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Optimization of Nutritional Parameters for Production of Alpha Amylase Using *Aspergillus oryzae* MTCC 3017 by Central Composite Design

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Abstract

Amylases are the most widely used enzymes that are used many sectors such as clinical, medical, analytical and industrial applications. Beside their use in starch saccharification they also find applications in food, baking, brewing, detergent, textile and paper industries. Increasing utility and consumption of amylase in different industries has placed a greater stress on increasing indigenous enzyme production and search of more rapid processes. The aim of the present investigation is to achieve optimal amylase production by using the culture Aspergillus oryzae MTCC 3107. Central composite design was used to optimize the condition for the six independent variables Starch, Peptone and the four mineral elements. The critical values obtained at the end of experimentation resulted in amylase yield of 4496.72 units while the expected was 4116.37 units. The critical condition revealed, when investigated had resulted in a 9.24% increase in product concentration. An R^2 value of 0.96172 indicates 96% fit of the model. This states that the chosen statistical model for the production of amylase is a powerful to get good product yield.

Keywords: alpha amylase, *Aspergillus oryzae* MTCC 3107, nutritional parameters, optimization,

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INTRODUCTION

The method of chemical hydrolysis has been effectively replaced by microbial amylases in starch processing industries. Amylase is produced by several fungi, yeasts, bacteria and actinomycetes, though, enzymes produced by certain fungi and bacteria have dominated applications in industrial sectors. Chief benefit of using fungi for production of amylase is the economical bulk production capacity. Many species of *Aspergillus* such as *A. niger*, *A. tamarii, A. awamori* and *A. oryzae* have received most attention to obtain many kinds of hydrolytic enzymes like alpha amylase, lipase and protease. However, *A. oryzae* is the organism of choice because of its ubiquitous nature, non-fastidious nutritional requirements and high productivity of alpha amylase. [1-4] Usually production of amylase from fungi has been carried out using well defined chemical media by submerged fermentation (SmF) and solid state fermentation (SSF).[5]

No defined medium has been established for optimum production of enzymes from different microbial sources and each organism has its own special condition for maximum enzyme production. The use of a good reliable statistical model is essential to develop better strategies for the optimization of the fermentation process.[6] Response surface methodology is an experimental strategy for seeking the optimization of production medium that involves usage of many variables.[7] It consists of a group of mathematical and statistical procedures that can be used to study relationships between one or more responses and a number of independent variables. Compared to classical methods of optimization, central composite design (CCD) is more effective in bioprocess optimization. A full factorial CCD was applied to study various effects of starch, peptone and mineral salts for production of α-amylase using *Aspergillus oryzae* MTCC3017 by submerged fermentation.

MATERIALS AND METHODS

Microorganism

Aspergillus oryze MTCC 3017 procured from MTCC Microbial Type Culture Collection), Institute of Microbial Technology, Chandigarh, Punjab, was used throughout the study. The organism is maintained in PDA slants and is subcultured once in a month and stored at 4^{o}C.

Preparation of Seed Culture

Potato infusion medium is prepared[8] and is used for inoculating the production medium. The inoculum levels are maintained at 2% and added to the CCD design generated keeping inoculums age as 48 hours.

Production Medium

The production medium used in the present investigation is the optimized medium obtained from previous studies. The basic composition of medium is as follows: Starch – 20g/L, Peptone – 45 g/L, inoculums level 2% (v/v) and inoculum age – 48 hours. In the present investigation optimization of production medium using CCD is the objective and apart from starch and nitrogen the elements that were observed to have significant role in amylase production by *Aspergillus oryzae* MTCC 3107 are $CaSO_4$, $MgSO_4$. $7H_2O$, $FeSO_4$. $7H_2O$ and $MnSO_4$ H_2O (Subhash *et al*. 2015).[8] The concentrations of mineral supplements added were as per the design under investigation. The other physical parameters that were maintained during the entire study are pH- 5, temperature- 30^{o}C and 120 rpm.

Optimization of α-Amylase Production Using Central Composite Design (CCD)

Optimization of various factors that contribute for optimal production of α-amylase production by *Aspergillus oryzae* using CCD is the aim of study. The six variables chosen in the present investigation and the range of study are mentioned in Table 1. This CCD design gave an output of 32 runs for 6 factors which include Starch, Peptone, $CaSO_4$, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, $MnSO_4.H_2O$ whose uncoded and coded values are mentioned in Table 2. The second order polynomial coefficients were calculated and analysed using STASTICA 6.0 software. The variables were incorporated for all the experimental runs as given by the CCD design in Erlenmeyer flasks with 100 ml of production medium were inoculated with 2% inoculum whose age is 48h. All the flasks were incubated at 30^{o}C and 120 rpm. Samples were withdrawn very 24h and amylase activity was determined.

Statistical analysis and modelling: The data obtained from running the CCD design was subjected to the analysis of variance (ANOVA). The results were used to fit a second order polynomial equation (1) as it represents the behaviour of such a system more appropriately.

$$Y = \beta_0 + \beta_1X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_6 X_6 + \beta_1 \beta_1X_1^2 + \beta_2 \beta_2 X_2^2 + \beta_3 \beta_3 X_3^2 + \beta_4 \beta_4 X_4^2 + \beta_5 \beta_5 X_5^2 + \beta_6 \beta_6 X_6^2 + \beta_1\beta_2X_1X_2 + \beta_1\beta_3X_1X_3 + \beta_1\beta_4X_1X_4 + \beta_1\beta_5X_1X_5 + \beta_1\beta_6X_1X_6 + \beta_2\beta_3 X_2X_3 + \beta_3\beta_4X_3X_4 + \beta_4\beta_5X_4X_5 + \beta_5\beta_6X_5X_6 + \beta_2\beta_4X_2X_4 + \beta_2\beta_5X_2X_5 + \beta_2\beta_6X_2X_6 + \beta_3\beta_5X_3X_5 + \beta_3\beta_6X_3X_6 + \beta_4\beta_6X_4X_6 \quad \text{Eq (1)}$$

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Where Y is response variable, β_0 is intercept, β_1, β_2 and β_3 are linear coefficients, $\beta_1\beta_1$ to $\beta_6\beta_6$ are squared coefficient and other are interaction coefficient while X_1 to X_6, and other are interactions among independent variables. Statistical significance of the model equation was determined by Fisher's test value, and the production of variance explained by the model was given by the multiple coefficient of determination, R squared (R^2) value.

Analytical Methods

The amylase produced during the submerged fermentation process was estimated using the protocol of Swetha *et al*, (2007).[9] One unit of amylase is expressed as milligrams of maltose released per ml of production medium per hour at 37°C.

Table 1. *Range of Values for CCD Design for the Production of Amylase by Aspergillus oryzae.*

Independent factors	**Coded factors levels**				
	–2	**–1**	**0**	**+1**	**+2**
Starch (g/L)	10	15	20	25	30
Peptone (g/L)	15	30	45	60	75
$CaSO_4$ (mg/L)	0.5	0.75	1.0	1.25	1.50
$MgSO_4.7H_20$ (mg/L)	0.5	0.75	1.0	1.25	1.50
$FeSO_4.7H_20$ (mg/L)	0.5	0.75	1.0	1.25	1.50
$MnSO_4.H_20$ (mg/L)	0.5	0.75	1.0	1.25	1.50

Table 2. *Central Composite Design Representing the Coded Values and Levels for the Six Independent Variables.*

S. no	**Starch (g/l)**	**Peptone (g/l)**	**$CaSO_4$(mg/l)**	**MgSO4.7H_2O (mg/l)**	**$FeSO_4.7H_2O$ (mg/l)**	**$MnSO_4.H_2O$ (mg/l)**	**Starch (g/l)**	**Peptone (g/l)**	**$CaSO_4$ (mg/l)**	**MgSO4.7H_2O (mg/l)**	**$FeSO_4.7H_2O$ (mg/l)**	**$MnSO_4.H_2O$ (mg/l)**	**Amylase units**
1	1	1	1	1	-1	-1	25.00000	60.00000	1.250000	1.250000	0.750000	0.750000	2360
2	1	1	1	-1	1	-1	25.00000	60.00000	1.250000	0.750000	1.250000	0.750000	2070
3	1	1	-1	1	-1	1	25.00000	60.00000	0.750000	1.250000	0.750000	1.250000	2700
4	1	-1	1	-1	1	1	25.00000	30.00000	1.250000	0.750000	1.250000	1.250000	2900
5	-1	1	-1	1	1	1	15.00000	60.00000	0.750000	1.250000	1.250000	1.250000	2850
6	1	-1	1	1	-1	1	25.00000	30.00000	1.250000	1.250000	0.750000	1.250000	2860
7	-1	1	1	-1	-1	-1	15.00000	60.00000	1.250000	0.750000	0.750000	0.750000	2200
8	1	1	-1	-1	1	1	25.00000	60.00000	0.750000	0.750000	1.250000	1.250000	2480
9	1	-1	-1	1	-1	-1	25.00000	30.00000	0.750000	1.250000	0.750000	0.750000	3310
10	-1	-1	1	-1	-1	1	15.00000	30.00000	1.250000	0.750000	0.750000	1.250000	2620
11	-1	1	-1	-1	-1	1	15.00000	60.00000	0.750000	0.750000	0.750000	1.250000	2250
12	1	-1	-1	-1	1	-1	25.00000	30.00000	0.750000	0.750000	1.250000	0.750000	2700
13	-1	-1	-1	1	1	-1	15.00000	30.00000	0.750000	1.250000	1.250000	0.750000	1860
14	-1	-1	1	1	1	1	15.00000	30.00000	1.250000	1.250000	1.250000	1.250000	1750
15	-1	1	1	1	1	-1	15.00000	60.00000	1.250000	1.250000	1.250000	0.750000	1700
16	-1	-1	-1	-1	-1	-1	15.00000	30.00000	0.750000	0.750000	0.750000	0.750000	2200

17	-1	0	0	0	0	0	10.00000	45.00000	1.000000	1.000000	1.000000	1.000000	1350
18	2	0	0	0	0	0	30.00000	45.00000	1.000000	1.000000	1.000000	1.000000	3400
19	0	-2	0	0	0	0	20.00000	15.00000	1.000000	1.000000	1.000000	1.000000	1830
20	0	2	0	0	0	0	20.00000	75.00000	1.000000	1.000000	1.000000	1.000000	3670
21	0	0	-2	0	0	0	20.00000	45.00000	0.500000	1.000000	1.000000	1.000000	2800
22	0	0	2	0	0	0	20.00000	45.00000	1.500000	1.000000	1.000000	1.000000	2400
23	0	0	0	-2	0	0	20.00000	45.00000	1.000000	0.500000	1.000000	1.000000	2350
24	0	0	0	2	0	0	20.00000	45.00000	1.000000	1.500000	1.000000	1.000000	2800
25	0	0	0	0	-2	0	20.00000	45.00000	1.000000	1.000000	0.500000	1.000000	2540
26	0	0	0	0	2	0	20.00000	45.00000	1.000000	1.000000	1.500000	1.000000	1940
27	0	0	0	0	0	-2	20.00000	45.00000	1.000000	1.000000	1.000000	0.500000	2590
28	0	0	0	0	0	2	20.00000	45.00000	1.000000	1.000000	1.000000	1.500000	3160
29 (C)	0	0	0	0	0	0	20.00000	45.00000	1.000000	1.000000	1.000000	1.000000	3680
30 (C)	0	0	0	0	0	0	20.00000	45.00000	1.000000	1.000000	1.000000	1.000000	3620
31 (C)	0	0	0	0	0	0	20.00000	45.00000	1.000000	1.000000	1.000000	1.000000	3650
32 (C)	0	0	0	0	0	0	20.00000	45.00000	1.000000	1.000000	1.000000	1.000000	3580

RESULTS AND DISCUSSION

The amylase activity produced at the end of 72 h is presented in Table 2. Maximum production was obtained for the centre point runs 29 to 32. The 20th experimental run resulted in maximum yield of 3670 amylase units while the central point's 29 and 32 resulted in 3680 amylase units.

Table 3 represents the predicted values, observed values and the deviation that is seen when the experimental runs were carried out for all the six chosen variables. Regression analysis for experimental data was carried out (Table 4) and is represented in the following second order polynomial equation shown amylase yield. The p values indicate that among the six variables except for $MnSO_4$ the rest of the variables were significant in the production of amylase by *Aspergillus oryzae*. The final response equation that represents the suitability of the model for α-amylase production is as follows:

Amylase activity (Y) = $3477.25 + 512.5 X_1 + 460 X_2 – 100 X_3 + 112.5 X_4 – 150 X_5 + 142.5 X_6 – 236.75 X_1^2 – 143.0 X_2^2 – 180.5 X_3^2 – 186.75 X_4^2 – 270.5 X_5^2 – 111.75 X_6^2 – 170.625 X_1 X_2 – 6.875 X_1 X_3 – 13.125 X_1 X_4 + 114.375 X_1 X_5 – 63.125 X_1 X_6 + 16.875 X_2 X_3 + 78.125 X_2 X_4 + 85.625 X_2 X_5 + 18.125 X_2 X_6 – 138.125 X_3 X_4 – 65.625 X_3 X_5 + 559.375 X_3 X_6 + 265.625 X_4 X_5 – 9.375 X_4 X_6 + 80.626 X_5 X_6$

An R^2 value of 0.96172 indicates 96% fit with the model. The profiles for predicted values and desirability are represented in Figure 1. The graphs of all the independent variables indicate their impact on the amylase yield. Starch has a negative impact beyond 25g/l while peptone reaches a stationary point beyond which further addition of the protein source has no impact on the product yield. The concentration range selected for the salts also indicate that an initial increase in salt concentration has a positive impact on amylase production, and addition beyond certain limit has a negative impact. As per the figure the centre points values correlate with the optimal values.

Table 3. *Observed Versus Predicted Values.*

Experimental run	Observed amylase activity	Predicted amylase activity	Deviation
1	2360.000	2282.375	77.625
2	2070.000	1992.375	77.625
3	2700.000	2622.375	77.625
4	2900.000	2822.375	77.625
5	2850.000	2772.375	77.625
6	2860.000	2782.375	77.625
7	2200.000	2122.375	77.625
8	2480.000	2402.375	77.625
9	3310.000	3232.375	77.625
10	2620.000	2542.375	77.625
11	2250.000	2172.375	77.625
12	2700.000	2622.375	77.625
13	1860.000	1782.375	77.625
14	1750.000	1672.375	77.625
15	1700.000	1622.375	77.625
16	2200.000	2122.375	77.625
17	1350.000	1505.250	-155.250
18	3400.000	3555.250	-155.250
19	1830.000	1985.250	-155.250
20	3670.000	3825.250	-155.250
21	2800.000	2955.250	-155.250
22	2400.000	2555.250	-155.250
23	2350.000	2505.250	-155.250
24	2800.000	2955.250	-155.250
25	2540.000	2695.250	-155.250
26	1940.000	2095.250	-155.250
27	2590.000	2745.250	-155.250
28	3160.000	3315.250	-155.250
29	3680.000	3477.250	202.750
30	3620.000	3477.250	142.750
31	3650.000	3477.250	172.750
32	3580.000	3477.250	102.750

Table 4. *Multiple Repression Analysis for Chosen Variables.*

	Coeff.	Std.Err.	t(4)	p
Mean/Interc.	3477.250	156.1292	22.27163	0.000024*
(1)Starch (g/l)(L)	512.500	246.8619	4.15212	0.014236*
Starch (g/l)(Q)	-236.750	129.4555	-3.65763	0.021625*
(2)Peptone (g/l)(L)	460.000	246.8619	3.72678	0.020354*
Peptone (g/l)(Q)	-143.000	129.4555	-2.20925	0.091702
(3)$CaSO_4$ (mg/l)(L)	-100.000	246.8619	-0.81017	0.463287
$CaSO_4$ (mg/l)(Q)	-180.500	129.4555	-2.78860	0.049382*
(4)$MgSO_4.7H_2O$(mg/l)(L)	112.500	246.8619	0.91144	0.413623
$MgSO_4.7H_2O$ (mg/l)(Q)	-186.750	129.4555	-2.88516	0.044780*

(5)$FeSO_4.7H_2O$ (mg/l)(L)	-150.000	246.8619	-1.21525	0.291092
$FeSO_4.7H_2O$ (mg/l)(Q)	-270.500	129.4555	-4.17904	0.013929*
(6)$MnSO_4.H_2O$ (mg/l)(L)	142.500	246.8619	1.15449	0.312576
$MnSO_4.H_2O$ (mg/l)(Q)	-111.750	129.4555	-1.72646	0.159341
1L by 2L	-170.625	174.5577	-1.95494	0.122262
1L by 3L	-6.875	174.5577	-0.07877	0.940998
1L by 4L	-13.125	302.3428	-0.08682	0.934986
1L by 5L	114.375	302.3428	0.75659	0.491411
1L by 6L	-63.125	174.5577	-0.72326	0.509553
2L by 3L	16.875	302.3428	0.11163	0.916495
2L by 4L	78.125	174.5577	0.89512	0.421319
2L by 5L	85.625	174.5577	0.98105	0.382114
2L by 6L	18.125	302.3428	0.11990	0.910346
3L by 4L	-138.125	174.5577	-1.58257	0.188685
3L by 5L	-65.625	174.5577	-0.75190	0.493934
3L by 6L	559.375	302.3428	3.70027	0.020831
4L by 5L	265.625	302.3428	1.75711	0.153734
4L by 6L	-9.375	174.5577	-0.10741	0.919632
5L by 6L	80.625	174.5577	0.92376	0.407891

**Indicates p Values < 0.05.*

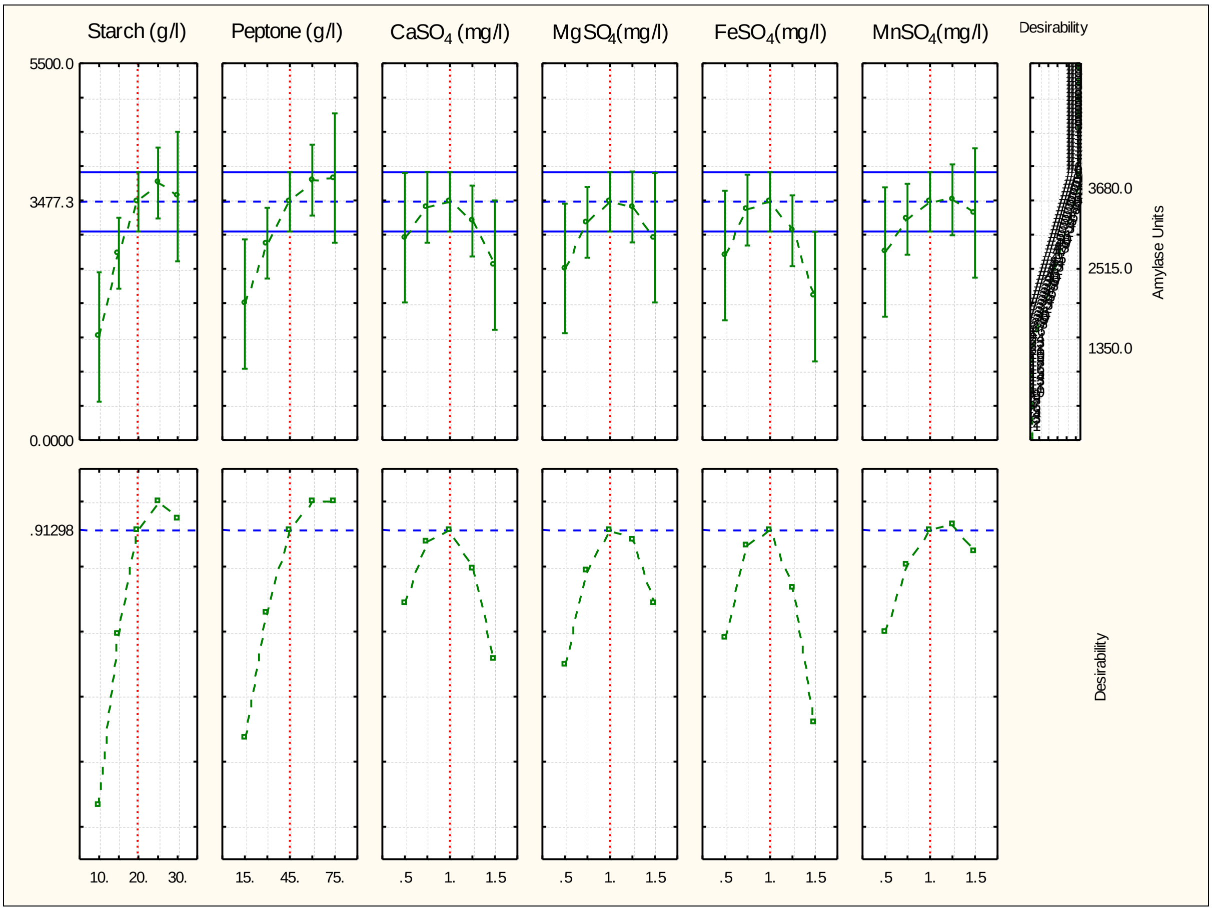

***Fig. 1.** Representation of Profiles for Predicted Variables and Desirability.*

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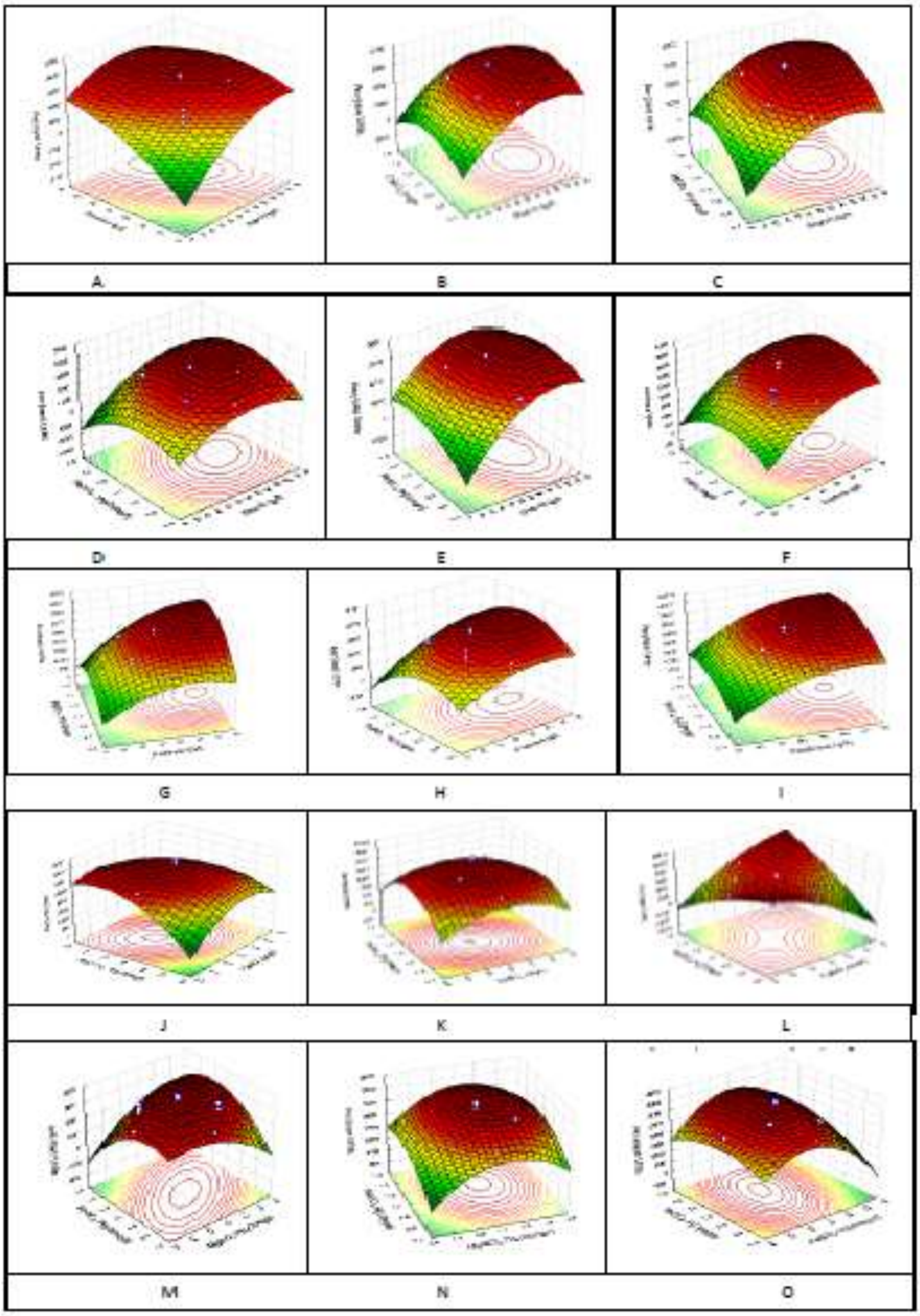

Fig. 2. *Response Surface Graphs of all the Independent Variables Under Investigation.*

Figure 1 indicates the effect of each variable independently on the production of amylase by *Aspergillus oryzae*. Starch has a direct contribution to the production of amylase until a concentration of 25g/L while further increase had a slight negative impact which may be due to increase in viscosity of the medium. Peptone had a linear effect till 60 g/L while further increase had no contribution to increase in product concentration. This may be due to the limitation with respect to substrate or other parameters under investigation. The four mineral elements i.e., $CaSO_4$, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$ and $MnSO_4.H_2O$, initially contributed to amylase production, while further increase had a negative impact. These indicate that a good range is taken for all the variables under investigation. The relationship between independent variable is represented in the form of 3D response surface plots. The Figure 2(A–O) indicate the response surface plot for the 6 independent variable and their interrelationship for the optimal production of the product amylase.

Figure 2A indicates the effect of starch and peptone on amylase production by *Aspergillus oryzae*. It may be seen that increase in starch content with a simultaneous increase in protein content has led to an increase in the product formation. Both have no negative impact and a peak is seen at central point substitutions of starch and peptone. Beyond the levels starch had a slight negative impact while peptone has no contribution to amylase production. It would have been used for biomass production.

Figure 2B indicates the effect of starch and calcium sulphate on amylase production. Calcium is a microelement substituted to the production medium to analyze its effect on final amylase activity. Calcium is known to have a stabilizing effect on amylases.[10] It can also act as in inducer for amylase production. The preliminary studies indicate that excess starch has a negative impact on amylase production due to its gelling effect, and Calcium in excess is slightly toxic to the microbial growth. The interaction among the two factors and their contribution to amylase production indicates that optimal supplementation of the two is essential for maximum product yield.

The effect of starch and Magnesium sulphate is represented in Figure 2C. As indicated by Najwa Mohammed (2013)[11], Magnesium has a positive impact on the growth of organism. It is divalent ion that has major role to play in the catalysis of biochemical reaction in vivo. Supplementation of Magnesium has led to an increase in amylase production upto 1 mg/L beyond which it had a negative impact just like starch that had positive impact upto 20 g/L. Increase in concentration of both the independent variables had a negative impact of the dependent variable and there was a decrease in the product formation.

The effect of Ferrous sulphate and starch on amylase production is Figure 2D and E shows the effect of manganese sulphate on amylase production. The works of Foster (1939), Bertrand et al (2004) and Steinberg (1936) indicated the significance of supplementation of Manganese and Iron to microorganisms.[12-14] Manganese at 1ppm was reported to be effective while a good balance of both the ions was required as they function synergistically. Both the figures indicate that supplementation of the salts beyond certain limits has an inhibitory effect on the production as indicated in by Foster (1939). [12]

The effect of the nutrient peptone that acts as the nitrogen source and other micronutrients is represented in Figures 2F–I. Peptone as such does not have a negative impact but excess supplementation adds to fungal biomass. All the figures indicate that the salts supplementation at optimal concentrations resulted in maximum production of amylases while further increase has a negative impact on the product formation. A clear peak is visible for all the four interaction effects.

Figure 2J indicates the effect of ferrous sulphate and calcium sulphate on amylase production. Both the salts are required for mycelial growth at low concentration and from the levels chosen a clear peak indicate that the centre point concentrations have resulted din maximum yield while value above and below had resulted in decrease in amylase concentration.

The response surface graph represented in Figure 2K is that of the effect of calcium sulphate and Magnesium sulphate. The saddle shaped graph indicates that the range chosen for any one of the variables does not fit in the study. Either an increase or a decrease in the salt concentration would be recommended as it will be given by the software as optimal values to be supplemented.

Figures 2L–O represent the interaction between the critical variables Ferrous sulphate, Calcium sulphate and Mangansese sulphate. As indicated by Foster (1939)[12] all the variables are critical for the growth of fungi. The peaks obtained in the response surface graphs indicate the fit of the study. Increase or decrease in supplementation of variables beyond the optimal values effected amylase production.

In the present investigation the optimal conditions to be maintained to get maximum production were as follows: Starch -22.43 g/l, Peptone -75.11 g/l, $CaSO_4$ -0.95mg/l, $MgSO_4.7H_2O$- 1.35 mg/l, $FeSO_4.7H_2O$ -1.23 mg/l and MnSO-$_4$.H_2O – 1.11mg/l. With the obtained critical values experiments were conducted in triplicate. As per the model the amylase yield expected was 4116.37 units. The average of triplicate run obtained after experimentation was 4496.72 units. An increase in amylase production by 9.24% could be obtained using central composite design at the end of 72hours of fermentation. The R^2 value and the increase in amylase production validate that the technique adopted to design the production medium for amylase production by central composite design is a powerful tool.

CONCLUSION

The aim of the present investigation is to achieve optimal amylase production by using the culture *Aspergillus oryzae* MTCC 3107. Central composite design was used to optimize the condition for the six independent variables Starch, Peptone and the four mineral elements. The critical values obtained at the end of experimentation and analysis are Starch -22.43 g/l, Peptone -75.11 g/l, $CaSO_4$ -0.95mg/l, $MgSO_4.7H_2O$- 1.35 mg/l, $FeSO_4.7H_2O$ -1.23 mg/l and $MnSO_4.H_2O$ – 1.11mg/l. As per the model the amylase yield expected was 4116.37 units. The average of triplicate run obtained after experimentation was 4496.72 units. The critical condition revealed, when investigated had resulted in a 9.24% increase in product concentration. An R^2 value of 0.96172 indicates 96% fit of the model. This states that the chosen statistical model for the production of amylase is a powerful to get good product yield.

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