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Optimization of Medium Components for Phytase Production by *Hypocrea lixii* SURT01 using Response Surface Methodology

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In monogastric animals the ability of digesting phytase is lacking which hydrolysis phytate to release phosphate. This paper concentrates on the commercial production of phytase which replenish the need of phosphate in livestock feed production. Response surface methodology was applied for the optimization of phytase production by *Hypocrea lixii* SURT01. Four factors such as carbon source (sucrose), nitrogen source (peptone), substrate (phytate) in different concentration and pH were optimized using central composite design (CCD). The optimum levels of variables that supported maximum enzyme activity were 1.5% sucrose, 0.2% phytate, 3% peptone and pH 7.5. The validity of the model in optimized conditions was verified. With this composition, the phytase production was 74.56IU (average of three repeats) after 96 h of cultivation, while the predicted maximum production was 73.42 IU. The optimized medium resulted in significant increase of the phytase yield by *Hypocrea lixii* in shake-flask cultivation.

Key words: Phytase, *Hypocrea lixii* SURT01, Central composite design, Response surface methodology.

Phytase which is produced by many microorganism and plant are capable of hydrolyzing phytate which is a major storage form of phosphate in plant seeds during maturation (Mitchell *et al*., 1997). Phytate in animal feedstuff is not digested by monogastric animals such as pigs, fish, and poultry, because they lack the microorganisms that produce phytases in their digestive tracts (Cromwell *et al*., 1991). The undigested phytate is then excreted in the manure, thereby causing serious phosphate pollutions,(Dae-Hee Lee *et al*, 2005) especially in the area of intensive livestock production (Cromwell and Coffey, 1993). This problem can be solved by supplementing phytase in feedstuff to improve the availability of both phosphates, and protein (Murry *et al*., 1997; Nelson *et al*., 1971).

Phytases convert phytate to partially phosphorylated *myo*-inositol and phosphate, making phosphorus available for absorption (Irving and Cosgrove, 1972). Supplementation of microbial phytase to animal diets alters the phytate complexes and also increases the bioavailability of proteins and essential minerals, providing growth performance equivalent or better than those with phosphate supplementation, and also reduces the amount of phosphorus in animal manure (Wodzinski and Ullah, 1996).

Conventional methods for optimization of phytase parameters are extremely time consuming and expensive. Response surface methodology (RSM) is efficient in handling large number of design parameters (Sonia Dahiya *et al*., 2009) In this present study optimum parameters such as

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carbon source concentration (sucrose), nitrogen source concentration (peptone), substrate concentration (phytate) and pH for phytase production from *Hypocrea lixii* SURT01 using RSM based on central composite design.

MATERIALS AND METHODS

Organism and culture maintenance

The fungal strain was isolated from poultry field soil by screen plate method was further identified by conventional method and confirmed by 18S rRNA T1 sequencing and had a similarity with *Hypocrea lixii*.

Inoculum preparation

The 7 days old culture from yeast extract agar slant was suspended with sterile distilled water with 0.1% Tween80 and the slant surface was scrapped with glass rod. The slurry was filtered through cheese cloth to remove mycelia debris and the resulting filtrate was used as source of inocula and the spore count was made using hemocytometer.

Cultivation medium and Fermentation technique.

The standard semi-synthetic fermentation medium(M1 medium)used in this work, contained in g/L: starch, 28; glucose, 5; peptone, 18; Potassium chloride (KCl), 0.5;Magnesium Sulfate ($MgSO_4$). $7H_2O$, 1.5; Potassium dihydrogenPhosphate (KH_2PO_4), 1; Calcium Chloride ($CaCl_2$). H_2O, 2 100 mL in 250 mL Erlenmeyer flasks, were sterilized at 120 ºC for 20 min (autoclave pressure: 0.14 MPa). (Maria Papagianni, SueNokes and Keith Filter 2001)

Phytaseassay

Phytase activity was measured in an assay mixture containing 44.1mM phytic acid and 200mM glycine buffer (pH 2.8) and suitably diluted enzyme. Reaction mixture is incubated at 37°C for 30 minutes, colour reagent was added and the developed colour was read colorimetrically at 400nm. One enzyme unit was defined as the amount of enzyme liberating 1µmol of inorganic phosphate in 1min under the assay conditions. (Heinomen and Lathi, 1981, Thyagarajan and Namasivayam, 2010).

Optimization of components - *Mathematical-statistic procedure.*

Response surface methodology (RSM) was employed to optimize the four significant factors such as carbon source - sucrose (A), phytic acid (B), nitrogen source - peptone(C), and pH (D), for enhancing phytase production. This four independent variables were studied at three different levels with the minimum and maximum ranges were investigated and listed in Table 1 and a set of 30 experimental runs were carried out (Table 2) using the statistical software package 'Design Expert 7.0.0 – Stat-Ease Inc. Minneapolis' was used to analyze the experimental data.

Upon completion of experiments, the average maximum phytase biosynthesis yield were taken as the responses (R). A multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response measured to the independent variables. A second order polynomial equation for a four factor system is:

$$R1 = +67.79 +1.55A -3.16B + 13.62C +1.97D -3.90AB +6.41 *AC+1.85 * AD -7.46 * BC -0.86 * B * D + 4.87 * C * D - 9.59 * A2 - 9.73 B2 -5.81 C2-14.89* D2$$

Where R1 is the predicted response, and A, B, C, D, A^2, B^2, C^2, D^2,AB, BC, CD, AC, AD, BD are levels of the independent variables. The response surface curveswere obtained the 'Design Expert 7.0.0 software for determining the optimum levels of the variables for maximum production of phytase.

RESULTS AND DISCUSSION

Fungal strain

The fungal strain was isolated from poultry field soil and identified by conventional method and confirmed by 18S rRNA T1 sequencing and had a similarity with as *Hypocrea lixii.* This sequences was submitted to Genebank it accession number HQ75779. The 7 days old culture from yeast extract agar slant was suspended with sterile distilled water with 0.1% Tween 80 and the slant surface was scrapped with glass rod. The slurry was filtered through cheese cloth to remove mycelia debris and the resulting filtrate was used as source of inocula and the spore count was made using hemocytometer.

Response surface methodology (RSM) using CCD was applied to determine the optimum

levels of the four selected variables that affects phytase production, and the mean predicted and observed responses are presented in Table 2.

The regression equations obtained after

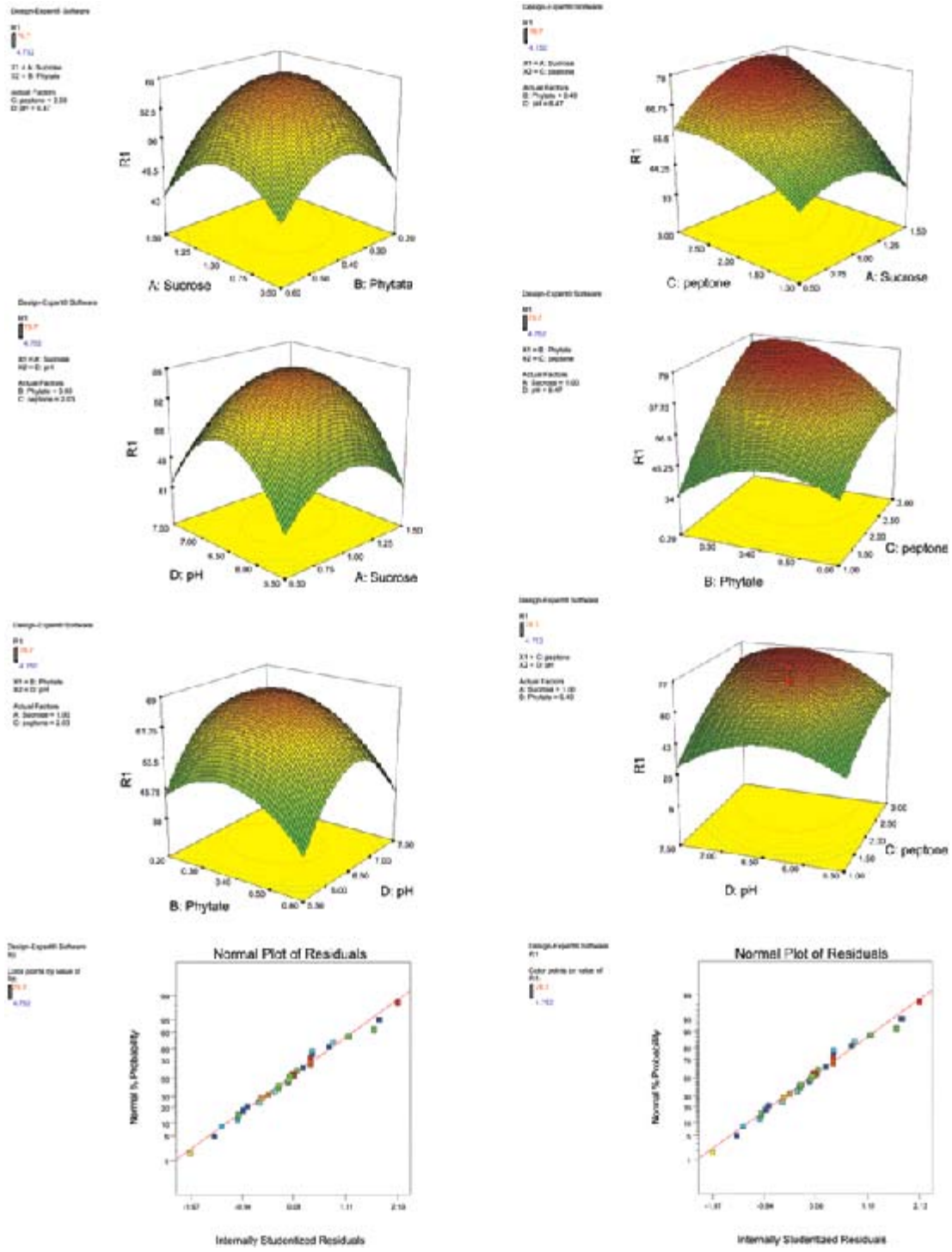

Fig. 1. Response surface plot of extracellular phytase production as a function of phytate and sucrose concentration.

the analysis of variance (ANOVA) provided the levels of phytase produced as a function of the values of sucrose concentration, phytate concentration, peptone concentration and pH. The production of phytase could be predicted by the model:

Response = -642.215 + 45.826 * Sucrose + 320.112 * Phytate +7.275 * peptone+183.812 * pH - 39.037 * Sucrose * Phytate + 12.811 * Sucrose * peptone +3.691 * Sucrose * pH - 37.275 * Phytate * peptone -4.280 * Phytate * pH + 4.873 * peptone * pH -38.361 * Sucrose2 - 243.133 * Phytate2 - 5.808 * peptone2 - 14.89 * pH2

Table 1. Ranges of the three independent variables variation used in RSM

Code	Independent variables	Levels		
A	sucrose	0.5	1	1.5
B	phytic acid	0.2	0.4	0.6
C	peptone	1	2	3
D	pH	5.5	6.5	7.5

The coefficient of determination (R^2) was calculated to be 0.98 for phytase production. This implies that 98% of experimental data of the phytase production was compatible with the data predicted by the model (Table 3). However, Pred R^2 of 0.932 is close to the Adj R^2 of 0.962, probably indicating a large block effect (Table 3).

The significance of the model was confirmed as the probability was less than 0.0001. Therefore, among the model terms in this study, Sucrose (A), Phytate (B), Peptone (C) and pH (D) are significant model terms. Table 3 also indicate that the interaction between A and A, B and B, C

Table 2. Experimental plan for optimization of phytase production using RSM

Std	Run	A:Sucrose	B:Phytate	C:peptone	D:pH	Response	
						Actual	Predicted
4	1	1.5	0.6	1	5.5	9.12	11.61
13	2	0.5	0.2	3	7.5	45.37	46.00
15	3	0.5	0.6	3	7.5	35.81	30.87
9	4	0.5	0.2	1	7.5	9.25	6.92
28	5	1	0.4	2	6.5	76.7	67.79
20	6	1	0.8	2	6.5	25.08	22.57
14	7	1.5	0.2	3	7.5	73.56	73.42
26	8	1	0.4	2	6.5	59.56	67.79
8	9	1.5	0.6	3	5.5	25.25	27.00
3	10	0.5	0.6	1	5.5	33.25	32.82
27	11	1	0.4	2	6.5	69.53	67.79
25	12	1	0.4	2	6.5	69.53	67.79
22	13	1	0.4	4	6.5	73.04	71.80
16	14	1.5	0.6	3	7.5	39.69	42.67
1	15	0.5	0.2	1	5.5	14.56	14.71
19	16	1	0.6	2	6.5	35.25	35.21
11	17	0.5	0.6	1	7.5	18.56	21.61
5	18	0.5	0.2	3	5.5	33.59	34.30
24	19	1	0.4	2	8.5	9.453	12.17
29	20	1	0.4	2	6.5	65.45	15.45
6	21	1.5	0.2	3	5.5	54.25	54.33
17	22	0	0.4	2	6.5	25.45	26.33
2	23	1.5	0.2	1	5.5	4.752	9.12
10	24	1.5	0.2	1	7.5	9.545	8.71
30	25	1	0.4	2	6.5	65.98	67.79
18	26	2	0.4	2	6.5	35.96	32.53
7	27	0.5	0.6	3	5.5	18.63	22.59
12	28	1.5	0.6	1	7.5	9.068	7.78
21	29	1	0.4	0	6.5	18.63	17.32
23	30	1	0.4	2	4.5	9.56	4.30

Table 3. Test of significance for regression coefficient

Source	Sum of Squares	df	Mean Square	F Value	p-value	Prob> F
Model	16098.26	14	1149.876	54.83087	< 0.0001	Significant
A-Sucrose	57.76855	1	57.76855	2.754645	0.1177	
B-Phytate	239.6481	1	239.6481	11.42742	< 0.0041	
C-peptone	4451.697	1	4451.697	212.2754	< 0.0001	
D-pH	92.97226	1	92.97226	4.433304	< 0.0525	
AB	243.8204	1	243.8204	11.62637	< 0.0039	
AC	656.4485	1	656.4485	31.30219	< 0.0001	
AD	54.505	1	54.505	2.599025	0.1278	
BC	889.2473	1	889.2473	42.40301	< 0.0001	
BD	11.72206	1	11.72206	0.558957	0.4662	
CD	379.9478	1	379.9478	18.11749	<0.0007	
A^2	2522.723	1	2522.723	120.2939	< 0.0001	
B^2	2594.247	1	2594.247	123.7045	< 0.0001	
C^2	925.1879	1	925.1879	44.1168	< 0.0001	
D^2	6081.204	1	6081.204	289.9771	< 0.0001	
Residual	314.5699	15	20.97133			
Lack of Fit	152.642	10	15.2642	0.471327	0.8542	Not significant
Pure Error	161.9279	5	32.38558			
Cor Total	16412.83	29				

Table 4. Analysis of variance (ANOVA) for regression

Std. Dev.	4.579446	R-Squared	0.980834
Mean	35.78093	Adj R-Squared	0.962946
C.V. %	12.79856	Pred R-Squared	0.932224
PRESS	1112.394	Adeq Precision	21.34564

and C, D and D, A and B, A and C, A and D, B and C, B and D, C and D, had very significant influence on phytase yield by the fungal strain used in this study. The Model F-value of 54.80 implies the model is significant. There is only a 0.01% chance that a "Model F-value" this large could occur due to noise.

Validation of the experimental model

All the data have shown that the optimal medium for phytase production contained 1.5% sucrose, 0.2% phytate, 3% peptone and pH 7.5. The results shows that 72.56 U/ml of phytase activity could be reached within 72 h of the fermentation, which was almost equal to the actual predicted value (73.42 U/ml). These results demonstrate that the *Hypocrea lixii* SURT01 could produce high yield of extracellular phytase in the simple medium and this may have wide uses in phytase production.

CONCLUSIONS

RSM method have been proved to be effective in optimizing phytase production by the *Hypocrea lixii* SURT01 isolated from poultry field soil in submerged fermentation, which resulted in an overall 17.5-fold enhancement in phytase production. This is the first report of *Hypocrea lixii* which produce extracellular enzyme. Optimized conditions by RSM method enhanced enzyme production many fold. Such a fungal strain may have highly potential application in animal feed industry for improving the nutritional status of feed and in combating environmental pollution.

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