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Research Article

Cellulase Production by *Trichoderma viride* in Submerged Fermentation using Response Surface Methodology

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Authors' Contributions
TZ conducted the experimental work and MN supervised it. MI designed the study and analysed the data. MG wrote the first draft while SA and MF revised it. QA, FS and ZY helped in literature review.

Keywords
Cellulase, Submerged fermentation, *Trichoderma* sp., *Bombax ceiba*, RSM

Abstract | The potential of *Trichoderma viride* for cellulase production using seed pods of silk cotton tree as substrate in submerged fermentation by response surface methodology has been investigated. Three variables like substrate concentration, peptone and KH_2PO_4 were optimized at three levels. The optimum carboxymethylcellulase (CMCase) activity and filter paper cellulase (FPase) activity was obtained after 96 hours of incubation with optimum conditions of media containing 5% substrate concentration, 0.05% peptone and KH_2PO_4 concentration of 0.5% with pH 5.0 at incubation temperature of 30°C. The model proposed was found significant. The cellulase produced could be potentially used in industries especially for biofuel production.

Novelty Statement | This is first report on cellulase production using *Bombyx cieba* as substrate in submerged fermentation by *Trichoderma viride*.

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Introduction

Cellulose is a copious as well as ubiquitous natural polymer, being principal constituent of plant cell wall. Glucose units linked via β-1,4-glycosidic bond form polymer of cellulose. Cellulase is a group of enzymes required to break this linkage to liberate these glucose molecules. (Ghazanfar *et al.*, 2019; Nazir *et al.*, 2019). These are inducible enzymes synthesized by microorganisms while propagating on cellulosic matter (Singh *et al.*, 2019). As described earlier that cellulase is a group of enzymes which consists of endoglucanases, exoglucanases or cellobiohydrolases, and β-glucosidases (Jayasekara and Ratnayake, 2019; Srivastava *et al.*, 2018; Thota *et al.*,

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2017). Biotechnologically cellulase is a chief enzyme due to its several applications in industries including beer and wine industry, textile industry, food industry, paper and pulp industry, biofuel production, agriculture and medical applications (Ibrahim *et al*., 2015; Jayasekara and Ratnayake, 2019). Various microorganisms (e.g. fungi and bacteria) have ability to degrade cellulose, some of them produce significant amount of extracellular enzymes. Fungi are mostly preferred over bacteria due to versatile substrate utilization and penetration ability. Fungi like *Trichoderma, Aspergillus*, *Cladosporium, Penicillium*, *Scopulariopsis*, *Stachybotrys*, *Verticillium* and *Chaetomium* have been investigated for cellulase production. Among them *Trichoderma* and *Aspergillus* and are mostly studied, as are used for industrial and agricultural purposes (Chinedu *et al*., 2011; Pradeep *et al*., 2012; Singh *et al*., 2019).

Conventionally there are two types of techniques for enzyme production namely submerged fermentation (SmF) and solid state fermentation (SSF). SmF contains free flowing nutrient media with microorganisms whereas SSF occurs on solids in the absence of free water. SmF is generally used for production of enzymes on large scale as this type of fermentation is easy to control, also offers ease of purification and product recovery. Different fungal strains have been used in SmF for cellulase production (Leghlimi *et al*., 2017; Srivastava *et al*., 2018). Various cellulosic substrates such as sugarcane bagasse, rice husk, wheat bran, coconut coir pith, tea production waste and rice bran etc. have been utilized for production of cellulase enzyme by employing different microbes (Ghazanfar *et al*., 2019). In present study cellulase production by *Trichoderma* strain in SmF was studied using seed pods of silk cotton tree (*Bombax ceiba*) as substrate employing Box Behnken design (BBD) of response surface methodology (RSM).

Materials and Methods

Substrate

Seed pods of silk cotton tree were used as substrate for production of cellulase. The substrate was collected, washed, dried and ground to powder form (2mm) and used for cellulase production in SmF.

Microorganism

Trichoderma viride was obtained from Fermentation Lab, PCSIR Ferozepur road Lahore, for cellulase production. The fungal strain was cultured on potato dextrose agar (PDA) slants and revived biweekly.

Inoculum preparation

Inoculum was prepared by shifting a loop of *Trichoderma viride* (in sterilized conditions) from slant to Erlenmeyer's flasks containing 100 mL of media, keeping flask at 30°C for 96 h in water bath shaker.

Submerged fermentation

Twenty-five milliliter of medium containing with different concentration of substrate (X1), peptone (X2) and KH_2PO_4 (X3) was taken (as per experimental design) in 250 mL Erlenmeyer flask and sterilized at 121°C for 15 minutes. Then 1 mL of inoculum suspension was added aseptically in sterilized media and incubated at 30°C at 120 rpm of agitation speed. Samples were taken after every 24 h for 4 days. Broth was centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant was used as a source of crude cellulase enzyme. All experimentations were run parallel in duplicate.

Enzyme assay

CMCase and FPase activity was measured as described by Irfan *et al*. (2011). Glucose was taken as standard and one enzyme unit is quantity of enzyme required to produce one micromole of glucose per milliliter per minute under standard assay condition.

Experimental design

BBD of RSM was used to carry out enzyme production. The parameters and their levels used are presented in Table 1.

Table 1: Coded and actual level of the three independent variables for cellulase production.

		Code and actual factor level		
Variables	Codes	-1	0	+1
Substrate (%)	X1	1	3	5
Peptone (%)	X2	0.275	0.05	0.5
KH_2PO_4 (%)	X3	0.1	0.3	0.5

Results and Discussion

For production of cellulase through *Trichoderma viride* using RSM, experiments were designed through BBD using three independent variables with their three levels. Significance of different percentage compositions of fermentation media components was evaluated by enzyme assay. The nutritional optimization of fermentation media was investigated to improve the cellulase production in SmF.

At different percentage concentrations of media components experiments were conducted, observed and predicted values for CMCase and FPase were compared and the residual obtained showed the relationship of variables (Tables 2, 3). The maximum observed value for CMCase (5.60 IU/ml) after 96 hours of incubation was closed to the predicted value 5.40 IU/ml that shows its validity at optimized conditions of medium components X1 (5%), X2 (0.05%) and X3 (0.3%). While the maximum observed value for FPase 8.70 IU/ml (at X1 3%, X2 0.3, X3 0.5%) after 96 hours of incubation was closed to the

predicted value 8.387 IU/ml that also shows its validity. The enzyme values were calculated using second order polynomial regression equation (Equations 1 and 2).

$$\text{CMCase (IU/ml)} = -0.951 + 1.175X_1 + 5.78X_2 - 2.81X_3 - 0.0521X_1^2 + 10.70X_2^2 + 0.42X_3^2 - 3.000X_1 \times X_2 + 2.063X_1 \times X_3 - 5.00X_2 \times X_3 \quad \ldots\ldots(1)$$

$$\text{FPase (IU/ml)} = 6.027 + 1.083X_1 - 13.73X_2 + 4.38X_3 - 0.2119X_1^2 + 5.38X_2^2 - 18.75X_3^2 + 1.611X_1 \times X_2 + 1.131X_1 \times X_3 + 16.06X_2 \times X_3 \quad \ldots\ldots(2)$$

Table 2: BBD results showing observed and predicted response for CMCase activity.

Run No.	X1 (%)	X2 (%)	X3 (%)	CM Case (IU/ml)		Residual
				Observed	Predicted	
1	3	0.5	0.1	3.50	3.262	0.2375
2	1	0.05	0.3	0.30	0.075	0.2250
3	5	0.275	0.1	2.50	2.512	-0.0125
4	3	0.275	0.3	2.90	2.666	0.2333
5	1	0.275	0.1	1.50	1.537	-0.0375
6	3	0.275	0.3	2.80	2.666	0.1333
7	1	0.275	0.5	0.80	0.787	0.0125
8	3	0.275	0.3	2.30	2.666	-0.3666
9	5	0.275	0.5	5.10	5.062	0.0375
10	3	0.5	0.5	3.90	3.712	0.1875
11	5	0.05	0.3	5.60	5.400	0.2000
12	1	0.5	0.3	3.10	3.300	-0.2000
13	3	0.05	0.1	2.10	2.287	-0.1875
14	5	0.5	0.3	3.00	3.225	-0.2250
15	3	0.05	0.5	3.40	3.637	-0.2375

Table 3: BBD results showing observed and predicted response for FPase activity.

Run No.	X1 (%)	X2 (%)	X3 (%)	FPase (IU/ml)		Residual
				Observed	Predicted	
1	3	0.50	0.1	5.60	5.661	-0.061
2	1	0.05	0.3	6.20	6.512	-0.312
3	5	0.275	0.1	6.00	6.251	-0.251
4	3	0.275	0.3	7.50	7.300	0.200
5	1	0.275	0.1	4.90	4.778	0.121
6	3	0.275	0.3	7.20	7.300	-0.100
7	1	0.275	0.5	4.50	4.248	0.251
8	3	0.275	0.3	7.20	7.300	-0.100
9	5	0.275	0.5	7.41	7.531	-0.121
10	3	0.50	0.5	7.29	7.481	-0.191
11	5	0.05	0.3	7.50	7.440	0.060
12	1	0.50	0.3	4.50	4.560	-0.060
13	3	0.05	0.1	7.80	7.608	0.191
14	5	0.50	0.3	8.70	8.387	0.312
15	3	0.05	0.5	6.60	6.538	0.061

All the data collected was statistically analysed and the analysis of variance showed that the proposed model was significant for both CMCase and FPase having F values of 26.59 and 24.56 with P- value of 0.001 respectively (Table 4). R^2 value for CMCase and FPase was 97.95% and 97.79% respectively. This showed the accuracy of the model (Figure 1). The adjusted R^2 (Adj) values were 94.27% and 93.81% for CMCase and FPase respectively.

Figure 2 demonstrated contour plots for CMCase and FPase production from *T. viride* in SmF. Different color patterns in these plots reflect different enzyme values at various concentrations. Results shows that concentration of each variable had significant effects on cellulase production in SmF at 30°C. This shows that cellulase production was mainly dependent on various nutritional components.

Desirability chart showed that if the value of substrate conc. (var1) is 3%, peptone (var2) is 0.275% and KH_2PO_4 (var3) is 0.3% then the maximum CMCase production would be 5.6846 and minimum value would be 0.02201 and FPase production would be 9.1317 and minimum value would be 4.0550 IU/ml. This was confirmed through repeated experiments as shown in Figure 3 and 4.

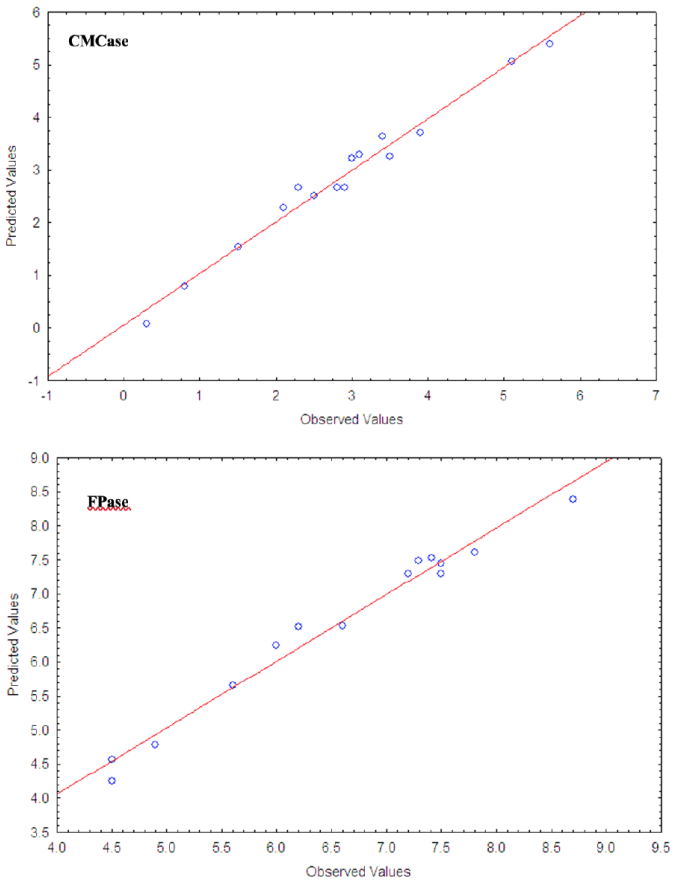

Figure 1: Predicted vs observed values for CMCase and FPase.

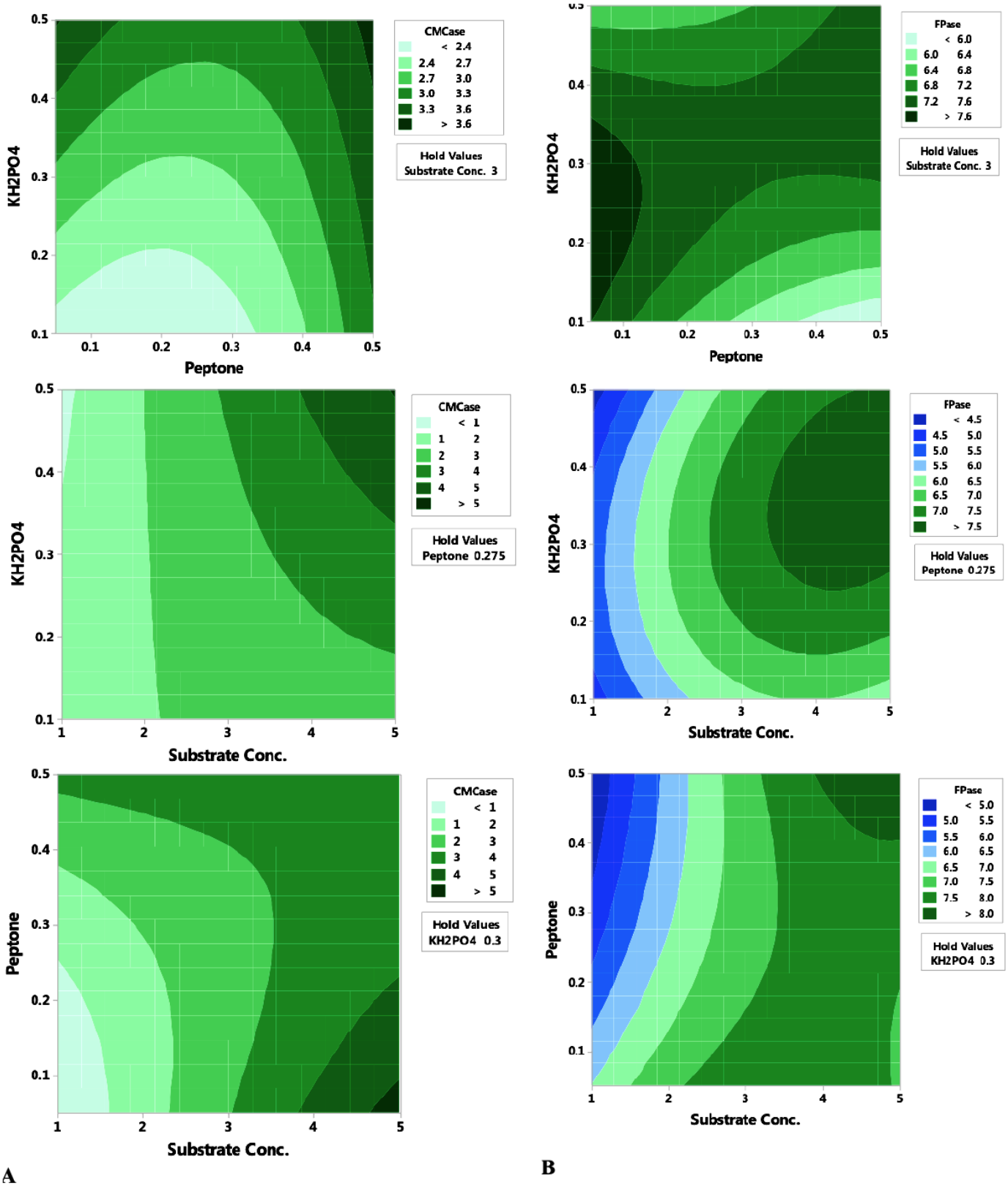

Figure 2: Contour plots for CMCase (A) and FPase (B) production from *Trichoderma viride* under SmF.

Bhoosreddy (2014) worked on *Trichoderma viride* and *Aspergillus niger* using corncob as substrate and revealed maximum cellulose production after 96-120 h of incubation. Khokhar *et al.* (2014) obtained maximum cellulase production from wheat straw after 96 h of incubation by *Trichoderma reesei*. Most important source for effective cellulase production is the carbon source. *Penicillium* sp. produced cellulase in SSF using leaves of *Agave salmiana* as carbon source (Silva-Mendoza *et al.* 2020). Neagu *et al.* (2012) employed wheat bran or sawdust as substrate for cellulase production. Peptone as nitrogen source resulted the maximum cellulase production as stated by Gautam *et al.* (2010). Highest CMCase activity (1.18 U/ml) was observed at 5.0 g L^{-1} lactose concentration (El-

Hadi *et al*., 2014). Balamurugan and Shankar (2018) used sorghum waste as carbon source for cellulase production and observed maximum enzyme titer (13.5 U/mL) after 4 days of incubation in SmF. Mrudula and Murugammal (2011) used *Aspergillus niger* for cellulase production in SmF and observed maximum yield after 96 h. Malik *et al*. (2010) reported $(NH_4)_2SO_4$ as nitrogen source for cellulase production. In nutritional optimization cellulase production is mainly dependent on substrate, carbon and nitrogen source utilizing in fermentation media.

Table 4: Analysis of variance for CMCase and FPase.

	Source	DF	SS	MS	F-Value	P-Value
CM-Case	Model	9	27.4832	3.0537	26.59	0.001
	Linear	3	15.9525	5.3175	46.31	0.000
	X_1	1	13.7812	13.7812	120.01	0.000
	X_2	1	0.5513	0.5513	4.80	0.080
	X_3	1	1.6200	1.6200	14.11	0.013
	Square	3	1.3157	0.4386	3.82	0.092
	X_1^2	1	0.1603	0.1603	1.40	0.291
	X_2^2	1	1.0833	1.0833	9.43	0.028
	X_3^2	1	0.0010	0.0010	0.01	0.928
	2-Way interaction	3	10.215	3.4050	29.65	0.001
	$X_1 \times X_2$	1	7.2900	7.2900	63.48	0.001
	$X_1 \times X_3$	1	2.7225	2.7225	23.71	0.005
	$X_2 \times X_3$	1	0.2025	0.2025	1.76	0.242
	Error	5	0.5742	0.1148		
	Lack-of-fit	3	0.3675	0.1225	1.19	0.488
	Pure error	2	0.2067	0.1033		
	Total	14	28.057			
FPase	Model	9	22.0527	2.4503	24.56	0.001
	Linear	3	12.0913	4.0304	40.40	0.001
	X_1	1	11.3050	11.3050	113.32	0.000
	X_2	1	0.5050	0.5050	5.06	0.074
	X_3	1	0.2812	0.2812	2.82	0.154
	Square	3	4.9519	1.6506	16.55	0.005
	X_1^2	1	2.6520	2.6520	26.58	0.004
	X_2^2	1	0.2742	0.2742	2.75	0.158
	X_3^2	1	2.0769	2.0769	20.82	0.006
	2-Way Interaction	3	5.0096	1.6699	16.74	0.005
	$X_1 \times X_2$	1	2.1025	2.1025	21.07	0.006
	$X_1 \times X_3$	1	0.8190	0.8190	8.21	0.035
	$X_2 \times X_3$	1	2.0880	2.0880	20.93	0.006
	Error	5	0.4988	0.0998		
	Lack-of-fit	3	0.4388	0.1463	4.88	0.175
	Pure error	2	0.0600	0.0300		
	Total	14	22.5515			

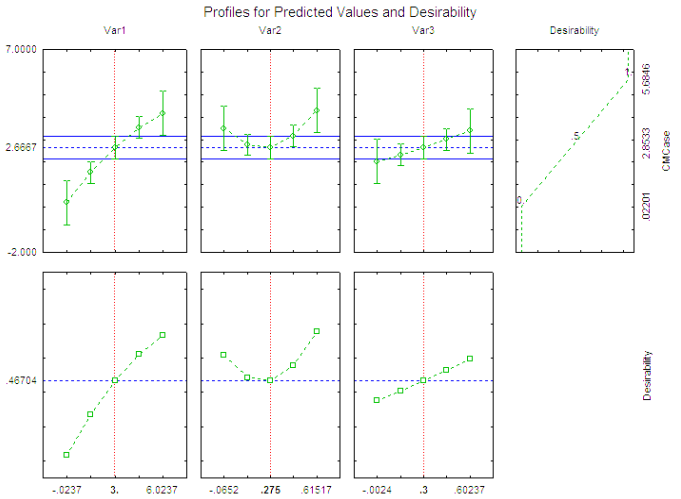

Figure 3: Desirability chart for CMCase production. Var1; substrate concentration, var2; Peptone, var3; KH_2PO_4.

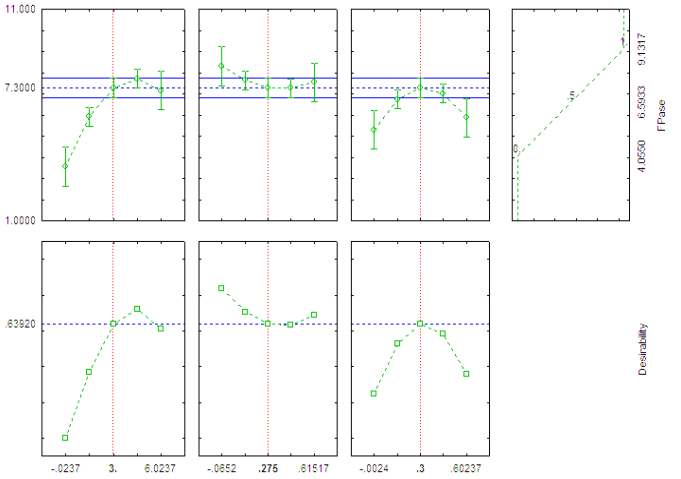

Figure 4: Desirability chart for FPase production. Var1; substrate concentration, var2; Peptone, var3; KH_2PO_4.

Conclusions and Recommendations

Results of this study concluded that *Trichoderma viride* had potential of utilizing seed pods of silk cotton tree as a surce of carbon for cellulase production in submerged fermentation. The optimized cellulase production was found at 5% substrate concentration, 0.05% peptone concentration and 0.5% KH_2PO_4 concentration with pH 5.0 at incubation temperature of 30°C. This crude enzyme production by cheaper process of SmF represents the good alternative for industrial applications.

Conflict of interest

The authors have declared no conflict of interest.

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