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Research Article

Utilization of *Bombyx ceiba* Seed Pods: A Novel Substrate for Cellulase Production through Solid State Fermentation using Response Surface Methodology

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SN conducted the research. MI conceived the sstudy design. SN and MI drafted the manuscript. MN analysed the data whilw MAS interpreted it. MK and SA reviewed the literature. QS reviewed the article.

Keywords
Cellulase, *Trichoderma* sp., Solid state fermentation, *Bombyx ceiba*, Response surface methodology

Abstract | In this work, *Trichoderma viride* was used for cellulase production in solid state fermentation (SSF) using seed pods of *Bombyx ceiba* as substrate. Three parameters were used such as inoculum size (1, 5.5 and 10%), medium volume (1, 3 and 5ml) and incubation period (3, 4, 5 days) to observed the maximum activity of cellulase using Box-Bhenken design of response surface methodology. Maximum activity of CMCase (0.700 IU/gds/min) and FPase (0.260 IU/gds/min) was observed on 4th day with inoculum size of 1% and medium volume of 1ml (for 5g substrate). The proposed model was highly significant as depicted by ANOVA. The results recommended that seed pods of *Bombyx ceiba* play important role in cellulase production, which is useful for the food industry, textile industry and ethanol production.

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Introduction

Cell wall of plant is composed of cellulose which is the chief component. When its composite structure is splited, it gives such components that are used by microorganisms. Due to its crystalline structure, it is difficult to degrade (Bras *et al*., 2011). Hydrolytic enzymes are peculiar for the acquisition of many other compounds and cellulase is one of the enzymes which digest the cellulose. There are three types of cellulase enzyme, which break the components of cellulose (Ozioko *et al*., 2013). Endo-1, 4-β-glucanase simply called endoglucanase, split randomly building block β-1, 4-glucosidic concatenation inside the long chains of glucose. The endoglucanases are frequently anatomized by glutinousness deductions in carboxymethyl

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cellulose (CMC) solution. Exo-1, 4-β-glucanase simply called exoglucanases, divide the open ends of cellulose element to free glucose.1, 4-β-Glucosidase catalyzed the dissolved pulp and polymers of glucose along degree of polymerization (DP) till six to prepare sugar molecules in the fluid stage. Cellulase can be obtained from bacteria, fungi and other termites. Fungi are proven to be best source for cellulase production. Contagious species from the sort *Trichoderma* are rendered by quick development and plenteous creation of conidial spores (Błaszczyk *et al*., 2014).

Organisms in the family *Trichoderma* have been known since from 1920s for the capacity to go about as biocontrol agents in contrast to plant microbe. Up to this point, the important components for control have been thought to be those basically following up on the pathogens and included mycoparasitism, antibiosis, and rivalry for assets and space. Late advances show that the impacts of fungus on plants, including incited systemic or confined resistance, are likewise critical. The cellulase production is obtained using SSF. The SSF is very significant because it is less costly. It is characterized as the maturation prepare in which microbes develop on strong substantial devoid of the absence of clear fluid. Idea of utilizing strong base material is most likely most seasoned strategy utilized for the production of enzymes to work for welfare of mankind (Bhargav *et al*., 2008).

Response surface methodology (RSM) was introduced in 1950. The objective to use response surface methodology is to enhance yield which is impacted by a few autonomous factors. Main purpose of RSM is to optimize process parameters thus reducing the production cost (Jiménez-Contreras *et al*., 2008). RSM is more efficient process of optimization as compared to other methods as it requires less experiment for calculation of numerous variables and their interaction (Kavitha *et al*., 2016). Cellulase has many applications like in food industry, paper and pulp industries, biofuel industry, and textile industry. The present study investigated the potential utilization of seed pods of *Bombyx ceiba* as carbon source in solid state fermentation by *Trichoderma viride* for cellulase production through response surface methodology.

Materials and Methods

Microorganism

Trichoderma viride was obtained from Fermentation Biotechnology Laboratory, PCSIR Labs. Complex Ferozepur road Lahore, and was used for the production of cellulase. The strain was maintained on PDA (potato dextrose agar) slants and revived biweekly.

Substrate

The substrate used in this study was seed pods of *Bombyx ceiba*. The substrate was washed and sun dried followed by oven drying at 70°C till constant weight. After that it was ground to fine powder (2mm) and sealed in airtight container for further processing.

Inoculum preparation

Inoculum was prepared by adding sterilized distilled water into the 5-days old slant of *Trichoderma viride*. With inoculating loop, the spores were mixed and one ml (2 x 10^8) of spore suspension was used as an inoculum.

Fermentation techniques.

Trichoderm viride strain was used to produce cellulase in SSF. Five gram powder of seed pods of *Bombyx ceiba* was added in media (g/l, $(NH_4)_2SO_4$ 10. $CaCl_2$ 0.5, $MgSO_4$ 0.5, KH_2PO_4 4) with ratio of 1, 3 and 5ml. After routine sterilization and inoculation, incubation was carried out at 30°C for 3, 4 and 5 days.

Extraction of enzyme

After incubation, samples were taken and citrate buffer was added with the ratio of 1:10 (solid: liquid ratio). In 2g of substrate, 20ml of citrate buffer was added. Samples were placed on shaker for 2 h with shaking speed of 150rpm. After shaking it was filtered and residue was discard and then it was centrifuged and further analysis purpose (Irfan *et al*., 2010).

Enzyme assay

CMCase and FPase activity was measured as described by Irfan *et al*. (2011). Glucose was used 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

Box-Bhenken design of RSM was used to carry out enzyme production. The variables and their levels used were presented in Table 1.

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

Independent Variable	Code	Code and actual factor level		
		-1	0	+ 1
Volume of media (ml)	A	1	3	5
Inoculum size (ml)	B	1	5.5	10
Incubation period (days)	C	3	4	5

Results and Discussion

Cellulase was produced using seed pods of *Bombyx ceiba* through SSF using BBD. Proximate analysis of substrate indicated that seed pods comprised of 34.0% cellulose, 25.6% hemicellulose and 23.7% lignin. Fifteen experiments were run with different variables i.e. inoculum

Table 2: Results of BBD showing observed and predicted response for cellulase activity.

Run #	A	B	C	CMCase activity (IU/gds/min)			FPase activity (IU/gds/min)		
				Observed	Predicted	Residual	Observed	Predicted	Residual
1	5.5	3	4	0.100	0.090	0.0100	0.180	0.140	0.0000
2	1	3	5	0.090	0.148	-0.058	0.160	0.160	-0.005
3	5.5	5	5	0.190	0.227	-0.037	0.150	0.135	0.0100
4	5.5	5	3	0.070	0.080	-0.010	0.160	0.055	-0.000
5	10	1	4	0.040	0.136	-0.096	0.140	0.135	0.0050
6	10	3	5	0.030	-0.05	0.0862	0.050	0.055	-0.005
7	5.5	3	4	0.060	0.090	-0.030	0.180	0.180	0.0000
8	1	5	4	0.120	0.023	0.0962	0.250	0.255	-0.005
9	10	3	3	0.050	-0.008	0.0587	0.090	0.085	0.0050
10	1	3	3	0.070	0.156	-0.086	0.185	0.185	0.0050
11	5.5	3	4	0.110	0.090	0.0200	0.180	0.180	0.0000
12	1	1	4	0.700	0.651	0.0487	0.260	0.255	0.0050
13	10	5	4	0.120	0.168	-0.048	0.160	0.165	-0.005
14	5.5	1	3	0.590	0.552	0.0375	0.140	0.150	-0.010
15	5.5	1	5	0.360	0.350	0.0100	0.120	0.120	0.0000

size (A), solid to liquid ratio (B) and time period (C). Highest value for CMCase was 0.19U/ml when inoculum size was 5.5%, solid to liquid ratio 5ml and time period was 5 days (Table 2). Highest value for FPase was 0.250 when inoculum size was 1%, solid to liquid ratio of 5ml and time period of 4 days. Result for CMCase and FPase was calculated by polynomial equation of regression as shown in Equation 1 and 2. There was very close resemblance between observed and predicted values (Fig. 1) indicating the accuracy of the model.

$$\text{CMCase ((IU/gds)/min)} = 1.862 - 0.0429\,A - 0.648\,B - 0.243\,C - 0.00216\,A2 + 0.0497\,B2 + 0.0137\,C2 + 0.01833\,A \times B - 0.0022\,A \times C + 0.0438\,B \times C \quad \ldots.(1)$$

$$\text{FPase (IU/gds/min)} = -0.5909 - 0.01262\,A - 0.0377\,B + 0.4568\,C + 0.000062\,A2 + 0.00531\,B2 - 0.05875\,C2 + 0.000833\,A \times B - 0.000556\,A \times C + 0.00125\,B \times C \quad \ldots.(2)$$

Statistical significance of data was evaluated by applying ANOVA for CMCase and FPase (Table 3). The proposed model for FPase production was found highly significant with P value of 0.000 having F-value of 54.57 while for CMCase the P and F-value was 0.03 and 6.13 respectively. The correlation (R^2 value) in model that the proposed model exhibited 98.99% and 91.69% accurately for FPase and CMCase, respectively. Coded coefficients for CMCase and FPase are shown in Table 4. Significant response for coefficient was found to be mainly dependent upon T value and P value. If P value is lesser then 0.05 and T value more than 0.05, then the model will be significant.

Figure 2 demonstrated contour plots for CMCase and FPase production from *T. viride* in SSF. These plots revealed that each variable had significant impact on enzyme production. Different color patterns in these plots reflect different enzyme values at various concentrations.

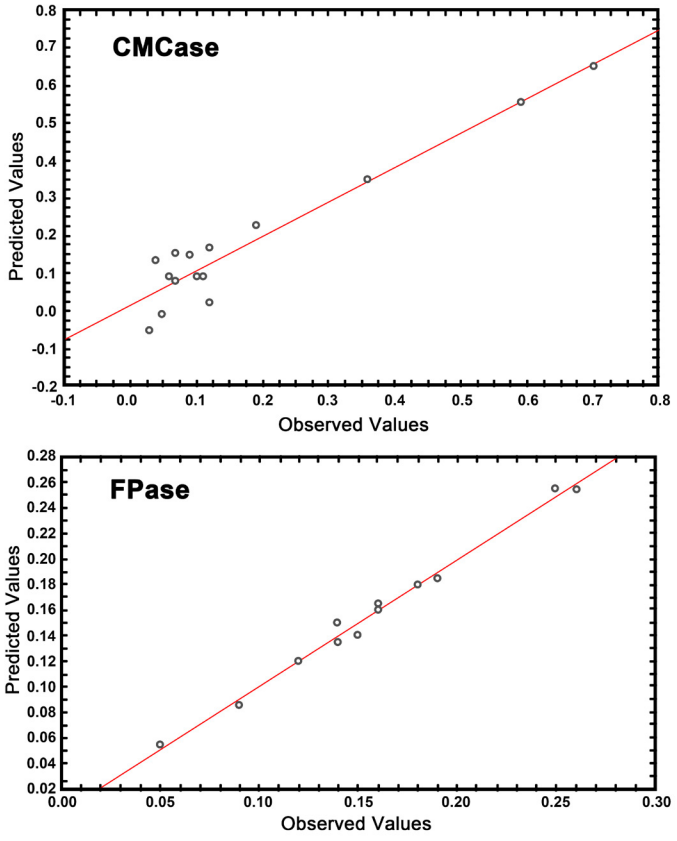

Figure 1: Observed and predicted values of CMCase and FPase production.

Desirability chart for CMCase and FPase are shown in Figure 3. These charts revealed that the enzyme values predicted in this chart was verified by repeating various experiments.

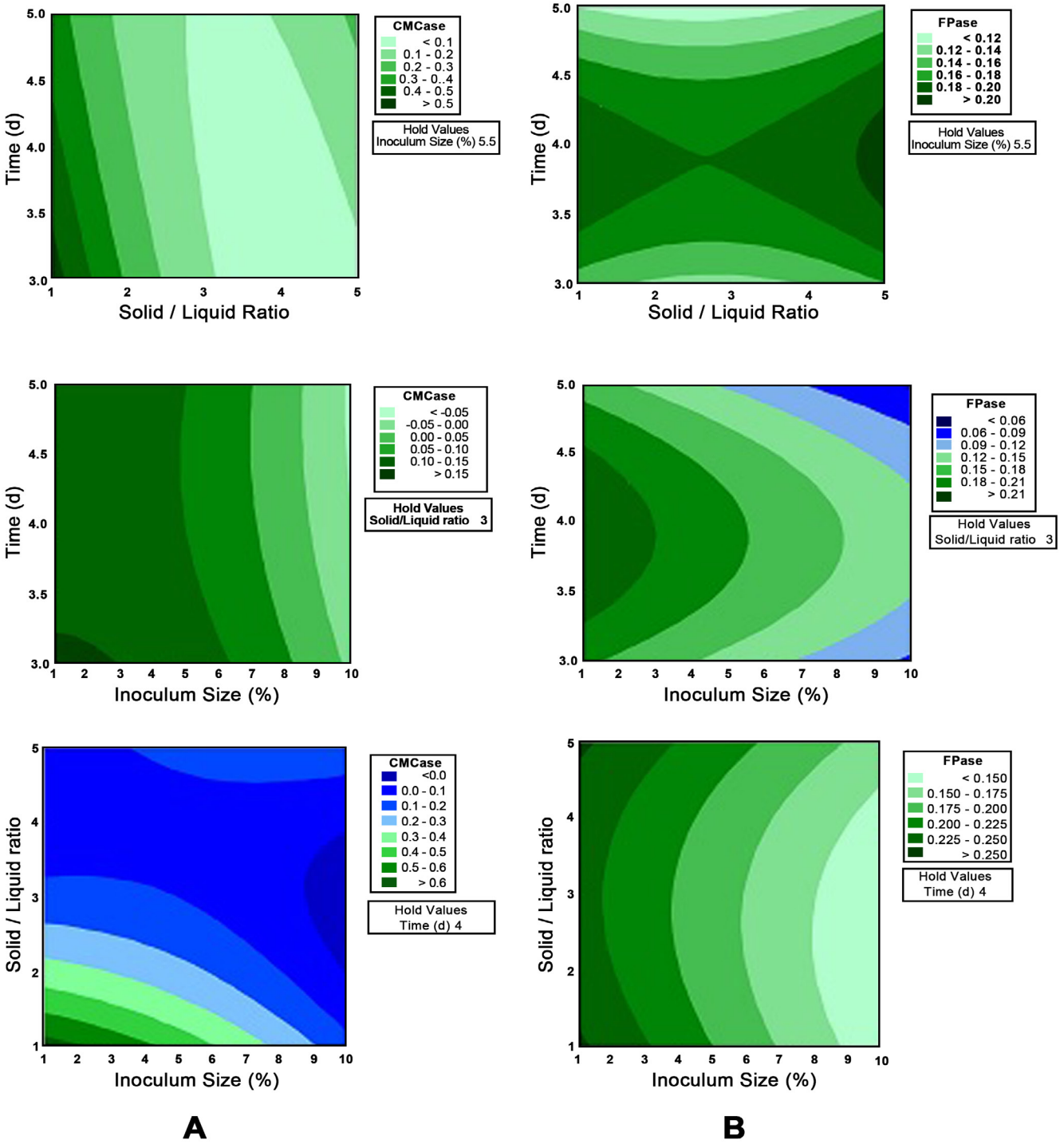

Figure 2: Contour plots for CMCase (A) and FPase (B) production from *Trichoderma viride* under solid state fermentation.

In this study inoculum size has been tested for maximum cellulase production by *T. viride* under SSF when inoculum size ranges between 5-15%. Inoculum size of 2.0×10^6 for *Purpureocillium lilacinum* has been reported for maximum cellulase production in submerged fermentation (Srilakshmi et al., 2017). The highest cellulase yield was obtained by *T. viride* FBL1 in SSF using 5 % inoculum size (Irfan et al., 2010).

Impact of time period on cellulase production was checked by inoculating the substrate with fungi and incubated for 3, 4 and 5 days of fermentation period. In our study, 4th day of fermentation period was found best for maximum cellulase production in solid state fermentation. Similar findings of 4th day was also reported for cellulase production by new mutant strain of *T. reesei* in SSF (Darabzadeh et al., 2018). Yadav et al. (2016) isolated fungi from soil which have capability to produce maximum cellulase after 6th day of fermentation period. *Purpureocillium lilacinum* yielded highest cellulase production after 7 days

of fermentation period (Srilakshmi et al., 2017). Pachauri et al. (2017) reported maximum cellulase production using 5g pretreated sugarcane bagasse from *T. koningii* after 10 days of incubation time. *T. stipitatus* MTCC 12687 had capability to secrete maximum cellulases on 5th day of incubation period (Bharti et al., 2018). Irfan et al. (2016) described optimum fermentation period of 3rd day for endoglucanase production by *T. harzianium* in submerged fermentation. Moosavi-Nasab and Majdi-Nasab (2008) worked on cellulase production by *T. reesei* using sugar beet pulp as substrate and maximum activity (0.46 IU/ml) was obtained on 4-6 days. Singhania et al. (2006) obtained maximum cellulase activity of 154.58U/gds from *T. reesei* NRRL 11460 after 72h in SSF strategy.

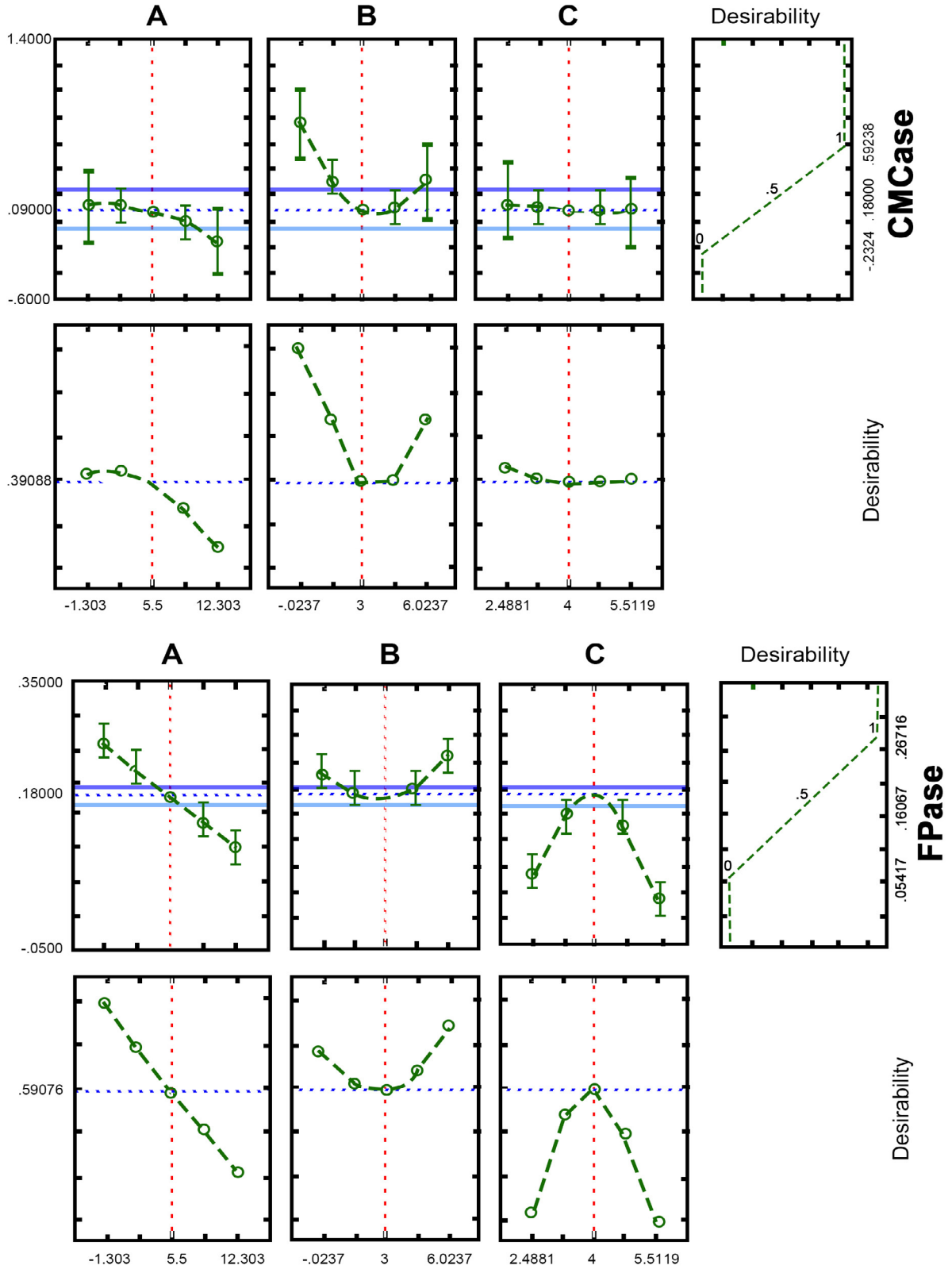

Figure 3: Desirability chart for CMCase and FPase production.

Table 3: Analysis of variance for CMCase and FPase.

	Sources	DF	Adj SS	Adj MS	F value	P value
CMCase (IU/gds/min)	Model	9	0.545725	0.060636	6.13	0.030
	Linear	3	0.246975	0.082325	8.32	0.022
	A	1	0.068450	0.068450	6.92	0.047
	B	1	0.177013	0.177013	17.89	0.008
	C	1	0.001513	0.177013	0.15	0.712
	Square	3	0.158825	0.052942	5.35	0.051
	A^2	1	0.007067	0.007067	0.71	0.437
	B^2	1	0.145852	0.145852	14.74	0.012
	C^2	1	0.000698	0.000698	0.07	0.801
	2 Way interaction	3	0.139925	0.046642	4.71	0.064
	A*B	1	0.108900	0.108900	11.01	0.021
	A*C	1	0.000400	0.000400	0.04	0.849
	B*C	1	0.030625	0.030625	3.09	0.139
	Error	5	0.049475	0.009895	22.89	0.042
	Lack of fit	3	0.048075	0.016025		
	Pure error	2	0.001400	0.000700		
	Total	14	0.595200			
FPase (IU/gds/min)	Model	9	0.039293	0.004366	54.57	0.000
	Linear	3	0.023750	0.007917	98.63	0.000
	A	1	0.022050	0.022050	275.63	0.000
	B	1	0.000450	0.000450	5.63	0.064
	C	1	0.001250	0.001250	15.63	0.011
	Square	3	0.015268	0.005089	63.62	0.000
	A^2	1	0.000006	0.000006	0.07	0.799
	B^2	1	0.001667	0.001667	20.84	0.006
	C^2	1	0.012744	0.012744	159.30	0.000
	2 Way interaction	3	0.000275	0.000092	1.15	0.416
	A*B	1	30.00027	0.000225	2.81	0.154
	A*C	1	0.000025	0.000025	0.31	0.600
	B*C	1	0.000025	0.000025	0.31	0.600
	Error	5	0.000400	0.00080	*	*
	Lack of fit	3	0.000400	0.000133		
	Pure error	2	0.000000	0.000000		
	Total	14	0.039693			

Table 4: Coded coefficients for CMCase and FPase production.

	Terms	Effect	Coef	SE Coef	T value	P value	VIF
CMCase (IU/gds/min)	Constant		0.900	0.0574	1.57	0.178	
	A(%)	-0.1850	-0.0925	0.0352	-2.63	0.047	1.00
	B	-0.2975	-0.1487	0.0352	-4.23	0.008	1.00
	C	-0.0275	-0.0137	0.0352	-0.39	0.712	1.00
	$A^2_{(\%)}$	-0.0875	-0.0437	0.0518	-0.85	0.437	1.01
	B^2	0.3975	0.1987	0.0518	3.84	0.012	1.01
	C^2	0.0275	0.0137	0.0518	0.27	0.801	1.01
	A*B	0.3300	0.1650	0.0497	3.32	0.021	1.00
	A*C	-0.0200	-0.0100	0.0497	-0.20	0.849	1.00
	B*C	0.1750	0.0875	0.0497	1.76	0.139	1.00
FPase (IU/gds/min)	Constant		0.18000	0.00516	34.86	0.000	
	A(%)	-0.10500	-0.05250	0.00316	-16.60	0.000	1.00
	B	0.01500	0.00750	0.00316	2.37	0.064	1.00
	C	-0.02500	-0.01250	0.00316	-3.95	0.011	1.00
	$A^2_{(\%)}$	0.00250	0.00125	0.00465	0.27	0.799	1.01
	B^2	0.04250	0.02125	0.00465	4.57	0.006	1.01
	C^2	-0.11750	-0.05875	0.00465	-12.62	0.000	1.01
	A*B	0.01500	0.00750	0.00447	1.68	0.1541	1.00
	A*C	-0.00500	-0.00250	0.00447	-0.56	0.600	1.00
	B*C	0.00500	0.00250	0.00447	0.56	0.600	1.00

Moisture content play an important role in production of enzymes during solid state fermentation. In this study, different volumes of liquid medium were used to moisten the solid substrate for maximum cellulase production. Maximum enzyme production was obtained when medium volume of 1ml for 5g of dry substrate powder was used. *T. viride* and *T. reesei* produced maximum cellulase in SSF with solid to liquid ratio of 1:1 using empty fruit bunch as substrate (Wonoputri *et al.*, 2018). Saini *et al.* (2017) reported maximum production for CMCase (11.65U/gds) and FPase (1.29U/gds) using *T. reesei* when solid to liquid ratio was 1:2.

Statement of conflict of interset

The Authors declare there is no conflict of interset.

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