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Research Article

Dilute Sulphuric Acid Pretreatment Optimization of Cotton Stalk for Cellulase Production through Box-Bhenken Design

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Authors' Contributions
NF conducted the experiments. MI conceived the study design. HAS provide technical assistance and JIQ critically evaluated the draft.

Keywords
Cellulase, Cotton stalk, Pretreatment, RSM, *Bacillus subtilis*, Submerged fermentation.

Abstract | In this study, cellulase enzyme production was assessed by pretreating cotton stalk with dilute sulphuric acid which was optimized through response surface methodology. Pretreatment conditions were optimized using three variable with three levels like sulfuric acid concentrations (0.6%, 0.8%, 1% v/v), biomass loading (5%, 10%, 15% w/v), and residence time (4, 6 and 8h). After pretreatment process, cellulase production was achieved by solid substrate which was conducted in 250mL capacity Erlenmeyer flask in which incubation of *Bacillus subtilis* was carried out for 24 h of fermentation period at temperature of 50°C. Results showed that cellulase production was greatly affected by the thermochemical pretreatment as compared to the chemical pretreatment. At the pretreatment condition of 1% H_2SO_4 conc, 6h residence time, 15% substrate concentration maximum CMCase production (0.858 IU/ml/min) was obtained while at pretreatment conditions of 1% H_2SO_4 concentration, 8h residence time, 10% substrate concentration at room temperature followed by routine autoclaving, FPase production up to 0.876 IU/ml/min was recorded. The results of present study were found significant. The cellulase enzyme produced in this process affectively hydrolyzes the pretreated substrate at temperature of 50°C and 53 h of incubation period by releasing reducing sugars of 0.74 mg/ml. This study ensures the effective usage of lignocellulosic biomass at large scale biofuel production.

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Introduction

Structural complexity and rigidity of lignocellulosic substrate have given rise to the diverse variety of degradative enzyme - the cellulases (Bayer *et al.*, 1998). A great deal of variety of lignolytic microorganisms mainly fungi and bacteria are identified and isolated among them *Trichoderma reesei* and its mutants, white rot fungi an efficient lignin degraders and *Phanerochaete chrysosporium* are most commonly used forcellulose and hemicellulases production (Baldrian and Gabriel, 2003; Falcón *et al.*, 1995; McCarthy, 1987; Zimmermann, 1990; Vicuña, 1988). The bond beta-1, 4-d-glucan in cellulose basically breaks down by the enzyme cellulases and glucose, cellobiose and cello-oligosaccharides produce as primary product.

Cellulose degradation is achieved by several methods. One of the important methods is the use of cellulase which includes three types of enzyme that degrade cellulose, by a phenomenon of synergism (Iqbal *et al.*, 2010). These three enzymes are endo-glucanases (EG), cellobiohydrolases also called exoglucanases (CBH) and β-glucosidases (BGL). Endo-glucanases and cellobiohydrolases by attacking on the reducing and non-reducing end of the cellulose structure produces the nicks at internal sites, oligosaccharides and new chain ends and cello-oligosaccharides and cellobiose respectively and glucosidases complete the hydrolysis by hydrolyzing the cellobiose and soluble cellodextrins to liberate glucose (Sukumaran *et al.*, 2005).

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Cellulases are produced by microorganisms when grown on cellulosic materials (Lee and Koo, 2001). Global production of cellulase enzymes has great interest in research field. One of the major concerns is low titers of cellulase production. Multifaceted approaches are adopted to improve enzyme production, such as for substrate using inexpensive raw materials, bioengineering the microorganisms, effective bioprocess technologies, *etc.* (Lynd *et al.*, 2002; Sukumaran *et al.*, 2005).

Cellulase secretion is largely effected by the lignocellulosic substrate. Some substrates not required any specific inducers to enhance the synthesis of lignocellulolytic enzyme (Elisashvili *et al.*, 2009). Solid state and submerged fermentation techniques are most commonly used for cellulase production. The SSF is carried out in absence of free water as it is close to the natural environment to produces the high titers of enzyme (Cen and Xia, 1999; Jha *et al.*, 1995).

Various parameters effected the cellulase production like the kind of the substrate, medium pH, nutrient availability, temperature of the fermentation, supply of inducer etc. Cellolulytic organisms like fungal species *Trichoderma*, *Penicillium*, *Humicola* and *Aspergillus* (Sukumaran *et al.*, 2005). Due to the capacity of producing the large quantities of extracellular enzyme *Bacillus* sp. is considered one of the important species due to their capacity of producing of large quantities of enzymes (Singh *et al.*, 2004). *Bacillus sphaericus* and *Bacillus subtilis* both species are reported to express high cellulose degradation activities (Mawadza *et al.*, 1996; Singh *et al.*, 2004).

Table I: Coded and actual levels of the factors for three factors Box-Behnken design.

Independent variables	Symbols	Coded and actual values		
		-1	**0**	**+1**
Acid concentration (%)	X_1	0.6	0.8	1.0
Substrate concentration (%)	X_2	5	10	15
Time (Hours)	X_3	4	6	8

Response surface methodology (RSM) is empirical and statistical analysis widely used for analyzing and modeling the problems by studying the aggregated effect of the several variables, mathematical technique in which several variables influences the response of interst (Kim *et al.*, 2008; Bas and Boyaci, 2007). It is used for optimization of different steps in the multivariable systems. Quantification of input levels and levels of selected response is carried out by using design of experiment. Box-Bhenken and central composite designs are common designs of RSM (Khuri and Cornell, 1987; Montgomery, 2005). The objective of the present study was production of cellulase enzyme from pretreated cotton stalk and its application in saccharification process.

Materials and Methods

Microbial strain

Bacillus subtilis K-18 was taken from repository of Microbial Biotechnology Laboratory and revive on nutrient agar plates and then used in present study.

Pretreatment of cotton stalk

Cotton stalk was collected from field of Shahkot, District Nankana, Punjab, Pakistan. Cotton stalks was washed to remove dust, then sundried for seven days and then oven drying at 70°C for 1 day. The dried cotton stalk was cut into small pieces and turn into powdered form. Pretreatment of powdered cotton stalk was performed as discussed earlier (Arshad *et al.*, 2017).

Fermentation methodology

Cellulase enzyme production was carried out in 25ml of fermentation medium (1% yeast extract and 2% pretreated substrate, pH of 5) in the 250ml capacity of Erlenmeyer flask. This medium was autoclaved and then flasks were inoculated employing 2% (v/v) of inoculum. The culture was incubated at 50°C with shaking 120 rpm for of 24 h. The cultures were filtered by muslim cloth at the end of fermentation period. After filtration, to obtain the clear filterate as crude source of enzyme, the filtrate obtained by muslim cloth was centrifuged at 10,000 rpm for 10 min at 4°C were carried out. Each fermentation experiments were carried out in triplicate.

Cellulase assay

CMCase and FPase were determined as described in our earlier reports (Irfan *et al.*, 2011). One unit of CMCase or FPase activity defined as the amount of enzyme required to liberate one micromole of glucose from substrate per milliliter per minute under standard assay conditions.

Experimental design

For cellulase production optimization of different pretreatment conditions was carried out by Box-Bhenken design (BBD) with three variables *i.e.*, sulfuric acid con (X_1), substrate con, (X_2) and time (X_3) (Table I). The response was calculated using STATISTICA software.

Results and Discussion

In this study dilute acid pretreatment of cotton stalk was performed with three factors *i.e.* dilute sulphuric acid concentration (X_1), substrate concentration (X_2) and residence time (X_3) with three levels as mentioned in Table I. After pretreatment, the solid residue was washed up to neutrality, dried and further used for the production of cellulase in submerged fermentation by *Bacillus subtilis*in at 50°C for 24 h. The experiments were conducted according to Box-Bhenken design of response surface methodology.

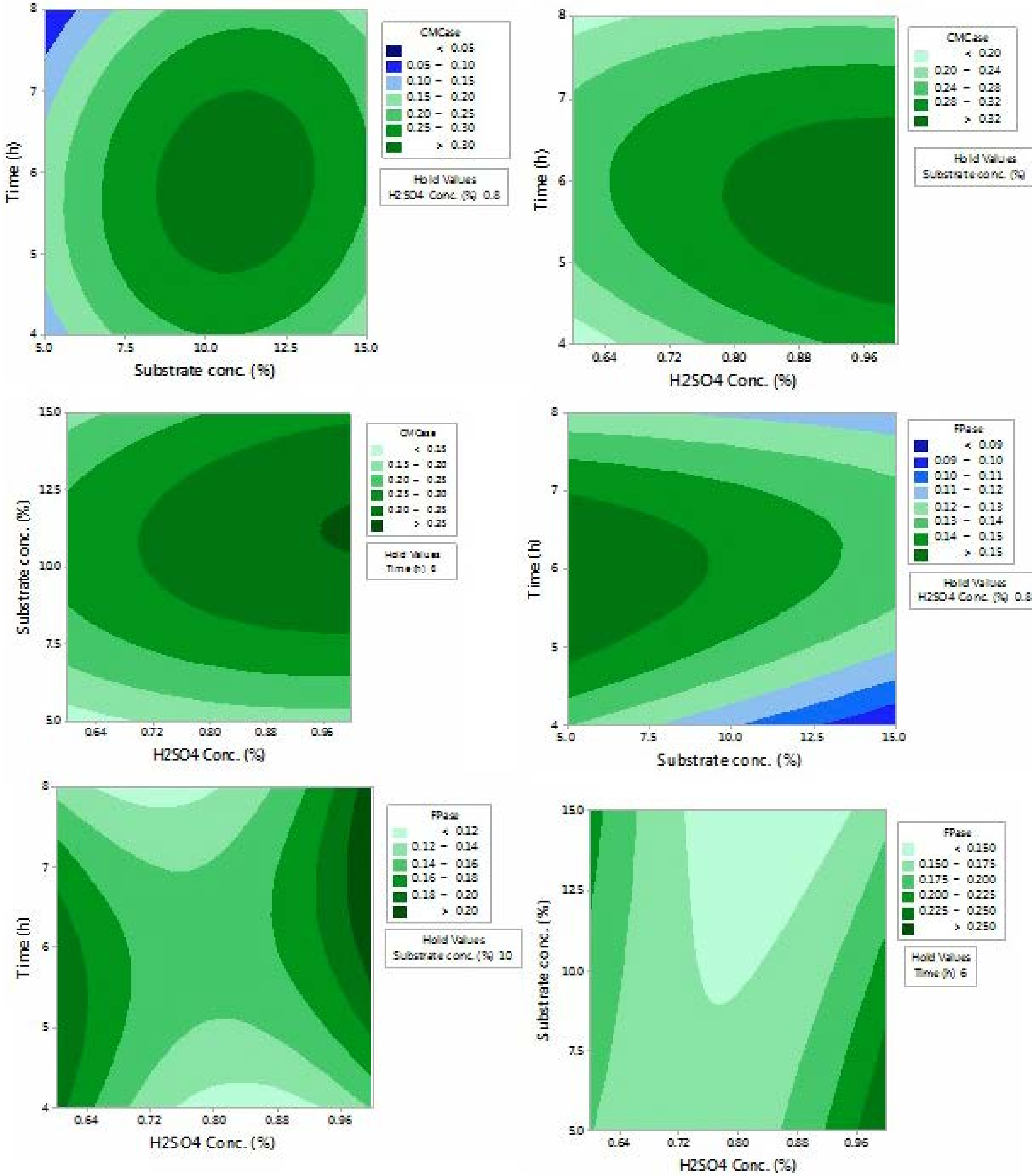

Figure 1: CMCase (IU/ml/min) and FPase (IU/ml/min) production from H_2SO_4 treated cotton stalk.

The calculation of the response was carried out according to polynomial regression equations (Equations 1 to 4). The cellulase enzyme production was found minimum in acid treated cotton stalk while acid followed by steam treated cotton stalk. In case of cellulase production, during fermentation process nature of substrate play a key role in influencing the production of enzyme (Kang *et al*., 2004). Box-Bhenken design results (Tables III, IV) revealed that under pretreatment conditions of 15% substrate concentration, 1% H_2SO_4, and time of 6 h. Maximum CMCase activity of 0.885 IU/ml/min was obtained. The highest FPase production (0.876 IU/ml/min) was noted with pretreatment condition of 1% H_2SO_4, 10% substrate concentration and residence time of 10h followed by steaming. Close matching of the observed and predicted values showed the accuracy of model.

Table II: Cellulase production by H_2SO_4 treated cotton stalk using Box-Bhenken design.

Run No.	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	0.322	0.322	0.000	0.148	0.148	0.000
2	1.0	10	8	0.246	0.226	0.020	0.219	0.211	0.008
3	1.0	15	6	0.303	0.289	0.013	0.129	0.167	-0.037
4	1.0	10	4	0.310	0.291	0.018	0.156	0.141	0.0147
5	1.0	5.0	6	0.124	0.177	-0.052	0.266	0.251	0.0149
6	0.6	15	6	0.231	0.178	0.052	0.195	0.210	-0.014
7	0.8	5.0	4	0.151	0.117	0.034	0.100	0.130	-0.029
8	0.6	10	8	0.162	0.181	-0.018	0.118	0.132	-0.014
9	0.8	15	8	0.137	0.172	-0.034	0.142	0.112	0.029
10	0.6	10	4	0.151	0.172	-0.020	0.180	0.188	-0.008
11	0.6	5.0	6	0.110	0.123	-0.013	0.214	0.176	0.037
12	0.8	5.0	8	0.078	0.046	0.032	0.098	0.121	-0.023
13	0.8	15	4	0.126	0.158	-0.032	0.113	0.089	0.023

Table III: Cellulase production by H_2SO_4 followed by steam treated cotton stalk using Box-Bhenken design.

Run No.	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	0.800	0.800	0.000	0.695	0.695	0.000
2	1.0	10	8	0.754	0.758	-0.003	0.876	0.751	0.124
3	1.0	15	6	0.885	0.858	0.027	0.341	0.415	-0.074
4	1.0	10	4	0.784	0.775	0.009	0.344	0.314	0.030
5	1.0	5	6	0.744	0.777	-0.032	0.348	0.429	-0.080
6	0.6	15	6	0.762	0.730	0.032	0.789	0.708	0.080
7	0.8	5	4	0.684	0.660	0.023	0.628	0.577	0.050
8	0.6	10	8	0.645	0.654	-0.009	0.657	0.687	-0.030
9	0.8	15	8	0.731	0.754	-0.023	0.738	0.788	-0.050
10	0.6	10	4	0.620	0.616	0.003	0.695	0.820	-0.124
11	0.6	5	6	0.616	0.643	-0.027	0.652	0.578	0.074
12	0.8	5	8	0.587	0.551	0.036	0.660	0.704	-0.043
13	0.8	15	4	0.587	0.624	-0.036	0.654	0.610	0.043

Equations for chemical treated cotton stalk

CMCase activity (IU/ml/min) = $-1.383 + 1.06\ X_1 + 0.0740\ X_2 + 0.2693\ X_3 - 0.453\ X_1^2 - 0.00449\ X_2^2 - 0.02168\ X_3^2 + 0.0145\ X_1{*}X_2 - 0.0466\ X_1{*}X_3 + 0.00210\ X_2\ X_3$ ………. (Eq. 1)

FPase activity (IU/ml/min) = $0.879 - 2.308\ X_1 + 0.0172\ X_2 + 0.0327\ X_3 + 1.358\ X_1^2 - 0.000036\ X_2^2 - 0.00846\ X_3^2 - 0.0296\ X_1{*}X_2 + 0.0784\ X_1{*}X_3 + 0.00078\ X_2{*}X_3$ ………. (Eq. 2)

Equations for thermochemical treated cotton stalk

CMCase activity (IU/ml/min) = $-0.446 + 0.424\ X_1 + 0.0140\ X_2 + 0.2754\ X_3 + 0.078\ X_1^2 - 0.002028\ X_2^2 - 0.02543\ X_3^2 - 0.0014\ X_1{*}X_2 - 0.0345\ X_1{*}X_3 + 0.00600\ X_2{*}X_3$ ………. (Eq. 3)

FPase activity (IU/ml/min) = $0.94 + 1.47\ X_1 + 0.0813\ X_2 - 0.388\ X_3 - 2.38\ X_1^2 - 0.00272\ X_2^2 + 0.0107\ X_3^2 - 0.0360\ X_1{*}X_2 + 0.356\ X_1{*}X_3 + 0.00130\ X_2{*}X_3$ ………. (Eq. 4)

All the response (CMCase and FPase activity) calculated was statistical analyzed with analysis of variance and results revealed for CMCase production, the proposed model was found significant in both treatments (H_2SO_4 treatment and H_2SO_4 followed steam treatment) while the model was not significant for FPase production. In H_2SO_4 treated cotton stalks the F and P value of the CMCase model was 4.75 and 0.050 while in H_2SO_4 followed by steam treated cotton stalks the values was 7.99 and 0.017, respectively. Coefficient of determination (R^2 value) of treated (89.54 %) and H_2SO_4 followed by steam treated (93.50%) further confirmed the fitness of the model by accurately showing the predicting response. Furthermore, the model also supported by adjusted R^2 value of 70.71% (H_2SO_4 treated) and 81.80% (H_2SO_4 followed by steam treated).

These results indicated that pretreatment of substrate is very effective in conversion of raw materials into valuable products with aid of microbes. In previous study, pretreatment of rice straw with 0.5M KOH followed by 0.1N

H_2SO_4 yielded better CMCase activity from *Bacillus* sp. 313SI (Goyal *et al*., 2014). Ghazanfar *et al.* (2018) reported maximum FPase production from *B. subtilis* K-18 when substrate *Sacharum spontaneum* pretreated with 1% H_2SO_4, substrate concentration of 10% and 4h of residence time followed by autoclaving. Anjum *et al.* (2017) obtained highest yield of cellulase from acacia dust pretreated with 0.8% H_2SO_4, 4 h residence time and 15% substrate concentration. Similarly, peanut shells treated with 0.6% and 0.8% H_2SO_4 yielded maximum CMCase and FPase production by *B. subtilis* K-18 in submerged fermentation, respectively (Arshad *et al*., 2017). Eucalyptus leaves treated with 0.8% and 1.0% H_2SO_4 gave maximum titer of CMCase and FPase production using *B. subtilis* K-18 in submerged fermentation, respectively (Iqbal *et al*., 2017). Arooj *et al.* (2017) stated that banana peduncle produced best cellulase under optimized pretreatment conditions of 0.4N H_2SO_4, substrate concentration of 15% and soaking time of 6h. Another study reported that pretreatment effectively improved cellulase production revealing correlation between physiochemical properties of substrates and enzyme production (Brijwani and Vadlani, 2011).

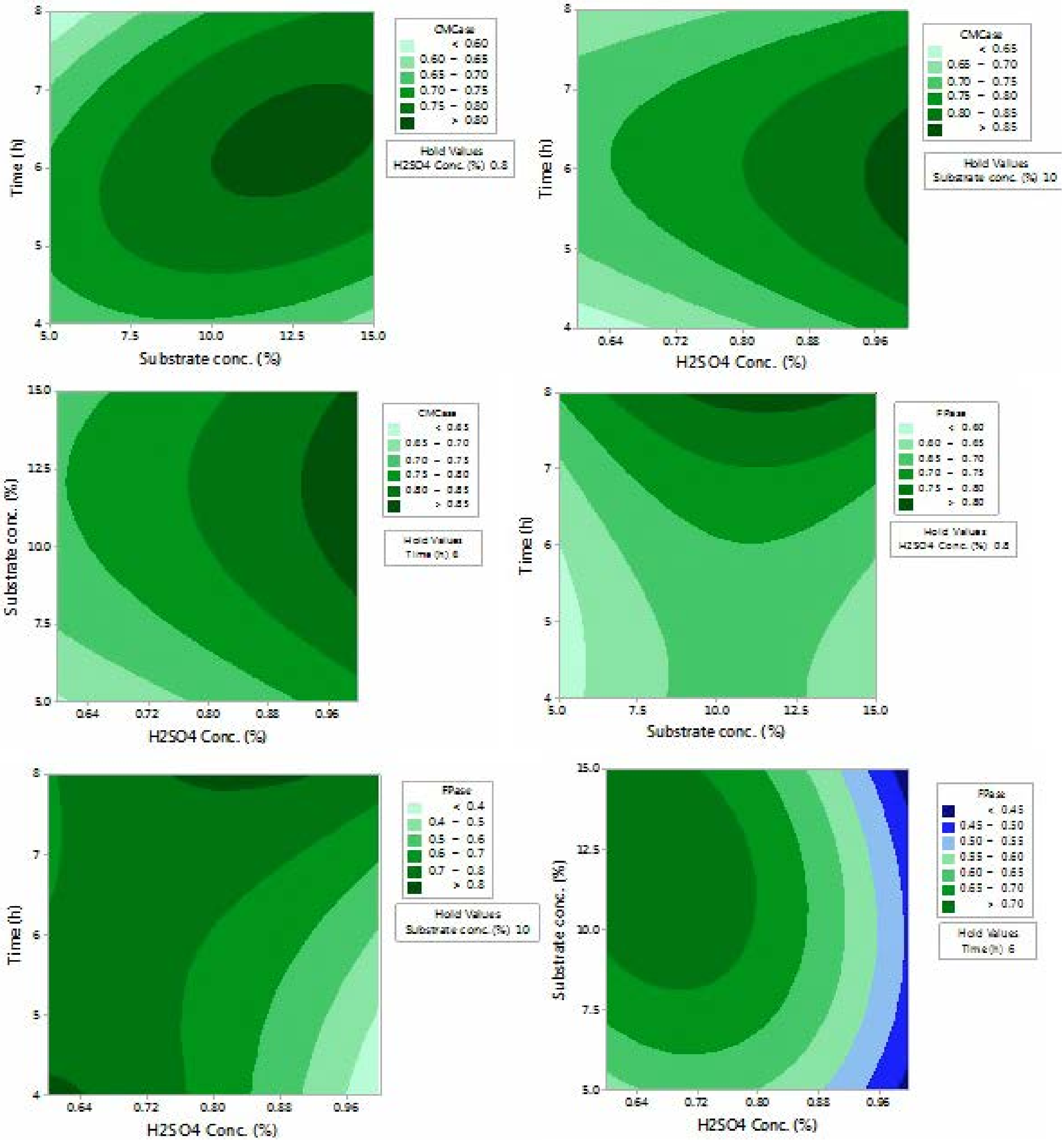

Figure 2: CMCase (IU/ml/min) and FPase (IU/ml/min) production from H_2SO_4 steam treated cotton stalk.

Table IV: ANOVA of chemical (H_2SO_4) and thermochemical treated cotton stalk.

	Sources	DF	Adj SS	Adj MS	F value	P value
Chemical (H_2SO_4) treated						
CMCase (IU/ml/min)	Model	9	0.102398	0.011378	4.75	0.050
	Linear	3	0.029160	0.009720	4.06	0.083
	X_1	1	0.013474	0.013474	5.63	0.064
	X_2	1	0.014046	0.014046	5.87	0.060
	X_3	1	0.001639	0.001636	0.68	0.446
	Square	3	0.069242	0.023081	9.65	0.016
	X_1^2	1	0.001210	0.001210	0.51	0.509
	X_2^2	1	0.046529	0.046529	19.45	0.007
	X_3^2	1	0.027778	0.027778	11.61	0.019
	2 Way interaction	3	0.003997	0.001332	0.56	0.666
	X_1*X_2	1	0.000839	0.000839	0.35	
	X_1*X_3	1	0.001387	0.001387	0.58	
	X_2*X_3	1	0.001770	0.001770	0.74	
	Error	5	0.011964	0.002393		
	Lack of fit	3	0.011964	0.003988		
	Pure error	2	0.000000			
Total		14	0.114362	0.000000		
FPase (IU/ml/min)	Model	9	0.025788	0.002865	2.12	0.210
	Linear	3	0.001837	0.000612	0.45	0.726
	X_1	1	0.000500	0.000500	0.37	0.569
	X_2	1	0.001239	0.001239	0.92	0.382
	X_3	1	0.000098	0.000098	0.07	0.798
	Square	3	0.016279	0.005426	4.02	0.084
	X_1^2	1	0.010892	0.010892	8.08	0.036
	X_2^2	1	0.00003	0.000003	0.00	0.964
	X_3^2	1	0.004226	0.004226	3.13	0.137
	2 way interaction	3	0.007672	0.002557	1.90	0.248
	X_1*X_2	1	0.003494	0.003494	2.59	0.168
	X_1*X_3	1	0.003936	0.003936	2.92	0.148
	X_2*X_3	1	0.000242	0.000242	0.18	0.689
	Error	5	0.006743	0.001349		
	Lack of fit	3	0.006743	0.002248		
	Pure error	2	0.000000			
Total		14	0.032531	0.000000		
Thermochemical treated						
CMCase (IU/ml/min)	Model	9	0.109443	0.012160	7.99	0.017
	Linear	3	0.048495	0.016165	10.63	0.013
	X_1	1	0.034350	0.034350	22.58	0.005
	X_2	1	0.013931	0.013931	9.16	0.029
	X_3	1	0.000214	0.000214	0.14	0.723
	Square	3	0.045775	0.015258	10.03	0.015
	X_1^2	1	0.000036	0.000036	0.02	0.884
	X_2^2	1	0.009490	0.009490	6.24	0.055
	X_3^2	1	0.038218	0.038218	25.12	0.004
	2 Way interaction	3	0.015173	0.005058	3.32	0.114
	X_1*X_2	1	0.000008	0.000008	0.01	0.946
	X_1*X_3	1	0.000761	0.000761	0.50	0.511
	X_2*X_3	1	0.014404	0.014404	9.47	0.028
	Error	5	0.007606	0.007606		
	Lack of fit	3	0.007606	0.007606		
	Pure error	2	0.000000	0.000000		
Total		14	0.117049	0.117049		

	Sources	DF	Adj SS	Adj MS	F value	P value
Thermochemical treated						
FPase (IU/ml/min)	Model	9	0.296322	0.032925	2.50	0.163
	Linear	3	0.151086	0.050362	3.83	0.091
	X_1	1	0.097806	0.097806	7.43	0.041
	X_2	1	0.006805	0.006805	0.52	0.504
	X_3	1	0.046475	0.046475	3.53	0.119
	Square	3	0.058044	0.019348	1.47	0.329
	X_1^2	1	0.033334	0.033334	2.53	0.172
	X_2^2	1	0.017100	0.017100	1.30	0.306
	X_3^2	1	0.006715	0.006715	0.51	0.507
	2 way interaction	3	0.087191	0.029064	2.21	0.205
	X_1*X_2	1	0.005194	0.005194	0.39	0.557
	X_1*X_3	1	0.081325	0.081325	6.18	0.055
	X_2*X_3	1	0.000672	0.000672	0.05	0.830
	Error	5	0.065803	0.013161		
	Lack of fit	3	0.065803	0.021934		
	Pure error	2	0.000000			
Total		14	0.362125	0.000000		

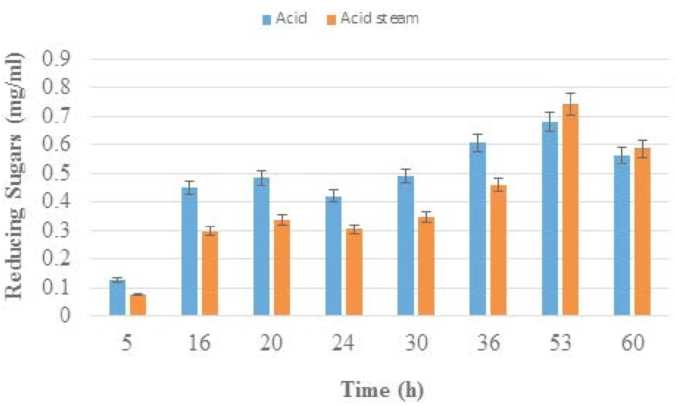

Figure 3: Enzymatic hydrolysis of H_2SO_4 and H_2SO_4 followed by steam treated cotton stalks.

The cellulase enzyme produced from these pretreated substrates was further used for enzymatic hydrolysis of best treated substrate (having maximum total phenolic compounds liberation). The enzymatic hydrolysis was performed at 50°C, pH 5.0 for various time periods to check the optimum time for maximum reducing sugar production. Results (Figure 3) reveals that reducing sugar production was increased with increase in time period, but maximum reducing sugar production was achieved at 53 h of incubation period. Various studies reported different optimum time for saccharification of various substrates like 8h for wheat straw (Asghar *et al*., 2014), pine needles (Irfan *et al*., 2017), 72h for rice straw (Kshirsagar *et al*., 2015) and 105 h for disposable wooden chopsticks (Phummala *et al*., 2015).

Conclusion

Results of this study concluded that H_2SO_4 pretreatment is efficient for cellulase production by *Bacillus subtilis* in submerged fermentation. The produced cellulase effectively hydrolyzed the pretreated substrate for sugar production which could be utilized for fermentation process for the production of different compounds like bioethanol.

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Conflicts of interest

The authors declare no conflicts of interest.

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