Full Length Research Article
Advancements in Life Sciences – International Quarterly Journal of Biological Sciences

ARTICLE INFO

Date Received: 06/09/2016;
Date Revised: 21/11/2017;
Date Published Online: 25/11/2017;

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How to Cite:
Anjum A, Irfan M, Tabbsum F, Shakir HA, Qazi JI (2017). Optimization of sulphuric acid pre-treatment of *Acacia* saw dust through box-bhenken design for cellulase production by *B. Subtilis* Adv. Life Sci. 5(1): 19 -24

Keywords:
Saw dust, Pre-treatment, Cellulase, RSM, *Bacillus* sp.

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Optimization of sulphuric acid pre-treatment of *Acacia* saw dust through box-bhenken design for cellulase production by *B. Subtilis*

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Abstract

Background: Cellulases are enzymes which are capable of degrading lignocellulosic biomass. The current study is centred on optimization of dilute sulphuric acid pre-treatment of *Acacia* saw dust for maximizing cellulase production (CMCase and FPase). Hydrolysis or saccharification of lignocellulosic biomass is brought about by cellulases and the sugar thus released can be used for further bioethanol production.

Methods: Box- Bhenken design (BBD) was employed for optimization of pre-treatment conditions for *Acacia* saw dust. Three variables *i.e.* sulphuric acid concentration (0.6%, 0.8% and 1.0% v/v), substrate concentration (5%,10% and15%) and reaction time (4h,6h and 8h) was optimized. The pre-treated saw dust was used in the study as a substrate for producing cellulase enzyme through submerged fermentation by *Bacillus subtilis* (K-18).

Results: An optimum conditions *i.e.* (0.8% H_2SO_4 conc., 15% substrate conc. and 4h of reaction time) yielded highest filter paper activity (1.3617 IU/ml/min) and CMCase activity (0.7783 IU/ml/min). The suggested model was significant as revealed by *F-value*, coefficient of determination (R^2) and *P-value*.

Conclusion: Results concluded that pre-treated substrate (*Acacia* sawdust) significantly increased cellulase production as compared to untreated substrate that could be utilized for further biofuel production.

Introduction

Kikar (*Acacia arabica*) is a lignocellulosic biomass, having about 40-50% cellulose, 18% hemicelluloses and 25% lignin [1]. Cellulose, the major part of plant cell wall has plentiful existence on earth. It is cheap raw material and can be used for bioethanol production [2]. Cellulose is homopolymer, unbranched polysaccharide formed of repeated β-1, 4 glucose units associated by β-1, 4-glycosidic bonds. Hemicellulose is a heteropolymer formed of different sugars such as D-glucose, D-galactose and others. Lignin contains phenylpropane units that are linked in 3- dimensional pattern. Lignin seals cellulose and hemicellulosic component of lignocelluloses and thus impedes enzymes accessibility to cellulose. Therefore, pre-treatment is necessary for delignification and subsequent enzymatic hydrolysis process [3]. Pre-treatment process must meet the characteristics such as it should avoid cellulose and hemicellulose degradation, it should avoid inhibitors formation and overall process cost must be less [3]. Different pre-treatment methods are available like physical method, chemical method (ammonia treatment, steam explosion, ozone treatment). Among different pre-treatment methods, acid and base pre-treatment is commonly employed for treating lignocellulosic biomass. The reason is that they give high reaction rate and the process is less costly than other processes.

Lignocellulosic biomass can be hydrolysed both by chemical or enzymatic method. Enzymatic hydrolysis is carried with fungi and bacteria that split complex cellulose polymers into glucose units by hydrolytic enzymes to utilize it as a carbon source. Complete hydrolysis of cellulose is brought about by synergistic action of three enzymes: i) Endoglucanase or CMCase destroys crystalline structure of celluloses by splitting internal bond. ii) Exoglucanase (also called avicelase or cellobiohydrolase) cleaves reducing and non-reducing ends of oligomers to produce cellobiose that is a disaccharide.iii) β- Glucosidases further cleaves cellobiose to release glucose units [4].

Cellulose digesting enzymes are produced from various bacterial and fungal species. *Aspergillus, Phanerochaete chrysosporium, Penicillium, Trichoderma* and *Fusarium* are some fungal species that possess high cellulase activity [5, 6]. Among bacterial genera, *Bacillus, Clostridium, Cellulomonas, Pseudomonas* possess high cellulase potential [7]. Cellulases have various applications: being used in pulp and paper industry, animal feed, textile, food, brewing, fuel and waste treatment [8].

Response surface methodology (RSM) is a mathematical and statistical mean for checking the fitness value of a variable for optimization of response in multivariable system. In RSM, association between input parameters and response can be studied. It is used whenever several variables are affecting response [9].The current study aims at optimization of dilute sulphuric acid pre-treatment of saw dust for producing cellulase enzyme through *Bacillus subtilis* K-18 through submerged fermentation.

Methods

Microorganism

Bacillus subtilis K-18 was procured from Microbial Biotechnology Laboratory, Department of Zoology, University of the Punjab. The culture was maintained on nutrient agar slants and then it was employed for producing cellulase enzyme through submerged fermentation.

Pre-treatment process of Saw Dust

Acacia saw dust was collected from local market of Ichra Lahore Pakistan. The saw dust was treated with varying concentration of dilute sulphuric acid according to Box-Behnken Design as described in our earlier reports [10]. Briefly, the specific amount of substrate was mixed with 100ml solution of specific concentration of sulphuric acid and left at room temperature for various time period as per design. After the termination of time period, the slurry was filtered and solid biomass was washed up to neutral pH and dried for further processing.

Enzyme Production

CMCase and FPase enzyme was produced in 250ml Erlenmeyer flask. Fermentation medium consists of 2% dilute sulphuric acid pre-treated saw dust and 1% yeast extract with pH 5. The medium was sterilized at 121°C, 15 Psi pressure for about 15 minutes. After sterilization, the flasks were cooled and inoculated with 2% (v/v) vegetative cells of 24hours old *Bacillus subtilis* and then it was incubated at 50°C. The agitation speed was maintained at 120 rpm for 24 h. Afterward fermented broth was filtered and centrifuged (Kokusan H-

1500ER) for 10 minutes at 10,000 x g and 4°C for getting clear filtrate as a crude enzyme source. Three readings were taken for every experiment.

Cellulase Assay

CMCase and FPase activity was observed according to the method described earlier [10]. One unit of CMCase / FPase activity is defined as the extent of enzyme needed to liberate 1µ mole of glucose from substrate per millilitre per minute under standard conditions.

Design of Experiment

For optimizing pre-treatment process for maximizing cellulase production, Box-Bhenken design (BBD) was followed in this study. Three factors were optimized *i.e.* sulphuric acid concentration (A), biomass loading (B) and retention time (C) (Table 1). The design of experiment is appropriate for quadratic response surface and produces second degree polynomial equation. Relationship between coded and actual values were expressed by the following equation (Eq. 1 & 2):

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

Here, xi represents coded value of independent variable.
X_i is encoded value of independent variable.
X_o is encoded value of independent variable at central point
ΔXi is the difference of X_i and Xo.

The response is calculated from the following equation using STATISTICA software (99th edition).

$$Y = \beta 0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC$$

Eq. (2)

Y is the response, A, B and C are the independent variables, ß0 is the intercept, ß1,ß2 and ß3 are linear coefficient, $ß_1^1$, $ß_2^2$ and $ß_3^3$ are square coefficients, ß12, ß13 and ß23 are interaction coefficients.

GDxy = l-dxy/dx+dy-dxy
Where GDxy = Genetic distance between two genotypes,
dxy= Total number of common loci (bands) in two genotypes,
dx= Total number of loci (bands) in genotypes,
dy = Total numbers of loci (bands) in genotypes

Independent variables	Symbols	Coded and actual values		
		-1	0	+1
H_2SO_4 (%)	X_1	0.6	0.8	1
Substrate concentration (%)	X_2	5	10	15
Time (h)	X_3	4	6	8

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

Results

Enzyme production

Rate of production of CMSase and FPase was analysed from second order polynomial equations as shown in Eq.3 and 4. Highest CMCase (0.7783 IU/ml/min) and FPase activity (1.3617 IU/ml/min) was observed at optimized condition *i.e.* 0.8% sulphuric acid concentration, 15g biomass loading and 4h reaction time (Table 2). The predicted and observed values were also found to be very close with values predicted by the model revealing the accuracy of the model (Fig. 1 & 2).

CMCase activity (IU/ml/min) = $-3.15 + 4.5\ X_1 + 0.2592\ X_2 + 0.321\ X_3 - 2.81\ X_1^2 + 0.00786\ X_2^2 - 0.0267\ X_3^2 - 0.0261\ X_1 * X_2 + 0.027\ X_1 * X_3 - 0.01038\ X_2 * X_3$ **Eq. (3)**

FPase activity (IU/ml/min) = $- 4.22 + 12.43\ X_1 + 0.3050\ X_2 - 0.420\ X_3 - 7.03\ X_1^2 - 0.00630\ X_2^2 + 0.0167\ X_3^2 - 0.01889\ X_1 * X_2 + 0.187\ X_1 * X_3 + 0.00101\ X_2 * X_3$ **Eq. (4)**

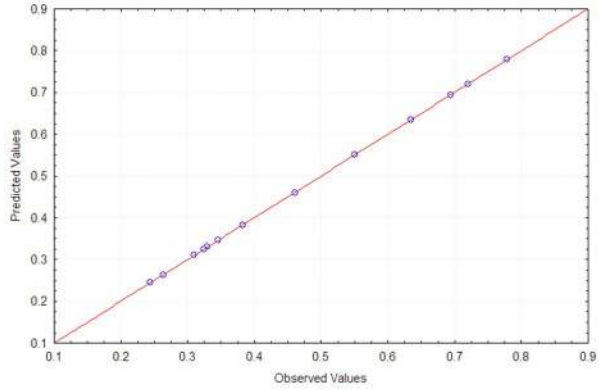

Figure 1: Observed and predicted values of CMCase produced by *Bacillus subtilis* K-18 through submerged fermentation

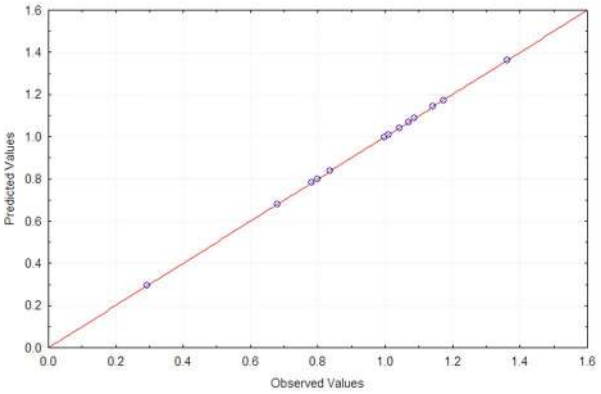

Figure 2: Observed and predicted values of FPase produced by Bacillus subtilis K-18 through submerged fermentation

Analysis of variance

Statistical significance of the data was analysed by ANOVA (Table 3). Proposed model for CMCase and FPase was significant with p-value of 0.017 and 0.005 respectively. Fitness of the model was further analysed by R^2 value which was found to be 93.44% and 96.19% for CMCase and FPase respectively for dilute sulphuric acid pre-treatment. While adjusted R^2 value for CMCase and FPase was found to be 81.64% and 89.34% respectively which further indicates model precision.

Run#	X_1	X_2	X_3	CMCase activity (IU/ml/min)		FPase activity (IU/ml/min)	
				Obs.d	Predicted	Obs.d	Predicted
1	0.8	10	6	0.7204	0.7204	1.173	1.173000
2	1.0	10	8	0.3298	0.3298	0.9966	0.996600
3	1.0	15	6	0.4606	0.4606	0.7981	0.798100
4	1.0	10	4	0.6346	0.6346	1.0866	1.086600
5	1.0	5	6	0.3244	0.3244	0.8365	0.836500
6	0.6	15	6	0.5508	0.5508	1.0099	1.009900
7	0.8	5	4	0.3823	0.3823	1.0425	1.042500
8	0.6	10	8	0.3462	0.3462	0.681	0.681000
9	0.8	15	8	0.2443	0.2443	1.1423	1.142300
10	0.6	10	4	0.6942	0.6942	1.0702	1.070200
11	0.6	5	6	0.3102	0.3102	0.2927	0.292700
12	0.8	5	8	0.2635	0.2635	0.7827	0.782700
13	0.8	15	4	0.7783	0.7783	1.3617	1.361700

Table 2: Cellulase production by sulphuric acid pre-treated saw dust by using Box-Behnken design (BBD).

CMCase (IU/ml/min)	Sources	DF	ASS	AMS	F value	P value
	Model	9	0.533	0.059	7.92	0.017
	Linear	3	0.283	0.094	12.58	0.009
	X_1	1	0.000	0.000	0.02	0.895
	X_2	1	0.071	0.071	9.52	0.027
	X_3	1	0.211	0.211	28.21	0.003
	Square	3	0.204	0.068	9.11	0.018
	X_1^2	1	0.047	0.047	6.23	0.055
	X_2^2	1	0.143	0.143	19.05	0.007
	X_3^2	1	0.042	0.042	5.61	0.064
	2 way interaction	3	0.046	0.015	2.06	0.224
	X_1* X_2	1	0.002	0.002	0.36	0.573
	X_1* X_3	1	0.000	0.000	0.06	0.815
	X_2* X_3	1	0.043	0.043	5.76	0.062
	Error	5	0.037	0.007		
	Lack of fit	3	0.037	0.012	*	*
	Pure error	2	0.000	0.000		
	Total	14	0.571			

Table 3 (A): Analysis of Variance of sulphuric acid treated saw dust.

FPase (IU/ml/min)	Sources	DF	ASS	AMS	F value	P value
	Model	9	0.962	0.107	14.03	0.005
	Linear	3	0.400	0.133	17.50	0.004
	X_1	1	0.053	0.053	6.94	0.046
	X_2	1	0.229	0.229	30.12	0.003
	X_3	1	0.118	0.118	15.43	0.011
	Square	3	0.397	0.132	17.36	0.004
	X_1^2	1	0.292	0.292	38.36	0.002
	X_2^2	1	0.092	0.092	12.04	0.018
	X_3^2	1	0.016	0.016	2.15	0.202
	2 way interaction	3	0.166	0.055	7.24	0.029
	X_1* X_2	1	0.143	0.143	18.75	0.007
	X_1* X_3	1	0.022	0.022	2.93	0.148
	X_2* X_3	1	0.000	0.000	0.05	0.826
	Error	5	0.038	0.008		
	Lack of fit	3	0.038	0.012	*	*
	Pure error	2	0.000	0.000		
	Total	14	0.999			

Table 3 (B): Analysis of Variance of sulphuric acid treated saw dust.

Contour plots

Contour plots for CMCase and FPase produced from dilute sulphuric acid treatment of saw dust is shown in (Fig. 3). These graphs indicated that each parameter had significant effect on cellulase production. The interaction between parameter X1.X2, X1.X3 and X2.X3 had more significant effect as they exhibited circular (dome shape) pattern.

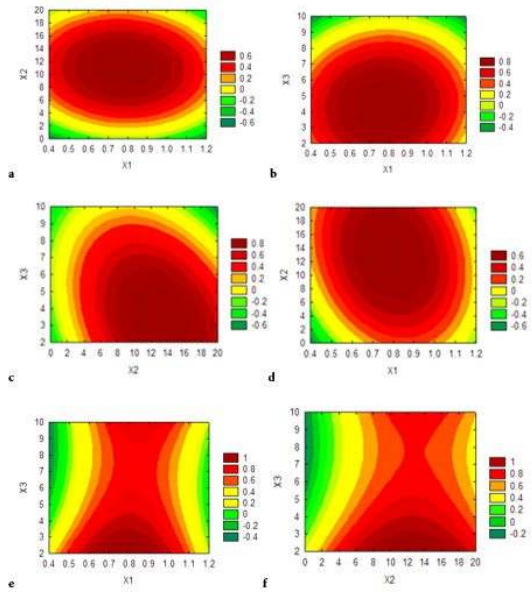

Figure3: Contour plots for CMCase (a-c) and FPase (d-f) production from sulphuric acid treated saw dust by Bacillus subtilis K-18 through submerged fermentation.

Discussion

Lignocellulosic waste is in abundance in our nature like saw dust, bagasse, rice hulls and corn stover. For efficient utilization of lignocellulosic biomass, their complex structure is needed to be disrupted. By pre-treatment, this complex structure disorganizes and further required hydrolysis of cellulose into simple sugars. Hydrolysis is brought about by cellulases which is produced from cellulase producing bacteria and fungi. Different bacterial strains have been explored for producing cellulase enzymes that can withstand high temperature, pH and have high growth rate. *Bacillus subtilis* (K-18) used in this study is selected owing to high stability, high growth rate, easy availability and good cellulase producing capability.

Cellulase enzyme production was higher in the present study as compared to previous reports. In the present study, dilute sulphuric acid pre-treated saw dust produced 0.773IU/ml/min of CMCase and 1.3617 IU/ml/min FPase by *Bacillus subtilis* K-18. In one study, pre-treatment of saw dust with 2N NaOH yielded 0.1813IU/ml of cellulase by *Aspergillus niger* [11]. Another study reported pre-treatment of bagasse, saw dust and corncob with caustic soda produced 0.0743IU/ml cellulase activity from saw dust using *Aspergillus flavus*. The corncob and bagasse produced 0.0502IU/ml and 0.0573 IU/ml of enzyme activity respectively [12]. The result of another study showed 0.037IU/ml of CMCase produced by *Bacillus sp.* C1AC5507 from bagasse in submerged fermentation [13]. In submerged fermentation, using *Acacia Arabica* pod as a substrate maximum cellulase production (0.440 IU/ml/min) was reported by *Bacillus cereus* [14].

In one study, cellulase production was optimized using 4% NaOH pre-treated saw dust and 0.28IU/g FPase and 5.8IU/g CMCase activity was reported by *Aspergillus fumigatus* under optimized conditions [15]. Dilute sulphuric acid treatment of eucalyptus leaves yielded CMCase activity of 1.811 IU/ml/min and FPase activity of 2.255 IU/ml/min using *Bacillus subtilis* K-18 in submerged fermentation [16]. Similarly, peanut shells pre-treated with dilute sulphuric acid gave CMCase units of 1.575 IU/ml/min and FPase units of 2.015 IU/ml/min by the same strain in submerged fermentation [17]. Using same strain and different substrate, the cellulase production become different, for instance, using potato peel as substrate, the CMCase units were 3.50 IU/ml in submerged fermentation [18]. *Bacillus aquimaris* isolated from the gut of *Labeo rhoita* has potential of cellulase production using bagasse as substrate in submerged fermentation [19].

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