.457500	0.032722
13	0.8	15	4	1.380296	1.399643	-0.01934	1.917481	1.950204	-0.03272

Cooefficent of determination (R^2) for alkaline pretreatment was 92.79% for CMCase and 99.57% for FPase stands for the same %age of the variations were well explained by the model. Validity of these results were further authenticated by adjusted R^2 values which are 79.82% and 98.79% for CMCase and FPase respectively. Contingent upon steam alkaline pretreatment R^2 value for CMCase and FPase was 98.13 and 96.12 symbolized that 98.13% and 96.12% variability were demonstrated by the model and only 1.87% and and 3.88% variations were credited to variables. Accuracy of R^2 was further proved by adjusted R^2 values as 94.75% and 89.13% for CMCase and FPase accordingly. In the current study thirteen experimental setups in accordance to BBD design were performed each for alkaline and steam alkaline pretreatment, here only optimal values will be discussed and compared.

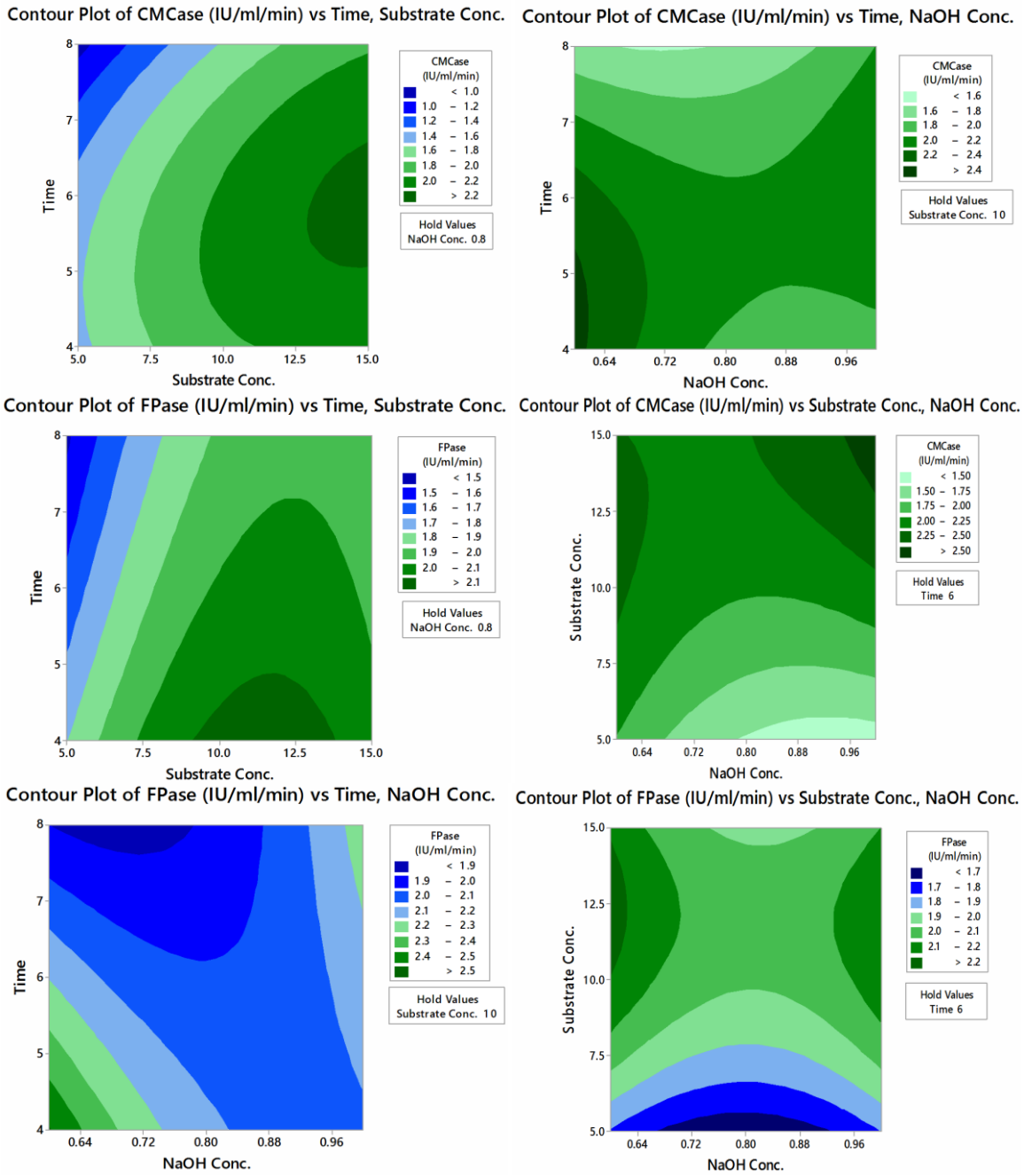

Figure 1. FPase and CMCase production from alkaline treated eucalyptus leaves

Abou-Taleb *et al.* (2009) reported CMCase 2.39U/ml and 1.18U/ml FPase activity from carboxymethyl cellulase using *B. amyloliquefaciens* C2. Sethi *et al.* (2013) reported CMCase production was 0.9U/ml from coconut cake by using *Bacillus subtilis* at 49 ºC lesser than the current study. Number of carbon sources were used for fermentation, posed a great impact on cellulase production. *B. licheniformis* 2D55 3% v/v hydrolyzed alkaline pretreated sugarcane bagasse and rice husk with 29.4U/ml CMCase and 12.9U/ml FPase activity at 60 ºC with 18h fermentation (Kazeem *et al.,* 2016). While alkaline pretreated rice straw hydrolyzed by*Trichoderma virens* produced lower CMCase (1669.65U/g) and FPase (93.75U/ml) (Rahnama *et al.,* 2014). Badhan *et al.,* (2007) produced 6.62U/g CMCase and 0.7U/g FPase from *Myceliophthora sp.* IMI387099 using bagasse as a carbon source. In the present study alkaline (NaOH) pretreated eucalyptus leaves peaked CMCase and FPase activities was 2.803IU/ml/min and 2.526IU/ml/min accordingly at pH-5, 50 ºC for 24h of fermentation by *Bacillus subtilis* K-18. While for steam alkaline steam pretreated eucalyptus leaves under the same fermentation conditions uppermost observed CMCase and FPase activities were 1.6544IU/ml/min and 2.264IU/ml/min respectively.

Table IV: ANOVA for cellulase from alkaline pretreated Eucalyptus leaves

CMCase (IU/ml/min)	**Sources**	**DF**	**Adj SS**	**Adj MS**	**F value**	**P value**
	Model	9	2.45803	0.27311	7.15	0.022
	Linear	3	1.41722	0.47241	12.38	0.009
	X_1	1	0.02633	0.02633	0.69	0.444
	X_2	1	1.16401	0.16401	30.49	0.003
	X_3	1	0.22688	0.22688	5.94	0.059
	Square	3	0.51546	0.17182	4.50	0.069
	X_1^2	1	0.16353	0.16353	4.28	0.093
	X_2^2	1	0.10475	0.10475	2.74	0.159
	X_3^2	1	0.21744	0.21744	5.70	0.063
	2 Way interaction	3	0.52535	0.17512	4.59	0.067
	X_1*X_2	1	0.23107	0.23107	6.05	0.057
	X_1*X_3	1	0.21588	0.21588	5.66	0.663
	X_2*X_3	1	0.07841	0.07841	2.05	0.211
	Error	5	0.19087	0.03817		
	Lack of fit	3	0.19087	0.06362		
	Pure error	2	0.00000	0.00000		
	Total	14	2.64890			
FPase (IU/ml/min)	**Sources**	**DF**	**Adj SS**	**Adj MS**	**F value**	**P value**
	Model	9	0.789215	0.087691	128.54	0.000
	Linear	3	0.336020	0.112007	164.18	0.000
	X_1	1	0.002337	0.002337	3.43	0.123
	X_2	1	0.240818	0.240818	352.99	0.000
	X_3	1	0.092865	0.092865	136.12	0.000
	Square	3	0.286174	0.095391	139.82	0.000
	X_1^2	1	0.102724	0.102724	150.57	0.000
	X_2^2	1	0.161484	0.161484	236.70	0.000
	X_3^2	1	0.000242	0.000242	0.35	0.578
	2 way interaction	3	0.167021	0.55674	81.61	0.000
	X_1*X_2	1	0.002340	0.002340	3.43	0.123
	X_1*X_3	1	0.154711	0.154711	226.77	0.000
	X_2*X_3	1	0.009970	0.009970	14.61	0.12
	Error	5	0.003411	0.000682		
	Lack of fit	3	0.003411	0.001137		
	Pure error	2	0.000000	0.000000		
	Total	14	0.792626			

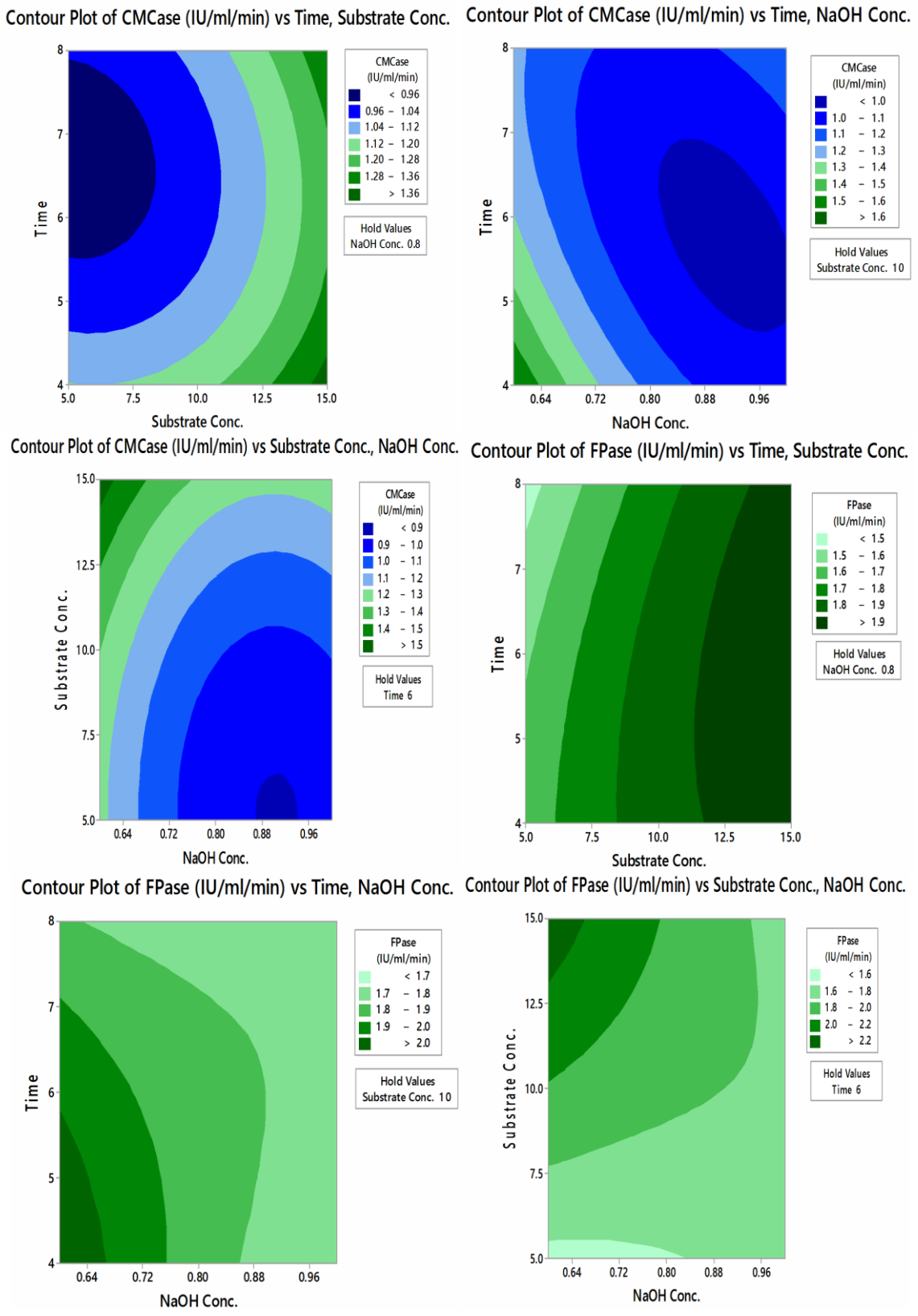

Figure 2: FPase and CMCase production from steam alkaline pretreated eucalyptus leaves

Table V: ANOVA for cellulase from steam alkaline pretreated Eucalyptus leaves

CMCase (IU/ml/min)	Sources	DF	Adj SS	Adj MS	F value	P value
	Model	9	0.618797	0.068755	29.08	0.001
	Linear	3	0.411349	0.137116	57.99	0.000
	X_1	1	0.173526	0.173526	73.39	0.000
	X_2	1	0.216327	0.216327	91.49	0.000
	X_3	1	0.021496	0.021496	9.06	0.030
	Square	3	0.131149	0.043716	18.49	0.004
	X_1^2	1	0.077001	0.077001	32.57	0.002
	X_2^2	1	0.027734	0.027734	11.73	0.019
	X_3^2	1	0.045036	0.045036	19.05	0.007
	2 Way interaction	3	0.076299	0.025433	10.76	0.013
	X_1*X_2	1	0.000057	0.000057	0.02	0.883
	X_1*X_3	1	0.073456	0.073456	31.07	0.003
	X_2*X_3	1	0.002786	0.002786	1.18	0.327
	Error	5	0.011822	0.002364		
	Lack of fit	3	0.011822	0.003941		
	Pure error	2	0.000000	0.000000		
	Total	14	0.630619			
FPase (IU/ml/min)	**Sources**	**DF**	**Adj SS**	**Adj MS**	**F value**	**P value**
	Model	9	0.617695	0.068633	13.76	0.005
	Linear	3	0.422795	0.140932	28.25	0.001
	X_1	1	0.088543	0.088543	17.75	0.008
	X_2	1	0.316160	0.316160	63.37	0.001
	X_3	1	0.018092	0.018092	3.63	0.115
	Square	3	0.023544	0.007848	1.57	0.306
	X_1^2	1	0.005826	0.005826	1.17	0.329
	X_2^2	1	0.011973	0.011973	2.40	0.182
	X_3^2	1	0.004614	0.004614	0.92	0.380
	2 way interaction	3	0.171356	0.057119	11.45	0.011
	X_1*X_2	1	0.131581	0.131581	26.37	0.004
	X_1*X_3	1	0.031447	0.031447	6.30	0.054
	X_2*X_3	1	0.008328	0.008328	1.67	0.253
	Error	5	0.025947	0.004989		
	Lack of fit	3	0.024947	0.008316		
	Pure error	2	0.000000	0.000000		
	Total	14	0.642642			

Figure 1 and 2 presented contour plots for cellulase production by *Bacillus subtilis* K-18 in submerged fermentation using alkali pretreated and alkali steam pretreated eucalyptus leaves. These plots clearly explained the significance of individual parameter of pretreatment on cellulase production. Vyas *et al.* (2016) stated that *Bacillus subtilis* M1 produced high titer of exoglucanase and endoglucanase by alkali treated ground nut shell as compared to untreated substrate. Mahalakshmi and Jayalakshmi (2016) described that alkali treated sugarcane bagasse yield better cellulase production from *Achromobacter xylosoxidans.* Assareh *et al.,* (2012) produced 143.50U/ml CMCase from *Geobacillus sp.* T1 AT 50 ºC using barely straw as substrate. Asha and Sakthivel (2014) reported 22.9U/ml CMCase activity from *Bacillus subtilis* using carboxymethyl cellulase (CMC) as substrate being lower than current study.

Conclusion

Results of this study indicated that alkali pretreatment of the eucalyptus leaves favored hyper cellulase production by *Bacillus subtilis* K-18 in submerged fermentation at 50^{o}C for 24h of fermentation period. The cellulase enzyme produced from this strain could be used in various industrial sectors especially in biofuels.

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