OPTIMIZATION OF CULTURE CONDITIONS FOR THE PRODUCTION OF XYLANASE IN SUBMERGE FERMENTATION BY *PENICILLIUM CITRINUM* USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

In the present study Response surface methodology (RSM) was used to investigate the combined effect of relevant process variables to maximize the production of xylanase in submerge fermentation by *Penicillium citrinum* MTCC 2553. The process variables include pH (6.5, 7.0, and 7.5); temperature (25, 30, and 35°C); agitation speed (190, 200, and 210 rpm); and, substrate (xylan) concentration (0.70%, 0.75%, and 0.80%). A 2^4 factorial central composite design (CCD) using response surface methodology (RSM) was employed to obtain interaction between the process variables and optimizing these variables. Total 22 experiments were carried out in shake flask and a three dimensional response surface was generated to determine the effect of process variables on xylanase production. The optimal calculated values of tested variables for maximal production of xylanase were: pH 7.49, temperature 29°C, agitation speed 209 rpm, and substrate concentration of 0.75%. This approach for process parameter optimization yielded enhance xylanase activity by three - folds compared to the unoptimized media.

Key words: *Xylanase; Penicillium citrinum MTCC 2553; RSM; CCD; Submerge fermentation; Process parameter optimization*.

1. INTRODUCTION

Xylanases are glycosidases (o-glycoside hydrolases, EC 3.2.1.8) which catalyse the endohydrolysis of 1,4-β-D-xylosidic linkages in xylan in a random manner and are extensively used in paper pulp, food, and animal feed industry. Xylan is a heterogeneous carbohydrate of β 1,4 linked D-xylopyranose units and short chain branches of O-acetyl, α L-arabinofuranosyl and α D-glucuronyl residues. Xylan is the major component of hemicelluloses which is the second most abundant renewable resources in nature. Monocotyls contain about 40% hemicelluloses whereas soft wood and hard wood contain 15-25% and 25-32% hemicelluloses respectively [1]. Conversions of hemicelluloses to valuable products by xylanases hold strong promise for the degradation of a variety of unutilized or underutilized agricultural residues for industrial applications including hydrolysis of lignocelluloses to fermentable sugars for fuel ethanol production, bread making, and clarification of beer and fruit juices [2]. Xylanases derived from microorganisms have immense potential applications in the food, feed, and paper pulp industries. Xylanases are produced by prokaryotes and eukaryotes. A large number of bacteria and fungi are known to produce xylanases [3-5]. Filamentous fungi are the most appropriate producer of xylanases and other xylan degrading enzymes as it gives higher yield compared to bacteria and yeast. As xylanases are extracellular enzymes, from industrial point of view, filamentous fungi are the most suitable one to produce xylanases and other xylan degrading enzymes.

In the present investigation, we report the production of xylanases by *P. citrinum* MTCC 2553 in submerged fermentation (SmF). Preliminary selection of the suitable medium formulation for xylanases by *P. citrinum* MTCC 2553 was conducted in SmF. Initially nutrient broth (NB) media, Mandels & Sternburg's basal (MS) media and Czapek yeast extract (CYE) media were used as growth media for *P.citrinum* MTCC 2553. The organism exhibited better activity in CYE media. Further experiments were conducted using the CYE media. Subsequently, the feasibility of using different levels of initial pH, temperature, agitation speed, and substrate concentration combinations were also investigated.

Optimization of media and process conditions are the most important factors to reduce the production cost. In preliminary study, optimization of xylanase production was done using conventional method, which involved varying one variable at a time while keeping the other variables constant. This method is lengthy and often does not produce the effect of interaction of different variables. To overcome this difficulty, response surface methodology (RSM) was used to optimize the media composition and few process variables [6-8].

RSM is the most commonly used statistical practice for bioprocess optimization. RSM is a compilation of numerical and statistical techniques useful for analyzing the effect of several independent variables. The process consists of a

low order polynomial equation in a predetermined region of independent variables. These independent variables are later analyzed to locate the optimum values of the independent variables for the best response [9]. It can be used to evaluate the relationship between a set of controllable experimental variables and observed results. The interaction among the possible influencing variables can be evaluated with limited number of experiments [10-11]. RSM and CCD are proved to be important tools to study the effect of multiple process variables with fewer experimental trials [11-19]. Although there are reports of optimization of xylanase using RSM [7-8,13,15] using different fungi and bacteria, but to the best of our knowledge no systematic study has been carried out on optimization of xylanase production using *Penicilium citrinum* MTCC 2553 using RSM.

2. MATERIALS AND METHODS

2.1 Materials

All chemicals used were of analytical grade and were obtained from Hi- media (Mumbai, India). Birchwood xylan was obtained from Sigma Chemical Co. (St Louis, MO, USA).

2.2 Microorganism and culture conditions

Penicilium citrinum MTCC 2553 was procured from Institute of Microbial Technology (IMTECH), Chandigarh. It was maintained on Potato Dextrose Agar (PDA) slant and stored at 4°C. Seed culture was developed by inoculating single colony of P.citrinum MTCC 2553 into 50 mL nutrient medium (CYE) (pH 7) containing sucrose 3.0%, yeast extract 0.5%, K2HPO4 0.1%, NaNO3 3.0%, KCl 0.5%, MgSO4 0.5%, FeSO4 0.01% for 24h at 30°C in an orbital rotary shaker (Innova 4230, Newbrunswick, USA) at 200 rpm. Erlenmeyer flasks (250 mL) containing 50 mL of production medium of same composition were inoculated with 2 mL of seed culture and grown for 5 days according to the experimental design showed in Table 2. Samples were withdrawn after 5 days, centrifuged at 10,000 rpm for 10 min at 4ºC. Supernatant was used for analysis of xylanase activity [20]. Precipitate was used to determine the dry cell mass by hot air oven drying method at 100°C until a constant weight was achieved [21].

2.3 Enzyme activity assays

Xylanase activity was measured using 1% Birchwood xylan (4-O- metyl glucuronoxylan) solution as a substrate [22]. The release of reducing sugars in 5 min at 50°C and pH 5.3 (0.05 M citrate buffer) was measured as xylose equivalents using dinitrosalisylic acid method [23]. One unit of enzyme activity (U) is defined as the amount of enzyme liberating 1μmol of xylose per min.

2.4 Experimental design and data analysis

Four experimental factors (temperature, pH, agitation speed and substrate concentration) were found to have significant effect on xylanase production as determined during preliminary optimization studies [24]. RSM using a three level central composite design was applied to optimize the response of four variables. A 2^4 factorial design was used in order to study the effect of pH, temperature, agitation speed (rpm) and substrate (xylan) concentration. Preliminary experiments revealed that optimum incubation time for xylanase production was 5 days. Therefore enzyme activity after 5 days of production was measured as responses. The statistical analysis of the results was performed using Design Expert ver. 6.0.9 statistical software (Stat-Ease Inc, Minneapolis, MN). Xylanase activity was analyzed using the analysis of variance (ANOVA) combined with the Fischer test to evaluate if a given term has a significant effect ($p \leq 0.05$). The optimum levels of the variables were obtained by graphical and numerical analysis using Design Expert program.

3. RESULTS AND DISCUSSION

3.1 Optimization of culture conditions by central composite design

Temperature, pH, agitation speed, and substrate concentrations were chosen as the process parameters to optimize the conditions for maximum xylanase production by a statistical design (CCD and RSM). A CCD with three coded levels for all the four factors pH (A), temperature (B), agitation speed (C) and xylan concentration (D) were used for this purpose. The levels of parameters for the CCD were based on preliminary experimental results and are represented in Table 1.

Table 1 Levels of factors chosen for the experimental design.

Factors	Unit	Symbols	Actual levels of coded factors		
			-1	0	1
pH	-	A	6.5	7.0	7.5
Temperature	^oC	B	25	30	35
Agitation speed	rpm	C	190	200	210
Xylan Concentration	%	D	0.7	0.8	0.9

The central composite design and the results of the CCD obtained for xylanase production are presented in Table 2.

Table 2 Central composite design for the experimental design and predicted results for xylanase activity. (U/mL).

Run number	Block	Factors				Enzyme production	
						Experimental results	Predicted results
		A	B	C	D	Xylanase activity(U/mL)	Xylanase activity(U/mL)
1	Block 1	7.5	35.0	190	0.70	87.267	89.471
2	Block 1	7.0	30.0	200	0.80	66.633	67.834
3	Block 1	7.5	25.0	190	0.90	73.856	76.061
4	Block 1	7.0	30.0	200	0.80	77.509	78.711
5	Block 1	7.0	30.0	200	0.80	54.330	55.532
6	Block 1	6.5	25.0	210	0.70	63.332	65.536
7	Block 1	7.0	30.0	200	0.80	87.267	89.472
8	Block 1	6.5	35.0	210	0.90	98.430	99.632
9	Block 1	7.5	25.0	210	0.90	97.740	94.334
10	Block 1	6.5	25.0	190	0.70	97.740	94.334
11	Block 1	7.5	35.0	210	0.70	97.740	94.334
12	Block 1	6.5	35.0	190	0.90	97.740	94.334
13	Block 2	7.0	30.0	183	0.80	83.655	81.246
14	Block 2	7.0	30.0	200	0.80	149.508	147.099
15	Block 2	7.0	38.4	200	0.80	119.396	116.987
16	Block 2	7.8	30.0	200	0.80	40.559	38.151
17	Block 2	7.0	30.0	200	0.63	124.632	123.416
18	Block 2	6.2	30.0	200	0.80	134.935	131.334
19	Block 2	7.0	30.0	200	0.80	65.506	63.098
20	Block 2	7.0	30.0	216	0.80	66.423	64.014
21	Block 2	7.0	21.6	200	0.80	97.740	107.373
22	Block 2	7.0	30.0	200	0.97	97.740	107.373

A second order polynomial (equation 1) fitted well the experimental data and the coefficients of the equation 1 are reported in Table 3.

Table 3 Analysis of variance (ANOVA) for response surface quadratic model obtained from experimental designs.

Source	Sum of squares	DF	F value	Prob>F	
Model	13169.15	14	18.42	0.0009*	Significant
A	2168.315	1	42.45	0.0006*	
B	3107.597	1	60.84	0.0002	
C	75.67431	1	1.84	0.2692	
D	0.420261	1	8.22E-03	0.9307	
A2	89.04664	1	1.74	0.2348	
B2	1710.771	1	33.49	0.0012*	
C2	770.5274	1	15.09	0.0081*	
D2	3697.669	1	72.40	0.0001*	
AB	32.04957	1	0.63	0.4584	
AC	536.2927	1	10.50	0.0177*	
AD	2420.929	1	47.40	0.0005*	
BC	264.0942	1	5.17	0.0633	
BD	2093.451	1	40.99	0.0007*	
CD	239.2392	1	4.68	0.0736	
Residual	306.4538	6			
Lack of fit	306.4538	2			
Pure error	0	4			
Cor. Total	14655.99	21			

* Significant at $p>0.05$, $R^2 = 0.9773$, Adjusted $R^2 = 9242$.

The fitted equation (in terms of coded values) for xylanase production (Y) is expressed as:

$$Y = +100.85+19.58\ A-23.44\ B+2.35\ C+0.27\ D+2.40\ A^2-10.54\ B^2+7.0\ C^2-15.49\ D^2+3.11\ A\ B+8.19\ AC-27.03\ AD+5.75\ BC+25.13\ BD+5.47\ CD \quad (1)$$

where A is pH; B is temperature (°C); C is agitation speed (rpm); and, D is xylan concentration (%).

The efficiency of fit of the model was checked by the determination coefficient (R^2). In this case , the value of the determination coefficient (R^2 = 0.977) indicates that only 3% of the total variations are not explained by the model. The value of the adjusted determination coefficient (Adj. R^2 = 0.9242) is also very high, which indicates a high significance of the model [25, 26]. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable in this case the model has an 'adequate precision value' of 17.876 and at the same time a relatively lower values of the standard deviation (SD = 7.15) and coefficient of variation (CV = 7.94) indicate improved precision and reliability of the conducted experiments.

3.2 The analysis of variance (ANOVA)

ANOVA for the refind model is summarized in Table 3. The model F-value and probality value (P_{model}> F) of 18.42 and 0.0009 respectively for xylanase production indicate that the applied model is highly significant. Values of Prob>F less than 0.05 indicate model terms are significant. In this case A, B, B^2, C^2, D^2, AC, AD, BD are significant model terms. The optimum experimental conditions were obtained by the differentiation of the quadratic model to achieve maximum enzyme production. The optimum conditions were A = 7.49, B = 29º C, C = 209 rpm. and D = 0.75%. The predicted enzyme production corresponding to these levels was 150.53 U/mL (Table 4).

Table 4 Predicted values vs experimental values for maximum xylanase production.

Variables	Culture conditions	Enzyme activity (U/mL)	
		Predicted value	Experimental value
pH	7.49	150.53	149
Temperature(°C)	29.0		
Agitation speed (rpm)	209		
Xylan Concentration (%)	0.75		

3.3 Validation of the Experimantal Model

Validation of the predicted results was accomplished by performing additional experiments in triplicate with the parameters suggested by the numerical modeling (suggested solution). These three sets of experiments yielded an average enzyme production of 149 U/mL. Good agreement between the predicted and experimental results confirmed the experimental adequacy of the model and the existence of the optimal point.

The response surface describing the quadratic effect of agitation speed and temperature on xylanase production by *Penicilium citrinum* MTCC 2553 is shown in Figure 1.

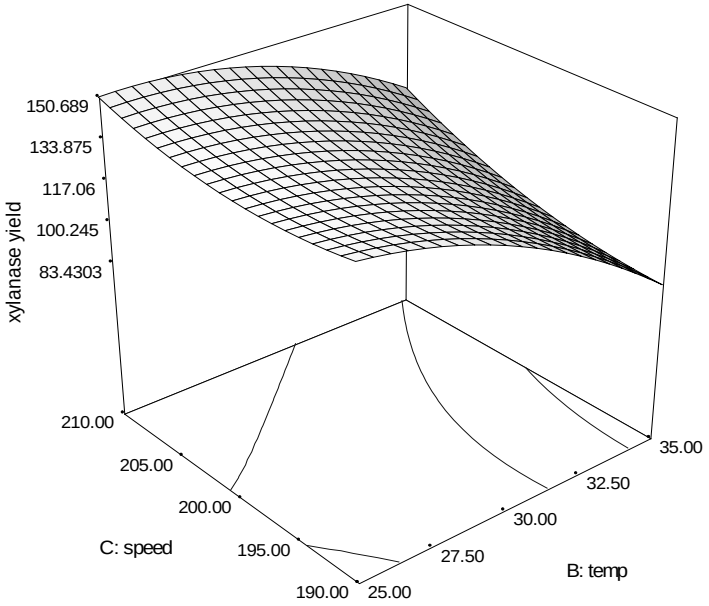

Figure 1. Effect of temperature and speed on xylanase production using P. citrinum.

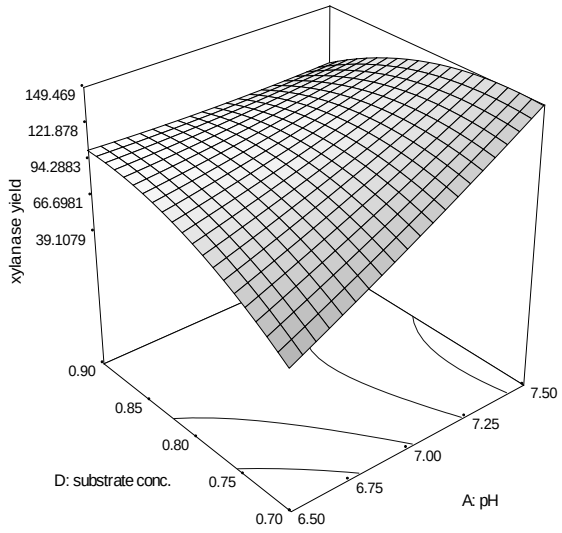

Figure 2. Eeffect of pH and substrate concentration on xylanase production using P. citrinum.

Here, the pH and substrate concentration were kept at the centre point values. Xylanase production inceased with agitation speed but decreased with increasing temperature. Maximum production of xylanase obtained at 209 rpm and 29°C. Figure 2 illustrate the effect of substrate concentration and pH on xylanase production in which agitation speed and temperature were kept at the centre point. Xylanase production increased with pH and substrate concentration. Maximum production of xylanase was obtained at pH 7.49 and 0.75% substrate concentration.

4. CONCLUSION

Penicillium citrinum MTCC 2553 is a promising organism for production of xylanase and statistical design like RSM can be used for optimization of process parameters and their interactions. RSM and CCD permitted studying and exploring fermentation conditions for the production of xylanase in just 22 experimental runs with overall three-fold increase in xylanase production. The optimum fermentation condition for the production of xylanase was established and the corresponding values of variables were: pH 7.49, temperature 29°C, agitation speed 209 rpm and substrate concentration 0.75%.

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