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A 2^3 Level Full Factorial Design to Optimize Cultural Conditions for Lipase Production by Consortium of *Aspergillus niger* and *Trichoderma viride*

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Abstract: In the presented research, lipase production was carried out using consortium of *Aspergillus niger* and *Trichoderma viride* (simultaneously) in shake flasks. The effect of three factors on the production rate of lipase was studied. The selected three factors were including different concentrations of rapeseed oil (1.0 and 1.5%), pH (5.0 and 6.0) and incubation temperature (25 and 30°C). These factors and their optimized levels were analyzed by using 2^3 level factorial designs. Rapeseed oil concentration and pH were found to have positive effects on the production and secretion rate of lipase from consortium. However, the effect of pH was being larger than concentration of Rapeseed oil. Temperature was also effective but with minor importance than the other two factors. Results indicated that optimum conditions for the production of lipase from *A. niger* and *T. viride* consortium are 30°C with 1.0% Rapeseed oil as carbon source and medium pH 5.0.

Key words: Rapeseed oil, pH, Incubation temperature, pareto chart, main effect plot

INTRODUCTION

Lipases (EC 3.1.1.3) are enzymes, the biological function of which is to catalyze the hydrolysis of tri-acylglycerol to fatty acids, mono- and di-acylglycerols and glycerol. Lipases act specifically at oil/water interfaces (Wang *et al*., 1999; Cihangir and Sarikaya, 2004). In the last few decades, lipases have received much attention with respect to their potential use in the laundry cleaning for the removal of fatty stains (Savitha *et al*., 2007).

A large number of microorganisms have been used for the production of lipases like *Fusarium oxysporum*, *Aspergillus niger*, *Trichoderma viride* and *Kluyveromyces lactis* (GutieÂrrez *et al*., 1999; Tamerler and Keshavarz, 2000). However, attempts are being made to over express lipase enzyme to commercially acceptable levels by using more than one strain synergistically.

In the present research, lipase production was carried out in the combined efforts of *Aspergillus niger* and *Trichoderma viride* consortium using Eggins and Pugh mineral salts solution as nutrient source and rapeseed oil as carbon source. A 2^3 full factorial design was employed to optimize the process in shake flasks. The results were analyzed using commercially available software, Minitab statistical software (2000) for Windows.

MATERIALS AND METHODS

Lipase Production and *Bioassay*

Aspergillus niger and *Trichoderma viride* obtained from the stock lab of Institute of Industrial Biotechnology, GC University, Lahore were grown at 30±1°C for 96 h on a solid medium composed of potato dextrose agar. The spore suspension in distilled water was made with a density containing 2.5×10^7 spore mL^{-1}. This suspension was used as an inoculum to carry out the fermentation process using sub-merged fermentation technique in shake flasks containing Eggins and Pugh (1962) mineral

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salts solution supplemented with 1.0% (v/v) rapeseed oil at 200 rpm. After 72 h of inoculation, the fermented broth was with drawn and centrifuged at 10,000 x g for 15 min and fungal free supernatant was then analyzed for lipase activity. Lipase activity was determined titrimatrically on the basis of olive oil hydrolysis, as reported by Kundu and Pal (1970). A lipase unit was defined as, the amount of enzyme which release one micromole fatty acids per minute under specified defined conditions.

Factorial Design

The experimental data obtained from three factors on the lipase production was analyzed using a 2^3 level full factorial design (Tamás *et al*., 2003). The major difference from a classical methodology (univariate analysis) is that this method enables to vary all of the factors. The classical method is laborious and time consuming, especially for a large number of variables. The factors to be studied were the effect of Rapeseed oil concentration (1.0 and 1.5%), pH (5.0 and 6.0) of the fermentation medium and temperature of incubation (25 and 30°C). Statistical analysis of the results was performed by fitting Eq. 01 to the response data (Y), i.e., lipase activity. Multilinear regression was performed using Minitab statistical software 13.20 (2000).

$$Y_u = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_1X_2X_3 \quad (1)$$

RESULTS AND DISCUSSION

These investigations are based upon the optimization of cultural conditions for the lipase production. Fermentation was carried out using consortium of *A. niger* and *T. viride* in the presence of rapeseed oil as substrate in addition with Eggins and Pugh (1962) mineral salts solution. The optimization was observed by varying three factors; different concentrations of Rapeseed oil, pH and incubation temperature. Two different values of each factor like rapeseed oil concentrations (1.0 and 1.5%), pH (5.0 and 6.0) and temperature (25 and 30°C) were followed.

The results obtained from the experimental work were analyzed using a 2^3 level full factorial design (Table 1). This design is an empirical modeling technique used to evaluate the relationship between a set of controllable experimental factors and their observed results. The design was applied after the analysis of experimental data by analysis of variance (ANOVA). The significance of each variable was determined by Fisher F-test and p-values, which are listed in Table 2. Here the double asteric signs (**) in the F and P column of Table 2 indicates that one-way interactions of the three factors are more significant than their 2-way and 3-way interactions (with single asteric) at 5.0% level of significance (Table 2 and 3).

All of the above considerations indicate an excellent adequacy of the regression model. The regression equation obtained after ANOVA gives the level production of lipase as a function of

Table 1: Source data and result of the creation of factorial design

					Coded values						
					----------						Lipase
No.	Standard order	Run order	Center point	Blocks	Temp	pH	Rapeseed oil (conc)	Temp (°C)	pH	Rapeseed oil (conc)	activity (U mL^{-1} min^{-1})
1	3	1	1	1	-1	1	-1	25	5	1.0	5.660
2	7	2	1	1	1	1	-1	25	5	1.5	6.870
3	6	3	1	1	1	-1	1	30	6	1.5	7.870
4	8	4	1	1	1	1	1	30	5	1.5	2.895
5	2	5	1	1	-1	-1	1	30	6	1.0	10.55
6	5	6	1	1	1	-1	-1	25	6	1.5	9.700
7	1	7	1	1	-1	-1	-1	25	6	1.0	5.400
8	4	8	1	1	-1	1	1	30	5	1.0	4.300

different factors: Rapeseed oil concentration, pH and temperature. All the terms regardless of their significance are included in the following equation:

$$Y_u = 6.656 - 0.252X_1 - 1.724X_2 + 0.178X_3 - 1.082\,X_1X_2 - 1.199X_1X_3 - 0.227X_2X_3 + 0.546X_1X_2X_3 \quad (2)$$

Where; Y_u is the fermentation response in terms of lipase activity (U mL^{-1} min^{-1}) and X_1, X_2 and X_3 are the coded values of the factors Rapeseed oil concentration, pH and temperature, respectively.

Regression model containing two linear and three interaction terms and one block term all the factors and their interactions have positive effects except the main effect of temperature, which gives a very low value of activity (0.178). These results were further interpreted in the form of interaction plot, main effect plot and Pareto chart at 5.0% level of significance. In Fig. 2 and 3, Pareto chart (general and standardized) shown that pH is the main parameter than other two main and all interactions of factors. Lipase bio-synthesis in the consortium of *A. niger* and *T. viride* is highly influenced by pH of the fermentation medium. On the other hand, temperature is almost neglectable as it does not exceed

Table 2: Analysis of variance (ANOVA) for the factorial design

Source	DF	Sq SS	Adj SS	Adj MS	F	p
Main effects	3	24.549	24.549	8.183	**	**
2-way interactions	3	21.283	21.283	7.094	*	*
3-way interactions	1	2.382	2.382	2.382	*	*
Residual error	0	0.000	0.000	0.000	-	-
Total	7	48.214	-	-	-	-

*Significant **more significant at 5% level of significant

Table 3: Estimated effect of different factors on lipase production

Factors	Effects
Rapeseed oil	-0.504
pH	-3.449
Temp	0.356
Rapeseed oil×pH	-2.164
Rapeseed oil×Temp	-2.399
pH×Temp	-0.454
Rapeseed oil×pH×Temp	1.091

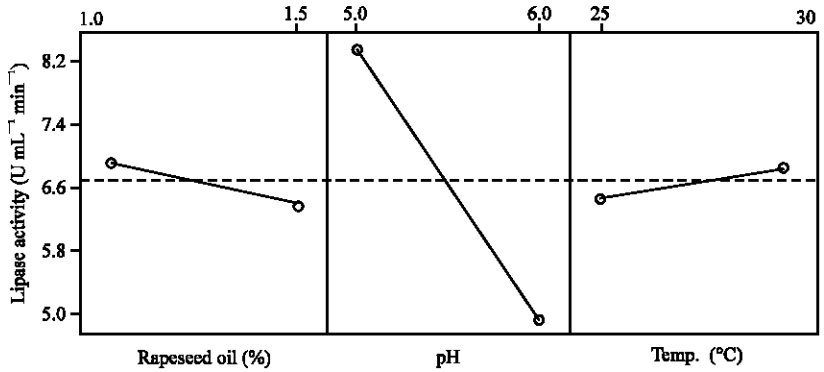

Fig. 1: Main effect plot of the three factors Rapeseed oil, pH and temperature on the lipase bio-synthesis. It summarizes the fact that at 1.0% Rapeseed oil, lipase activity is maximal but when the concentration increases further upto 1.5%, lipase activity decreases. Similar is the case with pH value, low pH (5.0) is the best rather than high pH (6.0). On the other hand, reversible effects are shown by the temperature that on low temperature lipase activity is low and higher temperature favors the lipase production

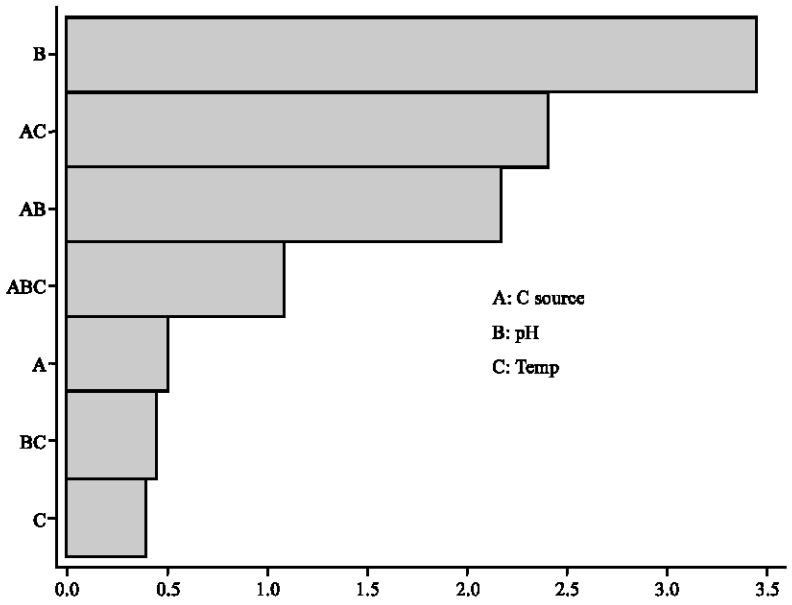

Fig. 2: Pareto chart of the general effects gives the profile of effective factors. It is pH > Rapeseed oil ×Temp>Rapeseed oil×pH>Rapeseed oil×pH×Temp>Rapeseed oil>pH×Temp and>Temp

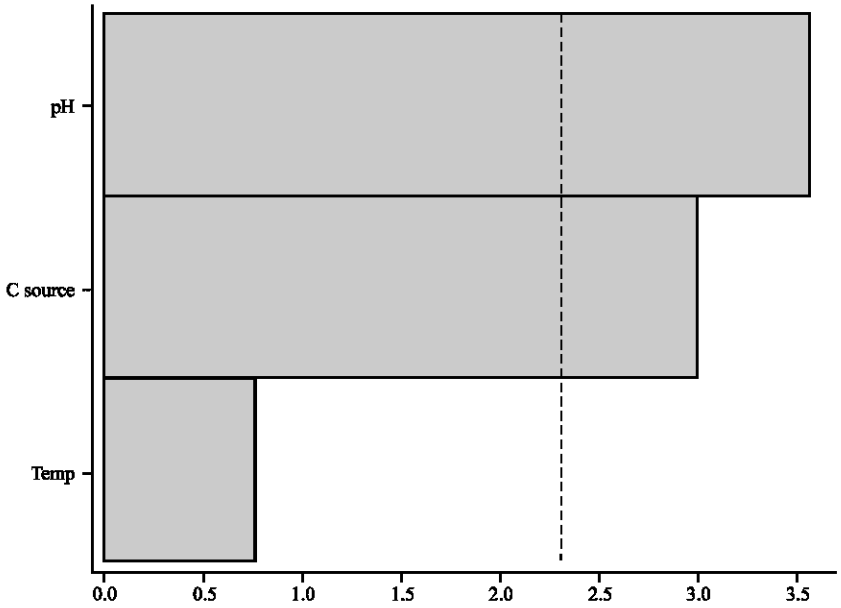

Fig. 3: Pareto chart of the standardized effects at 5.0% level of significance shows the effects of three factors Rapeseed oil, pH and temperature. Here is the indication that pH value highly affects the lipase production and it should be given the primary importance in the optimization of growth conditions of fungal consortium in lipase production

Table 4: Least square means for invertase production under different factors

Factors (Coded values)				Mean lipase activity (U mL^{-1} min^{-1})
Rapeseed oil				
-1				6.908
1				6.404
pH				
-1				3.380
1				4.931
Temp				
-1				6.478
1				6.834
Rapeseed oil		**pH**		
-1		-1		7.550
1		-1		9.210
-1		1		6.265
1		1		3.597
Rapeseed oil		**Temp.**		
-1		-1		5.530
1		-1		7.425
-1		1		8.285
1		1		5.382
pH		**Temp.**		
-1		-1		7.975
1		-1		4.980
-1		1		8.285
1		1		4.882
Rapeseed oil	**pH**		**Temp.**	
-1	-1		-1	5.400
1	-1		-1	10.55
-1	1		-1	5.660
1	1		-1	4.300
-1	-1		1	9.700
1	-1		1	7.870
-1	1		1	6.870
1	1		1	2.895

the reference line (Fig. 3), because any effect that exceeds reference line in the Pareto chart is more or less significant in the effectiveness point of view. The main effect plot for the experiment is given in Fig. 1. The use of the main effect is to determine which set of factors influence response and to compare the relative strength of the effects. The slope of the lines indicates pH effects are more as compared to Rapeseed oil concentration and temperature. If the slope of the line is parallel to the x-axis, then it implies that there is no main effect present. The larger the slope of the line, the stronger will be the main effect (Antony, 2002). Rosli *et al.* (2003) worked on CGase production using factorial design and reported similar kind of findings.

The results obtained from ANOVA, factorial design, main effect plot and Pareto chart shown that there is a relationship between lipase production and two variables of each factor. Their interactions first and secondly values directly effect the lipase production. Higher the concentration of Rapeseed oil and pH value, lower will be the production rate and vice versa. But production was seemed to be increased at higher temperature (optimum at 30°C). Hence, lipase bio-synthesis by consortium of *A. niger* and *T. viride* was found to be maximum with 1.0% Rapeseed oil at 30°C and medium pH 5.0 (Table 4).

CONCLUSIONS

Optimization of cultural conditions for the lipase production based on factorial design is numerically more complex as compared to the conventional univariate analysis. However, it provides better interpolation and more accurate prediction of optimal conditions when experimental data is

sufficiently accurate. This design enables the interpretation of factors or variables as these are related to the physiological and biochemical mechanisms of the fungal strains for the production and secretion of fermented products. This design also provides a time and cost effective strategy in order to check many factors and their multi-way interactions in a few instead of a chain of experiments for the production of industrial value products.

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