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Original Article

Optimization of dilute sulphuric acid pretreatment of peanut shells through Box- Bhenken design for cellulase production by *Bacillus subtilis* K-18

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Abstract

The present study includes the optimization of dilute sulphuric acid pretreatment of peanut shells as substrate for the production of cellulose enzyme using Box-Bhenken design of Response Surface Methodology. The pretreatment of substrate was conducted through Box- Bhenken design of Response Surface Methodology. Three factors with three levels such as H_2SO_4 conc. (0.6, 0.8, 1%), substrate conc. (5, 10, 15%) and residence time (4, 6, 8h) were employed for pretreatment with and without autoclaving at 121^{o}C for 15 min. The enzyme production was carried out in an Erlenmeyer flask of 250mL capacity using pretreated peanut shells as substrate by *Bacillus subtilis* K-18 in submerged fermentation at 50 °C for 24 h. Results revealed that acid pretreatment was found more effective for cellulase production as compared to thermochemical pretreatment. The maximum CMCase activity was 1.757 1U/ml/min under conditions of 0.6% acid concentration, 10% substrate concentration, and resident time of 4h. The maximum FPase activity was 2.015 1U/ml/min under conditions of 0.8% acid concentration, 10% substrate concentration, and time of 6h. This showed that peanut shells could successfully be used as substrate for cellulase production in submerged fermentation.

Key words: Acid pretreatment, peanut shells, cellulase, *Bacillus* sp. RSM

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INTRODUCTION

Cellulases (E.C 3.2.1.4) are a class of enzyme that is involved in hydrolyzing cellulose of industrial and agricultural wastes containing cellulose in them. Cellulose largely contains long chain polymers of glucose units having β 1-4, linkage and thus makes a crystalline structure (Shallom and Shoham, 2003). The complex of cellulase enzyme consists of three main components that are Endo-ß-glucanase- EC 3.2.1.4 or CMCase- Carboxymethyl cellualase, Exo-ß-glucanase- EC 3.2.1.91 and β-glucosidase- EC 3.2.1.21 (Kaur *et al.*, 2007; Thongekkaew *et al.*, 2008).

Cellulases have a varied variety of industrial applications for example in pulp and paper, laundry, textile, additives of animal feed, fruit juice extraction, and in production of bioethanol (Bhat, 2000). The enzymes have wide potential of saccharification of lignocellulosic biomass into fermentable sugars that can be used for the production of lactic acid, single cell protein, and bioethanol (Maki *et al.*, 2009).

A large study has been made upon the microbial cellulases production for several years. These studies have relatively less emphasis on cellulose production from bacterial sources as compared to fungal sources. These studies have more focus on fungi (Bhat, 2000). The reported bacterial genera for cellulases are *Clostridium, Bacillus*, *Ruminococcus*, *Acetivibrio, Bacteroides*, *Thermomonospora*, *Erwinia*, *Cellulomonas* and *actinomycetes* particularly

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Streptomyces species (Robson and Chambliss, 1989; Nascimento *et al.*, 2009).

Bacteria may work as highly effective sources of important industrial enzymes. Owing to their faster growth rate and high natural diversity, have the ability to produce alkali stable and extremely thermostable enzyme complement. The species of *Bacillus* genus act as dominant bacterial mainstays due to the ability of producing and secreting large amounts of extracellular enzymes (Aa *et al.*, 1994; Mawadza *et al.*, 1996; Schallmey *et al.*, 2004; Singh *et al.*, 2004; Ariffin *et al.*, 2006; Rastogi *et al.*, 2010). It has been reported that the expression of high activities of cellulose degradation belongs to the strains of species *Bacillus subtilis* and *Bacillus sphaericus* (Mawadza *et al.*, 1996; Singh *et al.*, 2004).

Mostly the enzymes having industrial importance are produced in submerged fermentation due to ease in handling and better monitoring. Mostly commercial cellulases are produced by the filamentous fungi *Trichoderma. Reesei* and *Aspergillus niger* in SmF (Cherry and Fidantsef, 2003; Kumar *et al.*, 2004). The production of cellulase in cultures is highly predisposed by several parameters together with the medium pH, cellulosic substrate nature, availability of nutrients and temperature of fermentation. So the formulation of media is of important concern as no ordinary medium composition can meet the optimal growth and prime cellulase production. Mostly the media used are specific for the concerned organism (Tholudur *et al.*, 1999).

Response surface methodology typically known as RSM is a statistical technique enormously used for studying the combined effect of some variables and to find the optimal conditions for the system that is multivariable (Kim *et al.*, 2008). It is also a set of mathematical techniques which are useful in the development, improvement, and optimization of processes wherein a response of interest is simultaneously influenced by some variables and the objective for the best response can be optimized as well (Bas and Boyaci, 2007).

In this study the design of RSM used is Box- Bhenken Design (BBD). BBD is of three variables and used to study the joined effects of acid (H_2SO_4) concentration, substrate concentration and resident time on the production of CMCase and FPase. In case of acid H_2SO_4 followed by steam we apply 121°C temperature and 15 Psi pressure. The main purpose is to optimize the pretreatment conditions of peanut shells for the production of cellulase in submerged fermentation by *Bacillus subtilis* K-18.

MATERIALS AND METHODS

Microbial Strain

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

Pretreatment of peanut shells

Chemical and thermochemical pretreatment were done as described in our earlier reports (Irfan *et al.*, 2010).

Fermentation Methodology

Enzyme production was done in a 250ml Erlenmeyer flask. It contained 25ml of fermentation medium comprising 2% pretreated substrate and 1% yeast extract with initial medium pH of 5. The medium was autoclaved at 121^{o}C, 15 Psi pressure for 15 minutes. After sterilization, the flasks were allowed to cool at room temperature. The medium was inoculated with 2% (v/v) of the vegetative cell culture aseptically and incubated at 50 ^{o}C for 24 h of fermentation period with agitation speed of 120 rpm. After accomplishment of the fermentation period, the fermented broth was filtered through muslin cloth followed by centrifugation (Kokusan H- 1500ER) at 10,000xg and 4 ^{o}C for 10 minutes 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.

Cellulase assay

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

Experimental design

In order to optimize different pretreatment conditions for cellulase production, Box-Bhenken design (BBD) was used for optimization study. The independent variables used were H_2SO_4 concentration (X_1), substrate concentration, (X_2) and residence time (X_3) and their levels are mentioned in Table I. 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:

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

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 Δ*Xi* is the change of *Xi.* The response is calculated from the following equation using Minitab software (version 17).

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

Y is the response, X_1, X_2 and X_3 are the independent variables, $ß_0$ is the intercept, $ß_1$, $ß_2$ 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.

RESULTS AND DISCUSSION

In the concerned study dilute acid pretreatment of peanut shells was executed with three factors that were dilute sulphuric acid concentration X_1, substrate concentration X_2 and residence time X_3 above three levels as shown in Table I.

Table I: Coded and actual levels of the factors for three factor Box-Bhenken design

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

In case of acid with steam we applied 121°C temperature and 15 psi pressure. After pretreatment of peanut shells substrate, the solid remainder or residue was washed up to neutralize. Then it was oven dried and used in submerged fermentation at 50 °C for 24h for production of cellulases by *Bacillus subtilis*.

The experiments were accompanied in accordance with Box-Bhenken design of RSM. The obtained response was calculated according to second order polynomial regression equations (Eq. 3-6). In case of enzyme production, during fermentation process substrate nature plays an effective role which influences the induction of enzyme production (Kang *et al.*, 2004).

The maximum Carboxymethyl cellulase production was found in acid treated peanut shells. The Table III-IV showed the CMCase and FPase activities of both acid and acid with steam treated substrate using BBD. The maximum CMCase activity (1.757 IU/ml/min) was observed by Run#10 under conditions of 0.6% acid concentration, 10% substrate concentration, and resident time of 4hrs.

The maximum FPase activity (2.015 IU/ml/min) was shown by Run No. 1 under conditions of 0.8% acid concentration, 10% substrate concentration, and time of 6hrs. The predicted and observed values for enzyme activity were close enough to show the accuracy of model.

Equations for cellulase from sulphuric acid treated peanut shells

$$\text{CMCase activity (IU/ml/min)} = 2.736 - 2.171 X_1 - 0.0082 X_2 - 0.0663 X_3 + 1.358 X_1^2 - 0.001869 X_2^2 - 0.00858 X_3^2 - 0.0241 X_1{*}X_2 + 0.0638 X_1{*}X_3 + 0.01135 X_2{*}X_3 \quad \textbf{Eq. (3)}$$

$$\text{FPase activity (IU/ml/min)} = 1.712 - 0.74 X_1 + 0.0343 X_2 + 0.171 X_3 - 0.350 X_1^2 - 0.00444 X_2^2 - 0.02638 X_3^2 + 0.0493 X_1{*}X_2 + 0.01225 X_1{*}X_3 - 0.00272 X_2{*}X_3 \quad \textbf{Eq. (4)}$$

Equations for cellulase from sulphuric acid followed by steam treated peanut shells

$$\text{CMCase activity (IU/ml/min)} = 1.14 + 0.46 X_1 + 0.0232 X_2 - 0.377 X_3 - 0.793 X_1^2 + 0.00090 X_2^2 + 0.02837 X_3^2 + 0.0052 X_1{*}X_2 + 0.1526 X_1{*}X_3 - 0.00483 X_2{*}X_3 \quad \textbf{Eq. (5)}$$

$$\text{FPase activity (IU/ml/min)} = 0.699 + 0.72 X_1 - 0.0837 X_2 + 0.080 X_3 - 1.167 X_1^2 + 0.00401 X_2^2 - 0.00518 X_3^2 + 0.0692 X_1{*}X_2 + 0.0648 X_1{*}X_3 - 0.00661 X_2{*}X_3 \quad \textbf{Eq. (6)}$$

Table II: Cellulase production by H_2SO_4 treated peanut shells using Box-Bhenken 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	1.685749	1.685749	-0.00000	2.015928	2.015928	-0.00000
2	1	10	8	1.705062	1.750413	-0.04535	1.913265	1.890062	0.023203
3	1	15	6	1.750585	1.713511	0.037074	1.896673	1.931931	-0.03525
4	1	10	4	1.703683	1.718340	-0.01465	1.845860	1.873470	-0.02761
5	1	5	6	1.753344	1.730410	0.022934	1.860378	1.820713	0.039665
6	0.6	15	6	1.681611	1.704545	-0.02293	1.823046	1.862711	-0.03966
7	0.8	5	4	1.703683	1.711960	-0.00827	1.848971	1.861026	-0.01205
8	0.6	10	8	1.656780	1.642122	0.014657	1.848971	1.821361	0.027610
9	0.8	15	8	1.732652	1.724375	0.008277	1.804380	1.792325	0.012055
10	0.6	10	4	1.757483	1.712132	0.045351	1.977559	2.000762	-0.02320
11	0.6	5	6	1.587805	1.624879	-0.03707	1.983781	1.948523	0.035258
12	0.8	5	8	1.488480	1.466064	0.022417	1.662311	1.725179	-0.06286
13	0.8	15	4	1.493998	1.516415	-0.02241	1.882155	1.819287	0.062868

Table III: Cellulase production by H_2SO_4 followed by steam treated peanut shells using Box-Bhenken 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	0.838736	0.838736	0.000000	0.851377	0.851377	0.000000
2	1	10	8	1.156021	1.087563	0.068458	0.738344	0.802897	-0.06455
3	1	15	6	0.927024	0.946854	-0.01983	1.048407	1.022352	0.026055
4	1	10	4	0.768382	0.814767	-0.04638	0.710345	0.738863	-0.02851
5	1	5	6	0.771141	0.773382	-0.00224	0.828563	0.761547	0.067016
6	0.6	15	6	0.877362	0.875120	0.002242	0.843081	0.910097	-0.06701
7	0.8	5	4	0.818044	0.769416	0.048627	0.759084	0.797583	-0.03849
8	0.6	10	8	0.950476	0.904090	0.046386	0.805749	0.777232	0.028518
9	0.8	15	8	1.034625	1.083252	-0.04862	0.970632	0.932133	0.038499
10	0.6	10	4	0.807008	0.875465	-0.06845	0.881450	0.816897	0.064553
11	0.6	5	6	0.742171	0.722341	0.019830	0.900116	0.926171	-0.02605
12	0.8	5	8	0.950476	1.016692	-0.06621	0.939522	0.941985	-0.00246
13	0.8	15	4	1.095323	1.029107	0.066216	1.054629	1.052166	0.002463

In literature the reported maximum CMCase and FPase activities by *Aspergillus terreus* were 1.023 IU/ml/min and 0.089 1U/ml/min respectively in submerged fermentation for groundnut shells pretreated with 0.25 N HCl (Ashish *et al.*, 2005). That activity was lower as compared to our obtained units. In another literature the maximum CMCase activity was observed to be 0.77 IU/ml/min at 50- 55 °C by thermostable *Bacillus staerothermophillus*-KGKSA40 using untreated ground palm leaf wastes (Bahobil *et al.*, 2014). Recently in literature, the reported maximum values for CMCase activity was 0.641U/ml/min at 0.32 N H_2SO_4, 15 % substrate (Banana peduncle) concentration, and resident time of 8 h. The maximum FPase activity was 0.95 IU/ml/min at 0.4 N H_2SO_4, 15 % substrate concentration, and resident time of 6 hrs. (Anam *et al.*, 2017). This activity was lower than the enzyme activity of this study. All data was analyzed statistically by ANOVA- Analysis of variance for checking the significance of model (Table IV-V). The proposed model was found only significant for CMCase production with model Fisher's F- test value of 5.31 and p- value of 0.04 in acid pretreatment (Table IV).

Table IV: Analysis of Variance of cellulase activity for acid treated peanut shells

	Sources	DF	Adj SS	Adj MS	F value	P value
CMCase activity (IU/ml/min)	Model	9	0.090733	0.010081	5.31	0.040
	Linear	3	0.009244	0.003081	1.62	0.296
	X_1	1	0.006555	0.006555	3.46	0.122
	X_2	1	0.001970	0.001970	1.04	0.355
	X_3	1	0.000720	0.000720	0.38	0.565
	Square	3	0.025056	0.008352	4.40	0.072
	X_1^2	1	0.010894	0.010894	5.74	0.062
	X_2^2	1	0.008063	0.008063	4.25	0.094
	X_3^2	1	0.004348	0.004348	2.29	0.190
	2 Way interaction	3	0.056433	0.018811	9.91	0.015
	X_1*X_2	1	0.002331	0.002331	1.23	0.318
	X_1*X_3	1	0.002605	0.002605	1.37	0.294
	X_2*X_3	1	0.051496	0.051496	27.14	0.003
	Error	5	0.009486	0.001897		
	Lack of fit	3	0.009486	0.003162	*	*
	Pure error	2	0.000000	0.000000		
	Total	14	0.100219			
FPase activity (IU/ml/min)	**Sources**	**DF**	**Adj SS**	**Adj MS**	**F value**	**P value**
	Model	9	0.117965	0.013107	3.99	0.071
	Linear	3	0.015293	0.005098	1.525	0.311
	X_1	1	0.001716	0.001716	0.10	0.502
	X_2	1	0.000323	0.000323	4.03	0.767
	X_3	1	0.013253	0.013253	8.16	0.101
	Square	3	0.080400	0.026800	0.22	0.023
	X_1^2	1	0.000724	0.000724	13.83	0.659
	X_2^2	1	0.045459	0.045459	12.51	0.014
	X_3^2	1	0.041108	0.041108	2.26	0.017
	2 way interaction	3	0.022273	0.007424	2.92	0.199
	X_1*X_2	1	0.009705	0.009705	0.90	0.146
	X_1*X_3	1	0.009603	0.009603		0.386
	X_2*X_3	1	0.002964	0.002964		
	Error	5	0.016430	0.003286		
	Lack of fit	3	0.016430	0.005477	*	*
	Pure error	2	0.000000	0.000000		
	Total	14	0.134395			

The model would be significant if p-value is < 0.05 while it would be insignificant if p-value is >0.10. But in both tables there were some linear terms and interaction terms that were found significant regarding CMCase and FPase activities. The model fitness was further assured by R^2 value- coefficient of determination. The R^2 value for CMCase and FPase activities was 90.93% and 87.78% respectively which revealed that only 10-13% variation was not explained by the model. Figure 1 and 2 illustrated contour plots for CMCase and FPase production from acid (H_2SO_4) pretreated peanut shells by *Bacillus subtilis* K-18 in submerged fermentation. In these plots different color patterns showed different levels of enzyme production by keeping one variable constant and two variables with different levels. These plots indicated that each parameters had significant effect on structure of substrate which ultimately affects cellulase production. Some studies (Brijwani and Vadlani, 2011) suggested that physiochemical properties of substrate had strong correlation with enzyme production. Oke *et al.* (2016) also investigated that pretreated substrate produced better titer of endoglucanase using *Bacillus aerius* S5.2 in submerged fermentation.

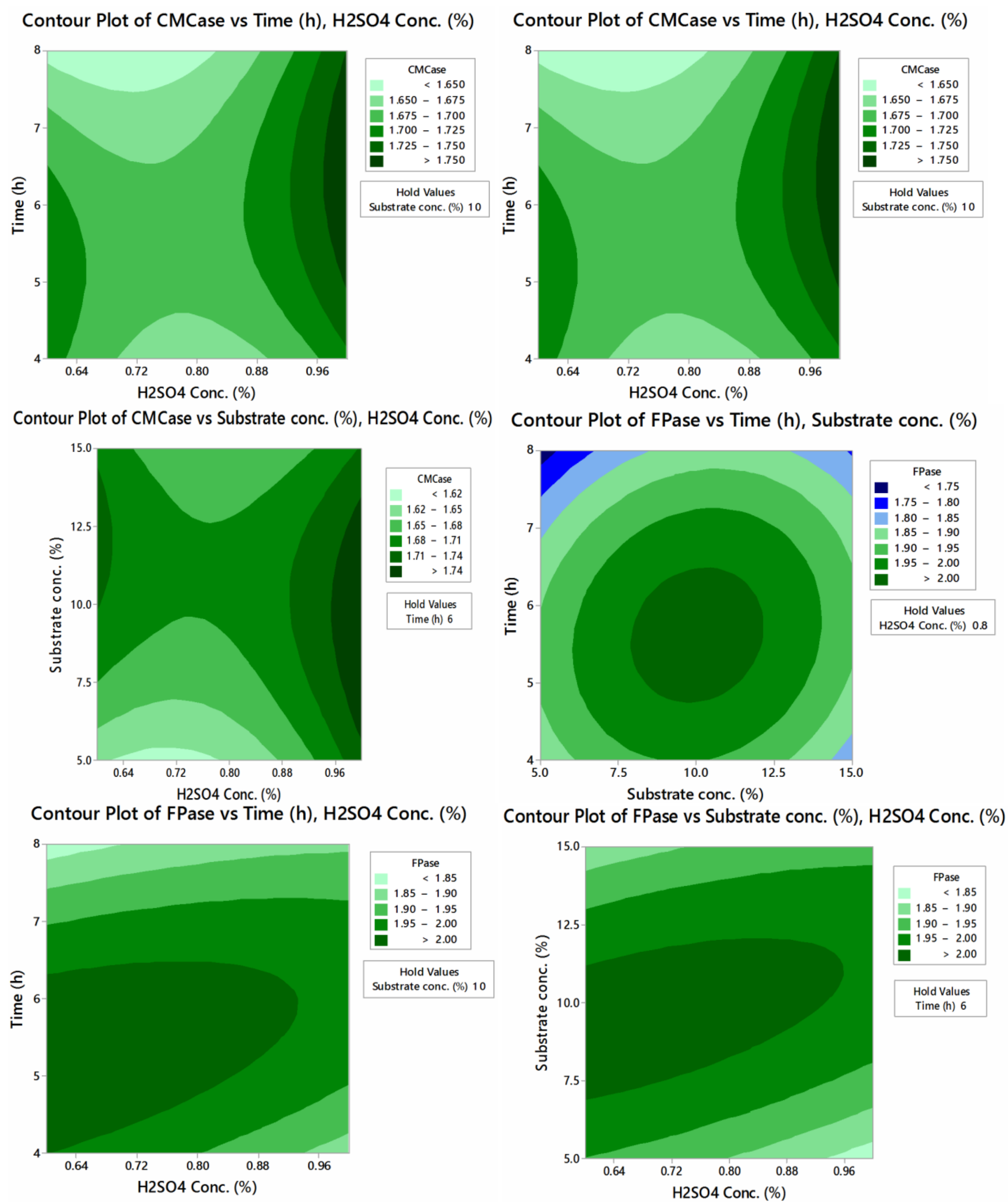

Figure 1. Contour plots for CMCase (IU/ml/min) and FPase (IU/ml/min) production from dilute sulphuric acid treated peanut shellsby *Bacillus subtilis* K-18 in submerged fermentation.

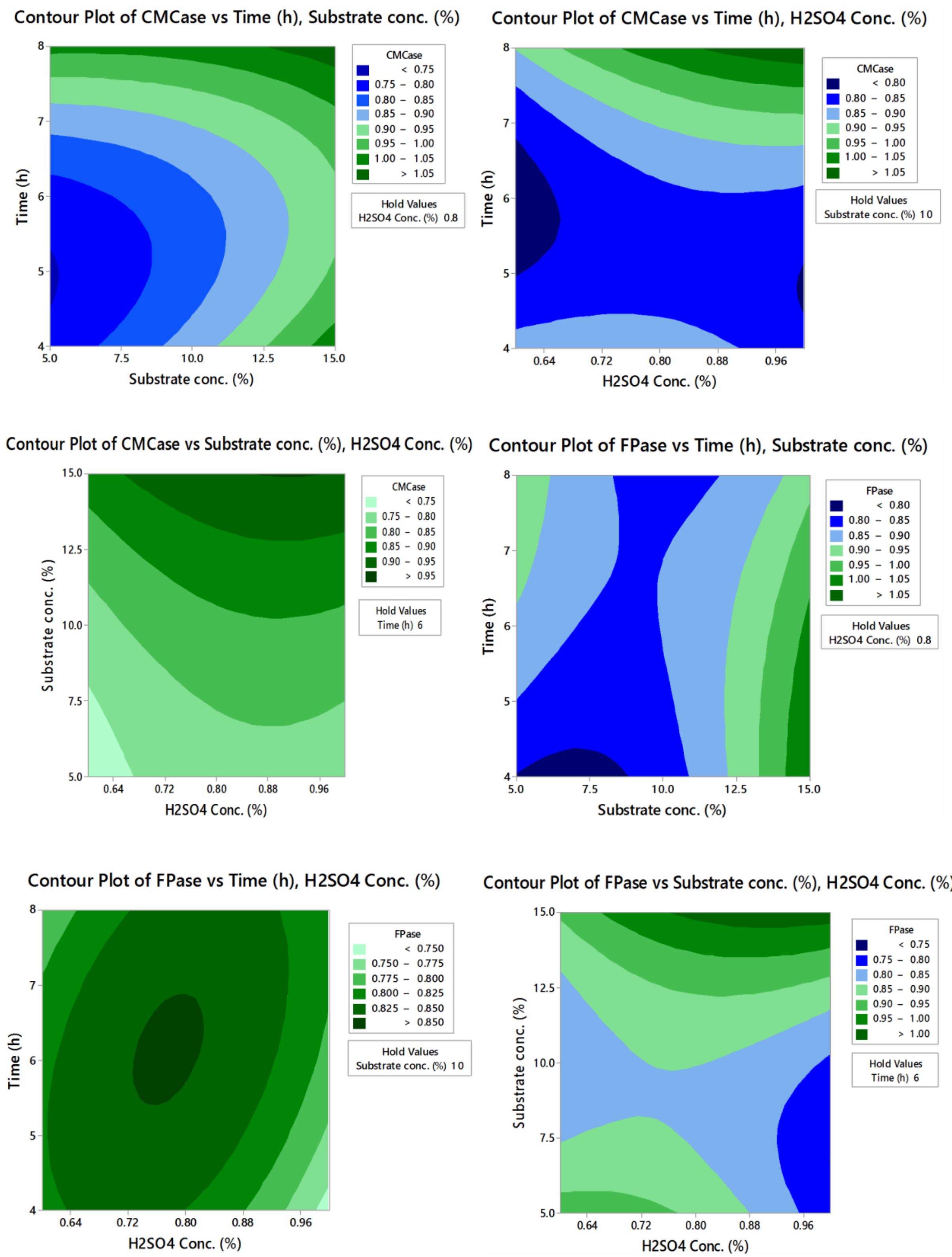

Figure 2. Contour plots for CMCase (IU/ml/min) and FPase (IU/ml/min) production from dilute sulphuric acid followed by steam treated peanut shellsby *Bacillus subtilis* K-18 in submerged fermentation.

Table V: Analysis of Variance for cellulase activity of acid followed by steam treated peanut shells

	Sources	DF	Adj SS	Adj MS	F value	P value
CMCase activity (IU/ml/min)	Model	9	0.185161	0.020573	3.68	0.083
	Linear	3	0.106184	0.035395	6.33	0.037
	X_1	1	0.007537	0.007537	1.35	0.298
	X_2	1	0.053220	0.053220	9.51	0.027
	X_3	1	0.045427	0.045427	8.12	0.036
	Square	3	0.054640	0.018213	3.26	0.118
	X_1^2	1	0.003717	0.003717	0.66	0.452
	X_2^2	1	0.001855	0.001855	0.33	0.590
	X_3^2	1	0.047535	0.047535	8.50	0.033
	2 Way interaction	3	0.024337	0.008112	1.45	0.334
	X_1*X_2	1	0.000107	0.000107	0.02	0.895
	X_1*X_3	1	0.014905	0.014905	2.66	0.164
	X_2*X_3	1	0.009325	0.009325	1.67	0.253
	Error	5	0.027971	0.005594		
	Lack of fit	3	0.027971	0.009324	*	*
	Pure error	2	0.000000	0.000000		
	Total	14	0.213132			
FPase activity (IU/ml/min)	**Sources**	**DF**	**Adj SS**	**Adj MS**	**F value**	**P value**
	Model	9	0.121357	0.013484	2.90	0.127
	Linear	3	0.031615	0.010538	2.26	0.199
	X_1	1	0.001371	0.001371	0.29	0.611
	X_2	1	0.029947	0.029947	6.43	0.052
	X_3	1	0.000297	0.000297	0.06	0.811
	Square	3	0.050406	0.016802	3.61	0.100
	X_1^2	1	0.008040	0.008040	1.73	0.246
	X_2^2	1	0.037167	0.037167	7.98	0.037
	X_3^2	1	0.001588	0.001588	0.34	0.585
	2 way interaction	3	0.039335	0.013112	2.82	0.147
	X_1*X_2	1	0.019165	0.019165	4.12	0.098
	X_1*X_3	1	0.002688	0.002688	0.58	0.482
	X_2*X_3	1	0.017481	0.017481	3.76	0.110
	Error	5	0.023277	0.004655		
	Lack of fit	3	0.023277	0.007759	*	*
	Pure error	2	0.000000	0.000000		
	Total	14	0.144634			

Conclusion

This study concluded that different pretreatment conditions significantly affect cellulase production by *Bacillus subtilis* K-18 in submerged fermentation. So proper pretreatment conditions are pre-requisite for enhanced enzyme production in submerged fermentation which could be helpful in industrial exploitation

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