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Optimization of aqueous extraction process to enhance the production of phytase by *Rhizopus oryzae* using response surface methodology coupled with artificial neural network

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Aqueous extraction process was optimized to reduce endotoxins from mixed substrate (1:1) for further phytase production by *Rhizopus oryzae* in solid state fermentation. 2^3 factorial design of experiment was combined with either a back-propagation artificial neural network (ANN) or the response surface methodology (RSM) for optimizing the process variables (length of extraction time, substrate loading and different pH of extraction solvent) to predict and simulate phytase production and phosphorus release. ANN was found to be a more powerful tool than RSM, for modeling and optimizing variables for the aqueous extraction process and can be used for predictive simulations of a process. A 2.37-fold increase in phytase production (37.65 U/gds) was achieved at the model predicted optimum concentration of extraction time of 29.78 min, substrate loading at 11.04 g and pH of extraction solvent at 7.1 as compared to the phytase yield in untreated substrate (15.91 U/gds). Moreover, the reduction in phytic acid after aqueous extraction of substrates was validated after high performance liquid chromatography (HPLC) characterization study. The results suggest that aqueous extraction process can be used efficiently for reducing the endogenous anti-nutritional factors from substrates eventually leading to enhanced phytase yield.

Key words: *Rhizopus oryzae*, high performance liquid chromatography (HPLC), phytic acid, solid state fermentation, optimization.

INTRODUCTION

Oil cakes/meals and agricultural by-products (various cereal brans and husks) have long been considered as the most inexpensive and high energy rich substrates for fermentation industry. Effective utilization of these residues not only helps to curb looming environmental pollution due to its disposal but also paves the way for solid waste management and minimizes the initial capital costs for the production processes. Linseed meal is a byproduct of linseed cold-pressing. The solid residue is used as a protein supplement and contains more Total Digestible Nutrients (TDN, 72%) than wheat bran (64%). Combination of these two substrates, with final moisture content of about 40%, was suggested to be an economic alternative substrate for phytase production by solid-state fermentation (Rani and Ghosh, 2011). Linseed and its byproducts have also been practiced in the animal feed industry and as the nutraceutical food for human due to its unlimited potential in reducing the risk of several diseases (Omah and Mazza, 1998; Ridges et al., 2001). Despite being a rich source of dietary proteins and fibres,

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presence of various endogenous antinutrients such as phytic acid, linamarin (a cyanogen) and linatine (an antipyridoxine factor) in linseed meal is the important factor limiting its use as value added substrate for economical production of industrial enzymes and as feed supplement at higher levels (>3%). Phytic acid (D-myo-inositol 1, 2, 3, 4, 5, 6 hexakisphosphate) is the principle source of phosphorus present in plant feedstuffs (Lott et al., 2000). Apart from containing a major portion of plant phosphorus, it also acts as a strong chelator, having the ability to form protein and mineral-phytic acid complexes and resulting into reduced protein and mineral bioavailability (Hossain and Jauncey, 1993; Erdman, 1979; Ketola, 1985; NRC, 1993; Spinelli et al., 1983). Phytase (myo-inositol hexakisphosphate phosphohydrolases), which hydrolyzes the phytate, helps in preserving the non-renewable phosphate source by replacing the lavish supplementation of additional phosphates into animal diets to meet their nutritional requirements. The negative effects of various anti-nutritional factors can be reduced by addition of exogenous phytase or by the removal of phytic acid from the oilseed meals and wheat bran by the use of appropriate feed processing techniques (Boutwell, 1917; Beleia et al., 1993; Han, 1988; Prendergast et al., 1994). For instance, Klosterman et al. (1967) showed that the linatine is a polar compound and can be extracted with water.

Similar results have also been found where the nutritive value of linseed meal was markedly improved by soaking the meal for 18 h prior to drying to remove HCN and phytic acid (Hossain and Jauncey, 1990). However, with the exception of this study, no comparative studies have been conducted to date, so as to optimize the process conditions for the reduction of phytic acid in linseed meal and its subsequent utilization for phytase production to ascertain the applicability of the aqueous extraction as well as the phytase production processes. Response surface methodology (RSM) has been thoroughly used as an optimization technique in a wide range of biotechnology applications including optimization of bioprocesses and enzyme production from microorganisms. Nowadays, many researchers have shifted towards artificial neural networks (ANN). The architecture of a multi-layered ANN, consisting of highly interconnected neurons, weights and biases, is normally composed of an input layer, a hidden layer and an output layer (Rumelhart et al., 1986). It offers an alternative to the RSM and can replace the quadratic polynomial models for solving regression problems in process modeling. In the present study, RSM and ANN were used to investigate the interdependence of the process parameters and models for the reduction of phytic acid in terms of phosphate release from mixed substrate (linseed meal + wheat bran, 1:1) for phytase production by *R. oryzae* in solid state fermentation.

To the best of our knowledge, there are no studies dealing with comparative analysis of RSM and ANN modeling techniques for the optimization of aqueous extraction process in substrate treatment and its affect on phytase production by *R. oryzae*. Furthermore, the reduction in phytic acid in the substrate was demonstrated by using an efficient and reproducible high performance liquid chromatography (HPLC) method.

MATERIALS AND METHODS

Microorganism and chemicals

Strain MTCC 1987 of *R. oryzae* was procured from microbial type culture collection (MTCC), Chandigarh, India. All chemicals were of analytical grade and at the highest purity, procured from Hi-Media laboratories P. Ltd., Mumbai, India. Agro-industrial by-products such as wheat bran (WB) and linseed oil cake (LOC) were purchased from local retail feedstuff outlets in Roorkee.

Inoculum preparation

The fungal strain was routinely grown on potato dextrose agar (PDA, Himedia, India) slants for 4 days at 30°C. Viable spores from slants were harvested by washing with 0.1% (v/v) Tween 80 (Himedia, India) and the spore suspension adjusted to ~1 × 10^6 cfu/ml (colony forming unit per milliliter) was used as inoculum for subsequent fermentations.

Pretreatment of mixed substrate and phytase production

The aqueous extraction of substrate was carried out to reduce endogenous anti-nutritional factors and to evaluate its effect on phytase production. The experiments were conducted in 250 ml Erlenmeyer flasks containing linseed meal and wheat bran (1:1) as the mixed solid substrate in SSF. The substrate was firstly subjected to a sieving procedure employing mesh-size sieves of 4, 8, 12, 16 and 20 prior to extraction with solvent of different pH. The smallest particles were of ~1.0 mm size, collected from fractions between meshes 16 and 20 (-16, +20), intermediate particles (~1.5 mm) were collected from fractions (-8, +12) and finally, heterogeneous oilcake (0.5 to 5.0 mm) was also used as substrate. Aliquots of mixed substrate (~1.0 mm) were soaked in five times their weight of extraction solvent of different pH (adjusted with HCl/NaOH) and were kept at room temperature for different length of time. The substrates were then filtered and dried at 37°C using an electric oven. The substrate free supernatants obtained were used for estimation of released inorganic phosphate and HCN using standard phytase assay and AOAC analysis method, respectively.

After pre-treatment, the dried substrate was supplemented with 20% (v/w) of mineral solution [(w/w), 0.3% NaCl, 0.3% $MgSO_4.7H_2O$, pH 5.6] with the moisture level adjusted to 40%. Medium sterilized at 121°C for 20 min was inoculated with 20% (v/w) inoculum and fermentation was carried out at 30°C for 96 h. The fermented medium was extracted with Tween 80 [0.1% (v/v)] at 30°C on an orbital shaker at 200 rpm for 1 h. Cell free extract was used for phytase activity assay. All experiments were performed in triplicate.

Phytase assay

Phytase activity was determined by estimating the inorganic phosphate released from sodium phytate (Bae et al., 1999). One unit of phytase is defined as the amount of enzyme required to release 1 nmol of inorganic phosphate (Pi) per second under the standard assay conditions. The phytase yield was expressed as a function of dry substrate weight (U/gds).

Table 1. Design matrix for PBD with coded levels of independent factors.

Run	A	B	C	D	E	F	G	Phytase activity[a] (U/gds)
1	1	1	1	-1	1	-1	-1	14.96
2	-1	1	1	1	-1	1	-1	21.78
3	-1	-1	1	1	1	-1	1	16.08
4	1	-1	-1	1	1	1	-1	12.36
5	-1	1	-1	-1	1	1	1	20.91
6	1	-1	1	-1	-1	1	1	13.54
7	1	1	-1	1	-1	-1	1	15.74
8	-1	-1	-1	-1	-1	-1	-1	16.99

[a]Results represent the mean of three experiments.

Inorganic phosphate and hydrocyanic acid determination

Spectrophotometric quantification of inorganic phosphate was performed according to Bae et al. (1999) in triplicate. HCN in the substrate was determined according to the AOAC method of analysis (1980).

HPLC method

Reduction in phytic acid content was validated by reversed-phase high performance liquid chromatography (RP-HPLC) using Agilent 1200 series (Hewlett Packard, Palo Alto, CA, USA) liquid chromatograph equipped with a variable wavelength detector (VWD 1200) and Agilent XDB eclipse C18 (250 × 4.6 mm) column (Rani and Ghosh, 2011). Phytate (IP6) dissolved in the 100 mM sodium acetate buffer (pH 5.1) was used to calibrate the standard curve. A 100 mM sodium acetate solution (pH 5.1) was used as mobile phase with a flow rate of 1.0 ml/min.

Identification of significant process variables using PBD

The prerequisite for optimization of a process involving multiple inputs is to screen out the most influential inputs to determine the model output. PBD was employed for screening the most significant variables in extraction process influencing the phytase production mostly. Based on single-factor experiment, suitable conditions and their ranges were preliminarily determined. In the present study, seven assigned factors were screened in a total of 8 runs. Dummy variables were introduced into the experiment to estimate the experimental error of an effect. The variables, whose effects were negligible under high and low concentrations, were considered as dummy variables. The detail of the design with the response (phytase activity) is given in Table 1.

Central composite designs (CCD)

To determine the mutual interactions among the selected variables (length of extraction time, substrate loading and different pH of extraction solvent) and their corresponding optimum levels, central-composite design (CCD) of response surface methodology (RSM) was used. A 2^3 factorial design having eight factorial points, six axial points and six replicates at the centre point with a total number of 20 runs was formulated. The details of experimental design with coded and actual levels of each factor are summarized in Table 2. A multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response to the independent factors. The complete second-order polynomial model (Equation 1) to be fitted to the yield values was:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \beta_{ii} x_i^2 + \sum_{i=1, i\neq j}^{n-1} \sum_{j=2}^{n} \beta_{ij} x_i x_j \quad (1)$$

Where Y is the observed value of the response (phytase production), x_i (i = 1, 2 and 3) is the controlling factors, b_0 is the offset term, and b_i (i = 1, 2 and 3), b_{ii} and b_{ij} (i = 1, 2 and 3, j = 2 and 3) are the model linear, quadratic and interaction coefficient parameters, respectively.

Artificial neural network (ANN)

The most commonly used network architecture of ANN that is, multilayer feed-forward neural network was used to build predictive models consisting of three inputs (extraction time substrate loading and extraction solvent pH) and two outputs (predicted phytase yield and released phosphate). The method used for the training phase was the back-propagation (BP) based on Levenberg-Marquardt algorithm (LMA) with the aim to fit the outputs of the network to be closer to the desired target and to minimize the performance function in terms of mean squared error (MSE). A backpropagation neural network typically uses sigmoid transfer function and a linear output layer. Therefore, in the present study, the tan sigmoid transfer function, 'tansig' and the 'purelin' transfer function were used for hidden layer and the output layer, respectively. Both input variables and targets were normalized to a range of (-1, 1) before being implemented in the ANN model to avoid any overflows due to very large or very small weights, by using Equation 2:

$$Y = \frac{(Y_{max} - Y_{min})(X - X_{min})}{(X_{max} - X_{min})} + Y_{min} \quad (2)$$

Where Y, Y_{max}, Y_{min}, X, X_{min}, and X_{max} denote the normalized value, maximum value of normalized values (+1), minimum value of normalized values (-1), value of variable, minimum value of variable, and maximum value of the variable, respectively.

The developed topology of ANN was trained several times until the network error (MSE) becomes sufficiently small or equal to the set error goal ($E_0 = 10^{-2}$). After successful termination of training phase of ANN, the predicted model was tested for statistical significance by using analysis of variance (ANOVA).

Table 2. Experimental design used in CCD and ANN with observed and predicted responses.

Run	Time	Substrate loading	pH	Phytase activity (U/gds)			Phosphate released (µmol/ml)		
				Observed	RSM predicted	ANN predicted	Observed	RSM predicted	ANN predicted
1	20(-1)	15(1)	5(-1)	28.34	28.66	29.21	0.184	0.190	0.187
2	20(-1)	15(1)	9(1)	26.57	27.37	26.64	0.106	0.120	0.107
3	30(0)	10(0)	10.36(2)	25.87	25.98	25.86	0.036	0.042	0.035
4	30(0)	18.41(2)	7(0)	31.78	31.39	31.70	0.208	0.207	0.209
5	30(0)	10(0)	7(0)	36.32	36.63	36.37	0.224	0.230	0.229
6	40(1)	5(-1)	5(-1)	25.09	24.25	25.14	0.218	0.230	0.217
7	40(1)	15(1)	5(-1)	25.98	26.04	26.08	0.212	0.220	0.205
8	30(0)	10(0)	7(0)	37.04	36.63	36.37	0.239	0.230	0.229
9	20(-1)	5(-1)	9(1)	25.19	25.09	25.35	0.101	0.096	0.097
10	46.82(2)	10(0)	7(0)	25.67	26.65	25.67	0.166	0.168	0.166
11	30(0)	10(0)	3.64(-2)	24.84	24.78	25.18	0.240	0.220	0.218
12	20(-1)	5(-1)	5(-1)	26.21	26.73	26.18	0.186	0.190	0.187
13	30(0)	10(0)	7(0)	36.79	36.63	36.37	0.207	0.230	0.229
14	30(0)	1.59(-2)	7(0)	27.53	27.97	27.52	0.197	0.192	0.208
15	40(1)	15(1)	9(1)	29.66	29.10	29.58	0.103	0.100	0.102
16	30(0)	10(0)	7(0)	35.98	36.63	36.37	0.226	0.230	0.229
17	40(1)	5(-1)	9(1)	27.32	26.97	27.24	0.095	0.095	0.098
18	30(0)	10(0)	7(0)	37.31	36.63	36.37	0.232	0.230	0.229
19	13.18(-2)	10(0)	7(0)	28.22	27.28	27.89	0.132	0.130	0.136
20	30(0)	10(0)	7(0)	36.34	36.63	36.37	0.230	0.230	0.229

Values in brackets show the corresponding coded values of each factors.

Table 3. Results of PBD analysis.

Factors (code, unit)	Low level (-1)	High level (+1)	SS[a]	Effect	Cont.[b] (%)	t-value
Extraction time (A, min)	20	60	45.89	-4.79	59.92	55.65**
Substrate loading (B, g)	5	10	25.99	3.61	33.94	41.74**
Solvent volume (C, ml)	30	50	0.016	0.09	0.021	1.98
Substrate particle size (D, mm)	1.0	>2.0	0.024	-0.11	0.032	2.23
Rotation of flask (E, rpm)	0	100	1.75	-0.94	2.28	10.69
Solvent pH (F)	3	7	2.90	1.21	3.79	13.92**
Extraction temperature (G,°C)	25	37	0.004	0.05	0.005	0.89

R^2 = 99.94%, R^2 (adj) = 99.86%, R^2 (pred) = 99.59%, coefficient of variation (CV) = 0.74%. [a]Sum of squares;[b]Contribution.

Statistical analyses

The statistical software package 'Design-Expert_8.0.5, Stat-Ease Inc., Minneapolis, MN, USA was used for experimental design and subsequent regression analysis of the experimental data. All experiments were done in triplicate, and the average phytase yield and released inorganic phosphate were taken as the responses. The Neural Network Toolbox V7.13 for MATLAB mathematical software was used for construction of the ANN model.

RESULTS

Selection of significant process variables using PBD

PBD was used for investigating the relative importance of seven independent factors for phytic acid reduction from substrate to be utilized for phytase production. The corresponding effects of these factors on the response (phytase activity) are given in Table 3. From the regression analysis, it was evident that A (extraction time), D (substrate particle size) and E (rotation of flask) enhanced the phytase production at their low level whereas, high level of B (substrate loading), C (solvent volume), F (solvent pH) and G (extraction temperature) supported high phytase yield. Based on analysis of total sum of squares and percent contribution, the most significant factors influencing phytase production were found to be A (extraction time), B (substrate loading) and F (solvent pH), respectively (Table 3). The regression

Table 4. ANOVA analysis for RSM model.

Source of variation	Phytase activity (U/gds)		Phosphate released (µmol/ml)	
	F– value	p–value Prob > F	F– value	p–value Prob > F
Intercept	85.52	< 0.0001	57.55	< 0.0001
A	0.84	0.3798	6.71	0.0269
B	25.07	0.0005	0.39	0.5460
C	3.06	0.1108	307.43	< 0.0001
AB	0.017	0.8977	0.000	1.0000
AC	16.79	0.0022	4.57	0.0582
BC	0.11	0.7484	0.47	0.5108
A^2	298.38	< 0.0001	94.25	< 0.0001
B^2	154.45	< 0.0001	11.45	0.0070
C^2	404.68	< 0.0001	121.06	< 0.0001

model gave a model F-value of 1291.33 with a corresponding model p-value (>F) of 0.0001, which shows the model to be highly significant. Also, the coefficient of determination (R^2) indicates that the model could explain 99.94% of the total variations in the response. A very low value of coefficient of variance (CV, 0.74%) further confirms the reliability of the model.

Predictive modeling using RSM

The experimental design output (Table 2) was analyzed using analysis of variance (ANOVA) which shows that the regression was statistically significant ($p<0.0001$) at 95% of confidence level. The results for ANOVA analysis for RSM for both responses have been summarized in Table 4. Application of multiple regression analysis on the RSM experimental data resulted in the following quadratic model (Equations 3 and 4) explicitly explaining the phytase production and phosphate released as a function of initial values of selected process parameters:

$$\text{Phytase activity } Y_1 = 36.63 - 0.19A + 1.02B + 0.36C - 0.035AB + 1.09AC + 0.088BC - 3.42A^2 - 2.46B^2 - 3.98C^2 \quad (3)$$

$$\text{Phosphate released } Y_2 = 0.23 + 0.008A + 0.002B - 0.054C + 0.000AB - 0.008AC + 0.00275BC - 0.029A^2 - 0.010B^2 - 0.033C^2 \quad (4)$$

Where Y_1 and Y_2 represents phytase activity (U/gds) and phosphate released (µmol/ml), respectively, and A, B and C are the coded factors of extraction time (min), substrate loading (g) and extraction solvent pH, respectively.

In this case, linear term (B), interaction terms (AC) and all the quadratic terms (A^2, B^2 and C^2) were found to be the most significant for phytase production. The statistical significance of the model equation was supported by the model high F-value of 85.52. Correspondingly, ANOVA for phosphate release indicated the F-value to be 57.55, which implied that the model is significant. Again, the quality of fit of the regression model was justified by high value of coefficient of determination (R^2) of 0.9872 and 0.9811, respectively, for responses Y_1 and Y_2, which indicates an excellent correlation between the independent factors. At the same time, the predicted R^2 (correlation coefficient) value of 0.9180 and 0.9073 were found in concordance with the adjusted R^2 value of 0.9756 and 0.9640, respectively, suggesting a strong agreement between the experimental and predicted values of phytase production and phosphate released. The coefficient of variation (CV) indicates the degree of precision with which the treatments are evaluated and a lower value of CV namely, 2.51 and 6.44%, respectively for Y_1 and Y_2, demonstrates that the performed experiments were highly reliable. Furthermore, high values of adequate precision (23.314 and 22.878, respectively, for Y_1 and Y_2) that represents signal (response) to noise (deviation) ratio, indicates an adequate signal and suggested that the model can be used to navigate the design space.

Predictive modeling using ANN

The feed-forward back propagation network used for fitting the same experimental data (Table 2) resulted in an optimum topology of ANN model with 3 inputs, one hidden layer with 4 neurons and 1 output layer involving single neuron in case of both responses (Figure 1). The results of the design of experiments and ANN are given in Table 2. The training phase was carried out for different set of conditions, for instance, number of neurons in the hidden layer, learning rate, random initialization etc. In the present study, the training was stopped after 9 epochs. At the final point of training, the performance function was observed to be 1.3×10^{-2}.

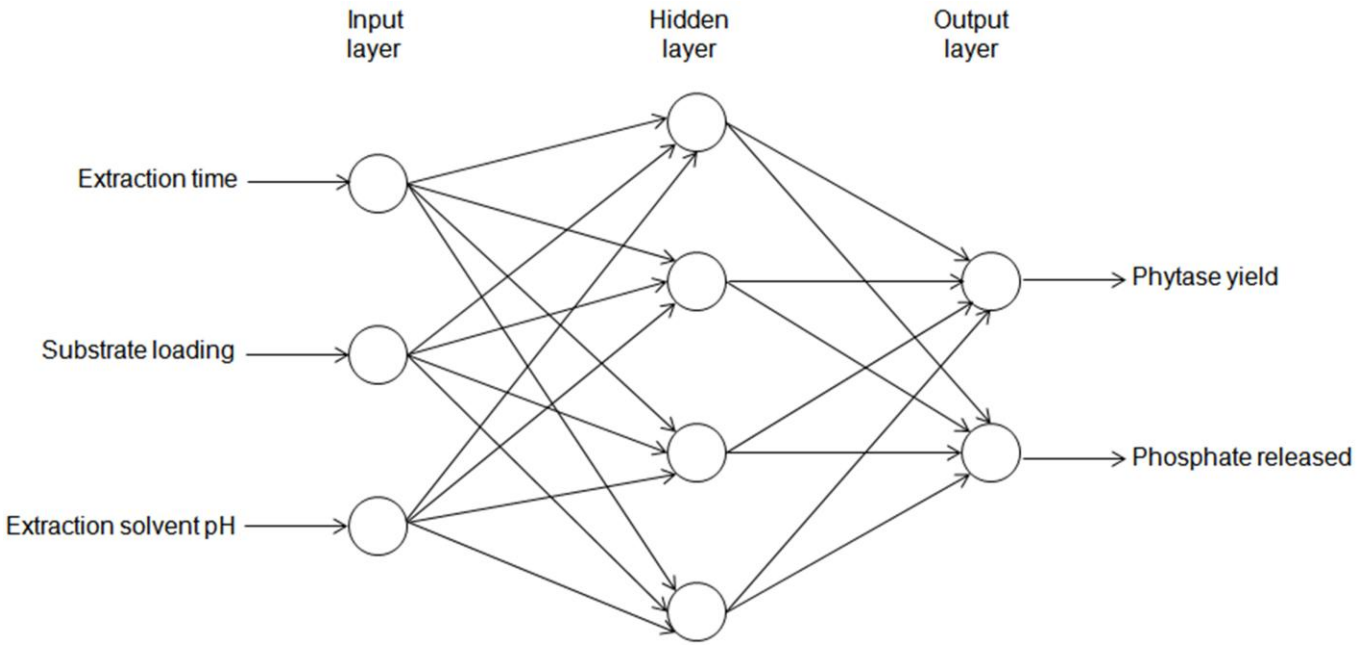

Figure 1. Schematic illustration of the ANN architecture.

Table 5. ANOVA analysis for ANN model.

Source	SS[a]	MS[b]	CV[c] (%)	F-value	p-value	R^2	R^2_{adj}	R^2_{pred}
For phytase yield								
Model	407.16	45.24	1.97	130.93	< 0.0001	0.992	0.984	0.931
Residual	3.46	0.69						
Total	410.62							
For released phosphate								
Model	0.067	7.49e-03	3.17	236.86	< 0.0001	0.995	0.991	0.965
Residual	3.17e-04	3.17e-05						
Total	0.068							

[a]Sum of squares. [b]Mean square; [c]Coefficient of variation.

The performance function (MSE) for training, validation and test data was found to have approached the set goal. The regression R values between the model predicted and experimental phytase yield related to the training, validation, test, and all datasets are illustrated in Figure 2A. The comparable values of MSE and R for each set outputs reveal that the feed-forward-based model possesses good approximation characteristics. Statistical results for the developed ANN model, calculated in a similar way as for the RSM model, have been summarized in Table 5, depicting a significant ANN model that can be used for predictive simulations of aqueous extraction process. The results also showed that the ANN based prediction were found to be more accurate as compared to the RSM model (Figure 2B).

In order to gain the better understanding of the effects of the significant factors on phosphate release and phytase production, the RSM predicted model and trained ANN model were represented as 3D response surface curves shown in Figure 3. The elliptical response surfaces implied that there were perfect interaction between the independent variables, however, the circular surfaces suggested that the optimized values may not vary widely from the single variable conditions. Significant interaction effect between extraction time and pH of extraction solvent for phytase production was predicted by both modeling techniques, however, neural network predicts a sharper ridge surface than RSM (Figure 3A). Figure 3B shows the interaction between extraction time and pH of extraction solvent on release of phosphate.

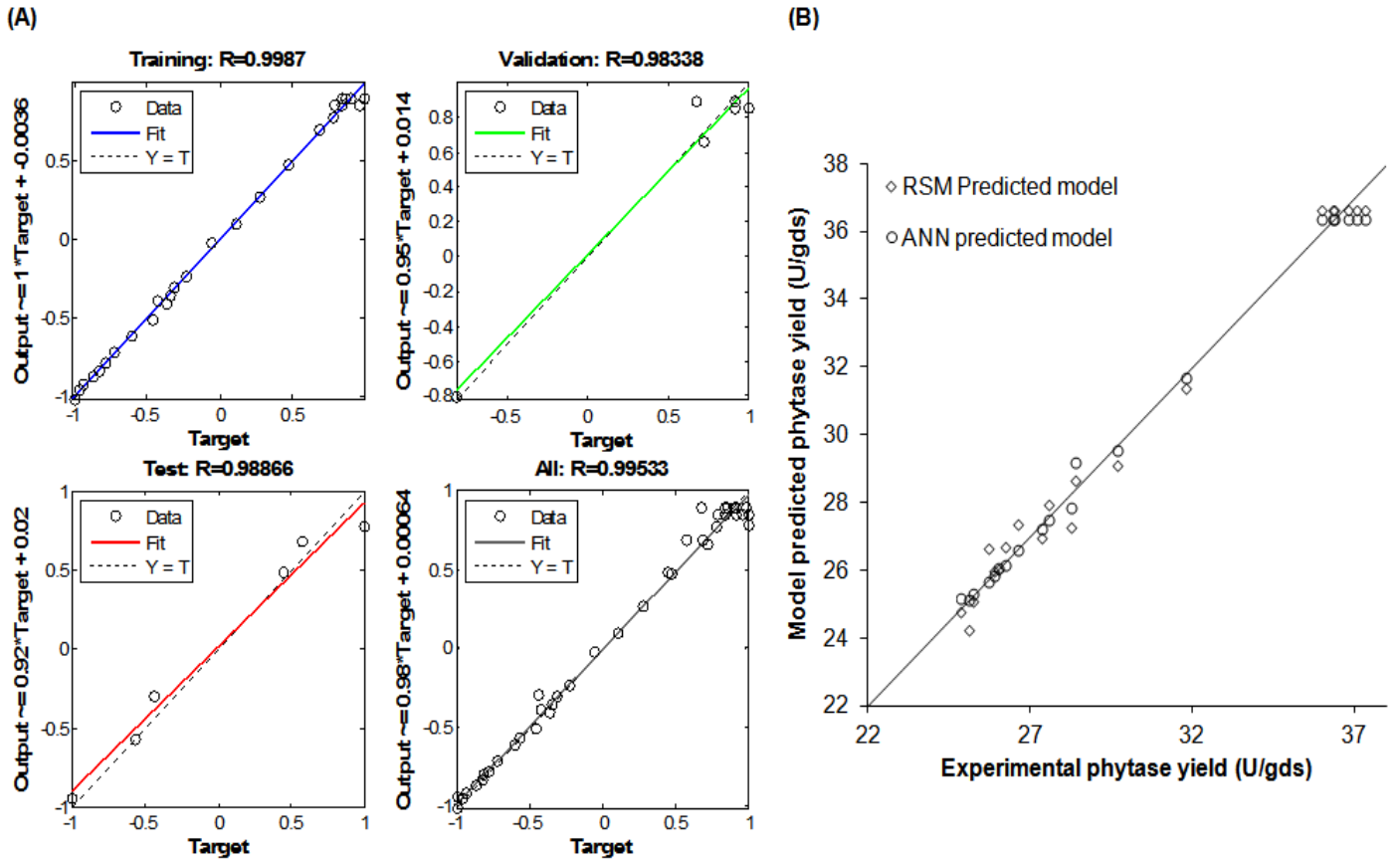

Figure 2. Regression analysis for training, validation, test, and all datasets for phytase production and phosphate released (A) and comparative analysis of RSM and ANN predicted models for phytase yield (B).

Increase in extraction time from 20 to 32 min with solvent of lower pH enhanced the release of phosphate from the substrate. It could be explained that, decreasing solvent pH may enhance the release of phosphate in aqueous extraction process.

As shown in Figure 3C, the interaction of substrate loading and pH of extraction solvent had a much weaker effect on the yield of phytase. The effect of combination of extraction time and substrate loading on the phosphorus release is shown in Figure 3D. It may be observed that increase of extraction time from 20 to 32 min and substrate loading from 5 to 11 g, the release of phosphorus was increasing gradually. How-ever, this interactive effect of extraction time and sub-strate loading on the phosphorus release was not very significant ($p = 0.465$). The maximum phytase production was observed after treating substrate (11.04 g) with extraction solvent adjusted at pH 7.1 for 29.78 min.

Validation of experimental model

The results from validation experiments showed a strong agreement between the maximum predicted response and the experimental response of 36.59 and 37.65 U/gds, respectively, thus supporting the high adequacy of the model. Moreover, the statistical optimization of aqueous extraction process for phytase production resulted in an overall 2.37-fold increase in phytase yield. Reduction in phytic acid content from mixed substrate was validated by the HPLC characterization study. The chromatogram of standard sodium phytate was found to be linearly proportional to the concentrations throughout, with R^2 value and retention time (R_t) of 0.991 and 1.23 ± 0.02 min, respectively, and was found to be in complete agreement with our previous report. Chromatogram profile for untreated and aqueous extracted substrate for phytic acid reduction is illustrated in Figures 4A and 4B, respectively.

DISCUSSION

The ANOVA analysis showed the effect of process variables on each response for both statistical models. Interestingly, the efficiency of each model for both responses was found to be different. The F-value of the RSM predicted model were 85.52 and 57.55 for the phytase

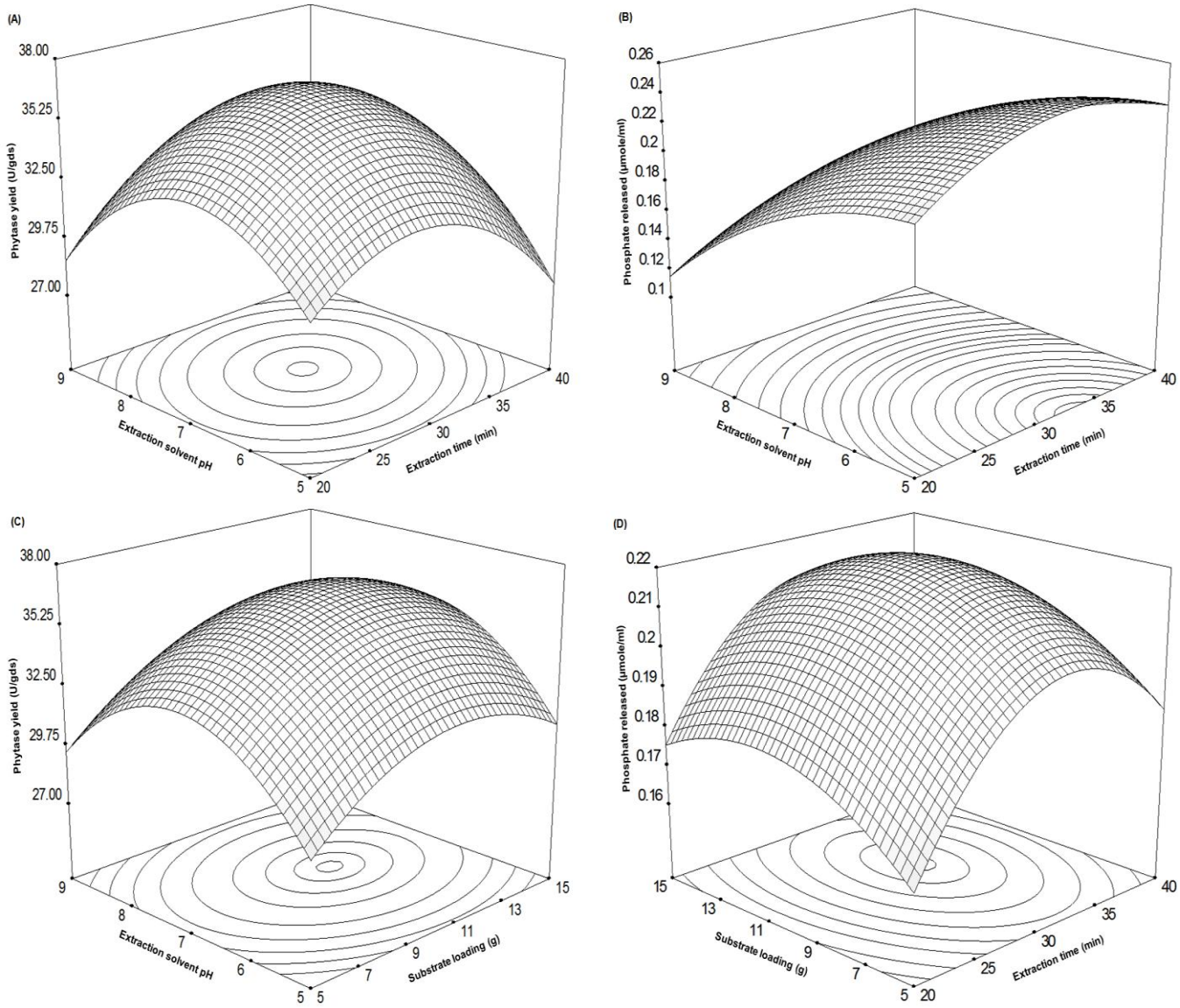

Figure 3. 3D response surface plots showing effect of interactions of, extraction time and extraction solvent pH on phytase production (A), extraction time and extraction solvent pH on phosphate released (B), substrate loading and extraction solvent pH on phytase production (C) and extraction time and substrate loading on phosphate released (D).

yield and phosphate released, respectively (Table 4), whereas, for ANN predicted model, the corresponding values were found to be 130.93 and 236.86, respectively. The comparative study between RSM and ANN, clearly revealed that the ANN based model for the extraction process was superior than the RSM model. Additionally, the experimental values were found to be very close to the ANN predicted theoretical values, which further showed that the ANN model could be used for the process optimization of the detoxification process in water. We conclude that aqueous treatment resulted in the reduction of HCN (data not shown) and inorganic phosphorus concentration and hence, reduction in phytic acid content from the substrate. The results were in full agreement with the findings by Hossain and Jauncey (1990), where a significant reduction in phytic acid along with HCN content was observed, with higher reduction efficiency in water extracted linseed meal as compared to the heat treated. Furthermore, an increasing trend in release of inorganic phosphate from the substrate treated with the extraction solvent at lower pH and for longer time, with no further increase in phytase yield suggested that initial inorganic phosphate concentration in the substrate is well correlated with the phytase production in SSF.

This result is in good conformity with the previously reported findings (Vats and Banerjee, 2002; Vohra and Satyanarayana, 2003) where, higher concentration of

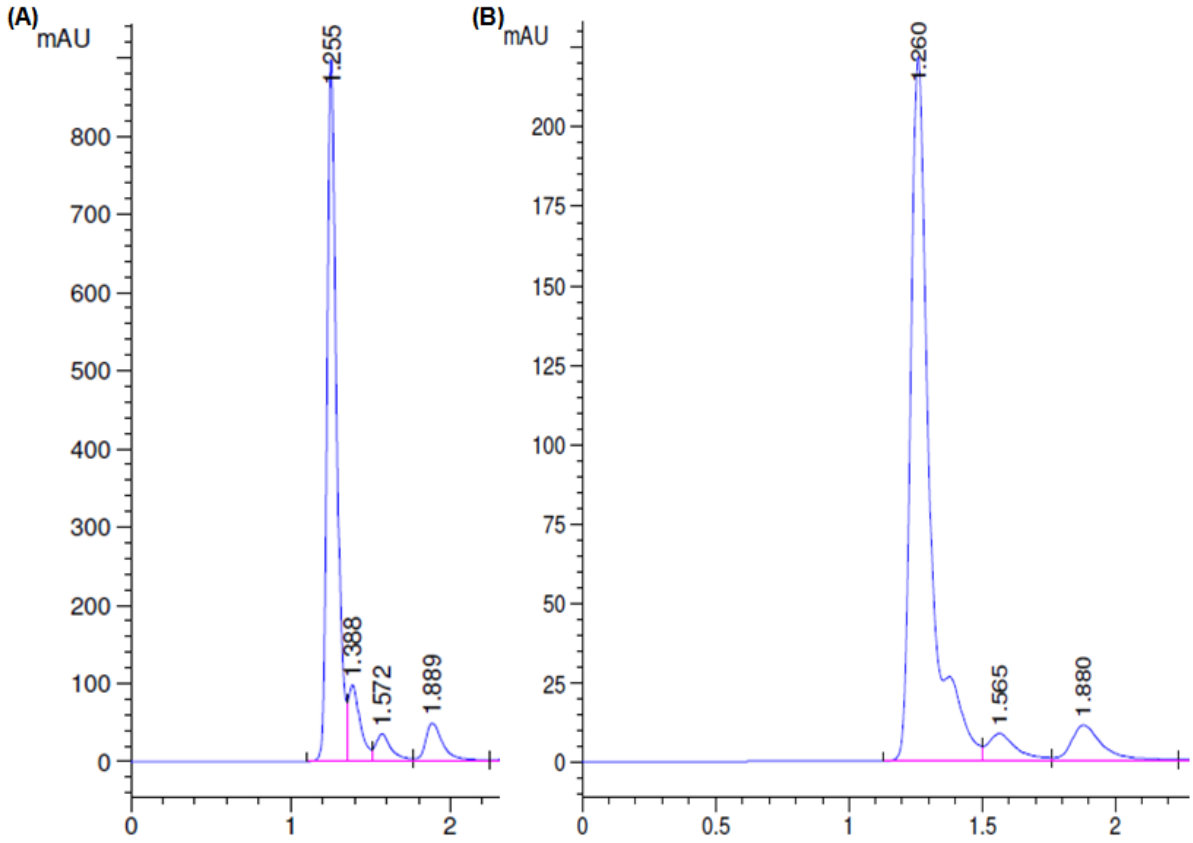

Figure 4. HPLC characterization studies showing phytic acid content in, untreated substrate (A) and aqueous extracted substrate (B).

inorganic phosphates resulted in a repression of phytase synthesis.

Longer extraction time, higher substrate loading and very high/low pH resulted in a significant lower phytase production during SSF. The results clearly suggested that the low inorganic phosphorus substrate stimulates phytase synthesis and excess of inorganic phosphorus causes repression of phytase synthesis, although the presence of traces of inorganic phosphorus is an essential ingredient of phytase production medium and induces its production (Soni and Khire, 2007).

Conclusion

Artificial neural network (ANN) and response surface methodology (RSM) methods were compared for their optimization efficiency in an aqueous extraction process. Both models preformed well and suggested stable responses in envisaging the interactions of the independent process variables and their optimal concentrations with respect to the responses, however, the ANN based approach was found to be more robust and accurate in fitting the computed responses when compared to the RSM based model. Treatment of the substrate prior to fermentation was shown to affect phytase production during solid-state fermentation with an overall increase in phytase yield.

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