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Research Article

Effect of Dilute Sulphuric Acid Pretreatment on Cellulase Production by *Bacillus subtilis* (K-18) through Response Surface Methodology

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Abstract: The present study investigated the optimization of dilute sulphuric acid pretreatment to maximize cellulase production from banana peduncle waste through Box-Behnken design of response surface methodology. Cellulase production was carried out in 250ml capacity Erlenmeyer flask using pretreated banana peduncle as substrate in submerged fermentation by *Bacillus subtilis* K-18 incubated at 50^{o}C for fermentation period of 24 h. Results indicated that chemical pretreatment using sulphuric acid favored cellulase production as compared to thermochemical pretreatment using sulphuric acid followed by autoclaving at 121^{o}C for 15 min and 15 psi. Maximum Filter Paper activity of 0.958 IU/ml/min was observed at optimal pretreatment conditions of 0.4 N H_2SO_4 concentration, 15% substrate concentration and residence time of 6h with chemical pretreatment. For thermochemical pretreatment optimal FPase activity of 0.63 IU/ml/min was recorded at 0.4 N H_2SO_4 concentration, 10% substrate concentration and residence time of 4 h. The proposed regression model for both types of pretreatments was found significant as revealed by *F-value*, *P-value* and coefficient of determination. These results indicated that banana peduncle can be successfully utilized as solid substrate in submerged fermentation for cellulase enzyme production.

Keywords: Cellulase, RSM, pretreatment, *Bacillus subtilis*, submerged fermentation

1. INTRODUCTION

Cellulose being the most abundant organic polymer from plant biomass can act as an inexhaustible and inexpensive raw material for a number of value added products like ethanol, organic acids and various chemical solvents, etc. [1]. Cellulose is a polysaccharide of repeated β-D-glucopyranose units interlinked by β-1,4-glycosidic bonds. Therefore, it needs to be depolymerized into its monomer glucose units which are further subjected to microbial fermentation leading to the production of various valuable products. The breakdown of glycosidic bonds in cellulose is done either by chemical or enzymatic hydrolysis. Since chemical breakdown of cellulose using acids under harsh conditions generates byproducts toxic to microbes, enzymatic hydrolysis through the activity of cellulases is more attractive. Complete hydrolysis of cellulose into its glucose monomers is done by synergistic activity of three different cellulases belonging to Glycoside Hydrolase (GH) family of enzymes [2].

These enzymes hydrolyze the glycosidic bond by acid/base catalysis method [3].Endo- β-1,4-glucanase also called CMCase randomly cuts glycosidic linkages particularly at internal amorphous sites of cellulose chain, generating long chain oligomers [2, 4]. These oligomers are further depolymerized by Exoglucanase or β-1,4-Cellobiohydrolase. Exoglucanases can hydrolyse both reducing and non-reducing ends in a highly processive manner producing Cellobiose units [2]. Finally β-Glucosidases which have a pocket shaped active site specifically bind to non-reducing glucose ends of cellobiose, hydrolyse it

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and liberate both glucose units [5].

Cellulases are produced from a vast diversity of microorganisms mainly Bacteria and Fungi. Most extensively studied fungal genera for cellulolytic activity include *Aspergillus, Trichoderma, Fusarium, Penicillium*. Some bacterial genera well known for cellulolytic activity *are Clostridium, Pseudomonas, Bacillus,* etc. *Streptomyces, Cellulomonas*, and *Thermomonospora* are major cellulase producing actinomycetes [1, 4]. Fungal Cellulases are commercially more attractive as they are robust and extracellular. They are having simple structure consisting of a Cellulose binding domain (CBD) and a Catalytic domain (CD) interlinked by a linker peptide. *Trichoderma reesei* is the most extensively used fungus for cellulase production [4]. Bacterial cellulases are present in the form of cellulosomes attached to the cell wall of bacterial cell [2].

Cellulases are known to have diverse industrial applications as they are being used in textile, food, brewing, pulp and paper industry as well as additives in detergents. Growing concerns over the depletion of fossil fuels have led the increased demand of cellulases to be used in lignocellulose based biorefinery [2, 4]. The high costof cellulases is the major bottleneck in commercialization of these biorefineries. A number of lignocellulosic wastes have been used to produce cellulases from various microbes using either solid state or submerged fermentation that leads to not only cost effective enzyme production but also waste management [6]. Solid state fermentation utilizes solid substrates like bagasse, bran, rice straw and is most applicable for Fungi and microbes requiring little water content. Submerged fermentation technology is based on using free flowing liquid substrates such as broth and is suited for bacteria requiring high water potential [7]. More than 70% of commercial enzyme production has been reported through the use of submerged fermentation technology due to the advantages of better monitoring, handling, ease of product purification and its greater extent to support the use of genetically modified organisms [2, 7, 8].

Different strains of *Bacillus subtilis* have been used to produce cellulases using a variety of lignocellulosic wastes [1, 6, 9]. Most of *Bacillus* species have shown to produce high cellulases on sugarcane bagasse [10], rice husk [8] and Corn stover [11]. Several studies have shown that Banana fruit stalk and other wastes as pseudostems found abundantly in tropical and subtropical regions have a great potential to be used as solid substrate for commercial production of cellulases employing *Bacillus subtilis*, *Trichoderma viride*, *Aspergillus niger*, *Neurospora sitophila and Pleurotus sp.* [6, 12-16]. The present study investigates the cellulolytic potential of *Bacillus subtilis* using pretreated banana peduncle.

2. MATERIALS AND METHODS

2.1. Microbial Strain

The bacterium *Bacillus subtilis* K-18 was obtained from Microbial Biotechnology Laboratory, Department of Zoology, University of the Punjab, New Campus, Lahore, Pakistan. The culture was maintained on nutrient agar slants and was used for production of cellulase in submerged fermentation.

2.2 Pretreatment of Banana Peduncle

Pretreatment of Banana Peduncle was done as described in our earlier reports [17]. For chemical pretreatment, the powdered banana peduncle samples were soaked in 0.24 N, 0.32 N, 0.4 N H_2SO_4 solutions with substrate loading of 5%, 10%, 15%w/v and pretreatment time of 4, 6, 8 h. Likewise thermochemical pretreatment was carried out by autoclaving the soaked biomass for 121°C, 15 psi, 20 min. After pretreatment the samples were filtered and solid residues were washed up to neutrality.

2.3. Enzyme production

Enzyme production was done in 250ml Erlenmeyer flask capacity having 25ml of fermentation medium containing 2% pretreated substrate and 1% yeast extract with initial medium pH of 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 24 h of fermentation period. After completion of the fermentation period, the fermented broth was filtered through muslin cloth followed by

Table 1. Coded and actual levels of the factors for three factor Box-Behnken design.

Independent variable	Symbol	Coded and actual values		
		-1	0	+1
Acid concentration (N)	X_1	0.24	0.32	0.40
Substrate concentration (%)	X_2	5	10	15
Time (h)	X_3	4	6	8

Table 2. Cellulase production by chemical treated banana peduncle 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	0.32	10	6	0.456657	0.456657	0.000000	0.551704	0.551704	-0.00000
2	0.40	10	8	0.431824	0.515637	-0.08381	0.860741	0.845833	0.014907
3	0.40	15	6	0.471833	0.448035	0.023799	0.958222	0.911556	0.046667
4	0.40	10	4	0.560130	0.515637	0.044493	0.698963	0.722944	-0.02398
5	0.40	5	6	0.376639	0.361118	0.015521	0.634667	0.672259	-0.03759
6	0.24	15	6	0.237296	0.252817	-0.01552	0.526815	0.489222	0.037593
7	0.32	5	4	0.531157	0.591171	-0.06001	0.522667	0.461093	0.061574
8	0.24	10	8	0.441481	0.485975	-0.04449	0.514370	0.490389	0.023981
9	0.32	15	8	0.641528	0.581514	0.060014	0.474963	0.536537	-0.06157
10	0.24	10	4	0.500806	0.416993	0.083812	0.572444	0.587352	-0.01490
11	0.24	5	6	0.404231	0.428030	-0.02379	0.556889	0.603556	-0.04666
12	0.32	5	8	0.550472	0.482181	0.068292	0.395111	0.372426	0.022685
13	0.32	15	4	0.335250	0.403542	-0.06829	0.399259	0.421944	-0.02268

Table 3. Cellulase production by thermochemical treated banana peduncle 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	0.32	10	6	0.21	0.218	0.00	0.55	0.55	0.00
2	0.40	10	8	0.24	0.24	-0.00	0.45	0.45	0.00
3	0.40	15	6	0.29	0.28	0.00	0.51	0.50	0.01
4	0.40	10	4	0.18	0.18	0.00	0.63	0.66	-0.02
5	0.40	5	6	0.19	0.20	-0.00	0.50	0.49	0.01
6	0.24	15	6	0.22	0.21	0.00	0.29	0.30	-0.01
7	0.32	5	4	0.14	0.13	0.00	0.39	0.37	0.01
8	0.24	10	8	0.15	0.15	-0.00	0.35	0.33	0.02
9	0.32	15	8	0.19	0.20	-0.00	0.30	0.31	-0.01
10	0.24	10	4	0.19	0.18	0.00	0.32	0.32	-0.00
11	0.24	5	6	0.18	0.18	-0.00	0.22	0.23	-0.01
12	0.32	5	8	0.15	0.14	0.00	0.34	0.35	-0.01
13	0.32	15	4	0.18	0.19	-0.00	0.50	0.49	0.01

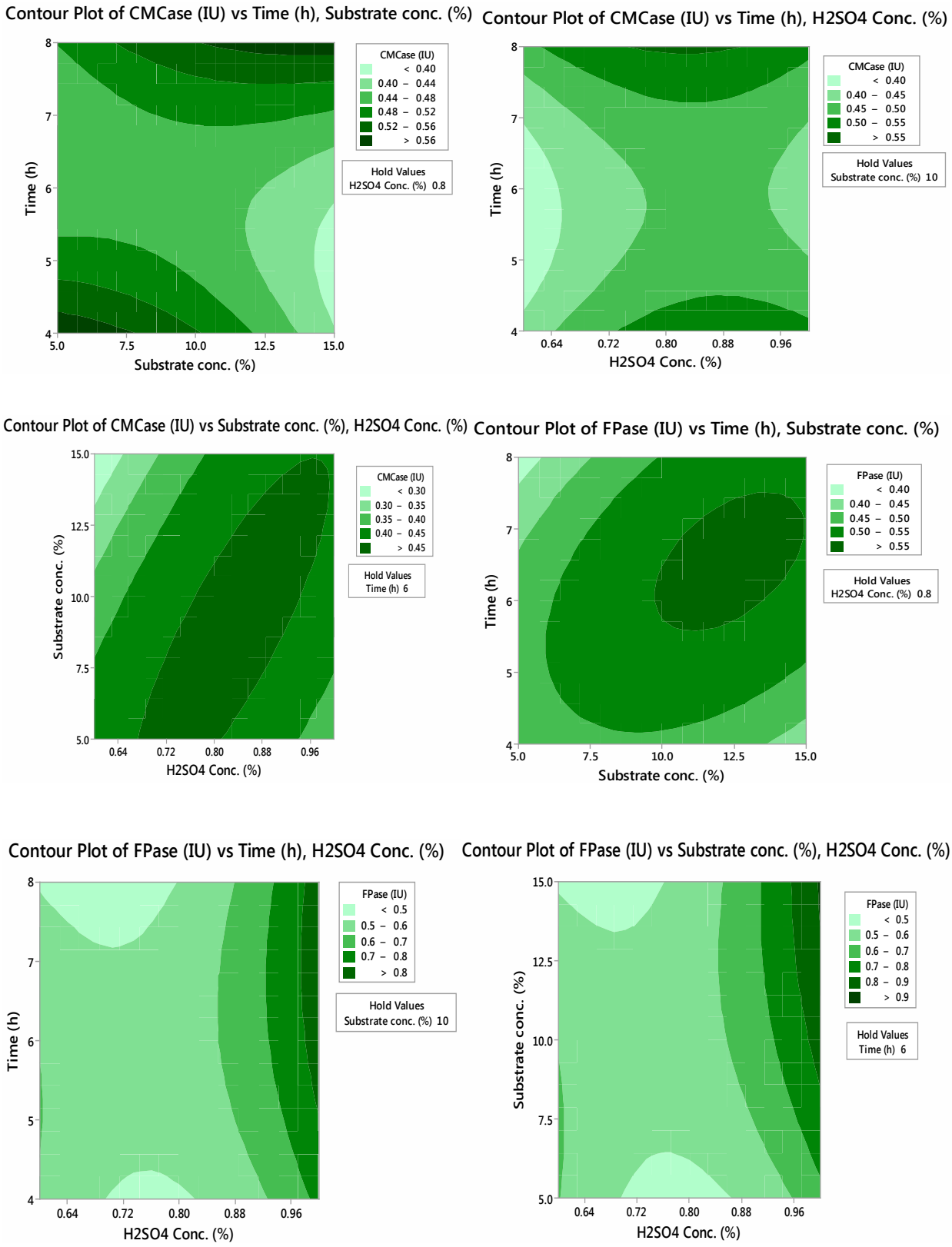

Fig. 1. Contour plots for CMCase (IU/ml/min) and FPase (IU/ml/min) production from sulphuric acid treated banana peduncle by *Bacillus subtilis* K-18 in submerged fermentation.

centrifugation (Sigma 2-16 PK) for 10 minutes at 10,000 x g and 4°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.

2.4. Cellulase assay

CMCase and FPase activity was determined as described in our earlier reports [18]. 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.

2.5. Experimental design

In order to optimize different pretreatment conditions for cellulase production, Box-Behnken design (BBD) was used in this study. The independent variables used were H_2SO_4 concentration (X_1), substrateconcentration, (X_2) and residence time (X_3) and their levels are mentioned in Table 1. This design is most suitable for quadratic response surface and generates second order polynomial regression model. The relation between actual and coded values was described by the following equation;

$$x_i = \frac{X_i - X_o}{\Delta X_i} \quad \text{Eq. (1)}$$

Where *xi* and *Xi*are the coded and actual values of the independent variable, *Xo* is the actual value of the independent variable at the center point and ΔXi is the change of *Xi.* The response is calculated from the following equation using STATISTICA software (99th edition).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad \text{Eq. (2)}$$

Y is the response, X_1, X_2 and X_3 are the independent variables, $ß_0$ is the intercept, $ß_1$, ß2 and $ß_3$ are linear coefficient, $ß_1^1$, $ß_2^2$ and $ß_3^3$ are square coefficients, $ß_{12}$, $ß_{13}$ and $ß_{23}$ are interaction coefficients.

3. RESULTS AND DISCUSSION

The present study investigated the effect of different pretreatment conditions for cellulase production from banana peduncle waste by *Bacillus subtilis* K-18 under submerged fermentation. Before carrying out enzyme production, biomass was pretreated chemically using H_2SO_4 and thermochemically using H_2SO_4 followed by autoclaving at 121°C for 15 min and 15 psi. Three experiment factors of H_2SO_4 concentration, substrate loading and residence time were optimized to maximize cellulose production. Second order polynomial equations were used to calculate enzyme production as shown in Eq. 3-6. Maximum Filter Paper activity of 0.958 IU/ml/min was observed at optimal conditions of 0.4 N H_2SO_4 concentration, 15% substrate concentration and residence time of 6 h with chemical pretreatment. For thermochemical pretreatment optimal FPase activity of 0.63 IU/ml/min was recorded at 0.4 N H_2SO_4 concentration, 10% substrate concentration and residence time of 4h. Sulphuric acid pretreatment resulted in higher values of enzyme production than sulphuric acid pretreatment followed by autoclaving. The results of cellulase production using Box-Behnken design for both types of pretreatments were shown in Table 2, 3.

Equations for CMCase and FPase production from acid treated substrate

$$\text{CMCase activity (IU)} = 3.58 - 4.58\ X_1 - 0.0543\ X_2 - 0.357\ X_3 + 2.19\ X_1^2 + 0.00023\ X_2^2 + 0.0203\ X_3^2 + 0.0552\ X_1 * X_2 + 0.054\ X_1 * X_3 - 0.00014\ X_2 * X_3 \quad \text{Eq. (3)}$$

$$\text{FPase activity (IU)} = 0.983 - 0.354\ X_1 - 0.0409\ X_2 - 0.1710\ X_3 + 0.240\ X_1^2 + 0.000902\ X_2^2 + 0.01381\ X_3^2 + 0.0207\ X_1 * X_2 - 0.0376\ X_1 * X_3 + 0.00036\ X_2 * X_3 \quad \text{Eq. (4)}$$

Equations for CMCase and FPase production from acid followed by steam treated substrate

$$\text{CMCaseactivity (IU)} = 0.291 - 1.009\ X_1 + 0.00275\ X_2 + 0.0792\ X_3 + 0.395\ X_1 * X_1 - 0.000392\ X_2 * X_2 - 0.01042\ X_3 * X_3 + 0.01300 X_1 * X_2 + 0.0591 X_1 * X_3 + 0.000100\ X_2 * X_3 \quad \text{Eq. (5)}$$

$$\text{FPase activity (IU)} = -2.678 + 3.811 X_1 + 0.1331 X_2 + 0.2915\ X_3 - 1.433 X_1 * X_1 - 0.004667\ X_2 * X_2 - 0.01430\ X_3 * X_3 - 0.0150 X_1 * X_2 + 0.1325 X_1 * X_3 + 0.00396 X_2 * X_3 \quad \text{Eq. (6)}$$

Statistical significance of data was evaluated by applying F-test in ANOVA. For chemical pretreatment, regression model for CMCase production was found to be insignificant with

Table 4. Analysis of variance of chemical treated banana peduncle.

	Source	DF	Adj SS	Adj MS	F value	P value
CMCase (IU/ml/min)	Model	9	0.098	0.010	1.51	0.339
	Linear	3	0.014	0.004	0.67	0.607
	X_1	1	0.008	0.008	1.14	0.335
	X_2	1	0.003	0.003	0.54	0.496
	X_3	1	0.002	0.002	0.33	0.591
	Square	3	0.044	0.014	2.06	0.224
	X_1^2	1	0.012	0.012	1.69	0.250
	X_2^2	1	0.002	0.002	0.36	0.575
	X_3^2	1	0.026	0.026	3.65	0.114
	2 Way interaction	3	0.038	0.012	1.80	0.265
	X_1*X_2	1	0.017	0.017	2.38	0.184
	X_1*X_3	1	0.001	0.001	0.16	0.702
	X_2*X_3	1	0.020	0.020	2.85	0.152
	Error	5	0.036	0.007		
	Lack of fit	3	0.036	0.012		
	Pure error	2	0.000	0.000		
	Total	14	0.134			
FPase (IU/ml/min)	Model	9	0.312	0.034	9.99	0.010
	Linear	3	0.128	0.042	12.34	0.010
	X_1	1	0.120	0.120	34.67	0.002
	X_2	1	0.007	0.007	2.25	0.194
	X_3	1	0.000	0.000	0.10	0.768
	Square	3	0.130	0.043	12.48	0.009
	X_1^2	1	0.101	0.101	29.09	0.003
	X_2^2	1	0.008	0.008	2.46	0.178
	X_3^2	1	0.011	0.011	3.28	0.130
	2 way interaction	3	0.053	0.017	5.14	0.055
	X_1*X_2	1	0.031	0.031	8.99	0.030
	X_1*X_3	1	0.012	0.012	3.47	0.121
	X_2*X_3	1	0.010	0.010	2.97	0.145
	Error	5	0.017	0.003		
	Lack of fit	3	0.017	0.005		
	Pure error	2	0.000	0.000		
	Total	14	0.329			

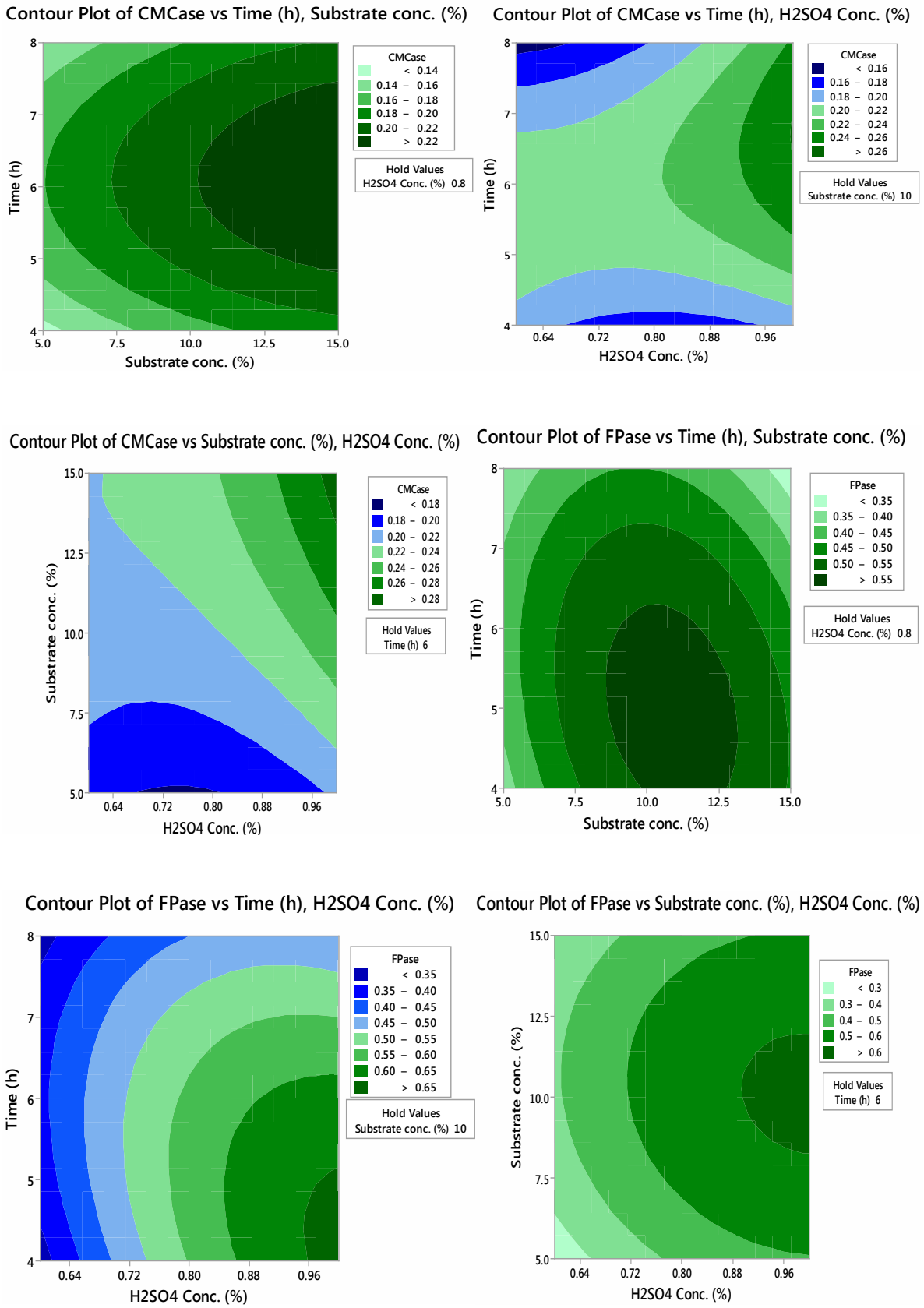

Fig. 2. Contour plots for CMCase (IU/ml/min) and FPase (IU/ml/min) production from sulphuric acid followed by steam treated banana peduncle by *Bacillus subtilis*K-18 in submerged fermentation.

Table 5. Analysis of variance of thermochemical treated banana peduncle.

	Sources	DF	Adj SS	Adj MS	F value	P value
CMCase (IU/ml/min)	Model	9	0.02	0.00	25.72	0.00
	Linear	3	0.01	0.00	38.51	0.00
	X_1	1	0.00	0.00	39.54	0.00
	X_2	1	0.00	0.00	74.01	0.00
	X_3	1	0.00	0.00	1.97	0.21
	Square	3	0.00	0.00	28.34	0.00
	X_1^2	1	0.00	0.00	9.81	0.02
	X_2^2	1	0.00	0.00	3.78	0.10
	X_3^2	1	0.00	0.00	68.22	0.00
	2 Way interaction	3	0.00	0.00	10.32	0.01
	X_1*X_2	1	0.00	0.00	7.19	0.04
	X_1*X_3	1	0.00	0.00	23.73	0.00
	X_2*X_3	1	0.00	0.00	0.04	0.84
	Error	5	0.00	0.00		
	Lack of fit	3	0.00	0.00		
	Pure error	2	0.00	0.00		
	Total	14	0.02			
FPase (IU/ml/min)	Model	9	0.21	0.02	50.84	0.00
	Linear	3	0.12	0.04	92.17	0.00
	X_1	1	0.10	0.10	224.84	0.00
	X_2	1	0.00	0.00	6.70	0.04
	X_3	1	0.02	0.02	44.96	0.00
	Square	3	0.06	0.02	47.26	0.00
	X_1^2	1	0.01	0.01	25.89	0.00
	X_2^2	1	0.05	0.05	107.31	0.00
	X_3^2	1	0.01	0.01	25.77	0.00
	2 way interaction	3	0.01	0.00	13.10	0.00
	X_1*X_2	1	0.00	0.00	1.92	0.22
	X_1*X_3	1	0.01	0.01	23.98	0.00
	X_2*X_3	1	0.00	0.00	13.41	0.01
	Error	5	0.00	0.00		
	Lack of fit	3	0.00	0.00		
	Pure error	2	0.00	0.00		
	Total	14	0.21			

Fisher's F-test value of 1.51 and p-value of 0.339. The proposed model for FPase production was found to be significant with p-value of 0.010 and F-value of 9.99. Sulphuric acid concentration (X_1) with p-value of 0.002 was the only linear term to influence FPase production significantly. Among square terms, $H_2SO_4$was significant factor as p-value was 0.05. Two way interaction between acid concentration and substrate loading was found to significantly influence results as p-value of 0.030 was lower than 0.05 (Table 4).The model fitness was further checked by coefficient of determination (R^2 value) which showed that the predicted model 94.73% and 73.07% accurately explained the predicted response for FPase and CMCase respectively for sulphuric acid pretreatment. Furthermore, the adjusted R^2 value supported the model with values of 85.25% and 24.61% for FPase and CMCase respectively.

The regression model for CMCase production by thermochemical pretreatment was significant with F-value of 25.72 and p-value of 0.00. The linear terms X_1, X_2, the quadratic terms, X_1^2, X32and interaction terms X_1X_2, X_1X_3 were found to be significant as probability value for all these was less than 0.05. High R^2 value of 97.89% and adjusted R^2value of 94.08% showed that there was a close agreement between experimental values and those predicted by model. A large F-value of 50.84% and the corresponding p-value of 0.00 implies that regression model for FPase production from sulphuric acid pretreatment followed by autoclaving was highly significant. X_1, X_2, X_3, X_1^2, X_2^2, X_2^2, X_1X_3, X_2X_3 were the linear, square and interaction terms to be significant with probability values of less than 0.05 as shown in Table 5. The coefficient of determination (R^2) of the model was 98.92% and adjusted R^2 value was 96.97%, which indicated that the model adequately represented the real relationship between FPase production and the tested variables. Fig. 1 and 2 depicted the contour plots for experimentally observed values of CMCase and FPase versus results predicted by quadratic model from H_2SO_4 treated and H_2SO_4 followed by steam treated banana peduncle waste.

Cellulase production in this study was higher as compared to Sreena *et al*. [6] who reported CMCase activity of 0.133 IU/ml from banana rachis incubated with 1% inoculum of *Bacillus subtilis*at 40°C for 48h. Krishna et al.[15] reported optimal filter paper activity of 2.8 $IUgds^{-1}$ and CMCase activity of 9.6 $IUgds^{-1}$ from banana fruit stalk pretreated by autoclaving at 121°C for 60min. Pretreatment by 2 N H_2SO_4 for soaking period of 6h resulted in FPase and CMCase activity of 1.04 and 2.30 $IUgds^{-1}$ respectively. In a comparative study of cellulase production using rice husk, banana peels, wheat bran, Millet bran, saw dust and coir waste, banana peels gave highest values of FPase and CMCase activities as 12.4IU/ml and 11.3 IU/ml, respectively, with *Aspergillis niger* at 30° C and incubation time of 4 days [19]. Kumar *et al*. [8] reported 100U/ml, 45U/ml and 3.5U/ml of CMCase, FPase and B-glucosidase by *Bacillus* sp. in submerged fermentation using rice husk as substrate. Shafiq et al. [16] reported that solid state fermentation of banana peduncle using *Bacillus subtilis* at 35°C, pH 7, for 72h generated FPase activity of 3.48IU/ml/min. This study indicates successful utilization of banana peduncle waste for the production of highly active cellulases. Sharma et al. [20] employed submerged fermentation of coconut water by *A. niger* to optimize cellulase production. Maximum value of FPase obtained was 0.531 IU/ml for 3 days of incubation period, 0.07% w/v glucose and 8% waste paper. The enzyme produced was then used for hydrolysis of acid and alkali treated mixture of cotton stalk and wheat straw. In one study submerged fermentation of corn husks using *Bacillus cereus* strain resulted to maximum cellulase activity of 0.213 IU/ml for temperature of 30°C, pH 5 and substrate concentration of 1% [21]. Vijayaraghavan et al. [22] used an RSM based experimental design to optimize the simultaneous production of CMCase and protease from solid state fermentation of cow dung with *Bacillus subtilis*. The resulted values were 2.1 and 2.5 fold higher for CMCase (473.01 U/g) and protease (4643 U/g protease) respectively than using non optimized medium, suggesting RSM as an effective methodology to enhance enzyme productions using cost effective substrates.

4. CONCLUSIONS

Results of this study revealed that dilute sulphuric acid pretreatment of banana peduncle effectively improved cellulase production by *Bacillus subtilis* K-18 under submerged fermentation. The produced cellulase enzyme could be industrially exploited with special emphasis on saccharification and bioethanol production.

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