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Optimization of cellulolytic enzyme production by thermophilic fungus *Thermoascus aurantiacus* using response surface methodology

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In the present study, thermophilic fungus *Thermoascus aurantiacus*, a local isolate, was used for production of cellulolytic enzymes using rice straw (RS) as substrate under submerged fermentation using Box-Behnken Design (BBD) of Response Surface Methodology (RSM). Themophilic fungus *T. aurantiacus* grew very well at 50-55°C temperature. BBD was used to study the influence of process parameters, pH (4-6), temperature (40-60°C) and substrate concentration (2-8 g/L) and their interactive effect on cellulase enzyme production. The R^2 value 0.94 for Filter paper activity and 0.95 for carboxymethyl cellulase activity indicate that the model is appropriate and replicated to predict the effect of pH, temperature and substrate concentration on both enzyme activities. Under optimized conditions, 6.1 (U/gds) FPase activity and 28.2 (U/gds) CMCase activity was observed.

Keywords: Biorefineries, Box-Behnken design (BBD), Submerged fermentation, Thermophilic Fungus

Lignocellulosic biomass is the most abundant and underutilized biomass on earth which can be used for production of value-added products like cellulase enzyme production. Cellulolytic enzymes are industrially important enzymes used in various industries including pulp and paper industry, detergent industry, food industry, brewery and wine industry, textile industry, fermentation industry, and biorefineries[1,2]. High costs of cellulases are one of the big challenge and obstacle for commercialization of biomass biorefineries[3]. Due to immense industrial importance, temperature stable enzymes produced from thermophilic microorganisms appear an ideal for the production of value-added products[4]. In the present study, production of cellulase enzyme (FPase and CMCase) through thermophilic fungus *Thermoascus aurantiacus* was statistically optimized under the influence of physiological parameters (pH and temperature) and carbon substrate from lignocellulosic rice straw biomass.

Material and Methods

Microorganism

The soil samples were collected in summer season (May, 2016) from Shiv Khori area (33°10'15″N and 74°35'55″E), District Reasi, Jammu (India) for isolation of thermophilic fungi. The major part of the sample collection area is covered with rocks, however, soil type is basically sandy loam and clay loam. As per the Indian Meteorological Department (IMD) data, the annual dry period occurrence is more frequent in the studied region, except few wet months starting from July to September. The average rainfall in Reasi area calculated for the last 65 years is ~1668 mm/yr[5].

Thermophilic fungi was isolated by using standard dilution method on potato dextrose agar (PDA) at 45°C for 8 days followed by sub culturing on PDA plates to obtain pure cultures. The microorganism was identified based on 18S rDNA sequencing method followed by White *et al*[6]. The ITS sequence of fungal strain had highest nucleotide similarities (99%) with *Thermoascus aurantiacus* (KC342033), indicating its taxonomic designation. The fungal culture was maintained on potato dextrose agar (PDA) at 4°C and sub-cultured regularly after 15 days.

Submerged fermentation

Cellulase enzyme production under submerged fermentation was carried out in 250 mL Erlenmeyer flask using rice straw (particle size 1 mm) as substrate. Rice straw (RS) was ground and sieved by 1mm mesh size to obtain desired particle size. The Czapeck mineral solution (CMS) containing, 2.0 g/L $NaNO_3$, 1.0 g/L K_2HPO_4, 0.5 g/L $MgSO_4$,

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0.5 g/LKCl, 0.01 g/L $FeSO_4$, was used for cellulase enzyme production. The substrate (as per design) and media (50 mL in each flask) were sterilized at 121°C for 15 min in autoclave. After sterilization, the flasks were inoculated with inoculums of 03 agar plugs (diameter 8 mm) of 5 days old *T. aurantiacus* culture, grown on PDA at 45°C. The fermentation was carried out at 120 rpm in incubator shaker and variable temperature as per design. The fermentation material was harvested after 5 days (optimized conditions, data not shown) and analyzed for enzyme activities.

Experimental design

The operating conditions and substrate concentration for cellulase enzyme production was optimized using statistical approach Response Surface Methodology (RSM). Box-Behnken design (BBD) of RSM was used to optimize the variables considered for the current study. Three variables including, pH (A), temperature (B), and rice straw concentration (C) were considered as most influential variables, following the literature review. All the variables were studied at three levels as revealed in (Table 1). In BBD, a total of 17 experiments were designed by the software representing each variable at three levels (Table 2). The overall second - order polynomial mathematical relationship was used to express cellulase enzyme activity as a function of independent variables, as depicted in the equation 1.

$$Y = b_0 + b_1A + b_2B + b_3C + b_{11}A^2 + b_{22}B^2 + b_{33}C^2 + b_{12}AB + b_{13}AC + b_{23}BC \quad \ldots (1)$$

where, Y is the predicted response, b_0 is the intercept, b_1, b_2, b_3, linear coefficients; b_{11}, b_{22}, b_{33}, squared coefficients; b_{12}, b_{13}, b_{23}interaction coefficients. The results of the experimental design were analyzed and interpreted using Design Expert Software (version 6.0 & 11.0, trial version).

Enzyme extraction and analytical methods

Erlenmeyer flasks were removed from incubator shaker after 5 days of incubation following the process of homogenization. The homogenate was filtered through muslin cloth and the filtrate was centrifuged at 7200 rpm for 20 min at 4°C. The supernatant was analyzed for enzyme activities as Filter Paper activity (exoglucanse) and endoglucanase (CMCase) activity. Both enzyme activities were determined by the method suggested by Ghose *et al*[7]. Enzyme activities have been expressed in International Units (IU), as the amount of enzyme which releases 1 μM of glucose in 1 min. The reduced

Table 1 — Actual level of variables tested with the Box-Behnken Design (BBD)

Code	Parameter name	Low level (−1)	Middle level (0)	High level (+1)
A	Initial pH	4	5	6
B	Temperature (°C)	40	50	60
C	Substrate concentration [Rice Straw, (RS)] (g/L)	2	5	8

Table 2 — Experimental design in term of actual factors and results of the Box-Behnken model (BBD)

Run	pH	Temperature (°C)	Substrate conc. (rice straw – RS) (g/L)	FPase activity (U/gds)	CMCase activity (U/gds)
1	4	40	5	4.7	20.4
2	6	40	5	4.2	21.1
3	4	60	5	3.8	19.2
4	6	60	5	4.1	18.5
5	4	50	2	3	17.2
6	6	50	2	2.9	21
7	4	50	8	4.1	20.7
8	6	50	8	3.8	22.1
9	5	40	2	3	19.4
10	5	60	2	2.9	18.2
11	5	40	8	3.1	19.1
12	5	60	8	3.45	22
13	5	50	5	5.5	27
14	5	50	5	5.3	28.2
15	5	50	5	6.1	28
16	5	50	5	5.1	26.8
17	5	50	5	5.9	26.1

sugars released were analyzed using the DNS (3,5-Dinitrosalicylic acid) assay[8].

Results and Discussion

Optimization of process parameters for high enzyme production and cost reduction is very important in cellulase enzyme research. RSM is a statistical tool that significantly design experiments to study the interactive effect of variables and finding the optimum process conditions. RSM was explored for the current study also for the optimization of factors influencing cellulase (FPase and CMCase) enzyme production. RSM technique is time and energy saving technique as compared to 'one factor at a time' method. Moreover, it also provides the interactive effect of process parameters on dependent outcome. In the present study, the interactive effect of three variables; pH, temperature and rice straw concentration on cellulase enzyme produced by *T. aurantiacus* was studied using BBD of RSM. Table 2 depicted the actual level of variables studied and response. The enzyme production (FPase and CMCase) was calculated using second order polynomial equation as shown by (equation 2 and 3).

$$\text{Filter paper activity (U/gds)} = +5.58 - 0.075*A - 0.094*B + 0.33*C - 0.52*A^2 - 0.86*B^2 - 1.61*C^2 + 0.20*A*B - 0.050*A*C + 0.11*B*C \quad \ldots (2)$$

$$\text{CMCase activity (U/gds)} = +27.22 + 0.65*A - 0.26*B + 1.01*C - 3.42*A^2 - 4.0*B^2 - 3.55*C^2 - 0.35*A*B - 0.60*A*C + 1.02*B*C \quad \ldots (3)$$

Analysis of variance was used to evaluate the data statistically. The proposed model was significant for both enzyme activities *i.e.* FPase and CMCase as highlighted (Table 3). Various statistical parameters like *F*-value, *P*-value, R^2 value *etc*. are used to check the quality of model and their values (Table 3). ANOVA revealed that the Lack of Fit for both enzyme activities are non-significant ($P > 0.5$), which indicates that the proposed model is significant. The value of R^2 plays an important role in predicting the aptness of the model and it varies between 0 and 1. The closer is the R^2 values to 1, more strongly the response could be predicted through RSM[9]. The R^2 value is also important in measuring the variability among the observed responses which can be explained by the independent experimental variables and their interactions[10]. The coefficient of determination (R^2 value) was 0.94 for FPase activity and 0.95 for CMCase activity, which showed that predicted model explained accurately 94% responses in case of FPase activity and 95% accurately in case of CMCase activity.

The cube plots were plotted to depict the interactive effect of independent variables on depedent outcome. The cube plots explain the interactive effect of studied variables on response along with prediction point. Figure 1A & 1B shows the the interaction between pH, temperature and substrate concetration on FPase and CMCase activities, resepectively. In the present study, highest enzyme activities were observed at middle level of studied independent variables as shown in (Fig. 1A & 1B and Table 2). The initial pH of the medium plays an important role towards stablisation of extracellular enzymes and moreover, any deviation from the particular pH may lead to denaturation of enzymes[11]. The pH and temperature also affects the mycelial

Table 3 — Estimated regression coefficient and corresponding *p* value for FPase and CMCaseenzyme activities by *T. aurantiacus*

Source	FPase activity		CMCase activity		
	F Value	Prob> F	F Value	Prob> F	
Model	12.33	0.001	15.59	0.0008	Significant
A	0.28	0.611	2.28	0.1745	
B	0.44	0.527	0.37	0.5610	
C	5.50	0.051	5.54	0.0508	
A^2	7.17	0.031	33.32	0.0007	
B^2	19.47	0.003	45.46	0.0003	
C^2	68.34	< 0.0001	35.80	0.0006	
AB	1.00	0.349	0.33	0.5830	
AC	0.062	0.809	0.97	0.3568	
BC	0.32	0.590	2.84	0.1358	
Lack of Fit	0.83	0.542	3.20	0.1454	Not-significant
R^2	0.94		0.95		

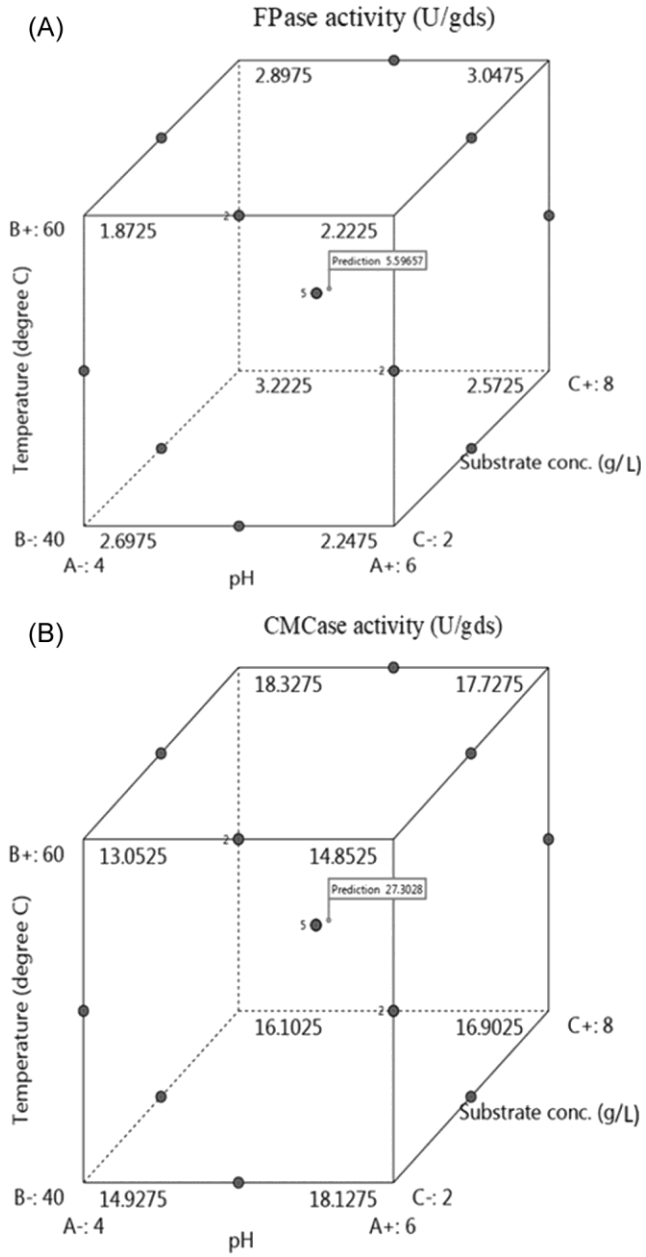

Fig. 1 — Cube plot showing interactive effects between pH, temperature (°C), substrate (RS) concentration (g/L) on (A) FPase enzyme; and (B) CMCase enzyme production (U/gds) by *T. aurantiacus*

growth and transport of various components across the cell membrane[4]. In the present study, pH 5.0 appear optimal both for FPase and CMCase enzyme production, further decrease and increase in pH, decreases enzyme production. Dave *et al.*[10] studied cellulase enzyme production from *T. aurantiacus* and found that highest endo, β-1,4, glucanase production at pH 4 and pH 5.0 was found optimal for highest FPase activity. Javanmard *et al.*[12] investigated cellulase enzyme production using *T. aurantiacus* under submerged fermentation and revealed highest CMCase, FPase and β-glucosidase activities at pH 5.0. Kalogeries *et al.*[3] observed that the fungus *T. aurantiacus* did not grew well at pH beyond 5.0 and resulted in low enzyme yield.

Temperature is also one of the significant factor which control the enzymatic activities. Temperature was varied from 40 to 60°C for the present study and highest enzyme activity was observed at 50°C temperature *i.e.* the middle level of studied temperature range. The temperature also have antagonistic effect on enzymatic yield like other variables and any deviation from their optimal temperature range, might lower down the enzyme yield. Jain *et al.*[4] studied thermostable cellulase and xylanase enzyme production from fungus *T. aurantiacus* RCKK and found maximum enzyme production at 45°C temperature. Kalogeries *et al.*[3] revealed that highest cellulolytic enzyme activities was observed at temperature 50°C, further increase in temperature leads to decrease in enzyme production. Jain *et al.*[13] have also reported highest cellulase enzyme production at 50°C from *T. aurantiacus* fungus. The cost of cellulase enzyme plays an important role in bio-ethanol production. Moreover, utilization of lignocellulosic biomass as substrate for enzyme production is gaining interest of researchers. Thermophilic fungus *T. aurantiacus* was exploited in the present study for cellulase enzyme production using rice straw as substrate, which support the fact for using agricultural residue (lignocellulosic biomass) for production of enzymes. Highest enzyme activities 6.1 (U/gds) FPase activity and 28.2 (U/gds) CMCase was obtained at pH 5, temperature 50°C and rice straw concentration 5 g/L as shown in (Table 2). Various lignocellulosic biomass like rice straw, wheat straw, rice husk, sugarcane bagasse, wheat bran were studied for enzyme production[3,4,10-14]. Utilization of lignocellulosic biomass as substrate for enzyme production solves the agro-industrial solid biomass residue problem as well as the cost of enzyme production.

Conclusion

In the present study, cellulase enzyme production from a local isolate thermophilic fungus *T. aurantiacus* using agricultural residue rice straw was demonstrated. The interactive effect of initial pH, temperature and rice straw concentration on cellulase

enzyme production was studied using statistical methodology. Highest enzyme activities were observed at initial pH 5, temperature 50°C and rice straw concentration 5 g/L. The current study concluded that the rice straw has significant potential to yield cellulase enzyme under optimized conditions which provide alternatives towards management of agricultural residue and control air pollution.

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