J. BIOL. ENVIRON. SCI.,
2015, 9(26), 93-100

Original Research Article

Optimization of Phytase Production by New Isolate *Bacillus* sp. EBD 9-1 Strain using Statistical Experimental Design

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Received: 07.09.2015; Accepted: 08.10.2015; Published Online: 30.11.2015

ABSTRACT

In this study, face centered central composite design (FCCCD) of response surface methodology (RSM) was applied to describe the relationship between the tested variables, pH, temperature, rpm, incubation period and phytase production by novel isolate Bacillus sp. EBD 9-1. The design was employed by selecting pH, temperature, rpm and incubation period as the model factors and to achieve maximum yield, interaction of these factors was studied by RSM. A second order quadratic model and response surface method showed that the optimum conditions for phytase production were pH, 8.0; temperature, 38.13°C; rpm, 113.64 and incubation period, 45 h. Under these conditions, phytase activity was found to be about 228 Uml-1.

Keywords: Enzyme, Phytase, Optimization, Bacillus, Response surface methodology, Face centered central composite design

İstatistiksel Deneysel Tasarım Kullanarak Yeni Izolat *Bacillus* sp. EBD 9-1 Suşundan Fitaz Üretiminin Optimizasyonu

ÖZ

Bu çalışmada, yeni izolat *Bacillus* sp. EBD 9-1'dan fitaz üretimi ile pH, sıcaklık, çalkalama hızı (rpm) ve inkübasyon periyodu arasındaki ilişkinin belirlenmesi amacıyla tepki yüzeyi metodolojisinin (RSM) yüzey merkezli merkezi bileşik tasarımı (FCCCD) uygulanmıştır. Bu tasarım, maksimum ürün elde etmek için pH, sıcaklık, rpm and inkübasyon periyodunun faktör olarak seçilmesine ve bu faktörlerin etkileşim etkilerinin RSM ile belirlenmesine olanak tanımaktadır. İkinci dereceden kuadratik model ve tepki yüzeyi metodu fitaz üretimi için optimum koşulların pH 8.0; sıcaklık 38.13°C; rpm 113.64 ve inkübasyon periyodu 45 saat olduğunu göstermiştir. Bu koşullar altında fitaz aktivitesi yaklaşık 228 Uml-1 olarak bulunmuştur.

Anahtar Kelimeler: Enzim, Fitaz, Optimizasyon, *Bacillus*, Tepki yüzeyi metodolojisi, Yüzey merkezli merkezi bileşik tasarım

INTRODUCTION

The main part of feedstuffs for poultry is derived from plants such as wheat, corn, and rye. Up to 80% of the grain phosphorus is bound in the phytic acid whose salt form, phytate, (Uhling 1998). The organically bound phosphate of phytic acid is not metabolized by monogastric animals such as pig, poultry and fish due to lack of phytase and consequently contributes to the phosphorus pollution problems in areas of intensive livestock production (Adeola 1999,Common 1989,Wodzinski 1996). And also, phytate is an antinutrient constituent in plant-derived food and feed, since it form complexes with proteins, amino acids (Pallauf 1997) and variety of metal ions such as calcium, magnesium, iron and zinc. Because of these problems, there is considerable interest in phytate degrading enzyme. This significant problems can be eliminated by hydrolysis of phytate using phytase (Simell *et al*. 1989). And thus, the phosphorus of phytate can be resorbed by the animal after hdyrolysis to inositol and inorganic phosphate. Phytases have been one of the focal enzymes for nutrition, environmental protection, and human health during the past two decades (Uhling 1998). Phytases (E.C.3.1.3.8. inositol hexaphosphate phosphohydrolase) sequentially cleave orthophosphate groups from the inositol core of phytate or phytic acid, the major chemical form (60–90%) of phosphorus in plants. These enzymes have been isolated from fungi, yeast, bacteria and protozoa (Lei *et al.* 2007) Bacterial phytases and phytase- producing bacteria is as well as their potential biotechnological applications. Especially, *Bacillus* sp., *E.coli*, *Pseudomonas sp*., *Citrobacter* sp., are the best alternative to produce the enzyme (Jorquera M 2008). *Bacillus* phytases have been studied extensively because of the immense potential of these enzymes having unique characteristics, feasibility of mass

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production for market and applicability in animal feed (Rao *et al.* 2008). Hence phytase has a great industrial significance, and there is an ongoing interest in isolation of new microbial strain producing phytase and optimization of this enzyme (Lan *et al.* 2002). Phytase has been marketed as a feed additive in the US since 1996 and by the close of the twentieth century annual sales of phytase, as animal feed additive, was about US$ 500 million (Vats and Banerjee 2004). This enzyme has, therefore, potential applications in feed and food industries (Singh and Satyanarayana 2010).

Response surface methodology (RSM) in biotechnological process is gaining excellent importance for optimization of enzymes production (Dahiya *et al.* 2009). RSM, which includes factorial designs and regression analysis, helps in evaluating the effective factors and building models to determine interaction and select optimum conditions of variables for a desirable response (Cotârlet and Bahrim 2011). Compared with conventional methods for optimization, RSM is a time- and labor-saving method, which consists mainly of the Central Composite Design (CCD), the Box Behnken Design (BBD). RSM has been used successfully for production optimization of many products, including enzyme, antibiotics and biofuel (Zhong *et al.* 2014). Series of experiments were carried out to determine critical variables level affecting enzymes production in this paper. Besides RSM have been used to optimize parameters (pH, temperature, rpm and incubation period) for phytase production by new isolate *Bacillus sp.* EBD 9-1 in the present study.

MATERIALS AND METHODS

Bacterial Strain

The microorganism used was isolated from soil. It was identified as *Bacillus sp.* EBD 9-1 by morphological and biochemical analysis (Demirkan *et al.* 2014).

Cultivation and media

Enzyme production medium contained (% wv^{-1}): Dextrose 0.5; peptone 1; yeast extract 0.5; $MgSO_4.7H_2O$ 0.1; $CaCl_2.2H_2O$ 0.1, sodium phytate 0.1 (TS medium) (Park 2001). Medium was autoclaved at 120˚C for 20 min. The precultures were cultivated in LB medium for 18h. Then, overnight cultures with OD_{600}: 0.3 were inoculated at 1% in enzyme production media (150 ml in 500 ml Erlenmeyer flasks). At the end of growth period, the cultures were centrifuged (3461 *xg*, 10 min) and the supernatants were used for determination of phytase activity.

Phytase activity assay

Phytase activity was determined according to an optimized enzyme ectivity method (Choi *et al.* 2001). The reaction was carried out 0.1 ml of enzyme solution with 0.9 ml of 2 mM sodium phytate in 0.1 M Tris–HCl buffer (pH 7.0) at 37 °C for 10min and then the reaction was stopped by adding 0.75 ml of 5% trichloroacetic acid. The liberated phosphate was measured at 700 nm after adding 1.5 ml of color reagent, which is prepared freshly before using by mixing four volumes of 2.5% ammounium molybdate solution in 5.5% sulfuric acid and one volume of 2.5% ferrous sulfate solution. One unit of phytase activity was defined as to liberate 1 µmol of phosphate per minute under the assay condition.

A phosphate calibration curve was made by treating standard phosphate solutions of 0–100 µM KH_2PO_4 without added phytase under the same conditions as described above.

Optimization of physical parameters for RSM

RSM which was developed by Box and Wilson (1951) has led to the start of a period considered to be the second period in experimental design literature. In this context, designed experiments have been applied first time in the chemical industry for the process of product development in America in the 1950s (Montgomery 2001). RSM is a collection of mathematical and statistical techniques that is useful for modeling, analysis and optimization of response variable (Myers and Montgomery 2002). RSM can be applied in various fields such as biological and clinical sciences, physics, engineering sciences, food science and social sciences.

In case of a product, process or system involving response y that depends on the controllable input variables $x_i, x_j, ...x_k$ the relationship between them can be shown as below:

$$y = f(x_i, x_j, ...x_k) + \varepsilon \quad (1)$$

The unknown true response function *f* can be complex and ε represents other sources of variability in equation 1 (Eq. 1). It has been assumed as statistical error term including response error of measurement, other error sources in the process or system ε has a normal distribution with a mean zero and variance σ^2. The relationship between the response and the input variables can be defined as a polynomial or modeled as a linear function.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i<j}^{k} \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \in \quad (2)$$

In Equation 2 (Eq. 2), β_i denotes linear effect, β_{ij} denotes linear interaction effect and β_{ii} denotes quadratic effect.

Three different pH, temperature, rpm, and the incubation period value is used in the experiments. Levels of the factors are presented in Table 1.

Table 1. Experimental range and levels of the factors.

	Range of levels					
	Low		Central		High	
Factors	Actual	Coded	Actual	Coded	Actual	Coded
pH	6	-1	7	0	8	1
Temperature (°C)	35	-1	40	0	45	1
rpm	50	-1	100	0	150	1
Incubation period (h)	35	-1	40	0	45	1

The factors considered to be effective for the production of phytase was determined as the pH, temperature, rpm, and incubation period. In this study, FCCCD has been used and a total of 31 trials with different combinations of factor levels have been implemented. The experimental layout of the work and observed and the predicted response values have been illustrated in Table 2.

Table 2. Central composite design (CCD) and observed – predicted responses.

Run	Factors				Response (U/ml)	
	pH	Temperature (°C)	rpm	Incubation period (h)	Observed	Predicted
1	8	45	150	35	67.990	82.660
2	7	40	100	45	186.620	182.580
3	8	35	150	35	96.900	94.620
4	6	45	50	35	59.880	51.770
5	7	35	100	40	86.110	107.630
6	6	45	50	45	117.370	116.210
7	6	35	150	45	206.590	199.320
8	6	35	150	35	79.400	75.190
9	8	35	50	35	58.390	35.290
10	6	35	50	35	32.720	47.670
11	7	40	100	40	106.000	122.800
12	7	40	100	40	116.000	122.800
13	7	45	100	40	88.270	74.220
14	7	40	150	40	100.760	91.160
15	8	45	50	35	86.150	89.990
16	7	40	100	40	116.000	122.800
17	7	40	100	40	126.000	122.800
18	8	35	150	45	193.780	203.450
19	8	40	100	40	161.470	167.420
20	7	40	100	40	126.000	122.800
21	8	45	50	45	133.340	139.130
22	6	35	50	45	184.150	171.050
23	7	40	100	35	84.430	95.940
24	7	40	100	40	136.000	122.800
25	8	45	150	45	150.940	132.550
26	7	40	50	40	63.620	80.690
27	6	40	100	40	144.730	146.250
28	6	45	150	35	19.890	12.620
29	6	45	150	45	53.140	77.810
30	7	40	100	40	156.000	122.800
31	8	35	50	45	139.520	143.360

RESULTS

Statistical analysis and response surface modeling

The relationship between phytase production and physical factors was expressed by a polynomial equation. Phytase production was accepted as response. The mathematical relationship of the factors and the response was calculated by the second order polynomial equation

$$\begin{aligned} Y &= 122.80 + 10.59A - 16.70B + 5.24C \\ &+43.32D + 34.00A^2 - 31.90B^2 - 36.90C^2 \\ &+16.50D^2 + 12.65AB + 7.95AC - 3.83AD \\ &-16.67BC - 014.73BD + 0.19CD \end{aligned} \quad (3)$$

where Y: phytase production, A: pH, B: temperature, C: rpm and D: incubation period. According to the calculated statistical results for the estimated model for phytase production, pH, temperature and incubation period were statistically significant model terms ($p<0.05$). It was observed that the most significant factor on the

phytase production is incubation period and the temperature and pH variable followed it, while rpm variable does not have significant effect on phytase production. The coefficient of determination was calculated as 0.8523 for phytase production. This showed that 85.23% of the total variation for phytase production was explained by factors and interactions.

When Analysis of variance (ANOVA) findings analyzed in Table 3, it is seen that the estimated model was significant for phytase production ($p<0.05$). In addition, lack of fit values that obtained insignificant ($p>0.05$) shows that the quadratic model was adequate.

Table 3. Analysis of variance (ANOVA) for quadratic model.

Source	DF	Seq SS	Adj MS	F	P
Regression	14	62491.4	4463.67	13.36	0.000
Linear	4	41305.8	2106.68	6.31	0.003
Square	4	9459.2	2364.81	7.08	0.002
Interaction	6	11726.4	1954.4	5.85	0.002
Residual Error	16	5345.0	334.06		
Lack-of-Fit	10	3745.0	374.5	1.4	0.351
Pure Error	6	1600.0	266.67		
Total	30	67836.4			

The surface plot reveals the effect of variables pH and temperature, pH and incubation period, temperature and incubation period for phytase production which is represented in Figure 1. Referring to the Figure 1a, the phytase production has maximum values in the region that has the highest pH and the temperature. Referring to the Figure 1b, the phytase production has maximum values in the region that has the highest incubation period and the highest and the lowest the pH. Referring to the Figure 1c, the phytase production has maximum values in the region that has the highest incubation period and the medium values of temperature.

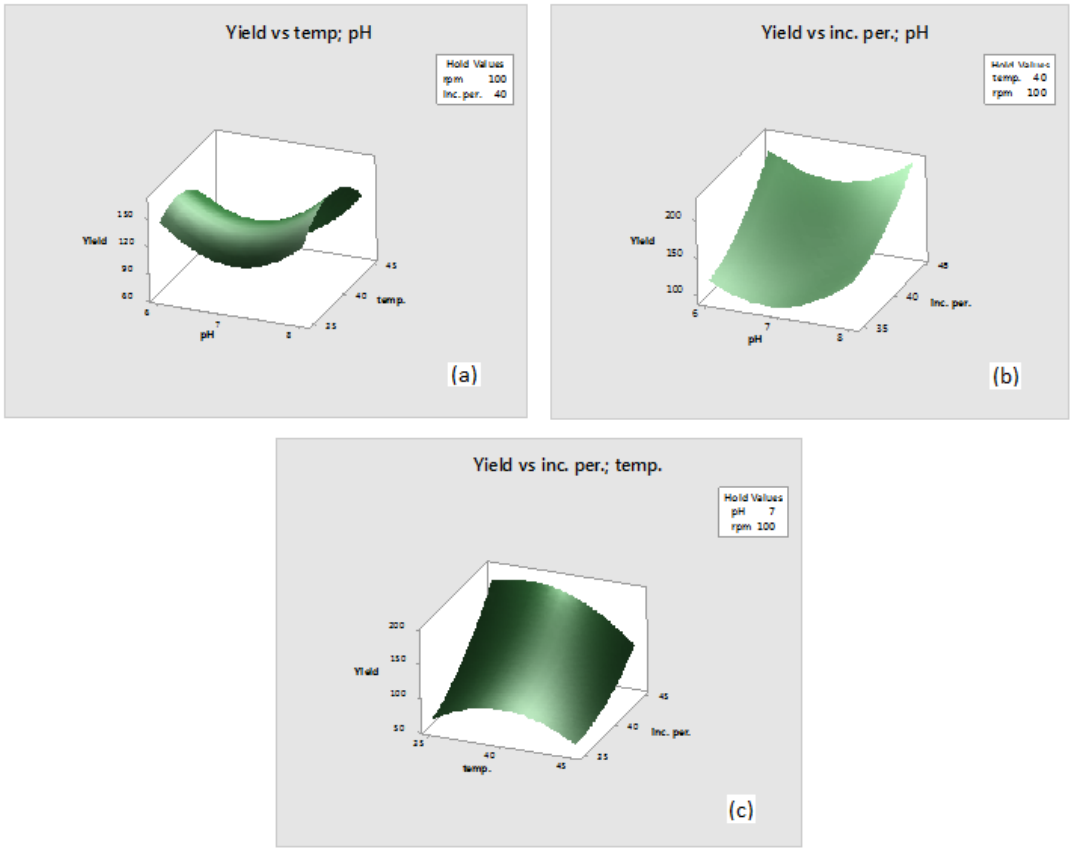

Figure 1. Surface plots of yield.

The observed and predicted response values can be seen in Figure 2. Also, Figure 2 shows that in the predicted values of phytase production in the 95% confidence interval are very close to the observed values of experiment. Therefore, it was been observed that the estimation performance of model was quite good. At the same time, this result would be accepted as an indicator of the goodness of fit of estimated model.

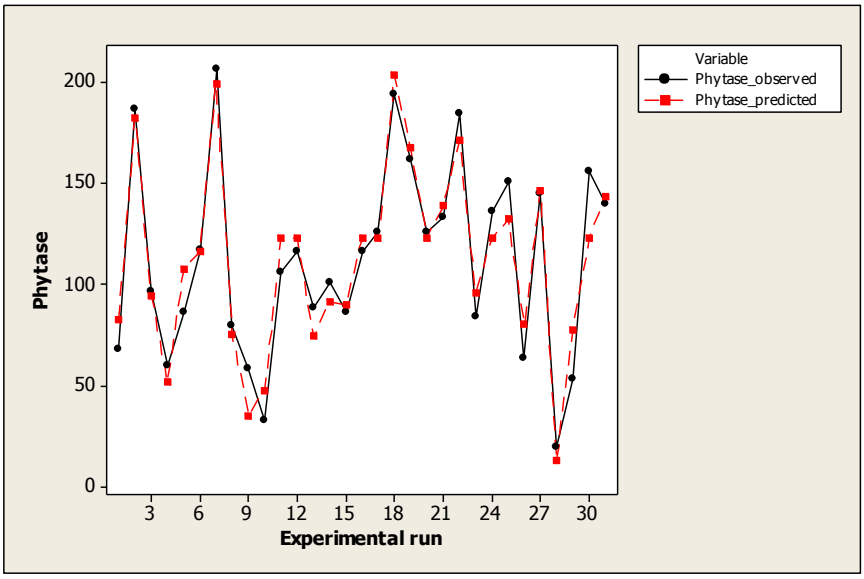

Figure 2. Plot of observed and predicted values for phytase.

Besides, high and significant correlation coefficient (r=0.960; p=0.000) between the observed and estimated values was showed that the estimated model was valid, and has an optimum point. As a result of optimization, obtained maximum value of phytase production was 228 Uml^{-1}. For this enzyme activity, suitable conditions were pH 8.0, 38.13 °C, 113.64 rpm and 45h.

Physical factors that affect phytase production by *Bacillus sp.* strains have studied by RSM. This study is report that indicates the parameters and its effects on phytase production in order to obtain best optimized model using RSM.

Both our and those of other researchers results showed that the nutritional and physical requirements of the microorganism differ from one another and therefore, need to be optimized for each microorganism.

DISCUSSION

It has been reported in literature to exert significant effects on phytase production, including carbon and nitrogen sources, inoculum level and age (Chadha *et al.* 2004, Krishna and Nokes 2001, Sunitha *et al.* 2000, Vohra and Satyanarayana 2002) by RSM.

Some researchers have been reported with different microorganisms (Chadha *et al.* 2004,Krishna and Nokes 2001,Sunitha et al. 2000,Vohra and Satyanarayana 2002), they showed that the optimum pH and temperature was found to be 4.0-4.7, 28-35°C for fungus (NSF-7 and NSF-9), respectively. The optimum operating conditions for production phytase by *Enterobacter sakazakii* ASUIA279 were at temperature of 37 ºC, initial pH of 7.0, rice bran of 15%, 300 rpm of agitation speed (Farouk *et al.* 2012) Phytate-degrading enzyme produced by *Aspergillus niger*, *Aspergillus ficuum* NRRL 3135 and *Aspergillus terreus* was carried out by shaking at 270 rpm (Shieh and Ware 1968).

The RSM resulted in a 1.09-fold increase in phytase production compared to the unoptimized medium (210 Uml^{-1}). Similar result has been shown by Awad *et al.* (2011), the activity of phytase increased 1.02-fold in *Penicillium funiculosum* NRC467. On the other hand, Ries and Macedo (2011) showed that the phytase activity obtained in unoptimized medium was 0.06 Uml^{-1} after 24 h of fermentation by *S. cerevisiae*. After optimization of the medium composition and the fermentation conditions, the activity of phytase in response to sodium

phytate was approximately 10-fold higher ($0.62\ Uml^{-1}$). It has also been reported in other studies that , after statistical optimization, the activity of phytase increased 1.75-fold in *P. anomala* cultured in synthetic medium (Vohra and Satyanarayana 2002), 1.7-fold in *Aspergillus ficuum* (Bogar *et al.* 2003a), 1.3-fold in *Rhizomucor pusillus* (Chadha *et al.* 2004), 3.73-fold in *Sporotrichum thermophile* (Singh and Satyanarayana 2008), and 1.8-fold in *M. racemosus* (Bogar *et al.* 2003b).

The present work demonstrates the feasibility of using experimental design tools to optimize physical parameters for phytase production. According to the phytase production model, it was observed that the parameters of pH, temperature and incubation period variables are effective while the rpm variable is not effective. While pH and incubation period were positively on the phytase production, temperature has negative effect. It was found that the most important factor was incubation period on phytase production and it suggests that this novel isolated *Bacillus* sp. EBD 9-1 strain has potential applications for the reduction of phytate in animal feed.

ACKNOWLEDGEMENTS

This research was funded by Uludag University Commission of Scientific Research Projects, Grant OUAP(F)-2012/11, which is highly appreciated.

REFERENCES

Adeola O (1999) Nutrient management procedures to enhance environmental conditions: an introduction Journal of animal science 77:427-429

Bogar B, Szakacs G, Linden JC, Pandey A, Tengerdy RP (2003a) Optimization of phytase production by solid substrate fermentation J Ind Microbiol and Biotechnol 30:183-189

Bogar B, Szakacs G, Pandey A, Abdulhameed S, Linden JC, Tengerdy RP (2003b) Production of Phytase by *Mucorracemosus* in Solid-State Fermentation Biotechnology Progress 19:312-319

Chadha BS, Harmeet G, Mandeep M, Saini HS, Singh N (2004) Phytase production by the thermophilic fungus *Rhizomucor pusillus* World Journal of Microbiology and Biotechnology 20:105-109

Choi Y, Suh H, Kim J (2001) Purification and Properties of Extracellular Phytase from *Bacillus sp.* KHU-10 J Protein Chem 20:287-292

Common FH (1989) Biological availability of phosphorus for pigs. Nature 143:370-380

Cotârlet M, Bahrim G-E (2011) Optimization of Cold-Adapted Amylases and Protease Production by Psychrotrophic *Streptomyces* 4 Alga Using Response Surface Methodology. Turkish Journal of Biochemistry 36:83-92

Dahiya S, Singh N, Rana JS (2009) Optimization of growth parameters of phytase producing fungus using RSM Journal of Scientific & Industrial Research 68:955-959

Demirkan E, Baygın E, Usta A (2014) Screening of phytate hydrolysis *Bacillus sp.* isolated from soil and optimization of the certain nutritional and physical parameters on the production of phytase Turk J Biochem 39 206–214

Farouk A-E, Greiner R, Hussin ASM (2012) Optimization of cultivation conditions for the production of phytate-degrading enzymes by *Enterobacter sakazakii* ASUIA279 isolated from Malaysian maize root. Journal of Biotechnology and Biodiversity 3:1-9

Jorquera M MO, Maruyama F, Marschner P, de la Luz Mora M. (2008) Current and Future Biotechnological Applications of Bacterial Phytases and Phytase-Producing Bacteria. Microbes and Environments 23: 182-191

Krishna C, Nokes SE (2001) Predicting vegetative inoculum performance to maximize phytase production in solid-state fermentation using response surface methodology. J Ind Microbiol Biotechnol 26:161-170

Lan GQ, Abdullah N, Jalaludin S, Ho YW (2002) Optimization of carbon and nitrogen sources for phytase production by *Mitsuokella jalaludinii*, a new rumen bacterial species Letters in Applied Microbiology 35:157-161

Lei X, Porres J, Mullaney E, Brinch-Pedersen H (2007) Phytase: Source, Structure and Application. In: Polaina J, MacCabe A (eds) Industrial Enzymes. Springer Netherlands, pp 505-529.

Montgomery DC (2001) Introduction to Statistical Quality Control. John Wiley & Sons, Inc, New York

Myers RH, Montgomery DC (2002) Response Surface Methodology: Process and Product Optimization Using Designed Experiments. John Wiley & Sons, Inc, New York

Pallauf J, Rimbach, G. (1997) Nutritional significance of phytic acid and phytase. Arch Tierernahr 50:301-319.

Park YJ (2001) Expression, characterization, and antifungal activity of phytase from *Bacillus subtilis* TS16-111. PhD Thesis, Faculty of the Graduate school of Seoul National University, Korea

Rao DECS, Rao KV, Reddy VD (2008) Cloning and expression of *Bacillus* phytase gene (phy) in *Escherichia coli* and recovery of active enzyme from the inclusion bodies. Journal of Applied Microbiology 105:1128-1137

Shieh TR, Ware JH (1968) Survey of Microorganisms for the Production of Extracellular Phytase Appl Microbiol 16:1348-1351

Simell M, Turunen M, Piironen J, Vaara T (1989) Feed and food applications of phytase. Lecture at 3rd Meet. Industrial Applications of Enzymes, Barcelona, Spain

Singh B, Satyanarayana T (2008) Improved phytase production by a thermophilic mould *Sporotrichum thermophile* in submerged fermentation due to statistical optimization Bioresource Technology 99:824-830

Singh B, Satyanarayana T (2010) Applications of phytase of thermophilic mould,*Sporotrichum thermophile*: A review Journal of Scientific and Industrial Research 69:411-414

Sunitha K, Kim Y-O, Lee J-K, Oh T-K (2000) Statistical optimization of seed and induction conditions to enhance phytase production by recombinant *Escherichia coli* Biochemical Engineering Journal 5:51-56

Uhling H (1998) Industrial enzymes and their applications. Wiley, New York

Vats P, Banerjee UC (2004) Production studies and catalytic properties of phytases (myo-inositolhexakisphosphate phosphohydrolases): an overview Enzyme and Microbial Technology 35: 3-14

Vohra A, Satyanarayana T (2002) Statistical optimization of the medium components by response surface methodology to enhance phytase production by *Pichia anomala* Process Biochemistry 37:999-1004

Wodzinski R, Ullah, AH. (1996) Phytase Adv Appl Microbiol 42:263-302

Zhong J, Zhang X, Ren Y, Yang J, Tan H, Zhou J (2014) Optimization of *Bacillus subtilis* cell growth effecting jiean-peptide production in fed batch fermentation using central composite design Electronic Journal of Biotechnology 17:132-136