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Application of response surface methodology for statistical optimization of carboxymethylcellulase by *Thermomyces dupontii* TK-19 using submerged fermentation

Aplicación de la metodología de superficie de respuesta para la optimización estadística de la fermentación sumergida de carboximetilcelulasa por *Thermomyces dupontii* TK-19

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Abstract

In present work, optimization of nutritional parameters for carboxymethylcellulase production was studied through experimental design. A central composite design, with 8 axial points, 16 factorial points and 6 center points were used for optimizing and predicting four independent variables such as Lactose, peptone, ammonium sulphate and Tween 80. By means of ANOVA significance of four independent variables and their possible interactions was tested with 95% of confidence level. A good agreement was obtained between predicted and experimental model. The predicted response 71.3 U/ml/min from the model showed close agreement with 72.0 U/ml/min of the experimental data which validates the effectiveness of the model while reducing the required number of experiments.

Keywords: cellulase, Tween 80, CMCase, coefficient, variable.

Resumen

En el presente trabajo, se estudió la optimización de los parámetros nutricionales para la producción de carboximetilcelulasa a través del diseño experimental. Un diseño compuesto central, con 8 puntos axiales, 16 puntos factoriales y 6 puntos centrales se utilizaron para optimizar y predecir cuatro variables independientes como la lactosa, la peptona, el sulfato de amonio y El Tween 80. Mediante la importancia de ANOVA de cuatro variables independientes y sus posibles interacciones se probó con el 95% del nivel de confianza. Se obtuvo un buen acuerdo entre el modelo predicho y el modelo experimental. La respuesta pronosticada 71.3 U/ml/min del modelo mostró un estrecho acuerdo con 72 U/ml/min de los datos experimentales que valida la eficacia del modelo al tiempo que reduce el número requerido de experimentos.

Palabras clave: celulasa, Tween 80, CMCase, coeficiente, variable.

1 Introduction

Lignocellulosic material is mainly consists of cellulose, hemicellulose and lignin. These materials are considered the most abundant renewable carbon source on the earth. Cellulose is a linear chain of β-D-glucose units linked together by 1, 4-β-linkages.The lignocellulosic biomass is converted in to simple sugars with the aid of cellulases. Cellulases belong to group of hydrolytic enzymes that mediate the complete hydrolysis of cellulose. Endoglucanase (EC 3.2.1.4) randomly break the internal O-glycosidic bonds, exoglucanase (EC 3.2.1.91) acts on reducing and non-reducing ends of cellulose chain and releases end product as β-cellobiose. β-glucosidase (EC 3.2.1.21) hydrolyzes the cellobiose residues and release β-D-glucose units (Mojsov, 2016; Oliveira *et al*., 2019).

Cellulases have various industrial applications for instance, food, textile, paper and pulp, animal feed and pharmaceutical industry, etc. Cellulases are mainly produced by different bacterial and fungal strains. Due to higher rate of enzyme production fungal cellulases are more advantageous as compared to other microorganisms. The most efficient cellulolytic fungal genera are *Trichoderma, Penicillium, Humicola* and *Aspergillus* (Gupta *et al*., 2015).

Liquid medium is used in submerged fermentation for the growth of fungi and this type of fermentation is preferred over solid state fermentation due to better control on the process parameters including pH, aeration, temperature etc. for efficient growth of fungi consequently better production of enzyme (Mohan *et al*., 2013).

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The optimization of physiochemical parameters is important for enhanced production of the enzyme. For this purpose two approaches have been employed i.e. one factor at a time (OFAT) and the other is response surface methodology (RSM). The first approach is more time consuming and interaction between the medium components cannot be studied. On the other hand RSM overcomes this problem and interaction among the medium components is being accurately studied. The objective of RSM is to hypothesize an analytical form of the response surface that manages the experimental data. RSM is a statistical tool used to build models and design experiments. It also evaluates the effects of different factors and finds the optimal conditions required for the responses by reducing the number of experiments (Tabssum *et al*., 2018).

2 Materials and methods

2.1 Microorganism

The previously isolated strain of *Thermomyces dupontii* TK-19 was obtained from the culture collection of Department of Biotechnology, LCWU and used in present study.

2.2 Inoculum preparation

For inoculum preparation 3 to 4 days old slant of *Thermomyces dupontii* TK-19 were used. In each slant 10 ml of saline water was added and without damaging the medium, conidia was slowly scratch and shaken vigorously to get the homogenous suspension.

2.3 Submerged fermentation

1.0 ml of conidial inoculum ($2x10^7$ conidia/ml) was added in 25ml of sterilized Mandel and Weber (1969) fermentation medium g/l: $(NH_4)_2SO_4$ 1.4, KH_2PO_4 2.0, Urea 0.3, $MgSO_4{\times}7H_2O$ 0.3, $CaCl_2$ 0.3, $FeSO_4{\times}7H_2O$ 0.005, $ZnSO_4{\times}7H_2O$ 0.0014, $MnSO_4{\times}H_2O$ 0.0016, $CoCl_2$ 0.002,Tween 80 2.0 ml, CMC 10.0 . All the inoculated flasks were kept at 40 °C on shaking incubator for 3 days. After fermentation the fermented broth was centrifuged at 8000 rpm for 20 min and supernatant was used for the estimation of CMCase.

2.4 Determination of CMCase

Determination of CMCase was carried out following Gao *et al*. (2008). 0.5ml of 1% CMC prepared in citrate buffer (0.1 M; pH5) was added in 0.5 ml of supernatant. A blank was also run side by side in which enzyme is substituted by distilled water. The reaction mixture was incubated at 60 °C for 30 minutes. The reducing sugar was estimated according to Miller (1959). One unit activity was defined as the "amount of enzyme needed to liberate 1 μmol of glucose from the appropriate substrate per ml per min under standard assay conditions".

2.5 Experimental design

In present study Central composite design (CCD) was used to optimize nutritional parameters for cellulase production. Four independent variables were used including Lactose conc. (X_1), Peptone conc. (X_2) NH_4SO_4 (X_3) and Tween 80 (X_4). In RSM, Central composite design is most suitable design for fitting the second order polynomial equation.

Nutritional factors which affect the CMCase production were designed by using central composite design (CCD) in RSM with the aid of Design Expert (Version 11.0). A set of 30 experimental runs with 8 star points, 16 factorial and 6 center points were obtained from design expert in order to optimize the response (CMCase activity). Following quadratic equation (1) was developed that predict the interaction between independent and dependent variables.

$$\begin{aligned} Y = {} & b0 + b1x1 + b2x2 + b3x3 + b4x4 + b11x12 \\ & + b22x22 + b33x32 + b4x42 + b12x1x2 \\ & + b13x1x3 + b14x1x4 + b23x2x3 + b24x2x4 \\ & + b34x3x4, \end{aligned} \tag{1}$$

where Y is the response variable (CMCase activity), $b0$ is constant, $b1$, $b2$, $b3$, and $b4$ are coefficient for linear effects, $b11$, $b22$, $b33$, and $b44$ are quadratic coefficient, and $b12$, $b13$, $b14$, $b23$, $b24$, and $b34$ are interaction coefficients, respectively.

3 Results and discussion

3.1 Optimization of nutritional parameters in SmF by the response surface methodology

Optimization of process parameters are mainly categorized into two approaches i.e. Univarate and multivariate. The first approach which is univariate is also known as OVAT (one variable at a time) optimizes the single factor at a time. On the other hand multivariate approach gained more attention and has more advantages as compared to OVAT. RSM (which is multivariate) has possibly reduces the number of experiments that are required for optimization; In addition to this the interaction between the variables and non-linear relationship with the response was studied. RSM comprises the group of statistical and mathematical techniques which construct the empirical models based on experimental data.

Basically CCD contains three points in its design (a) two-level factorial points, that consist of +1 and -1 levels of each factor (b) star points (sometimes called axial points) are fixed axially and it shows the distance from the center point that create quadratic terms (c) replicates in the CCD design are represented by center points that helps to estimate the error in the experiment (Asghar *et al*., 2014).

The CCD that is second-order design, used for parallel experimentation that allows reasonable information to check the lack of fit without including large number of design points. Mainly four stages of optimization are involved in CCD. First is the implementation of statistically designed experiments, second is the accurate prediction of the mathematical model that is based on the experimental data third is to control the efficiency of the predicted model with diagnostic plots and last is to estimate the response followed by validation of model.

Table 1. Variables for CMCase Production by CCD.

Factor	Name	Low Level	High Level
$X1$	Lactose	0.50	1.25
$X2$	Peptone	0.10	0.40
$X3$	Ammonium sulphate	0.10	0.40
$X4$	Tween 80	0.75	1.50

In order to improve the CMCase production via submerged fermentation four nutritional factors were selected. To achieve this goal, each factor with minimum (-1) and (+) maximum value were designed in CCD (Table 1).

A set of 30 runs with possible combined effect of independent variables were performed in triplicates (Table 2). A second order polynomial equation (Eq. 2) was derived to represent the CMCase production.

CMCase activity

$$\begin{aligned} Y = {} & +71.33 + 0.3333X1 + 0.5833X2 + 0.5000X3 \\ & - 0.0833X4 - 0.7500X1X2 - 0.8750X1X3 \\ & + 0.0000X1X4 + 0.0000X2X3 - 0.3750X2X4 \\ & + 0.7500X3X4 - 2.19X1^2 - 1.44X2^2 - 0.9375X3^2 \\ & - 0.9375X4^2 \end{aligned} \quad (2)$$

where Y = Predicted response (CMCase activity), $X1, X2, X3$ and $X4$ are coded values of independent variables such as Lactose, peptone, ammonium sulphate and Tween 80, respectively.

Predicted response was calculated from regression equation (Table 2). Actual and predicted values show that the data obtained from the experiment is in reasonable agreement. Statistical analysis of the model by means of ANOVA was presented in Table 3. High 'F' value with low 'P' value probability specifies the high significance of regression model. The computed F-value 15.61 indicates the significance of quadratic regression model. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. P-value of the regression model not only evaluates the significance of the each term but also indicate the interaction between the independent variable. Table 3 also gives the P-values of each variable and their quadratic and interaction terms. Values of "Prob>F" less than 0.05 indicates that the model terms are significant. In this case B, C, AB, AC, CD, A^2, B^2, C^2, D^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. Lack of Fit F-value of 1.85 implies the Lack of Fit is not significant relative to the pure error. There is a 25.78% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant in the model indicates that the lack of fit is good. The closer the value of R to 1 indicates the better correlation between the observed and predicted values suggesting a good fit for SmF. In addition to this R^2 closer to 1 also indicate that the model is stronger to predict the response.

Table 2. The Central Composite Design Variables with the CMCase Production

Run	X1: Lactose %	X2: Peptone %	X3:Ammonium sulphate %	X4:Tween 80 %	**Actual Value** U/ml/min	**Predicted Value** U/ml/min
1	0.875	-0.05	0.25	1.125	65	64.42
2	0.5	0.4	0.1	0.75	66	66.67
3	1.625	0.25	0.25	1.125	64	63.25
4	0.5	0.1	0.1	0.75	63	63.25
5	0.5	0.1	0.1	1.5	62	62.33
6	1.25	0.1	0.1	1.5	65	66.25
7	1	0.25	0.25	1	71	71.33
8	0.875	0.55	0.25	1.125	67	66.75
9	0.5	0.1	0.4	1.5	66	66.58
10	1.25	0.4	0.1	0.75	67	67.58
11	0.5	0.4	0.4	1.5	68	68.5
12	0.875	0.25	-0.05	1.125	67	66.58
13	1	0.25	0.25	1	70	71.33
14	1.25	0.1	0.1	0.75	68	67.17
15	1.25	0.1	0.4	1.5	68	67
16	1	0.25	0.25	1	72	71.33
17	0.5	0.4	0.4	0.75	68	67.92
18	1.25	0.4	0.4	0.75	66	65.33
19	1.25	0.4	0.4	1.5	65	64.42
20	0.5	0.1	0.4	0.75	66	66.67
21	1.25	0.4	0.1	1.5	64	63.25
22	1	0.25	0.25	1	63	63.25
23	0.875	0.25	0.55	1.125	62	62.33
24	0.875	0.25	0.25	1.875	65	66.25
25	0.5	0.4	0.1	1.5	71	71.33
26	0.125	0.25	0.25	1.125	67	66.75
27	1	0.25	0.25	1	66	66.58
28	0.875	0.25	0.25	0.375	67	67.58
29	1.25	0.1	0.4	0.75	68	68.5
30	1	0.25	0.25	1	67	66.58

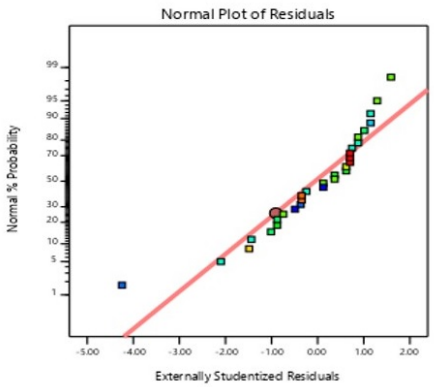

Fig. 1. Distribution of predicted and Experimental values on diagonal plot.

Different values of R-square help to analyze the Fitness of the model such as R^2 represents the percentage of total difference that is attributable to the variables under consideration.

Higher value of R^2 signifies to better fit. R^2 value obtained for CMCase productivity under submerged fermentation by *T. dupontii* was 0.9358 which indicate that 93.58% of variance is attributed to the variables and only 6.42% occurred due to the chance.

Adjusted R^2 is the value that is adjusted for the number of parameters in the given model. Predicted R^2 is a measure how finely the model predicts the observations. The Predicted R^2 of 0.6890 is in reasonable agreement with the Adjusted R^2 of 0.8758; i.e. the difference is less than 0.2. The coefficient of variation (CV) in the model measures the residual variation of the data relative to the size of the mean; the lower CV values provides the better reproducibility.

Table 3. ANOVA Quadratic model for CMCase activity.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	228.2	14	16.3	15.61	< 0.0001	significant
A-Lactose	2.67	1	2.67	2.55	0.1309	
B-Peptone	8.17	1	8.17	7.82	0.0136	
C-Ammonium sulphate	6	1	6	5.74	0.03	
D-Tween 80	0.1667	1	0.1667	0.1596	0.6952	
AB	9	1	9	8.62	0.0102	
AC	12.25	1	12.25	11.73	0.0038	
AD	2.84E-14	1	2.84E-14	2.72E-14	1	
BC	5.68E-14	1	5.68E-14	5.44E-14	1	
BD	2.25	1	2.25	2.15	0.1628	
CD	9	1	9	8.62	0.0102	
A^2	126.93	1	126.93	121.53	< 0.0001	
B^2	58.37	1	58.37	55.89	< 0.0001	
C^2	25.44	1	25.44	24.36	0.0002	
D^2	25.44	1	25.44	24.36	0.0002	
Residual	15.67	15	1.04			
Lack of Fit	12.33	10	1.23	1.85	0.2578	not significant
Pure Error	3.33	5	0.6667			
Cor Total	243.87	29				

The CV value 1.53% clearly indicated the higher degree of precision and reliability of the experimental values (Table 4). Besides the relationship between predicted and the actual experimental values, figure 1 showed that plotted points cluster around the diagonal line, which represents the good fitness of the present model and they are in reasonable agreement.

The response surface in the model indicates the interaction between each factor and also identifies the optimal levels in order to attain the maximum enzyme production. The actual CMCase activity 72 U/ml/min was well agreed with predicted value 71.33 U/ml/min. The optimum production was observed at 1% Lactose, 0.2% Ammonium sulphate, 0.2% Peptone and 1% Tween 80 concentration.

The Figure 2a represents the interaction between peptone and lactose concentration on CMCase production. Cellulases are the inducible enzymes and their productivity increases in the presence nitrogen and carbon sources. The interactive effect of these factors on CMCase production was significant. Maximum CMCase activity was observed at 1% and 0.2% concentrations of lactose and peptone, respectively. Any change above or below the optimal concentration decreases the enzyme production. Lactose is inexpensive and widely used as a soluble carbon source for cellulase production. Fungi do not uptake lactose directly, it first break disaccharide into glucose and galactose and these monomers move in the cell which is possibly beneficial for enhanced enzyme production (Bazafkan *et al*., 2014; da Silva *et al*., 2015). Our findings are in accordance to Muthuvelayudham and Viruthagiri (2006) who reported lactose as a best inducer for cellulase production. Choice of suitable concentration of organic and inorganic nitrogen source is an important factor for enhanced production of enzymes. Nitrogen sources also have inducible effect just like carbon sources. Different studies reported that the additional supplementation of nitrogen in the medium leads to enhancement in the microbial growth and improve the physiology (Yadav *et al*., 2016). The enhancement in CMCase activity by peptone might be due to its composition which is the source of nitrogen, vitamins and additional carbon minerals as these nutrients plays a significant role in the growth of fungi (Karim *et al*., 2015).

Table 4. Summary of the ANOVA for CMCase production by *T.dupontii* TK-19.

	R^2	Adjusted R^2	Predicted R^2	C.V.%
T.dupontii	0.9358	0.8758	0.6890	1.53

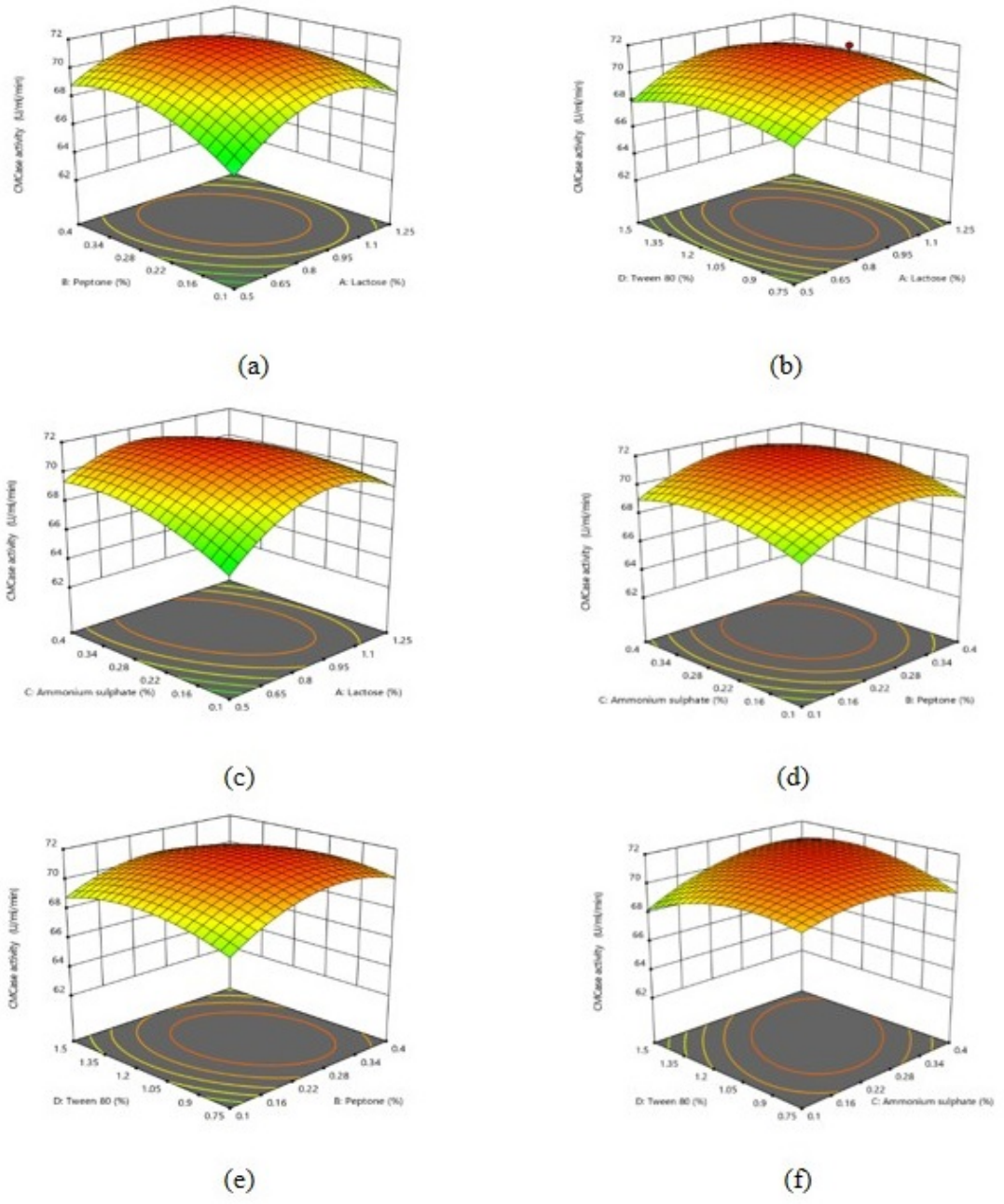

Fig. 2. Contour plots and response surface plots of nutritional factors for CMCase production by *T.dupontii* TK-19. (a) Impact of peptone and lactose concentration (b) Impact of Tween 80 and lactose concentration (c) Impact of ammonium sulphate and lactose concentration (d) Impact of ammonium sulphate and peptone concentration. (e) Impact of peptone and Tween 80 concentration (f) Impact of Tween 80 and ammonium sulphate concentration.

The interaction between lactose and Tween 80 concentration for CMCase production was evaluated (Fig.2b) Lactose and Tween 80 at a concentrations of 1% found to be effective for CMCase production whereas low levels of CMCase production was observed above or below the optimal levels. Effect of surfactant is well known on fungal growth and cellulase production.

In present study, Tween 80 at a concentration of 1% has a positive effect on CMCase activity because it doesn't denature the enzyme (Saini *et al*., 2017). Similar results were observed for cellulase production by *Trichoderma reesei* and *Aspergillus phoenicis* (Wen *et al*., 2005).

Addition of surfactants (Tween 80) in the fermentation medium increases the permeability of the microbial membranes which eventually affects enzyme production cost.

Figure 2c depicts the significant interaction of lactose and ammonium sulphate on enzyme production. The optimal CMCase production was achieved at 1% of lactose and 0.2% ammonium sulphate. Concentration below or above the optimal level was unable to produce optimal CMCase. Our results are similar to Sethi and Gupta (2014) who reported ammonium sulphate as a best nitrogen source for cellulase production. The stimulating effect of ammonium salts can be characterized to the direct entry of ammonium in protein synthesis which influences the enzyme production (Vyas *et al*., 2005; Muthukrishnan. 2017).

Majority of microorganism used in industry possess the ability to utilized organic and inorganic nitrogen sources. Figure 2d shows the significant effect of peptone and ammonium sulphate concentration on the CMCase production. However, negative impact on CMCase production was noticed at higher concentrations of both the sources. The better enzyme production in the presence of ammonium suphlate was probably due to the reason that it provides both ammonium and sulphate ions for the growth of conidia and subsequently for enzyme production (Malik *et al*., 2010). The complex nitrogen sources (like peptone) in contrast to simple nitrogen sources gave better enzyme production by supplying minerals and other growth factors which improves the growth of fungi as well as enzyme production (Bharti *et al*., 2018).

Figure 2e depicts the correlation between peptone and Tween 80. Nitrogen source and surfactant significantly influence the carboxy methyl cellulase production when used in appropriate concentration. Maximum production was achieved at 0.2% and 1.0% concentration, respectively. Any change in this concentration leads to decrease in CMCase production. Nitrogen sources at higher concentrations may cause vitrification *i.e.* yellow and glassy appearance of medium which is generally not suitable for microorganisms (Malik *et al*., 2010). Similar studies were reported by Mehboob *et al*. (2014) and Bharti *et al*. (2018) who reported enhanced cellulase production by addition of Tween-80 and peptone. Figure 2f represents the effect of ammonium sulphate and Tween 80 concentration on CMCase production. Highest CMCase production was obtained at 0.2% of ammonium sulphate and 1% of Tween 80 and reached a plateau phase beyond the optimal levels. Our findings follow the results of Guoweia *et al*. (2011) who have found the combination of ammonium sulphate and Tween 80 enhance cellulases production.

Conclusions

Thermomyces dupontii TK-19 possess the ability for considerable production of CMCase (72.0 U/ml/min) in submerged fermentation after optimizing nutritional parameter using RSM which is well agreed with predicted value 71.33 U/ml/min. Optimization of nutritional parameters using CCD proved to be more reliable, accurate and satisfactory as it is less time consuming, reduces the number of experiments and provide better interaction among the medium components.

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