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CELLULASE FUSARIUM

Optimization of Culture Conditions for the Production of Alkaline Cellulase Enzyme Produced from *Fusarium oxysporum* VSTPDK

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ABSTRACT

Alkaline cellulase producing *Fusarium oxysporum* VSTPDK was isolated and screened from the soil of Kapurthala district Punjab, India. This organism produced endoglucanase (CMCase) and exoglucanase (FPase) when grown at a different range of parameters pH (6, 7, 8, 9, 10 and 11), temperature (30, 40, 50 and 60^{O}C), incubation time (4th 6th 8th 10th and 12th day) and nitrogen source ($(NH_4)_2SO_4$, NH_4Cl, $NaNO_3$ and NH_4HCO_3). Carboxymethylcellulose (CMC) and cellulose powder were used as the sole carbon source. In this research, statistical tools called Response Surface Methodology (RSM) was used for the optimization of cellulase enzyme by selecting three important parameters after one factor at a time (OFAT) approach. Using OFAT, optimum production of both CMCsase (3.52U/mL) and FPase (4.07U/mL) were achieved after 8 days incubation at pH 8, temperature 30^{O}C and 1.0g/L ammonium sulphate while RSM produced CMCase 3.91U/mL and FPase 4.26U/mL respectively when incubated for 8 days at pH 8.5, temperature 45 and 3% ammonium sulphate concentration. Optimization of the culture conditions using RSM leads to an increase of 0.39U/mL (CMCase) and 0.19U/mL (FPase). The use of RSM has gained considerable attention in the past decade in the optimization of various physicochemical parameters and nutritional factors. Its application in different industries may find ways of selecting different factors influencing cellulase activity. The fungus can produce a considerable amount of cellulase enzyme at a pH of up to 10 and 50^{O}C. To our knowledge, this is the first report of alkalothermophilic *oxysporum* VSTPDK from Punjab, India.

INTRODUCTION

Many agro-industrial and plant-related food processing wastes are abundant in large quantities globally and their availability can be a threat to environmental pollution. These wastes which comprise hemicelluloses, cellulose and lignin are chemically bonded by non-covalent forces and cross-linkages. This strong and complex molecular polymeric structure makes lignocelluloses highly resistant to chemicals attack and bioconversions [1]. Cellulase is a family of three (3) groups of enzymes called endoglucanase, exoglucanase and β-glycosidase. At random, endoglucanse (EG) break the inner O-glycosidic bonds leading to the release of glucan chains in different lengths; this is followed by an attack on the ends of the cellulose chains by releasing β-cellobiose as an end product by exoglucanase (CBH) while β-glycosidase act specifically on the breaking down of β-cellobiose disaccharides to glucose [2]. Cellulase enzyme can be produced from different microorganisms like bacteria (*Clostridium spp, Cellulomonas spp*) and fungi (*Trichoderma, Aspergillus spp*) when they grow on cellulosic materials. Many microorganisms such as bacteria and fungi produce these cellulase enzymes when they grow on cellulosic materials [3].

The enzyme is relatively costly which has a great advantage for its commercial use. Low enzyme yield and substrate cost are some of the problems associated with cellulase production which mainly affect its large-scale production. However, these limitations can be overcome by the application of optimizing parameters controlling enzyme yield. This can be done by either optimizing the physical factors like pH, incubation time and

temperature [4] or nutrient composition of the media such as carbon and nitrogen source [5];[6]. One factor at a time approach (OFAT) is a conventional method for enzyme production which is time-consuming, laborious and overlooks interactions between different variables, leading to misinterpretation of the data obtained. In order to examine the effects of various factors that influence responses by changing them simultaneously, a statistical approach called Response Surface Methodology (RSM) through central composite design (CCD) can be used to replace one factor at a time (OFAT) approach [7]. RSM is a collection of approaches, statistical interferences and mathematical techniques for exploring and constructing an estimated useful interaction between a set of design experiments and response variables. *F. oxysporum* are a ubiquitous soil inhabitant that can leave as saprophytes and can degrade lignin as well as carbohydrates associated with soil debris [8]. This present study involves the optimization of some important parameters necessary for the production of cellulase by alkaliphilic *Fusarium oxysporum* VSTPDK (*F. oxysporum*) using both OFAT and RSM, to test its potential as a new means of removing ink from wastepaper.

MATERIALS AND METHODS

Chemicals

Chemicals and reagents used in this research are of analytical grades (AR) and were purchased from HiMedia (India) and Sigma (USA) unless otherwise stated.

Isolation, Screening and Identification

Alkaline *Fusarium oxysporum* (VSTPDK) was isolated and screened in our Laboratory from soil sample of Samana village in Kapurthala District, Punjab India. Based on the method of Vega [9], the fungus was kept in potato dextrose agar (PDA) and stored in refrigerator at 4 ºC. it was then screened for the ability to produce cellulase using Mandel and Reese media. The broth media containing in (g/l) of the following composition: Proteose peptone 1.0, Ammonium sulphate (NH_4SO_4) 1.4, Potassium dihydrogen phosphate KH_2PO_4 2.0, Urea NH_2-CO-NH_2 0.3, Magnesium sulphate $MgSO_4.7H_2O$ 0.3, Calcium chloride $CaCl_2$0.002, Ferrous sulphate $FeSO_4.7H_2O$ 0.005, Manganese sulphate $MnSO_4.H_2O$ 0.001, Zinc chloride $ZnCl_2$ 0.017, Carboxy Methyl Cellulose (CMC) 10 and Cellulose powder (CP). The pH of the medium was adjusted to different alkaline level using NaOH and HCl.

The fungal organism was identified as *F. oxysporum* by Indian Agricultural Research Institute (IARI) New Delhi. The organism has 97.77% similarity with *Fusarium oxysporum* LD01 with GenBank ID MH752591.1. Fasta format of the partial sequence of this *F. oxysporum* strain internal transcribed region is given below.

>VSTPDK_NS1_B09.ab1 *F. oxysporum*:
TACCCGCGAAACTGCGAATGGCTCATTATATAAGTTATCGTTTATTTGATAGTAC
CTTACTACTTGGATAACCGTGGTAATTCTAGAGCTAATACATGCTAAAAATCCCG
ACTTCGGAAGGGATGTATTTATTAGATTAAAAACCAATGCCCTTCGGGGCTCACT
GGTGATTCATGATAACTCCTCGAATCGCATGGCCTTGTGCCGGCGATGGTTCATT
CAAATTTCTTCCCTATCAACTTTCGATGTTTGGGTATTGGCCAAACATGGTTGCA
ACGGGTAACGGAGGGTTAGGGCTCGACCCCGGAGAAGGAGCCTGAGAAACGGCTA
CTACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGG
TAGTGACAATAAATACTGATACAGGGCTCTTTTGGGTCTTGTAATTGGAATGAGT
ACAATTTAAATCCCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGC
CGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGTGGTTAAAAAGCTC
GTAGTTGAACCTTGGGCCTGGCTGGCCGGTCCGCCTCACCGCGTGTACTGGTCCG
GCCGGGCCTTTCCCTCTGTGGAACCCCATGCCCTTCACTGGGTGTGGCGGGGAAA
CAGGACTTTTACTGTGAAAAAATTAGAGTGCTCCAGGCAGGCCTATGCTCGAATA
CATTAGCATGGAATAATAGAATAGGACGTGTGGTTCTATTTTGTTGGTGTCTACG
ACCGCCCTCATGATTATTAGGGACAGTCAGTGGCATCAGTATTCACTTGTCAGAG
GTGAAATTCTTGGATGTATTGAAAACTAACTACTGCGACGCCGTTAGCGAGGATG
TTTTCATTATTAAGAACGACCGTACGGG

Alkaline cellulase production using different carbon sources

The secondary screening was conducted according to the method by [1]. Liquid cellulase enzyme production medium containing 100 mL of the modified culture media in which 1%CMC was replaced with 1% of three different agro cellulolytic waste which includes Rice straw (RS), wheat straw (WS) and sugarcane bagasse (SB). This media was prepared, autoclave and prepared with *F. oxysporum* in a 250 mL Erlenmeyer flask after alkaline pretreatment of the agro-waste. The liquid culture medium was incubated at 150 rpm in a rotary for 12 days with an interval of 2 days (2, 4, 6, 8, 10 and 12). In each interval, reducing sugar was measured as per the method of [10].

Enzyme assay

The cellulase enzyme (endoglucanase and exoglucanase) were measured using Dinitrosalicyclic acid method [10]. In this method, 0.5 mL diluted enzyme in 0.05 M citrate buffer (pH 8.5) was mixed with 0.5 mL of 1% CMC for endoglucanase and a Whatman #1 filter paper (6 x 1 cm) was striped in the same buffer for exoglucanase. After incubation at 50 ºC for 30min, the reaction was immediately stopped by the addition of 3mL dinitrosilicyclic acid and heating at 100 ºC for 10min followed by immediate cooling to stop the further reaction. Absorbance was measured by a spectrophotometer at 540 nm. One unit of CMC and filter paper were defined as the amount of enzyme produced by releasing 1µmole of reducing sugar equivalent to glucose per minute under standard conditions [1].

One factor at a time (OFAT) approach

Important cellulase enzyme production parameters such as nitrogen source, temperature, pH, and incubation time were optimized while determined while keeping others constant. The effects of temperature (30, 40, 50 and 60 ºC), incubation time (4^{th} 6^{th} 8^{th} 10^{th} and 12^{th}) and pH (6, 7, 8, 9, 10 and 11) were examined. The nitrogen sources tested are ammonium carbonate, ammonium chloride, ammonium sulphate and sodium nitrate. Ammonium sulphate was found to be the best and thus, its percentage concentrations ranging from (1, 2, 3, 4 and 5%) was subsequently tested. All experiments involving these parameters were carried out in triplicate with mean standard deviation [11].

Optimization using statistical approach on the production of cellulase

Design Expert windows vision 6.0.8 portable was the statistical software package used during the tabulation and processing process that allows a quick and simple data appraisal. Combined physical and nutritional factors were optimized by response surface methodology using central composite design (CCD). Three major factors selected were ammonium sulphate concentration (1.0 - 5.0%), temperature (30 - 60ºC) and pH (6 - 11) as indicated in **Table 1**. A total of 20 different experiments randomLy selected by the software were conducted. All experiments are in triplicate and mean enzyme production was used as the variable responses *Y*. Equation indicate the second-order model used in describing the relationship between an independent variable and the response. Experiments are carried out in triplicate while the mean production was used as response variable *Y*. Final RSM predicted response was further validated experimentally [6] and [12].

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC$$

Eqn.… (1)

Key: *Y* is the predicted response parameter, β_0, β_1, β_3, β_{11}, β_{22}, β_{33}, β_{12}, β_{13} and β_{23} are constant regression coefficients of the model, β_0 means intercept term, β_1, β_2 and β_3 are linear coefficients, β_{11}, β_{22} and β_{33} are squared coefficients, β_{12}, β_{13} and β_{23} are interaction coefficients and A, B, C, A^2, B^2, C^2, AB, AC and BC are independent parameters

Table 1. Level of independent variables and experimental range from CCD for optimization of CMCase and FPase produced from *Fusarium oxysporum* VSTPDK.

Variable	Factors	Range	Level of experimental variables Low (-1)	Medium (0)	High (+1)
A	pH	6-11	6	8.5	11
B	Temperature (^{O}C)	30-60	30	45	60
C	Ammonium Sulphate (%)	1-5	1	3	5

Statistical Analysis

The statistical tool used in the research was an analysis of variance (ANOVA) using data generated from central composite design (CCD) experiments for CMCase and FPase production. All $p < 0.05$ are considered statistically significant.

RESULTS AND DISCUSSION

Effect of different temperature

One of the essential physical factors affecting the survival and growth of microorganisms is temperature. Any specific enzyme-mediated degradation process will have an optimum temperature [13]. The temperature for fungal growth and cellulase production was measured between the ranges of 30, 40, 50 and 60 ºC as shown in **Fig. 1a**. The optimum temperature for both CMCase and FPase was found to be at 30 ºC with the production of 3.50U/mL and 3.70U/mL respectively. This result is in agreement with that of [14]) and [15] where they also found an optimum temperature of cellulase enzyme production at 30^{O}C and 33^{O}C from *Aspergillus niger* and *Fusarium oxysporum* respectively. However, the result was in parallel with the work of Basak and Rangan [16] and Remaz [11] that produced optimum cellulase from *Fusarium oxysporum* and *Aspergillus niger* at a temperature of 60^{O}C and 50^{O}C respectively. [1] Also produced optimum CMCase from *Fusarium oxysporum* at 50 ºC. Despite having an optimum production temperature of 30 ºC as seen in **Fig. 1a**, higher temperature values also yield a significant amount of both CMCase and FPase as such, *Fusarium oxysporum* VSTPDK can be termed as thermophilic fungi.

Effect of initial pH

Another essential physical factor influencing microbial growth and metabolism is pH [13]. It is of the medium shows the potentials for a microbial activity where an increase or decrease, may affect microbial growth and enzyme production [17]. Being the cardinal factor of our research, the effects of pH on fungal growth and cellulase enzyme production was studied between the range of 6 to 11 (6, 7, 8, 9, 10 and 11). Based on the result in **Fig. 1b**, it was found that enzyme production decreases with an increase in pH value.

Optimum cellulase production for both CMCase and FPase (3.50 U/mL and 4.07) was found at pH 6. The work is similar to the work of [18] where cellulase was optimum at pH 6. This may be because most fungi grow best at acidic pH. At a pH of and 7, [16] as well as [19] also produced optimum cellulase from *Fusarium oxysporum* and *Mucor circinelloides* which is in line with our work. However, a significant amount of enzyme was produced across an alkaline environment (pH 7-10). Having such an amount of enzyme in an alkaline environment indicated that this newly isolated *Fusarium oxysporum* as alkaline cellulose producing fungi. Similar results in an alkaline environment from different fugal strains are reported [9,20-22].

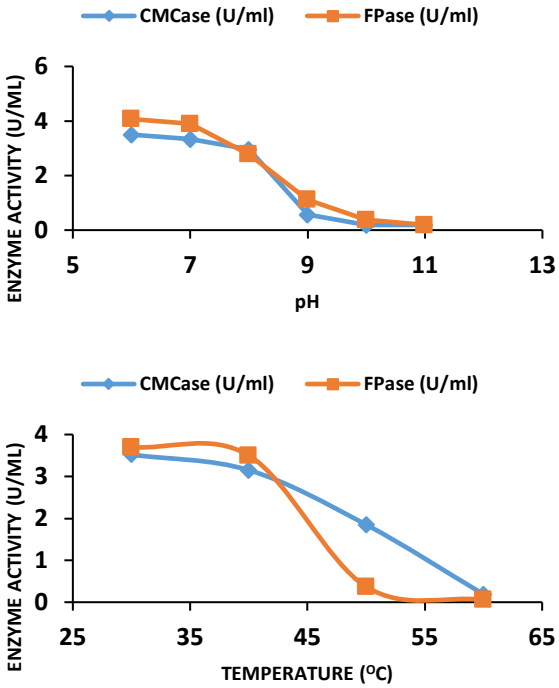

Fig 1. Effect of temperature (a, lower figure) and pH (b, upper figure) on the production of endoglucanase (CMCase) and exoglucanase (FPase) from *Fusarium oxysporum* VSTPDK.

Effect of different carbon sources

Three different types of agricultural waste within the University farm which includes Rice straw (RS), wheat straw (WS) and sugarcane bagasse (SB) were selected and subjected to drying as well as alkaline pre-treatment. These agro cellulolytic wastes were used to substitute carboxymethylcellulose (CMC) and cellulase powder as sole sources of carbon from the fermentation media. Maximum CMCase (2.78 U/mL) and FPase (3.70 U/mL) activity were found in rice straw on 8th day incubation at 30^{O}C and pH 8.5 as shown in **Fig. 2**. This activity were accordance with work of [23] were maximum CMCase of 0.128g/mL was found in rice bran followed by wheat brand (0.097g/mL) and sugarcane bagasse (0.019) respectively. [24] also reported a maximum CMCase production of 7.4 U/mL from banana stem followed by rice straw 5.4 U/mL using fungal cellulase.

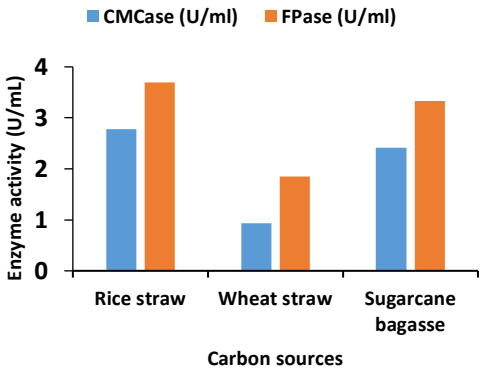

Fig 2. Effect of different carbon sources on the production of endoglucanase (CMCase) and exoglucanase (FPase) from *Fusarium oxysporum* VSTPDK

Effect of Nitrogen source

One of the most important nutritional factors for influencing microbial growth and cellulase production is the nitrogen source. Different nitrogen sources may have inhibitory or stimulatory effects on fungal growth and cellulase production. Ammonium sulphate ((HN_4SO_4)) was found with maximum enzyme production (CMCase 3.50U/mL and FPase 3.70U/mL) when four different inorganic nitrogen sources which includes ammonium sulphate (HN_4SO_4), ammonium carbonate (HN_4HCO_3), ammonium chloride (HN_4Cl) and Sodium nitrate ($NaNO_3$) were used (**Fig. 3**). The results of the effects of different nitrogen sources obtained here are similar to one reported by [23] who found that ammonium sulphate increase the number of cellulase enzymes produced from *Aspergillus flavus* as well as [25] where optimum cellulase was found when ammonium sulphate was used as nitrogen source. However, it disagrees with work of [26] and [27] where ammonium nitrate found have maximum cellulase followed by ammonium sulphate. Urea was also found to be the best nitrogen source for the production and optimization of cellulase enzyme from *Fusarium oxysporum* [1].

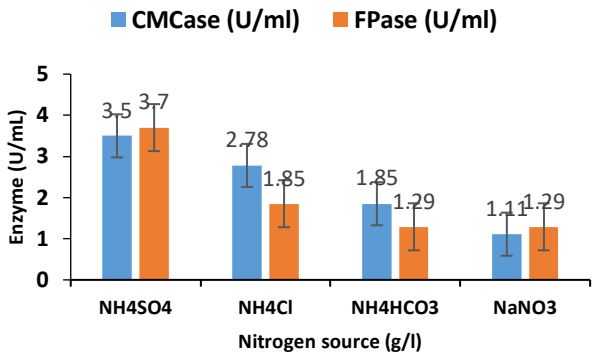

Fig 3. Effect of different inorganic sources of nitrogen on the production of CMCase and FPase from *Fusarium oxysporum* VSTPDK. Error bars represent mean±standard deviation.

Effect of incubation Time

Production of cellulase enzyme was determined based on their incubation period by inoculating the flasks on different incubation days (4^{th} 6^{th} 8^{th} 10^{th} and 12^{th}). It was observed that the initial increase in fermentation day increases enzyme production. Optimum enzyme production for both CMCase and FPase (3.52 U/mL and 3.88 U/mL) respectively were observed on 8^{th} day incubation which was later given to decline as indicated in **Fig. 4a**. This result complies with the work of [1] where a maximum CMCase (1.92±0.005) and FPase (1.34±0.003) were produced from *Fusarium oxysporum* after eight days of incubation. [27] were able to produce maximum xylanase from *Trichoderma viride* IR05 at seven days incubation period. However, maximum production at low incubation time was also conducted were [26] has found a maximum enzyme at 9hrs incubation time for the production of CMCase on *Aspergillus hortai*

Effect of Ammonium Sulphate concentration

The effect of various ammonium sulphate concentrations on cellulase production was investigated. The data obtained indicated that 3.0% was the optimum concentration supporting cellulase production for CMCase and FPase (3.70U/mL and 3.89 U/mL) respectively (**Fig. 4b**).

An increase or decrease of ammonium sulphate from the optimum concentration will lower the rate of enzyme production. This result is similar to the result obtained by [25] and [23] where ammonium sulphate was reported to be optimum.

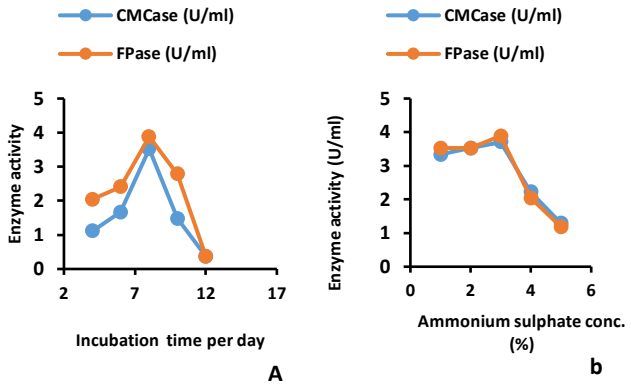

Fig 4. Effect of incubation time (a) and ammonium sulphate concentration (b) on the production of endoglucanase and exoglucanase from *Fusarium oxysporum* VSTPDK.

Central Composite Design (CCD) of experiment

Three important parameters identified in one factor at a time (OFAT) were further optimized using CCD in Response System Methodology (RSM). A total of 20 experiments were randomLy designed by the software and later conducted in our laboratory as predicted. The independent variables were pH (A), temperature (B) and concentration of ammonium sulphate (C). The predicted and actual responses from central composite experimental plan for both CMCase and FPase were summarized in **Table 2**. An estimated endoglucanase and exoglucanase as a function of independent variables were obtained after ANOVA has provided a regression equation. Models' precisions were normally determined by coefficient (R^2). Its values always range between 0 to 1, where the order of magnitude suggest goodness of the model [28].

As indicated in **Table 3**, R^2 value of CMCase and FPase were found to be 0.9933 and 0.9894 respectively, which is close to 1, and it signifies that the 99.33% and 98.94 behavior of the model can be interpreted for cellulase enzyme production while only 0.67% and 1.06% full variance cannot be explain by the models. According to [29], for high accuracy and ability of polynomial model to be good, R^2 value must be close to 1. A similar R^2 value of 0.9873 and 0.8770 for CMCase and FPase respectively was reported by [30]. The Adjusted R^2 from this model was 0.9873 and 0.9798 respectively. This is an indication of a good relationship between actual and predicted values. From the result obtained, predicted R^2 values for CMCase (0.9710) and FPase (0.9300) agreed with Adjusted R^2 values of 0.9873 and 0.9798 respectively. Hence, the model provides clarity on the relationship between response and independence variables. Adequate precisions of the model measured the signal to noise ratio for CMCase and FPase was found to be 48.790 and 39.373 respectively indicated an adequate signal while the result showed that the model is significant. This result agreed with that of [31], with adequate precision values of 17.4 and 14.4 respectively while optimizing nutrient supplements on the removal of Cr (VI) by *Aspergillus lentulus* AML05.

Significant of the model is generally measured based on P-value of F-value (prob > F). the higher the F-value and corresponding lower prob > F value, the better the importance of the corresponding coefficients (R^2) [32]. For maximum cellulase enzyme production, second-order response surface models in the form of ANOVA are summarized in **Tables 4** and **5** for CMCase and FPase respectively. The result indicated that the high models *F*-value for CMCase and FPase are 165.31 and 103.28 with their respective small prob >F values (P-value) of <0.0001 signify that the models were significant. This means the probability where the F value model could happen due to noise was 0.01%. To ensure the importance of each coefficient, P-values are adopted are the tools.

The prob>F<0.05 values showed that the models were significant. This means that A, B, C, A^2, B^2, C^2, AB, AC as well as BC are the significant model terms. The lack of fit *F*-value of the models for both CMCase and FPase are 1.83 and 4.29 while lack of fit P-values was 0.2613 and 0.0679, respectively, indicated that lack of fits was not significant and indicated models as very accurate without any noise. [28] reported that, a lack of fit must be estimated to examine the analysis of variance (ANOVA) on each model coefficient and ensure an in-depth model fit. [33] and [34] reported a non-significant lack of fits by describing it as an excellent fit. Based on the result obtained, there was a strong relationship between actual and predicted model values for both CMCase and FPase as depicted in **Eqns 2** and **3** for CMCase as well as **Eqns. 4** and **5** for FPase which described actual and coded factors respectively.

CMCase = (0.70) + (-0.63xA) + (1.00xB) + (0.22xC) + (0.21xA^2) + (0.90xB^2) + (0.21xC^2) + (0.65xAB) + (0.094xAC) + (0.14xBC) **(Eqn. 2)**

CMCase = (19.07785) + (-0.51475*A) + (-0.58746*B) + (-0.16350*C) + (-0.033600xA^2) + (4.00000E-003*B^2) + (-0.052500*C^2) + (0.017300*AB) + (0.018750*AC) + (4.62500E-003*BC)
(Equatin 3)

FPase = (0.71) + (-0.76xA) + (-1.02xB) + (-0.24xC) + (-0.15xA^2) + (0.77xB^2) + (-0.15xC^2) + (0.79xAB) + (0.14xAC) + (0.14xBC)
(Eqn. 4)

FPase = (20.92858) + (-0.91753xA) + (-0.5693xB) + (-0.34291xC) + (-0.024436xA^2) (-0.56937xB) + (-0.34291xC) + (-0.024436xA^2) + (3.43232E-003xB^2)+ (-0.036932xC^2) + (0.021033xAB) + (0.027750xAC) + (4.62500E-003xBC)
(Eqn. 5)

The 3D response surface as well as their contour plots in central composite design (CCD) shows an interaction between the two different factors while keeping other factors constant (**Fig. 5**). This visualization helps to understand the interaction between two factors and pinpoint the optimum level of each parameter for maximal response [12]. The interaction between ammonium sulphate concentration and enzyme production showed an important effect.

Increases in ammonium sulphate concentration will increases enzyme production from 1 to 3% and where subsequent increases showed a decrease in enzyme production. The optimum enzyme production was indicated at 3% ammonium sulphate and pH 8.5 (**Fig. 3a** and **5c**). An excellent correlation was also observed between pH and temperature as well as temperature and ammonium sulphate concentration.

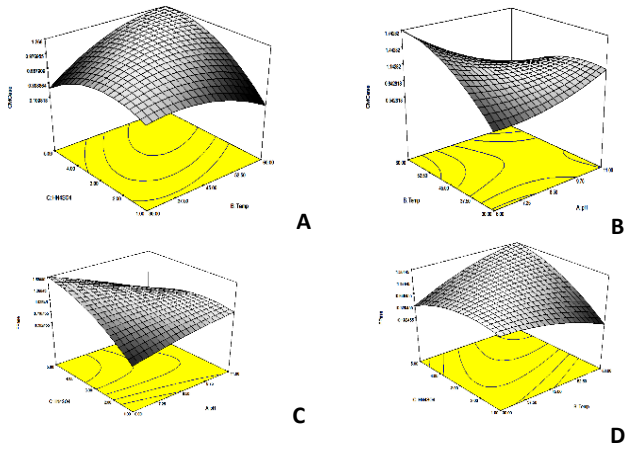

Fig 5. A three 3D response surface plots for the optimization of endoglucanase (a and b) and exoglucanase (c and d) from *Fusarium oxysporum* VSTPDK having interactive effects of the three different parameters.

Table 2. Experimental design having coded levels of variables used in Central Composite Design with experimental and predicted value for CMCase and FPase activity from *Fusarium oxysporum* VSTPDK.

Standard Order	Run	Factor 1 pH	Factor 2 Temperature (^oC)	Factor 3 NH_4SO_4 (%)	CMCase (U/mL)		FPase (U/mL)	
					Actual	Predicted	Actual	Predicted
1	16	0	0	0	3.89	3.91	4.26	4.26
2	3	1	0	0	1.11	1.16	0.74	0.89
3	11	1	-1	1	0.37	0.33	0.37	0.37
4	4	-1	-1	-1	0.19	0.19	0.19	0.16
5	7	0	0	0	2.96	3.00	3.15	3.23
6	20	-1	1	-1	0.56	0.63	0.37	0.41
7	5	0	0	0	0.00	-0.02	0.00	-0.11
8	6	0	0	1	0.19	0.21	0.19	0.23
9	15	1	-1	-1	1.11	1.11	1.29	1.31
10	12	1	1	-1	0.00	-0.14	0.00	-0.02
11	17	-1	1	1	2.77	2.59	2.77	2.50
12	8	0	0	0	0.56	0.60	0.37	0.46
13	13	-1	-1	1	0.74	0.71	0.93	0.80
14	9	0	1	0	0.37	0.26	0.37	0.32
15	10	-1	0	0	0.74	0.70	0.56	0.71
16	14	0	0	-1	0.74	0.70	0.74	0.71
17	19	0	-1	0	0.74	0.70	0.74	0.71
18	1	1	1	1	0.56	0.70	0.74	0.71
19	18	0	0	0	0.56	0.70	0.56	0.71
20	2	0	0	0	0.56	0.70	0.56	0.71

Table 3. Summary of ANOVA result from Central composite design (CCD) for CMCase and FPase from *Fusarium oxysporum* VSTPDK.

Parameters	Result		Remark
	CMCase	FPase	
F- value	165.31	103.28	
Prob > F	<0.0001	<0.0001	Significant
R^2 value	0.9933	0.9894	
Adjusted R^2	0.9873	0.9798	
Predicted R^2	0.9710	0.9300	
Adequate precision	48.790	39.373	Adequate signal to noise ratio
Lack of fit F value	1.83	4.29	
Lack of fit prob > F	0.2613	0.0679	Not significant

Table 4. Analysis of variance (ANOVA) for endoglucanase (CMCase) from *Fusarium oxysporum* VSTPDK.

Source	Sum of square	DF	Mean square	*F* value	Prob>F value	
Model	20.48	9	2.28	165.31	< 0.0001	Significant
A = pH	3.94	1	3.94	286.57	< 0.0001	
B = temperature	9.96	1	9.96	723.72	< 0.0001	
C = NH_4SO_4	0.49	1	0.49	35.81	0.0001	
A^2	0.12	1	0.12	8.81	0.0141	
B^2	2.23	1	2.23	161.86	< 0.0001	
C^2	0.12	1	0.12	8.81	0.0141	
AB	3.37	1	3.37	244.66	< 0.0001	
AC	0.070	1	0.070	5.11	0.0473	
BC	0.15	1	0.15	11.19	0.0074	
Residual	0.14	10	0.014			
Lack of fit	0.089	5	0.018	1.83	0.2613	Not significant
Pure error	0.049	5	9.720E -003			
Cor Total	20.61	19				

Table 5. Analysis of variance (ANOVA) for exoglucanase (FPase) from *Fusarium oxysporum* VSTPDK.

Source	Sum of square	DF	Mean square	*F* value	Prob > F value	
Model	23.91	9	2.66	103.28	< 0.0001	Significant
A = pH	5.75	1	5.75	223.41	< 0.0001	
B = Temperature	10.34	1	10.34	402.17	< 0.0001	
C = NH_4SO_4	0.58	1	0.58	22.58	0.0008	
A^2	0.064	1	0.064	2.49	0.1453	
B^2	1.64	1	1.64	63.77	< 0.0001	
C^2	0.060	1	0.060	2.33	0.1576	
AB	4.98	1	4.98	193.52	< 0.0001	
AC	0.15	1	0.15	5.99	0.0344	
BC	0.15	1	0.15	5.99	0.0344	
Residual	0.26	10	0.026			
Lack of fit	0.21	5	0.042	4.29	0.0679	Not significant
Pure error	0.049	5	9.720E-003			
Cor total	24.16	19				

Validation of statistical experiments

The accurate prediction and model's fitness evaluation of each variable investigated requires validation of the model from both graphical and numeric approaches. Interpretation of results in such matter as graphic and numeric have a key role in variable combination as well as concluding the effect of each variable. To examine the significance of the facts, simple statistical and mathematical tests such as F test, P value and ANOVA were used in validating the experimental design. Analysis of variance (ANOVA) was used which is the best reliable statistical test to examine the goodness of the model fitness by comparing changes based on the treatment and random inherent errors in the determination of analyzed responses [6].

Based on the RSM-CCD results, the optimum conditions for enzyme production were 8.5% initial pH, 45^{O}C temperature and 3% ammonium sulphate concentration, while incubation time was kept constant (8 days). RSM experiment produced an actual enzyme production of 3.91 U/mL and 4.26U/mL for CMCase and FPase respectively. These experiments were conducted based on the different predicted experiments from three factors identified as pH, temperature and NH_4SO_4 concentration in order to validate the results previously conducted with CCD. Comparison between OFAT and RSM revealed an increase enzyme production 0.39 U/mL (CMCase) and 0.19 U/mL (FPase) using RSM (**Table 6**) as compared to that of OFAT, with the OFAT having 3.52 U/mL (CMCase) and 4.07 U/mL (FPase) as against 3.91 U/mL (CMCase) and 4.26 U/mL (FPase) with RSM. This indicates that RSM can be used to increase cellulase enzyme production as compared to OFAT and other conventional methods of enzyme productions.

CONCLUSION

Presently, Response Surface Methodology (RSM) has been mainly preferred over conventional methods like one factor at a time (OFAT) in the optimization of cellulase. This is due to the fact that RSM generates a high amount of information from a small number of experiments. RSM help in identifying relationships between factors and responses using a mathematical model. In recent years, central composite design is the most widely used second-order experimental design for optimization of cellulase production. RSM is suitable for the production and optimization of alkaline cellulase from *Fusarium oxysporum* VSTPDK isolated in our laboratory from the soil. We believe that *Fusarium oxysporum* VSTPDK is the first alkaliphilic fungi isolated from the region of Punjab India and will be tested for its application in the removal of ink from wastepaper.

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CONFLICT OF INTEREST

Authors have non to declare

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