World Applied Sciences Journal 15 (11): 1635-1644, 2011
ISSN 1818-4952

Optimization of Phytase Production by *Penicillium funiculosum* NRC467 under Solid State Fermentation by Using Full Factorial Design

[1]Ghada E.A. Awad, [2]Magdy M.M. Elnashar and [1,3]Enas N. Danial

[1]Chemistry of Natural and Microbial Products Department,
Advanced Materials & Nanotechnology Laboratory, National Research Center, Giza, Egypt
[2]Centre of Scientific Excellences, Polymers Department,
Advanced Materials & Nanotechnology Laboratory, National Research Center, Giza, Egypt
[3]Department of Biochemistry, Faculty of Girls Science, King Abdulaziz University, Jeddah, Saudi Arabia

Abstract: Nine substrates were investigated for phytase production by *Penicillium funiculosum* NRC467 fine particles of crushed fava bean has been selected as the best substrate using solid state fermentation (SSF). The SSF conditions were optimized by using one-variable-at-a-time (OVAT) strategy. Penicillin G has been used to enhance phytase production instead of the traditionally used Tween 80 and Triton X100. 3^3 Full factorial design of response surface methodology was applied to describe the relationship between the tested variables, cane molasses, penicillin G, pH and phytase activity. The results showed that the maximum phytase activity of 392.4 U/g ds was attained when concentrations of both cane molasses and penicillin G were 20% (w/v) and 40 U/g ds, respectively at fermentation medium of pH 8. This result represent an improvement in phytase production of 8.2 folds when compared to that previously obtained using the basal medium under the same cultivation conditions. The generated model was found to be very adequate for phytase production (95% accuracy) as the experimental value was 392.4 U/g ds compared to 401 U/g ds for the predicted value. In brief, the use of fava bean and penicillin G for production of phytase are novel and will open new way for researchers to discover and explore this arena.

Key words: Industrial enzymes · Solid state fermentation · Penicillium *funiculosum* NRC467 · Factorial design · Penicillin G · Biopolymers

INTRODUCTION

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 millions [1]. This enzyme is capable of hydrolyzing phytic acid to myo-inositol and phosphoric acid in a stepwise manner forming myo-inositol phosphate intermediates [2]. Therefore, the supplementation of microbial phytase to animal diets tends to increase the bioavailability of proteins and essential minerals and consequently improve growth performance. It also reduces the amount of phosphorus in animal manure, thereby helping in combating phosphorus pollution [3].

Phytic acid (myo-inositol hexakisphosphate) comprises 50–80% of the total phosphorus in most foods of plant origin [3]. It is the organic form of phosphorus that is largely unavailable to monogastrics such as poultry, pigs, fish and humans due to the lack or inadequate levels of phytate hydrolyzing enzymes in their gastrointestinal tract [4].

Phytase act as nutritional factor in more than one way, but their excess in the environment lead to phosphorus pollution problems [5]. These problems can be overcome by using phytase enzyme (myo-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8 and 3.1.3.2.6 which belong to a family of histidine acid phosphatases. In our laboratory, we succeeded in the past few years to produce some industrial enzymes such as penicillin G acylase, β-galactosidase and most recently inulinase [6, 7].

Corresponding Author: Enas N. Danial, Chemistry of Natural and Microbial Products Department, Advanced Materials & Nanotechnology Laboratory, National Research Center, El-Behooth St. Dokki, Cairo, Egypt.
Tel: +2 02 29188174, Fax: +2 02 33370931.

The traditional process involving one-variable-at-a-time (OVAT) optimization strategy is simple and easy and the individual effects of medium components can be seen on a graph without the need to revert to statistical analysis. Unfortunately, it frequently fails to locate the region of optimum response because the joint effects of factors on the response are not taken into account in such procedures. It was reported that the complexities and uncertainties associated with large-scale fermentation usually come from lack of knowledge of the sophisticated interactions among various factors [8]. Statistical experiment design provides an efficient approach to optimization. Fractional factorial design (FFD) is especially suitable to account for the interactions and identify the most significant components in the medium formula [9]. A combination of factors generating a certain optimum response can be identified through factorial design and the use of response surface methodology [8].

During recent years, an economical alternative for enzyme production and application by solid state fermentation (SSF) has gained much interest [10, 11]. Since the latter has several advantages over submerged fermentation (SmF) such as lower wastewater output, reduced energy requirements, simpler fermentation media, easier aeration and reduced microbial contamination[(10], SSF is particularly useful for the production of industrial enzymes [12].

Although several strains of bacteria and yeast can produce phytase, the use of filamentous fungi has been preferred in SSF systems due to high production yields and acid tolerance enzyme for feeding purpose [13]. The crude product is mixed in feed rations as a value-added supplement [14, 15].

The present investigation could be considered as the first report that exploited the use of fava bean as a locally available and inexpensive agro-substrate for phytase production. Penicillin G has been also used for the enhancement of phytase production using *Penicillium funiculosum* NRC467 under solid-state fermentation. Cane molasses solution as an important biopolymer was also added to the fermentation medium. The latter, which is a by-product of the sugar refinery process, is the most economical source of carbohydrate for various industrial fermentations [16].

MATERIALS AND METHODS

Different substrates (black-eyed peas, navy beans, fava beans, soya beans, red beans, wheat bran, barley wheat from maize and wheat from rice) were purchased from the local markets; beans were crushed uniformly and passed through a mash of 1mm diameter. Phytic acid was purchased from Sigma-Aldrich. All other chemicals were of analytical grades.

Experimental Techniques

Isolation of Phytase Producing Fungi and Culture Conditions: Among different 9 fungal strains isolated from Egyptian soil, a potent strain which produces a clear zone around its colonies when cultivated in a solid medium containing 0.5% phytic acid and 2% g/L agar was chosen for further study. The strain was kindly identified by Assuit University, mycological Center AUMC as *Penicillium funiculosum* and designated as *P. funiculosum* NRC467. The isolate was routinely grown on potato dextrose agar medium at 28°C and preserved at -80°C in 50% (v/v) glycerol.

Solid State Fermentation: Experiments were performed in 250 ml Erlenmeyer flasks with 5g of crushed substrate moistened with 5ml distilled water containing various nutritional factors. The percent of moisture content was calculated as volume of distilled water / total weight of dry solid substrate. The substrate was sterilized at 121°C (1.5 atm.) for 20 min. After cooling, the substrate was inoculated with 1.0 ml containing (5×10^6 spores/ml). The contents of the flasks were well mixed and incubated in rotary shaker (200 rpm) at 28°C for predetermined time period. For investigation of inoculums level, the inoculums volume was increased accordingly whenever required.

Analytical Methods for Enzyme and Protein Assay: The SSF samples were extracted with distilled water (20 ml water /g substrate), by shaking for 1 h at 200 rpm at room temperature. The suspension was centrifuged (5000 rpm for 20 min) and the supernatant was used in enzyme assay. Phytase activity was assayed by measuring the inorganic phosphorus released from sodium phytate as described earlier [17-19]. One unit of phytase is defined as the amount of enzyme releasing 1 ìmol of inorganic phosphorus per ml per minute under the assay conditions. The activity of the enzyme is expressed as units per gram dry substrate (U/g ds). Soluble proteins were determined according to Lowry [20]. and expressed as mg/gds.

Fungal Biomass Estimation in SSF: Fungal biomass estimation was carried out by determining the N-acetylglucosamine released by the acid hydrolysis of

chitin present in the cell wall of fungi [21] whereas 0.5 g (dry weight basis) of fermented substrate was mixed with concentrated sulphuric acid (2 ml) and the reaction mixture was kept for 24 h at room temperature (30°C). This mixture was diluted with distilled water to make 1N solution, autoclaved (1.5 atm, 1 h), neutralized with 10% NaOH and made to 100 ml with distilled water. The N-acetylglucosamine content was estimated using dinitrosalicylic acid method using N-acetylglucosamine as the standard [22]. The results obtained are expressed as mg glucoseamine per gram dry substrate (mg/g ds).

Optimization Procedure and Experimental Design: The optimization of medium components for phytase production by *P. funiculosum* NRC46 was carried out in two stages as described below: Six kind of carbon sources, two kinds of surfactants and penicillin G were investigated using one-variable–at-a-time strategy. Carbon sources were biopolymers as glucose, lactose, sucrose, soluble starch, glucose syrup and cane molasses, all at tow levels (10-20% w/v in distilled water). These solutions were added to the substrate in appropriate concentrations to perform the desired moisture content. The surfactants tween 80, triton X100 were added at two levels (40 and 120 mg/g ds). Penicillin-G was also added at two concentrations (20 and 60 U/g ds). Solutions of carbon source and surfactants were sterilized separately and were added before inoculation to the fermentation medium.

Optimization of Selected Components Using Full Factorial Design: Full factorial design was followed for production of phytase by *P. funiculosum* NRC46. This method is useful for optimizing a small number of variables at a few levels [23] accordingly, the three variables, namely concentration of cane molasses, penicillin G and initial pH were found to be the most important. Thus, a total of 27 set of experiment, including all possible combination of the three variables at three levels (3^3) experiments were performed in duplicate. Based on preliminary studies the variables and their levels were presented in details in Table 1. A second-order polynomial model was fitted to the response data obtained from the design. The polynomial equation was in the following form:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3 \quad (1)$$

Whereas Y is the predicted production of phytase (U/g ds) and X_1, X_2 and X_3 are the independent variables corresponding to the concentration of cane molasses, Penicillin G and pH, respectively; β_0 is the intercept, β_1, β_2, β_3 are linear coefficient, β_{11}, β_{22}, β_{33} are square coefficients, β_{12}, β_{23}, β_{23}, β_{123} are cross product coefficients.

Data Analysis and Optimization: Statistical software SPSS (version 16) was used to fit the response surface model to the experimental data through the multiple regression analysis. Optimization of the culture conditions in terms of can molasses, penicillin G and pH were analyzed using the predictive polynomial model through solver function. Using *Statistica V* software, three dimensional plots were created by holding two variables of the model as constants. All data are the mean of triplicates unless it is stated otherwise and their±SD were almost negligible.

RESULTS AND DISCUSSION

Evaluation of Different Substrate for Phytase Production: The selection of ideal substrate/support materials for enzyme production in a solid-state fermentation process depends upon several factors, mainly related with cost and availability of the substrate material and thus may involve screening of several substrates [10]. The results in the present study indicated that phytase production pattern varied with the type of substrate (Fig.1).

Table 1: Concentration range of the three independent variables used in 3^3 full factorial designs

		levels		
Factor	Name	-1	0	+1
X1	Cane molasses (%)	10	20	40
X2	Penicillin G (U/g ds)	20	40	60
X3	pH	7	8	9

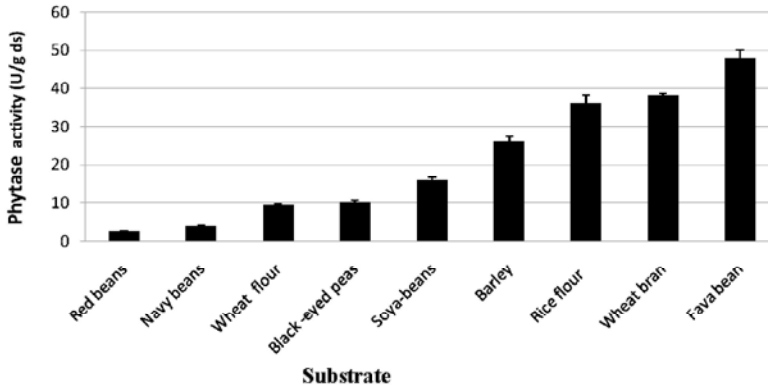

Fig. 1: Effect of different substrates on phytase production by *P.funiculosum* NRC467

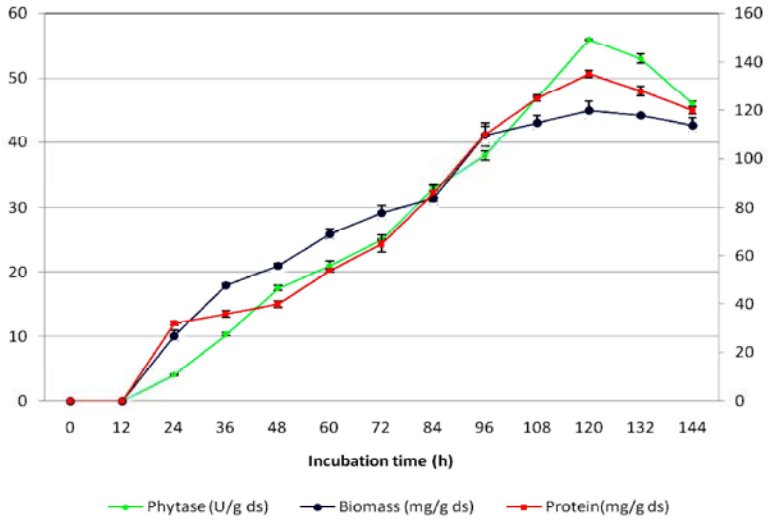

Fig. 2: Effect of different incubation time on phytase production by *P.funiculosum* NRC467

This could be attributed to solid materials dual role-supply of nutrients to the microbial culture growing and anchorage for the growing cells. Maximum enzyme production (48 U/g ds) was observed with fava beans, while minimum phytase production (9.4 U/g ds) was noticed with flour from maize as substrate/support material. Residuals of crushed Fava bean are obtained during the preparation of feeding meal of cheeps and some domestic animals. Crushed fava bean is considered as a new substrate/support materials for SSF of phytase production that is revealed to storage of different minerals in fava beans, including magnesium, potassium, phosphorus, iron and molybdenum, as well as β-vitamins and also high phytate content.

Biomass Growth and Phytase Production at Different Periods of Fermentation: The fungal biomass growth and phytase production as well as the soluble protein content at different time intervals were shown in (Fig. 2). It shows that the enzyme production increased with incubation time and maximal enzyme production of 56 U/g ds was obtained after 120 h. Apparently, enzyme production was growth associated and the maximum growth of 135.7 mg/g ds was also observed after 120 h. The enzyme yield declined during further incubation, which could be due to the reduced nutrient level of the medium. Results also showed that the soluble protein content increased along with the enzyme production and a maximum of 102.1 mg/g ds soluble protein was obtained after 120 h. Similar finding in production of phytase was reported with *Mucor racemosus* [24].

Influence of Inoculum Level and Moisture Content: There was a gradual increase in the enzyme synthesis with increase in inoculums concentrations up to 2.0 ml, but thereafter a steady decline was observed for further inoculum concentrations. Fig. 3 showed that 2 ml of the spore suspension was found to be the best for phytase

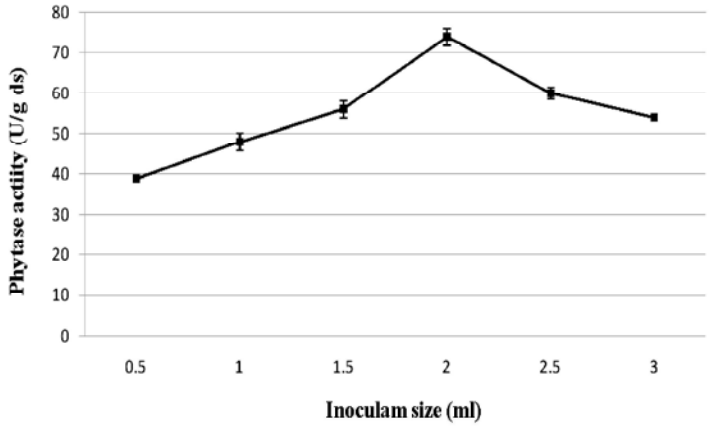

Fig. 3: Effect of different inoculums size on phytase production by *P.funiculosum* NRC467

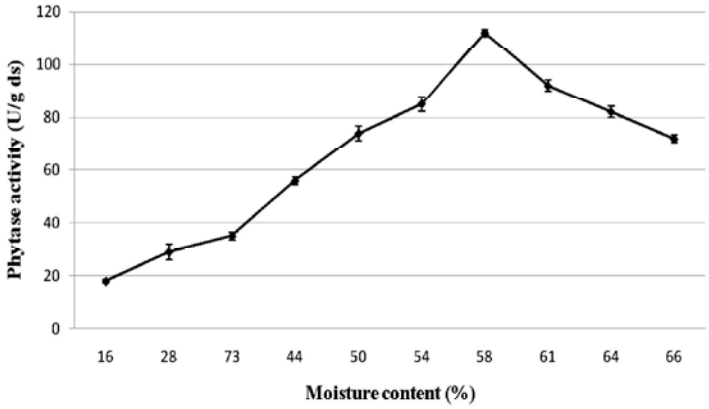

Fig. 4: Effect of different moisture content on phytase production by *P.funiculosum* NRC467

production, giving the maximum enzyme yield of 74 U/g ds. The effect of higher concentrations of inoculums was inhibitory for phytase production and 54 U/g ds were obtained with the highest inoculums concentration of 3 ml. This may be due to the high concentrations of inoculums could lead to an increase in the fungal biomass production. That results in increased competition for carbon source and nutrients, which lead to exhaustion of nutrients and this imbalance results in reduced enzyme production. Similar findings were reported with *Mucor racemosus* [24].

Among several factors that are important for microbial growth and enzyme production under SSF using a particular substrate, moisture level content/water activity is one of the most critical factors [10, 25]. Since, solid-state fermentation processes are different from submerged fermentation culturing, the microbial growth and product formation occur at or near the surface of the solid substrate particle having low moisture contents [10]. Thus, it is crucial to optimize moisture content for the fermenting substrate in order to achieve maximum production. Fig. 4 showed that maximum enzyme production of 112 U/g ds was observed with 58% moisture content. Linearity between moisture content and enzyme production was observed up to 58% and thereafter, further increase in moisture level in the fermentation medium resulted in reduction of production. By increasing the water content at constant substrate volume, the air content of substrate decreased. At the lowest and the highest water contents, the decomposition rate of the total organic matter was found to decrease [26].

Influence of pH: Phytase production by microbial strains strongly depends on the extra-cellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes. The later support the cell growth and product production [27] Results showed that enzyme synthesis was increased with increase of medium pH towards alkaline range from neutrality; maximum phytase production of 154 U/g ds was achieved at pH 8.0 and then decreased at pH 9.0–10.0 as shown in (Fig. 5).

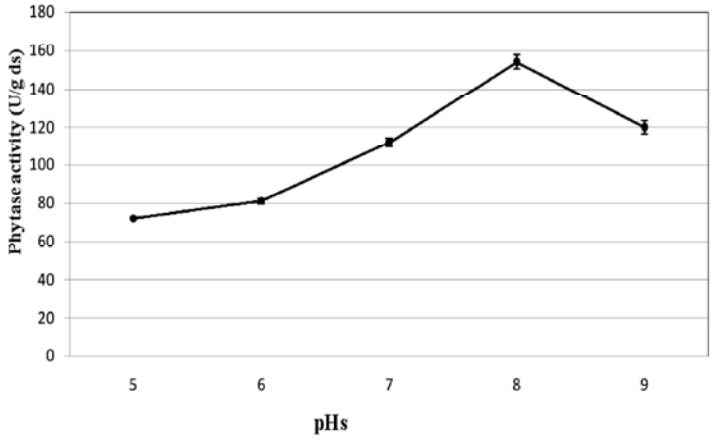

Fig. 5: Effect of different pHs on phytase production by *P.funiculosum* NRC467

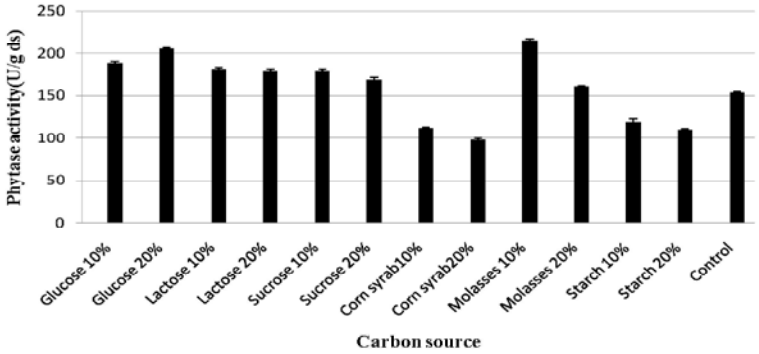

Fig. 6: Effect of addition of different carbon sources on phytase production by *P.funiculosum* NRC467

Effects of Supplementation of Additional Carbon Sources on Production of Phytase: The impact of supplementation of external carbon sources at two concentration 10% (w/v) and 20% (w/v) on phytase production were studied and the results are shown in (Fig. 6). All the supplemented carbon sources were found to enhance the phytase production except starch. Apparently, the fungal strain had a preferential choice towards monosaccharide, i.e. glucose and sucrose produced high titers of enzyme 189.9 U/g ds and 212 U/g ds, respectively when supplemented at 10% (w/v). On the other hand molasses support phytase produced 216 U/g ds in comparison to 154 U/g ds for the control at the same condition. It was reported that addition of molasses enhanced the phytase production. Cane molasses, the by-product of the sugar refinery process, containing 45–50% (w/v) sugars, is the most economical source of carbohydrate for various industrial fermentations [28].

Effects of Supplementation of Surfactants and Penicillin G on Production of Phytase: Effect of surfactants such as tween 80 and triton X100 on phytase production by *P.funiculosum* NRC467 were studied by addition of surfactants to crushed fava bean in two concentrations (40 and 120 mg/g ds) after inoculation. The effect of addition of Penicillin G on phytase production was firstly tested by addition of penicillin G on two concentrations (20 and 60 U/g ds) to crushed fava bean after inoculation. The results shown in (Fig. 7) indicated that addition of tween 80 to fava bean enhance phytase production by *P. funiculosum* NRC467, phytase production reached to 188 U/gds with addition of (40 mg/g ds) of tween 80 in comparison to 154 U/g ds for the control. Similar results by other authors indicated that tween-80 enhance the phytase production by *Sporotrichum thermophile* [18, 19].

To the best of our knowledge, This report is the first report indicated that addition of penicillin G enhances phytase production by *P. funiculosum* NRC467. The results showed that addition of 60 U/g ds penicillin G enhanced phytase production to 207 U/g ds compared to154 U/gds for the control. The effect of penicillin G on phytase production may be explained by its effect on fungal cell wall permeability similar to the effect of tween 80 on *Aspergillus fiucum* [29] or it may be explained by partial inhibition of cell wall synthesis [30, 31].

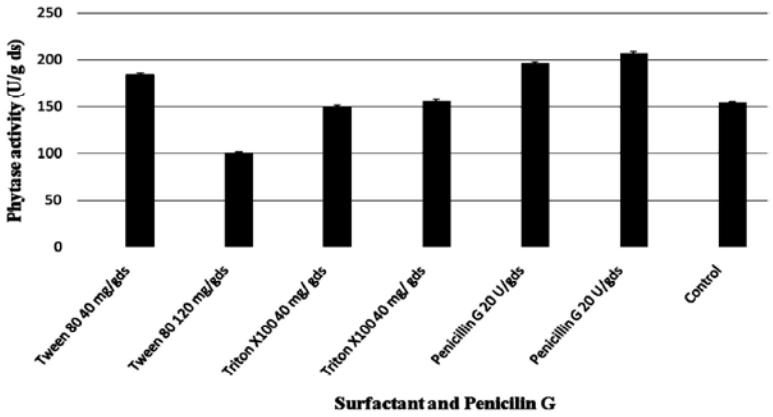

Fig. 7: Effect of addition of surfactants and penicillin G on phytase production by *P.funiculosum* NRC467

Table 2: Experimental plan for optimization of phytase using 3^3 full factorial designs

Number of trials	X1	X2	X3	Experimental Phytase activity (U/g ds)	Predicted Phytase Activity (U/g ds)
1	-1	-1	-1	236±2.1	216
2	-1	0	-1	337± 4.9	304
3	-1	+1	-1	234±7.1	261
4	0	-1	-1	212± 3.5	493
5	0	0	-1	347±4.9	347
6	0	+1	-1	318±1.2	318
7	+1	-1	-1	210±4.2	222
8	+1	0	-1	377±2.1	346
9	+1	+1	-1	334±3.5	343
10	-1	-1	0	283±4.9	301
11	-1	0	0	386±1.4	374
12	-1	+1	0	324±4.2	318
13	0	-1	0	306±4.2	324
14	0	0	0	392±5.6	401
15	0	+1	0	367±4.9	349
16	+1	-1	0	325±2.1	282
17	+1	0	0	336±2.4	367
18	+1	+1	0	326±4.2	324
19	-1	-1	+1	274±2.8	263
20	-1	0	+1	289±6.3	320
21	-1	+1	+1	246±4.2	249
22	0	-1	+1	307±4.9	277
23	0	0	+1	330±2.1	330
24	0	+1	+1	271±0.7	256
25	+1	-1	+1	201±6.3	219
26	+1	0	+1	263±2.1	264
27	+1	+1	+1	182±1.4	180

Optimization of the Screened Variables: The experimental design and results are shown in Table (2). Maximum phytase activity was observed with run number 14 using 20% cane molasses and 40 U/g ds of Penicillin G at pH 8. The maximum experimental phytase production by *P.funiculosum* NRC467 was 392.4 U/g ds while the predicted value was 401 U/g ds. The experimental results are approximately 95% of the predicted value. This indicates that the generated model is an adequate prediction of the enzyme activity. Regression analysis was performed to fit the response function (phytase activity) with the experimental data (Table 3). The analysis of variance for the three variables (cane molasses, penicillin G and pH) indicated that enzyme activity can be well described by a polynomial model.

$$Y = 4557.093 + 19.621X_1 + 23.429X_2 + 1075X_3 + 0.144X_1^2 + 0.161X_2^2 + 62.333X_3^2 + 0.021X_1X_2 + 1.683X_1X_3 + 1.296X_2X_3 \quad \text{Eq. 2}$$

Table 3: Model coefficient estimated by multiplies linear regression

Factor	Coefficient	Stander Error	*t*-Value	*p*-Value
Intercept	- 4557.093	726.212	-6.275	0.000
X_1	19.621	5.168	3.797	0.001
X_2	23.429	3.914	5.986	0.000
X_3	1075.000	179.243	5.997	0.000
X_1^2	-0.144	0.057	-2.543	0.021
X_2^2	-0.161	0.028	-5.782	0.000
X_3^2	-62.333	11.127	-5.602	0.000
X_1X_2	0.021	0.026	0.821	0.423
X_1X_3	-1.683	0.515	-3.268	0.005
X_2X_3	-1.296	0.393	-3.294	0.004

Table 4: Analysis of variance (ANOVA) test for 3^3 full factorial designs

Terms	Values
F value	11.490
P>F	0.000
R	0.927
R^2	0.859
Adjusted R^2	0.784
Sundered Error of the Estimate	27.255
Sum of square	76818.294
Mean of square	8535.366
Degree of freedom	9

Table 4 showed ANOVA for phytase activity units/gds (Y) indicated the F value of 11.490 which implied the model to be significant. Model terms having values of Prob >F (0.000) which considered highly significant. ANOVA indicated the R^2 value of 0.859 for response Y, which indicated that 85.9% of data variability can be explained by the model. A 95% correlation between observed and predicted results for phytase activity reflected the accuracy and applicability of the design for process optimization.

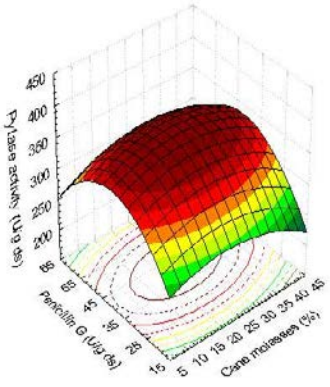

Fig. 8: Response surface plot and its contour plot of phytase production by *P.funiculosum* NRC467 showing the interaction between cane molasses and penicillin G concentrations at X3 = 0

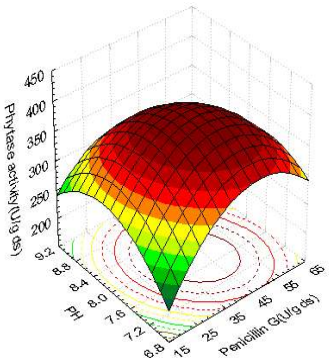

Fig. 9: Response surface plot and its contour plot of Phytase production by *P.funiculosum* NRC467 showing the interaction between penicillin G concentration and pH at X1= 0

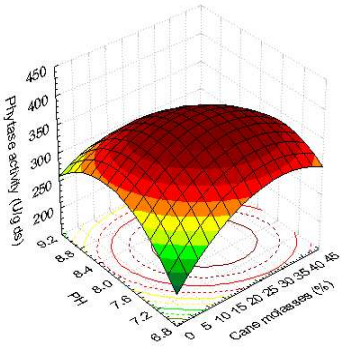

Fig. 10: Response surface plot and its contour plot of Phytase production by *P.funiculosum* NRC467 showing the interaction between cane molasses concentration and pH at X2 = 0

Figs 8-10 showed the response surface plots of phytase production. Obviously, phytase yield varied significantly upon changing the initial pH, cane molasses or penicillin G concentration. It indicated that the optimum values of each variable was identified based on the hump in the three dimensional or from the central point of the corresponding contour plot.

The statistical optimization resulted in about 8.2 fold compared to the non optimized medium. The statistical optimization revealed an increase of phytase production by 1.3 fold for *Rhizomucor pusillus* [32], 1.8 fold for *Mucor racemosus* [33], 1.7 fold for *Aspergilis ficuum* [33], 1.75 fold for yeast *Pichia anomala* in synthetic medium [34] and 5 folds for cane molasses medium [28]. These observations clearly suggested that the nutritional and physical requirements of the microbes differ from one another and therefore, need to be optimized for each strain.

CONCLUSIONS

Residual crushed fava bean as a wastes of some prepared food is considered as a new substrate/support materials for SSF of phytase production by *P.funiculosum* NRC467. Addition of penicillin G was also used for the first time for enhancement of phytase production. 3^3 full factorial design has been demonstrated to be effective in the optimization of phytase production (95% accuracy). Maximum enzyme production was achieved by using 20% cane molasses, 40 U/g ds penicillin G and pH 8. The optimization conditions revealed an increase in the enzyme production by 8.2 folds compared to the control.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the financial assistance from the Department of Chemistry of Natural And Microbial Products at the National Research Center.

REFERENCES

1. Vats, P. and U.C. Banerjee, 2004. Production studies and catalytic properties of phytases (myo-inositolhexakisphosphate phosphohydrolases): an overview. Enzyme and Microbial Technol., 35: 3-14.
2. Jareonkitmongkol, S., M. Ohya, R. Watanbe, H. Takagi and S. Nakamori, 1997. Partial purification of phytase from a soil isolate a cterium, *Klebsiella oxytoca* MO-3. J. Ferment. Bioeng. 83: 393-394.
3. Harland, B.and, E. Morris, 1995 Phytate: a good or a bad food component. Natur. Res. Lett., 15: 733-754.
4. Vohra, A. and T. Satyanarayana, 2003. production, purification and potential biotechnological applications. Crit. Rev. Biotechnol., 23: 29-60.
5. Mallin, M.A., 2000. Phytase for food application. Am. Sci., 88: 26-37.
6. Elnashar, M., E. Danial and G. Awad, 2009. Novel Carrier of Grafted Alginate for Covalent mmobilization of Inulinase Ind. Eng. Chem. Res., 48: 9781-9785.
7. Danial, E., M. Elnashar and G. Awad, 2010. Immobilized inulinase on Grafted Alginate Beads Prepared by the One-Step and the Two-Steps. Ind. Eng. Chem. Res., 49: 3120-3125.
8. Kennedy, M. and D.J. Krouse, 1999. Strategies for improving fermentation medium performance: a review. J. Ind. Microbiol. Biot., 23: 456-475.
9. Dean A. and D. Voss, 1999. Strategies for improving fermentation medium performance: a review. J. Ind. Microbiol Biot. New York: Springer-Verlag, 23: 483-529.
10. Pandey, A., C.R. Soccol, P. Nigam, D. Brand, R. Moha and S. Roussos, 2000 Biotechnological potential of coffee pulp and coffee husk for bioprocesses. Biochem. Eng. J., 6: 153-162.
11. Papagianni, M. and S.E. Nokes, 1999. Production of phytase by Aspergillus niger in submerged and solid-state fermentation. Proc. Biochem., 35: 397-402.
13. Sabu, A., A. Pandey, M. Daud and G. Szakacs, 2005. Tamarind seed powder and Palm kernel cake: two novel agro residues for the production of tannase under solid-state fermentation by *Aspergillus niger* ATCC 16620. Biores. Technol., 96: 1223-1228.
14. Bogar, B., G. Szakacs, J.C. Linden, A. Pandey and R.P. Tengerdy, 2003. Optimization of phytase production by solid substrate fermentation. J. Ind. Microbiol. Biotechnol., 30: 183-189.
15. Krishnan, R., R. Sumitra, N. Madhavan, S. George and A. Pandey, 2006. A Comparison of phytase production on wheat bran and oilcakes in solid-state fermentation by *Mucor racemosus*. Bioresource Technol., 97: 506-511.
16. Najafpour, G. and C. Shan, 2003. Enzymatic hydrolysis of molasses. Biores. Technol., 86: 91-94.
17 Fiske, C.H. and Y. Subbarow, 1925. The colorimetric determination of phosphorus. J. Biol. Chem., 66: 376-406.
18. Singh, B. and T. Satyanarayana, 2008a. Improved phytase production by a thermophilic mould *Sporotrichum thermophile* in submerged fermentation due to statistical optimization. Bioresource Technol., 99: 824-830.
19. Singh, B. and T. Satyanarayana, 2008b. Phytase production by a thermophilic mould *Sporotrichum thermophile* in solid state fermentation and its potential applications. Bioresource Technol., 99: 2824-2830.

20. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 265-275.
21. Sakurai, Y., T.H. Lee and H. Shiota, 1977. On the convenient method for the glucosamine estimation in koji. Agric. Biol. Chem., 4: 619-624.
22. Miller, G.L., 1959. Use of dinitrosalicylic reagent for determination of reducing sugars. Annal. Chem., 3: 426428.
23. Gupte, M.D. and P.R. Kulkarni, 2002. A study of antifungal antibiotics production by *Streptomyces chattanoogensis* MTCC 3423 using full factorial design. Appl. Microb., 35: 22-26.
24. Roopesh, K., S. Ramachandran, K.M. Nampoothiri, G. Szakacs and A. Pandey, 2008. Comparison of phytase production on wheat bran and oilcakes in solid-state fermentation by *Mucor racemosus*. Biores. Technol., 97: 506-511.
25. Nigam, P. and D. Singh, 1994. Solid states (substrate) fermentation system and their applications in biotechnology. J. Basic Microbiol., 34: 405-523.
26. Gautam, P., A. Sabu, A. Pandey, G. Szackacs and C.R. Soccol, 2002. Microbial production of extra-cellular phytase using polystyrene as inert support. Biores. Technol., 83: 229-233.
27. Ellaiah, P.B. Srinivasulu and K. Adinarayana, 2002. A review on microbial alkaline proteases. J. Sci. Ind. Res., 61: 690-704.
28. Kaur, P. and T. Satyanarayana, 2005. Production of cell-bound phytase by *Pichia anomala* in an economical cane molasses medium: optimization using statistical tools. Process Biochem., 40: 3095-3102.
29. Pandey, A., C.R. Soccol and D.A. Mitchell, 2002. Strategies for improving fermentation medium performance: a review. J. Ind. Microbiol Biot. Proc. Biochem., 35: 1153-1169.
30. Cooper, P.D., 1956. Site of action of penicillin changes in *Micrococcus pyogenes var aureus* during the first two hours growth in penicillin media. Bacterial review, 20: 28-39.
31. Kutseva, L. and N. Klyueva, 1969. Controlled biosynthesis of lysine and glutamic acid by *Brevibacterium* 22 Appli. Biochem. And Microb., 5: 556-562.
32. Chadha, B.S., H. Gulati, M. Minhas, H.S. Saini and N. Singh, 2004. Phytase production by the thermophilic fungus *Rhizomucor pusillus*. World J. Microbiol. Biotechnol., 20: 105-109.
33. Bogar, B., G. Szakacs, A. Pandey, S. Abdulhameed, J.C. Linden and R. Tengerdy, 2003. Production of phytase by *Mucor racemosus* in solid-state fermentation. Biotechnol. Progr., 19: 312-319.
34. Vohra, A. and T. Satyanarayana, 2002. Statistical optimization of the media components by response surface methodology to enhance phytase production by *Pichia anomala*. Process Biochem., 37: 999-1004.