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Optimization, Purification and Characterization of Phytase from Isolated Probiotic *Pediococcus acidilactici* BNS5B

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A B S T R A C T

Microbial phytases are of immense importance due to their application in food/feed industry by enhancing the availability of essential minerals such as phosphorous, iron, calcium etc. required for normal human physiology and also have commercial and environment significance. Therefore, in present study an attempt was made to enhance the production of phytase from isolated probiotic *Pediococcus acidilactici* BNS5B by employing both one variable at a time approach and statistically based design of experiments such as Plackett-Burman and Response Surface Methodology. Interestingly, phytase production was enhanced 94 fold at an optimised condition of 0.8% galactose, 1.25% yeast extract, 1.25% beef extract and 1.25% ammonium sulphate. Further, the phytase enzyme was purified and had apparent molecular weight of 43 KDa, pH optima of 5.5 with pH stability in the range of 2.5-6.5, temperature optima of 40°C and retaining an activity of 76% at a temperature range of 20-80°C and followed normal Michael-Menten curve with the kinetic parameter K_m and V_{max} of 0.5455mM and 33.927µmol/min respectively. Taken together, it is suggested that phytase from probiotic *Pediococcus acidilactici* BNS5B can be employed to enhance mineral bioavailability in food/feed industry, but needs to be correlated both experimentally and clinically.

Introduction

Plant based diet such as vegetables, cereals, legumes and oilseeds contain 80% of total phosphorous in the form of phytic acid-cation complexes, bound phosphorous being excreted in manure due to unavailability of phytate degrader in the gastrointestinal tract of monogastric animals (Ashraf *et al.*, 2013). The undegraded phytate leads to phosphorous deficiency in animals, elevated levels of phosphorous in soil and eutrophication of water bodies and renders phytic acid as the anti-nutritive factor by decreasing the bioavailability cations such as iron, calcium, magnesium, phosphorous, zinc, iodine, etc (Madsen, 2019; Singh *et al.*, 2013). Most of these cations are involved in various physiological functions as their deficiency may lead to conditions such as anemia,

neurological disorders, immune disorders (Black *et al.,* 2013; Christian and Stewart., 2010). The enzymatic degradation of organically bound phosphate by phosphohydrolases and phytases, reduces the need of feed supplementation with calcium phosphate resulting in reduced phosphorous excretion and environmental pollution (Almeida *et al.,* 2013).

Phytases are used as feed supplement to manogastric animals for the reduction of phytate and have been isolated from various sources like plant, animals, bacteria, fungi for improving the nutritional quality of food and feed products (Rasul *et al.,* 2019; Shah *et al.,* 2017; Menezes-Blackburn *et al.,* 2015). However, microbial phytases are more efficacious due to their substrate specificity, resistance to proteolysis and catalytic efficiency for animal nutrition, environment protection as well as for human health (Qvirist *et al.,* 2017; Sreedevi and Reddy, 2013; Saravanamuthu, 2010). The various commercially available phytases have been produced synthetically from genetically modified organisms such as Quantum^{TM} being produced from *Escherichia coli*, Natuphos^{TM} from *Aspergillus niger*, Ronozyme from *Peniophora lycii* and Phyzyme is derived from yeast *Schizosacchromyces pombe* and are used for in-vitro degradation of livestock feed products (Menezes-Blackburn *et al.,* 2015; Nam-Soon Oh and Man-Jin In, 2009). Since, recombinant phytases are costly and are under legal issues, thus the need of hour is that a phytase to be used as the feed additives should be more economical and effective in releasing phytate phosphorous in the digestive tract (Sreedevi and Reddy, 2013a). Therefore, an attempt was made to isolate an organism with phytase activity from the human microbiome as gut microbiota is the least explored source of microorganisms capable of producing enzymes of industrial importance (Feng *et al.,* 2018; Haefner *et al.,* 2005). In this context, we have isolated a phytase producing probiotic *Pediococcus acidilactici* BNS5B from neonatal feces with dephytinising ability on both food/feed products (Sharma and Shukla, communicated).

Due to the commercial importance of phytase and to enhance the yield of phytase being produced by isolated probiotic *Pediococcus acidilactici* BNS5B, different optimisation strategies have been employed (Qvirist *et al.,* 2017). Therefore, designing an appropriate medium is of crucial importance because medium composition significantly affects the growth of organism vis-à-vis enzyme yield (Gao *et al.,* 2009). However, the traditional one variable at a time technique used for optimisation is not only time consuming and employs number of experiments to determine the optimum levels but also misses the alternative effect between the nutrients (Kumar and Satynarayan, 2007). To overcome these problems Plackett- Burman (Plackett-Burman, 1946) and Central Composite Design using Response Surface Methodology (RSM) was employed where levels can be easily evaluated. Therefore, in the present study physico-chemical parameters were optimised to design a medium for enhanced yield of phytase from isolated well characterized probiotic *Pediococcus acidilactici* BNS5B as well as to characterise the purified phytase.

Materials and Methods

Bacterial strain

Pediococcus acidilactici BNS5B (Accession No. MH916767) was grown and maintained in chemically defined medium (CDM). Briefly, media contained glucose (1.5%), yeast extract (1%), beef extract (0.5%), peptone (1%), sodium acetate (2.5%), $FeSO_4$ (0.001%), $MgSO_4.7H_2O$ (0.01%), $CaCl_2.2H_2O$ (0.01%), $MnSO_4$ (0.001%), NH_4SO_4 (0.5%), KCl (0.05%), NaCl (0.01%), calcium phytate (1%)

and pH 6.5 was inoculated, incubated at 37°C for 24h for the production of enzyme phytase and was optimised to enhance the production of phytase.

Phytase assay

Activity of phytase produced by probiotic *P. acidilactici* BNS5B was assayed as per Raghavendra and Halami, 2009. Briefly, 100µL of the supernatant containing enzyme was mixed with 900µL of substrate (2mM calcium phytate in sodium acetate-acetic acid buffer, pH 5.5) and incubated 15 min at 37°C for catalytic reaction. The reaction was stopped by addition of 500µL trichloroacetic acid (10%), followed by addition of 1mL coloring reagent (prepared by mixing 1 volume of 2.5% ferrous sulphate to 4 volumes of 2.5% ammonium molybdate in 5.5% sulphuric acid) was added. The released inorganic phosphorous was measured spectrophotometrically at 700nm as the phytase activity is the amount of enzyme liberating 1µmol of inorganic phosphate from calcium phytate under standard assay conditions (Nielsen *et al.*, 2008).

Hyperproduction of phytase from *P. acidilactici* BNS5B in submerged fermentation (SmF)

The various physico-chemical factors were optimized for hyperproduction of phytase from probiotic *P. acidilactici* BNS5B employing both one variable at a time (OVAT) and statistical method (Plackett-Burman and Response Surface Methodology) in submerged fermentation.

Optimisation of phytase production in SmF by One-variable-at-a-time method (OVAT).

Various nutritional (carbon source, nitrogen source) and physical parameters (incubation time, incubation temperature, inoculum age, inoculum percentage, pH, agitation) are known to affect the enzyme yield. Therefore, the optimisation of phytase was performed by varying the nutritional and physical parameters one at a time keeping other variables constant in production media. The optimized condition in each step was taken as constant for subsequent steps and the phytase activity was assessed after every optimisation step as described above.

Incubation time

To assess the effect of time on phytase production, the production medium was inoculated with 1% inoculum of 18 h old log phase culture and incubated at 37°C for 120 h. After every 24 h the culture was cold centrifuged at 7826g and the cell free supernatant was analyzed for phytase activity.

Inoculum age and Inoculums density

The inoculum age was optimized by inoculating the production medium with 1% inoculum of different age (12, 24, 48, 72 and 120 h) and incubated at 37°C for 72 h.

Thereafter, the phytase activity was assessed in the cell free supernatant. However for inoculum size, medium was inoculated with different concentration (1%-5%) of 24 h culture by all other variables in optimum conditions. After incubation the cell free supernatant was obtained and analyzed for phytase activity.

Effect of different carbon sources

Effect of different carbon sources (Glucose, Galactose, Sucrose, Lactose, Mannose, Xylose, Arabinose) was estimated by replacing glucose in the production medium with 1% respective sugar and incubating at 37°C for 72h, cold centrifuged and cell free supernatant was analyzed for phytase activity.

Effect of different concentration of galactose

Concentration of galactose was varied from 0 to 5% in the production medium and incubated at 37°C for 72 h. Thereafter, the culture was cold centrifuged and phytase activity was assessed in the supernatant.

Production pH

To determine the effect of pH on the production of phytase, the pH of the production medium was varied from 3.5 to 7.5 with 1M HCl and 1N NaOH. The medium was then assessed for phytase production after 72 h at 37°C keeping all other conditions constant.

Effect of Incubation Temperature

To find out the optimal incubation temperature for maximum phytase production, the medium was inoculated with 1% inoculum and incubated at various temperatures i.e. 27°C, 37°C, 47°C, 57°C and 67°C for 72 h while other conditions were kept optimum and phytase activity was monitored.

Effect of different nitrogen sources

The effect of nitrogen source was determined by replacing ammonium sulphate in the production medium with 1% of different nitrogen sources (Beef extract, Yeast Extract, Peptone, Urea, Ammonium sulphate, Potassium nitrate, Sodium nitrite, Ammonium ferricitrate).

Optimisation of phytase production in SmF by statistical method.

Selection of significant factors by Plackett-Burman design

On the basis of OVAT, the 11 variables i.e. Galactose, Beef Extract, Yeast extract, Proteose Peptone, Ammonium sulphate, Inoculum density, Manganese sulphate, Magnesium sulphate, Potassium chloride, Sodium chloride and Calcium chloride were screened using Plackett- Burman Design (Design expert 11.03, Stat-Ease Inc., Minneapolis, USA at two levels (high and low; +1 and -1) for the preliminary screening of significant cultural parameters that may further affect the production of enzyme phytase from *P. acidilactici* BNS5B (Table 1). Factors were analysed using normal probability plot or pareto-chart of model where factors showing maximum positive effect were selected for further optimisation using central composite design of response surface methodology.

Response surface methodology using Central composite design

The variables affecting positively on the enzyme production were further optimised by central composite design (CCD) using design expert software 11.0.3. The four most significant factors i.e. Galactose, Ammonium sulphate, Beef Extract and Yeast Extract were optimized at five different levels (-2, -1, 0, +1, +2) in an experimental plan of 30 trials keeping other factors constant. A multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response measured to the independent variables. The following equation explained the behaviour of the system and was used to construct 3D plots (Eq. 1).

$$Y = ß_o + \Sigma\, ß_i X_i + \Sigma ß_{ii} X_i^2 + \Sigma\, ß_{ij} X_i X_j \qquad \text{(Eq. 1)}$$

Where Y is predicted response (Phytase activity U/mL), $ß_o$ is constant, $ß_i$ is coefficient of linear effect, $ß_{ii}$ is coefficient of quadratic effect, $ß_{ij}$ is coefficient of interaction effect.

The 3D and counter plots generated with the statistical software were employed to analyse the trend of phytase activity and the interactive effect of the significant variables on the activity response. The resulting model was analysed using ANOVA and the significance of each coefficient was determined by p and f value.

Validation of the Experimental model

The validation of the statistical model was performed using the optimal conditions predicted by the model and response (enzyme yield) was measured by phytase assay and compared with the predicted value. Each experiment was performed in triplicates.

Purification and characterization of enzyme phytase from *P. acidilactici* BNS5B

The phytase from the probiotic strain was purified using standard protein purification protocol. Optimized production media was inoculated with 1% inoculum of 24 h old MRS broth culture and incubated for 72 h at 37°C. The crude enzyme was obtained after cold centrifugation of 15 min at 9000g. The cell free supernatant obtained was employed for purification of extracellular enzyme.

Enzyme purification

The cell free supernatant was filtered through a 0.45µm pore size filter and then the equal volumes of 70% ethanol was added to the filtrate and incubated overnight at -20°C. After ethanol precipitation, extracellular enzyme and alcohol was separated by cold centrifugation (9000g for 15 min). The concentrated extracellular enzyme was suspended in 0.1 M sodium acetate-acetic acid buffer, pH 5.5, and a volume of 1mL was loaded onto a DEAE- Cellulose ion-exchange column. The fractions were eluted with linear gradient of 0 to 0.5M NaCl in 0.1M sodium acetate- acetic acid buffer (pH 5.5) at a flow rate of 1ml/min. The eluted fractions were assayed for protein at 280nm and phytase activity. The phytase active fractions were pooled and dialyzed against 10mM sodium acetate acetic acid buffer (pH5.5) and stored at -20°C for further characterization. The protein content was estimated by Lowry's method at each purification step so as to assess specific activity, fold purification and yield percentage of enzyme (Parhamfar *et al.,* 2015).

Molecular mass determination

The enzyme purified at each step was analyzed by SDS-PAGE (Laemmli, 1970). The molecular weight of the purified phytase was determined with the BLUeye Prestained protein ladder with wide range of 11 to 245 KDa.

Characterization of purified phytase

The purified phytase enzyme was characterized for pH optima, temperature optima, temperature stability and kinetic properties

pH optima and stability

The purified enzyme was assessed for the optimum pH by measuring the enzyme activity at different pH (2.5-8.5) using Glycine-HCl (2.5), Sodium acetate-Acetic acid (3.5-6.5) and Glycine-NaOH (7.5-8.5) buffers. The stability was assessed by pre-incubating the enzyme in buffer for one hour and the estimating the residual activity at optimum pH under standard assay conditions (37°C, 15 min).

Temperature optima and stability

The optimum temperature of purified enzyme was determined at different temperature (20 to 70°C) and thermal stability was determined by

pre-incubating the enzyme at different temperature for 30 min followed by measuring the enzyme activity under standard conditions at optimized pH.

Effect of metal ions on purified phytase enzyme activity

The effect of metal ions was determined by measuring phytase activity in the presence of metal ions (Cu^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, Ca^{2+}, Mn^{2+}) at concentration of 0.5mM and 1mM. To assess the effect 10µL of purified phytase was incubated with the metal ion solution at 37°C for 15 min. Thereafter, the phytase activity at 40°C for 15 min was assessed and the enzyme without metal treatment was used as control.

Substrate specificity and Kinetic parameters

The substrate specificity of phytase was tested with 2mM concentration of sodium phytate, p-nitrophenyl phosphate, sodium pyrophosphate and calcium phytate in 0.1M sodium acetate acetic acid buffer (pH 5.5). The kinetic parameters of enzyme were studied for the substrate with maximum specificity.

The Michaelis-Menten constant (K_m) and the maximum attainable velocity (V_{max}) of phytase at different concentrations of sodium phytate (0.5mM to 5mM) was determined using Lineweaver- Burke plot and applying Michaelis-Menten equation (Eq. 2)

$$\frac{1}{V_o} = \left(\frac{Km}{Vmax}\right)\left(\frac{1}{[S]}\right) + \frac{1}{Vmax}$$

(Eq. 2)

Where V_o is initial velocity and [S] is the substrate concentration

The phytase activity was measured at 40°C for 15 min by the standard enzyme assay. All the experiments were performed in triplicates, and results show the mean values of the activities.

Statistical analysis

All the experiments were repeated in triplicates and the results are expressed as mean ± standard deviation.

Results and Discussion

Lactic acid bacteria are not naturally optimized for maximal production of biotechnologically important compounds, therefore it is of significant important to optimize nutritional and physical conditions with regard to desired end products (Wood and Holzapfel, 1995).

A variety of nutritional (carbon source, nitrogen source) and physical parameters (incubation time, incubation temperature, inoculum age, inoculum percentage, pH, agitation) were optimised by conventional "one variable at a time" approach. The significant factors were then further optimised by statistical software package 'Design expert 11.1, Stat-Ease Inc., Minneapolis, USA'.

Effect of incubation time on phytase production

To assess the time course for enzyme production by *P. acidilactici* BNS5B, the medium was incubated upto 120 h and maximum phytase production was found after 72 h (0.33U/mL). Thereafter, phytase activity started declining due to catabolic repression or reduction in nutrient availability (Singh and Satyanarayan, 2006).

Inoculum age and Inoculums density

To investigate the optimum inoculum age and density for phytase production, inoculum of different ages (12, 24, 48, 72 and 120 h) was employed and found to have maximum

phytase activity (0.42U/mL) with 24 h inoculum of 3% v/v density.

Effect of different carbon sources and its concentration

The phytase production was assessed with different carbon sources i.e. Glucose, Galactose, Sucrose, Lactose, Mannose, Xylose, Arabinose and was found that galactose at a concentration of 0.5% exhibited maximum activity of 0.72U/mL.

Production pH

To assess the effect of pH on phytase activity, medium with different pH (3.5 to 7.5) was employed and maximum phytase yield of 0.82U/mL was obtained at pH 5.5. This may be due to increase in pH that affects the active site resulting into decreased enzyme activity vis-a-vis enzyme-substrate complex formation (Roy *et al.*, 2012).

Effect of Incubation Temperature

The phytase was produced at all the temperatures but maximum activity of 1.32U/mL was obtained at 37°C. As production of enzyme depends on the growth of microorganisms, since the optimum temperature for the growth of most organism lies in the range of 25°C - 37°C, resulting into enhanced enzyme production (Tungala *et al.*, 2013).

Effect of different nitrogen sources

Since, LAB have limited capacity to synthesize amino acids from inorganic nitrogen source thereby to assess the optimum nitrogen source combination of both organic (Yeast extract, beef extract and peptone, urea) and inorganic nitrogen source (Ammonium sulphate, Potassium nitrate, Sodium nitrite, Ammonium Ferricitrate) was analysed. It was found that maximum phytase activity (1.54U/mL) was observed with the combination of both organic (1% of Yeast extract, 1% beef extract and 1% peptone) and inorganic nitrogen source (1% ammonium sulphate) compared with organic (0.44U/mL) and inorganic (0.57U/mL) sources used alone (Fig. 1a,1b,1c). Therefore, it is crucial to include balanced amounts of yeast extract, beef extract and peptone in LAB culture media to ensure suitable level of growth and better functionality (Hayek and Ibrahim, 2013).

Selection of significant factors by Plackett-Burman design

The influence of 11 parameters i.e. Galactose, Beef Extract, Yeast extract, Proteose Peptone, Ammonium sulphate, Inoculum density, Manganese sulphate, Magnesium sulphate, Potassium chloride, Sodium chloride and Calcium chloride on phytase production was assessed in 12 runs using Plackett- Burman Design. The Length of columns in pareto chart represented the significance of studied parameters on enzyme activity (Fig. 2) where 4 factors (Galactose, Ammonium sulphate, Beef extract, Yeast extract) were found to have positive effect and were selected for further optimization using Central Composite Design (CCD) of Response surface methodology.

Optimisation using Response Surface Methodology

RSM using Central Composite Design (CCD) was employed to optimize and understand the interaction between 4 selected variables i.e. galactose, ammonium sulphate, beef extract and yeast extract in an experiments of 30 runs.

The coded levels of variable and experimental and predicted results of 30 runs for phytase activity are shown in Table 2.

The results were analyzed by ANOVA (Table 3) and the second order regression equation was obtained which showed phytase activity as a function of galactose, ammonium sulphate, beef extract and yeast extract which can be predicted in terms of coded factors as:

$Y = +54.00 +0.2421 A +0.0879 B +0.2921 C +0.1971 D -0.8744 AB +0.7106 AC +1.68 AD +1.13 BC +0.6644 BD -1.28 CD -2.13 A^2 -1.69 B^2 -1.30 C^2 -1.33 D^2$

Where Y is phytase (U/mL), A is galactose (%), B is ammonium sulphate (%), C is beef extract (%) and D is yeast extract (%).

The "lack of fit" value of 0.8740 and p value of <0.001 indicated the model to be highly significant. The coefficient of determination (R^2) was 0.9663 which implies data variability in the response of 96.63%. The coefficient correlation depicted by predicted R^2 was 88.32% which suggests good agreement between predicted and experimental values of phytase production. The model showed AB, AC, AD, BC, CD, A^2, B^2, C^2 and D^2 to be significant. The 3D response surface plots showed optimal levels from the peak and non linear interaction between the variables for phytase production from shape of the curve and the elliptical counter plots indicate that the interaction between related variables are significant (Fig 3). All these factors depict that model could be used for prediction of phytase yield under given ranges.

The model predicted that maximum phytase production (55.53U/mL) was obtained with 0.8g galactose, 1.25g each of yeast extract, beef extract and ammonium sulphate per 100g after an incubation of 72 h at 37°C under static condition. The model was validated as phytase activity for optimum medium was in close agreement with predicted value. The phytase production under un-optimized conditions was 0.59U/mL which increased to 55.53U/mL resulting in approximately 94 fold increase.

Purification of enzyme phytase from *Pediococcus acidilactici* BNS5B

The phytase activity was eluted as a single sharp peak from ion- exchange column after application of the gradient (Fig. 4). A summary of the purification scheme is given in Table4. A 23 fold purification of the phytase was achieved with a recovery of 47.8% and enzyme exhibited an activity of about 66.5U/mg.

Table.1 Experimental range and 5 levels of independent test variables used in Central Composite Design for phytase production from *Pediococcus acidilactici* BNS5B

Code	Factor	Units	LEVELS				
			-2	-1	0	+1	+2
A	Galactose	%	0.4000	0.60	0.8000	1.00	1.20
B	Ammonium sulphate	%	0.7500	1.00	1.25	1.50	1.75
C	Beef Extract	%	0.7500	1.00	1.25	1.50	1.75
D	Yeast Extract	%	0.7500	1.00	1.25	1.50	1.75

Table.2 Experimental Design and result of Central Composite Design of Response Surface methodology.

Run Order	Galactose (%)	Ammonium sulphate (%)	Beef Extract (%)	Yeast Extract (%)	Experimental Value	Predicted Value	Residual
1	0	0	0	0	53.32	54.00	-0.6783
2	+1	+1	-1	+1	48.43	48.68	-0.2533
3	-1	-1	+1	1	48.81	48.21	0.5967
4	0	+2	0	0	47.56	47.41	0.1471
5	-1	-1	-1	+1	47.40	47.01	0.3867
6	+1	-1	-1	+1	51.21	51.18	0.0312
7	-1	+1	-1	+1	47.24	48.02	-0.7754
8	-2	0	0	0	44.85	44.98	-0.1279
9	0	0	0	0	52.31	54.00	-1.69
10	+1	+1	+1	-1	47.82	47.86	-0.0433
11	0	0	-2	0	48.56	48.21	0.3521
12	0	0	0	0	54.76	54.00	0.7617
13	-1	-1	-1	-1	48.66	48.75	-0.0904
14	+1	-1	-1	-1	45.37	46.21	-0.8383
15	0	0	0	0	53.90	54.00	-0.0983
16	+1	+1	-1	-1	41.33	41.06	0.2746
17	-1	+1	-1	-1	47.35	47.10	0.2550
18	+2	0	0	0	46.38	45.95	0.4337
19	0	-2	0	0	47.22	47.06	0.1587
20	-1	+1	+1	+1	48.06	46.88	1.18
21	0	0	+2	0	49.33	49.38	-0.0463
22	+1	-1	+1	-1	48.64	48.51	0.1262
23	+1	+1	+1	+1	49.83	50.39	-0.5588
24	0	0	0	0	55.53	54.00	1.53
25	0	0	0	-2	48.33	48.28	0.0471
26	0	0	0	0	54.17	54.00	0.1717
27	-1	+1	+1	-1	50.38	51.06	-0.6804
28	-1	-1	+1	+1	40.45	41.37	-0.9237
29	+1	-1	+1	+1	48.47	48.38	0.0883
30	0	0	0	+2	49.33	49.07	0.2587

Std. Dev. = 0.8868, Mean = 48.83, C.V.% = 1.82, R^2= 0.9663, Adjusted R^2 = 0.9348, Predicted R^2 = 0.8832, Adeq Precision = 20.6405

Table.3 Analysis of Variance and regression analysis for phytase production by *Pediococcus acidilactici* BNS5B by CCD.

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	337.86	14	24.13	30.69	< 0.0001	significant
A-Galactose	1.41	1	1.41	1.79	0.2010	
B-Ammonium sulfate	0.1855	1	0.1855	0.2359	0.6342	
C-Beef	2.05	1	2.05	2.60	0.1275	
D-Yeast Extract	0.9322	1	0.9322	1.19	0.2934	
AB	12.23	1	12.23	15.55	0.0013	
AC	8.08	1	8.08	10.27	0.0059	
AD	44.99	1	44.99	57.21	< 0.0001	
BC	20.27	1	20.27	25.78	0.0001	
BD	7.06	1	7.06	8.98	0.0090	
CD	26.04	1	26.04	33.11	< 0.0001	
A^2	124.92	1	124.92	158.84	< 0.0001	
B^2	78.37	1	78.37	99.65	< 0.0001	
C^2	46.47	1	46.47	59.09	< 0.0001	
D^2	48.54	1	48.54	61.72	< 0.0001	
Residual	11.80	15	0.7864			
Lack of Fit	5.52	10	0.5520	0.4398	0.8740	not significant
Pure Error	6.28	5	1.26			
Cor Total	349.66	29				

Table.4 Purification and yield of phytase from *Pediococcus acidilactici* BNS5B

	Total protein (mg)	**Total activity (U)**	**Specific activity (U/mg)**	**Purification fold**	**Yield (%)**
Crude supernatant	1590	4580	2.88	1	100
Ethanol precipitated	379	3583	9.45	3.28	78.2
DEAE- cellulose ion exchange chromatography	33	2195	66.51	23.09	47.8

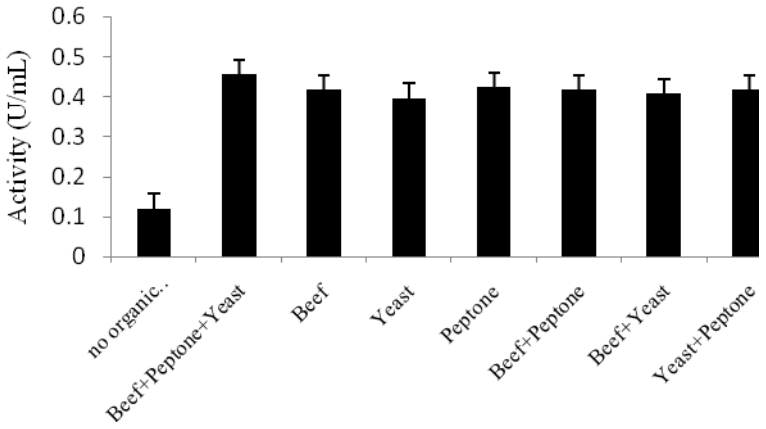
Table.5 Effect of metal ions on the activity of *Pediococcus acidilactici* BNS5B phytase

Metal ions	Relative activity (%)	
	0.5mM	1mM
Control	100	100
Cu^{2+}	57.43	55.57
Mg^{2+}	62.7	54.51
Fe^{2+}	60.95	54.14
Zn^{2+}	58.67	54.6
Ca^{2+}	59.8	56.61
Mn^{2+}	55.47	54.7

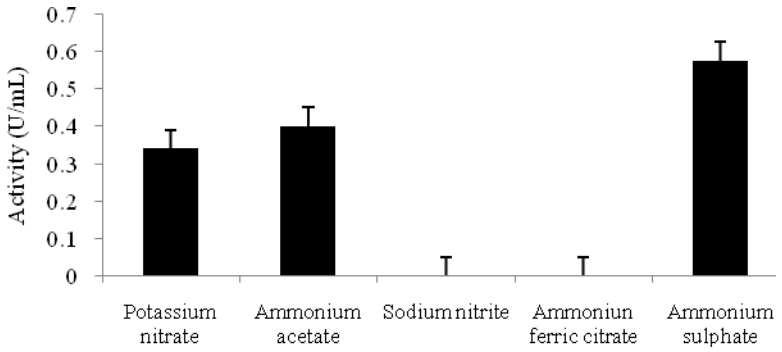
Phytase activity with no metal ions (Control) was considered 100%.

Fig.1 Effect of different nitrogen sources on the phytase production: a) Organic sources (1% each); b) Inorganic sources; c) Combination of organic (Beef extract +Yeast extract + peptone) and inorganic (Urea , Potassium nitrate, Ammonium acetate, Ammonium sulphate).

a)



b)



c)

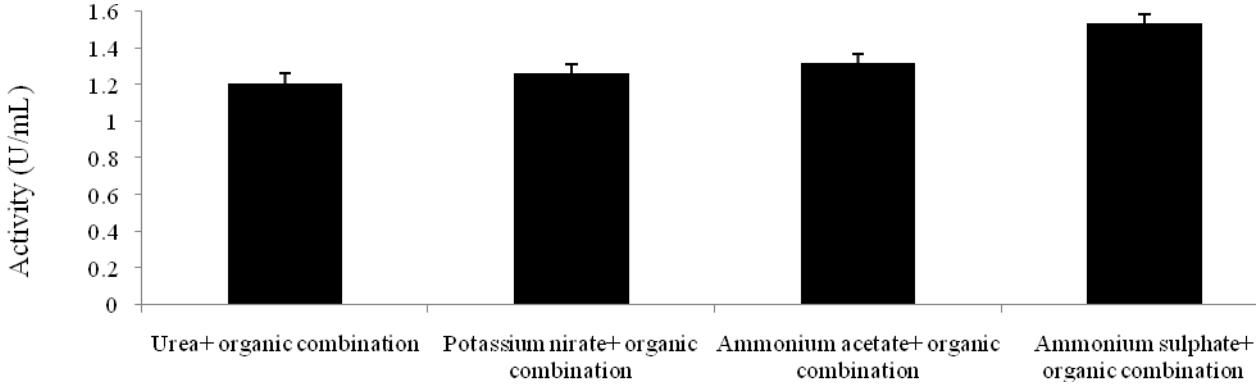


Fig.2 Pareto chart showing effect of different parameters in the Plackett-Burman design for phytase production from *Pediococcus acidilactici* BNS5B

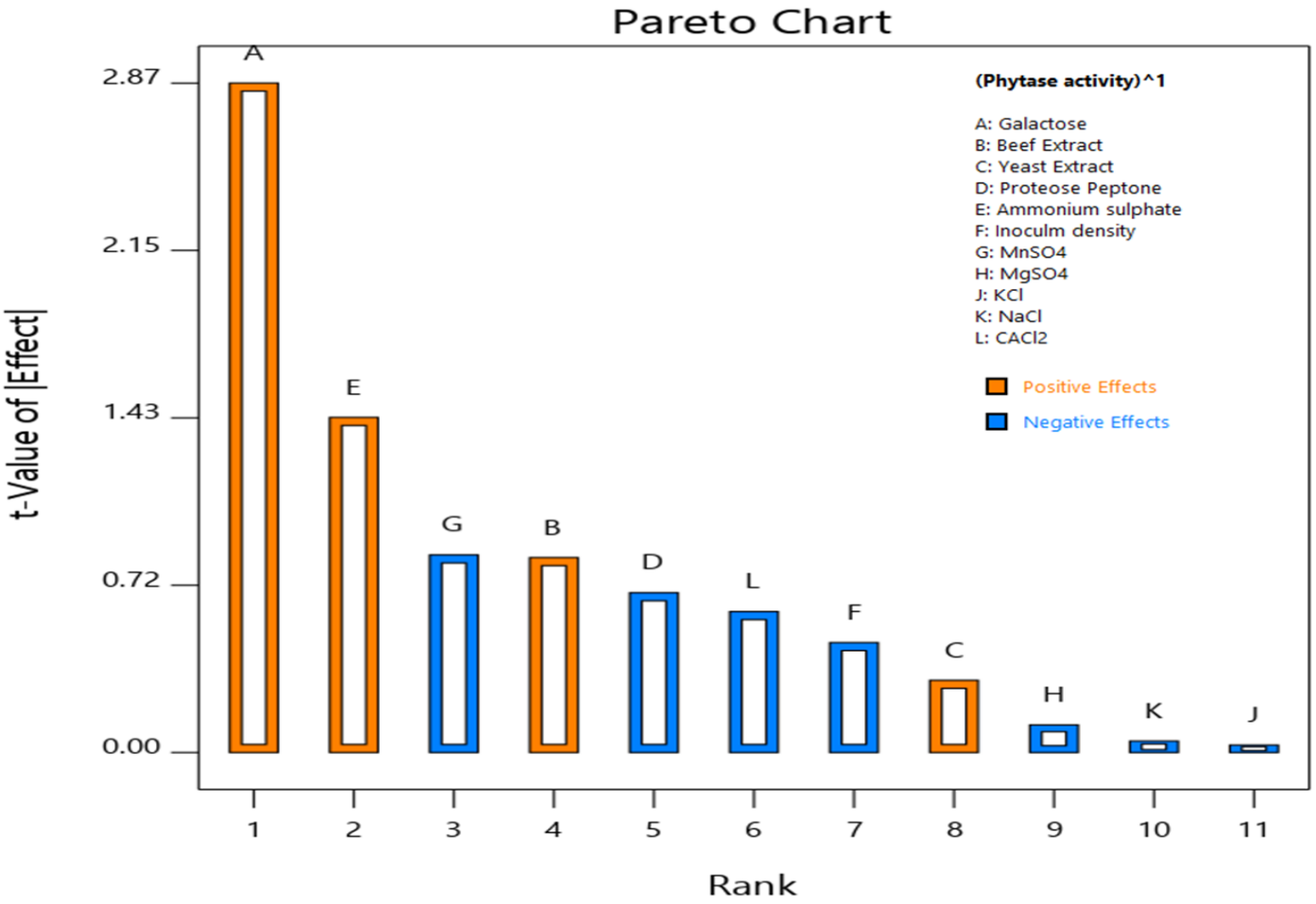
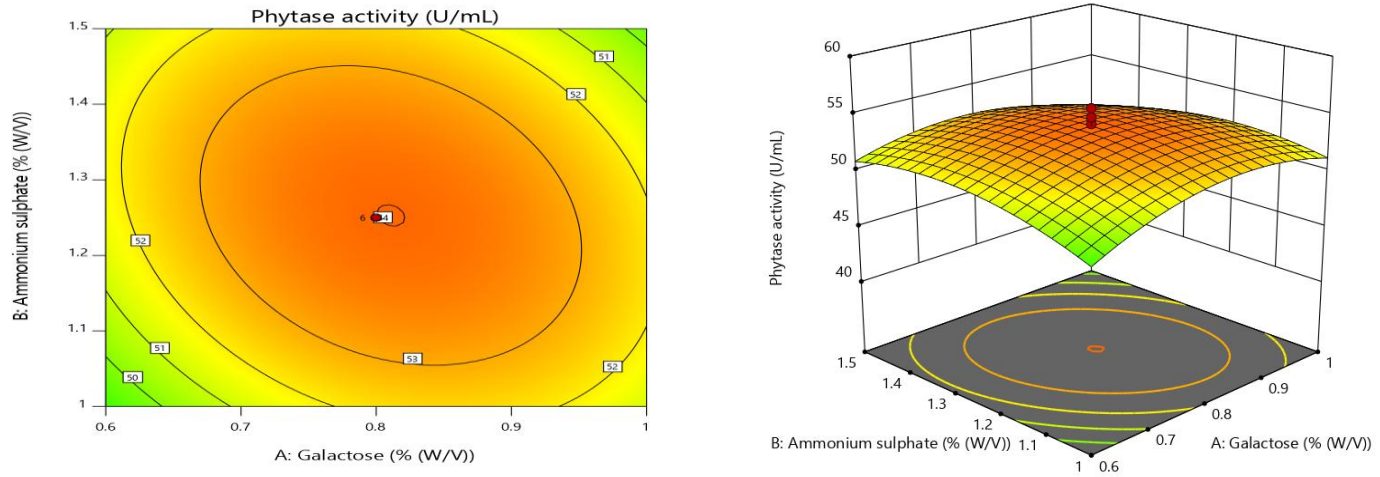
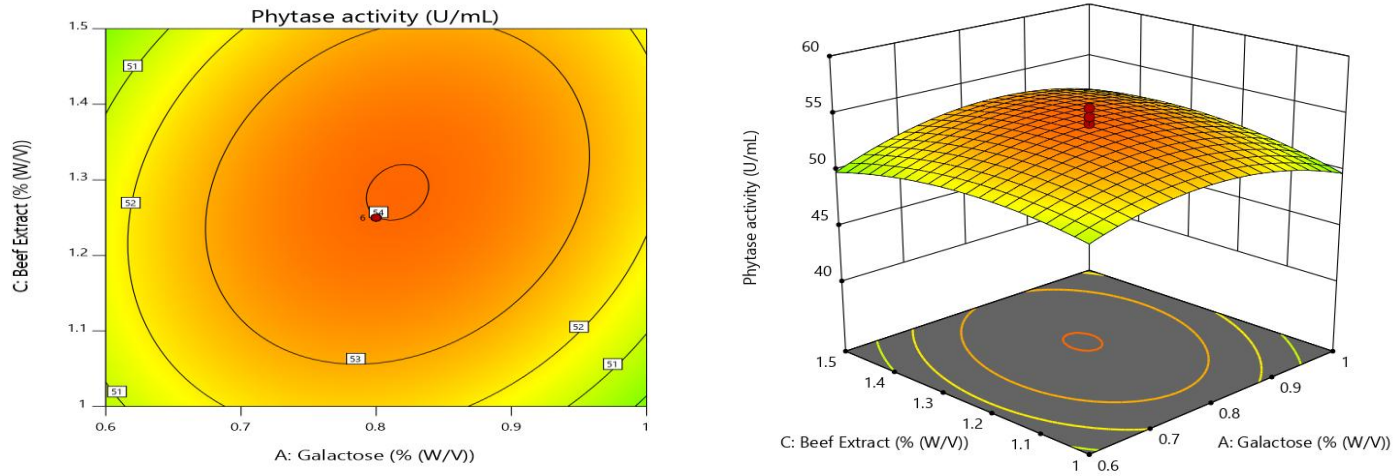


Fig.3 3D plots and contour plots of interaction between 2 variables when the third was kept at its optimum value: A) Ammonium sulphate and Galactose; B) Beef extract and Galactose; C) Yeast extract and Galactose; D) Beef extract and Ammonium sulphate.

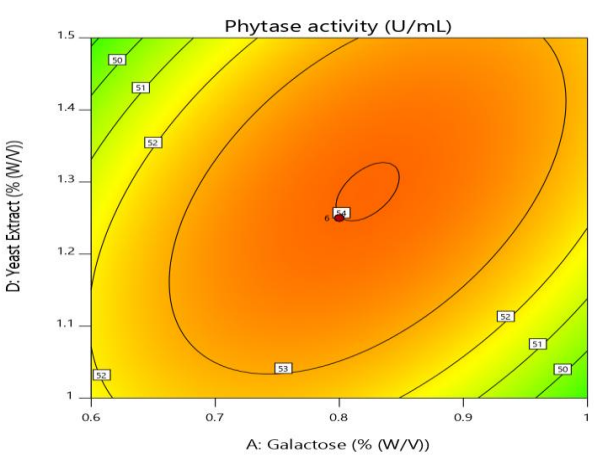
A)

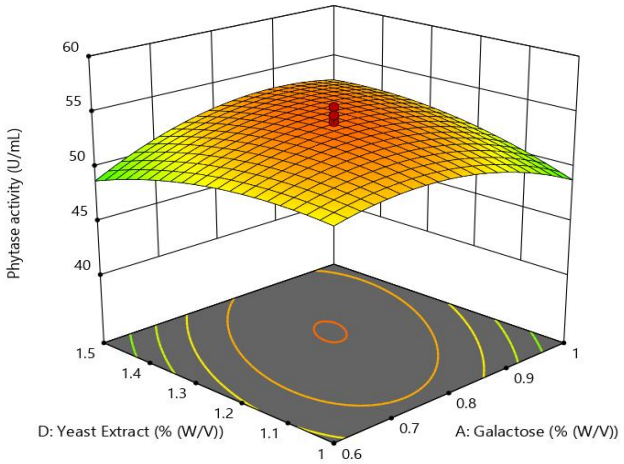


B)

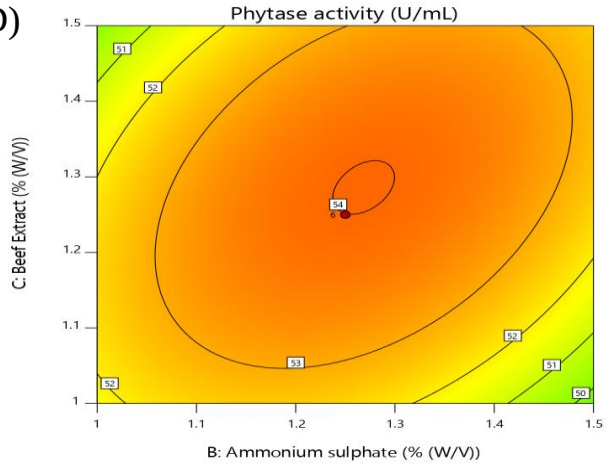


C)


Phytase activity (U/mL)
D: Yeast Extract (% (W/V))
A: Galactose (% (W/V))


Phytase activity (U/mL)
D: Yeast Extract (% (W/V))
A: Galactose (% (W/V))

D)


Phytase activity (U/mL)
C: Beef Extract (% (W/V))
B: Ammonium sulphate (% (W/V))

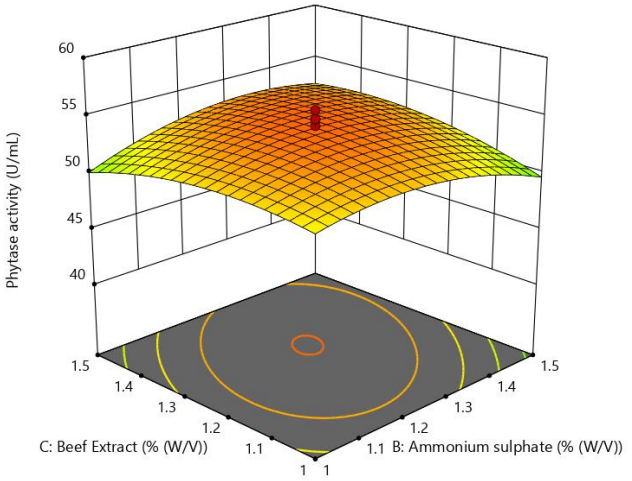

Phytase activity (U/mL)
C: Beef Extract (% (W/V))
B: Ammonium sulphate (% (W/V))

Fig.4 Elution profile of phytase on DEAE-Cellulose

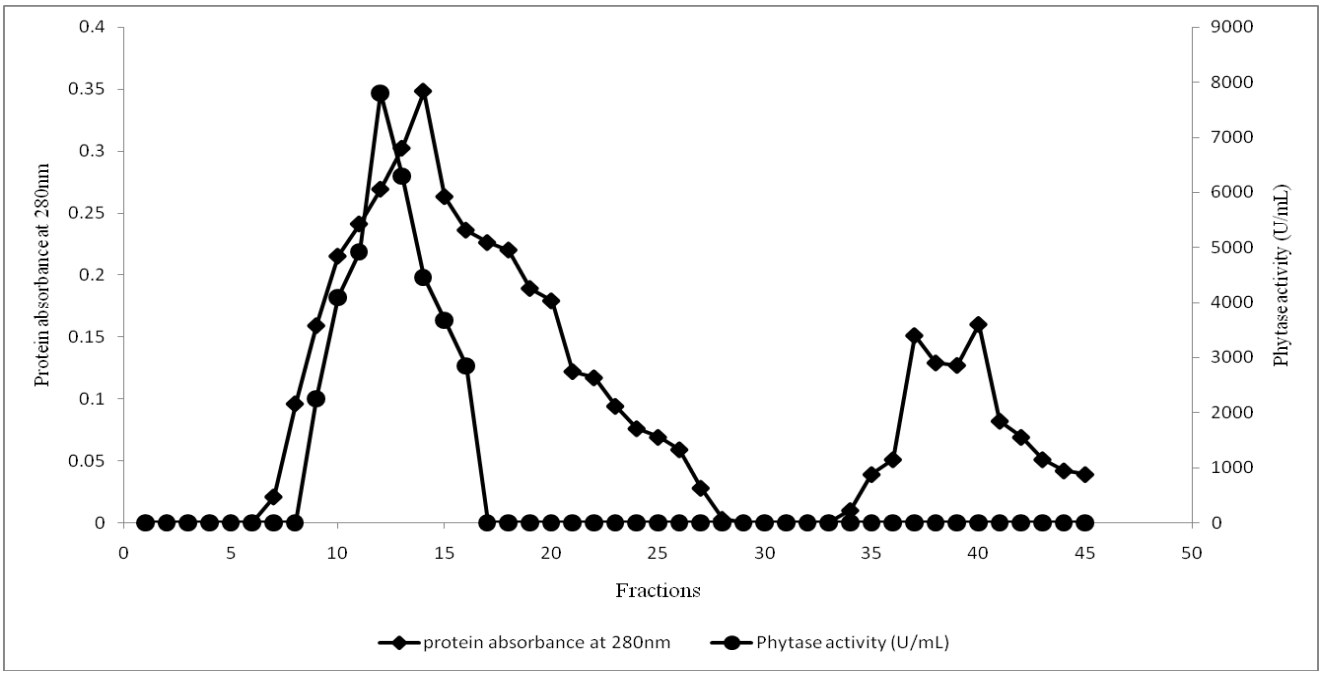


Fig.5 SDS-PAGE: A) Protein marker; B) DEAE-Cellulose elute

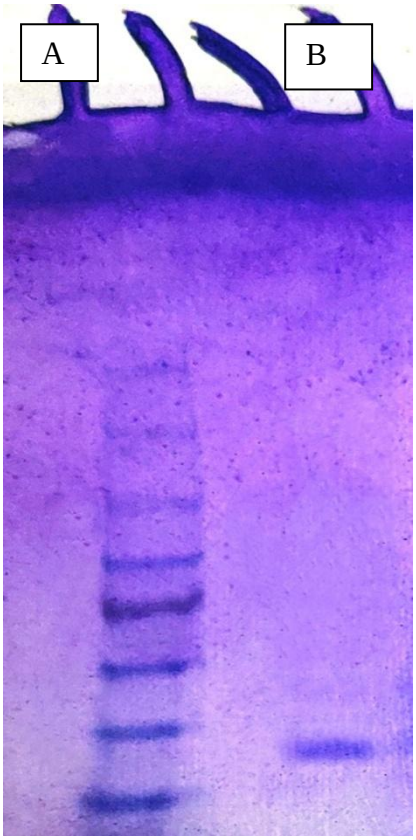


Fig.6 Effect of pH on purified phytase activity and stability

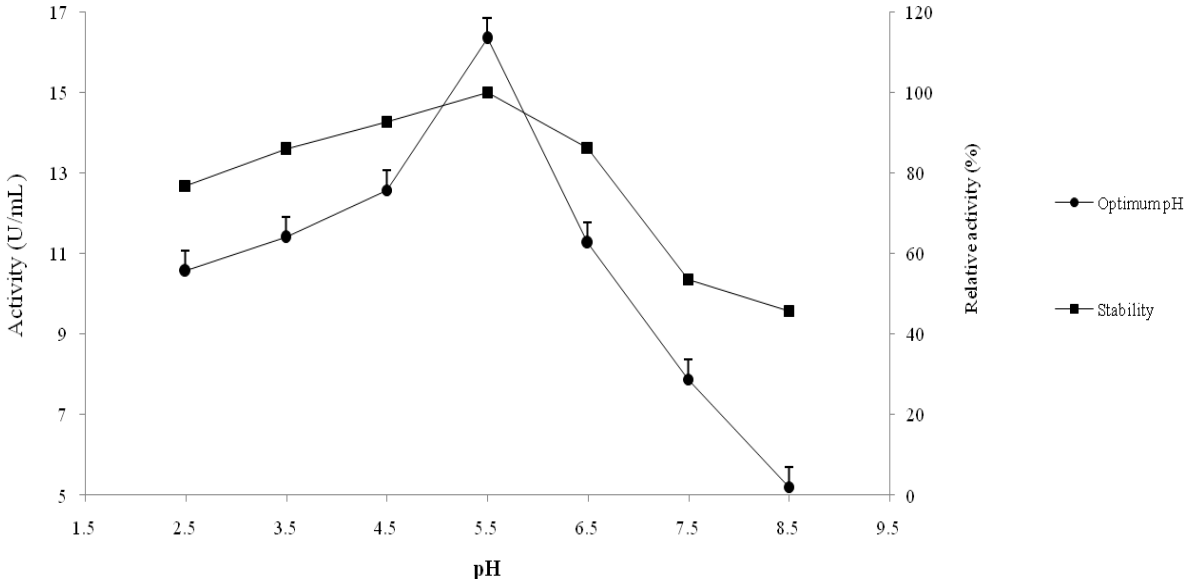


Fig.7 Effect of temperature on purified phytase activity and stability

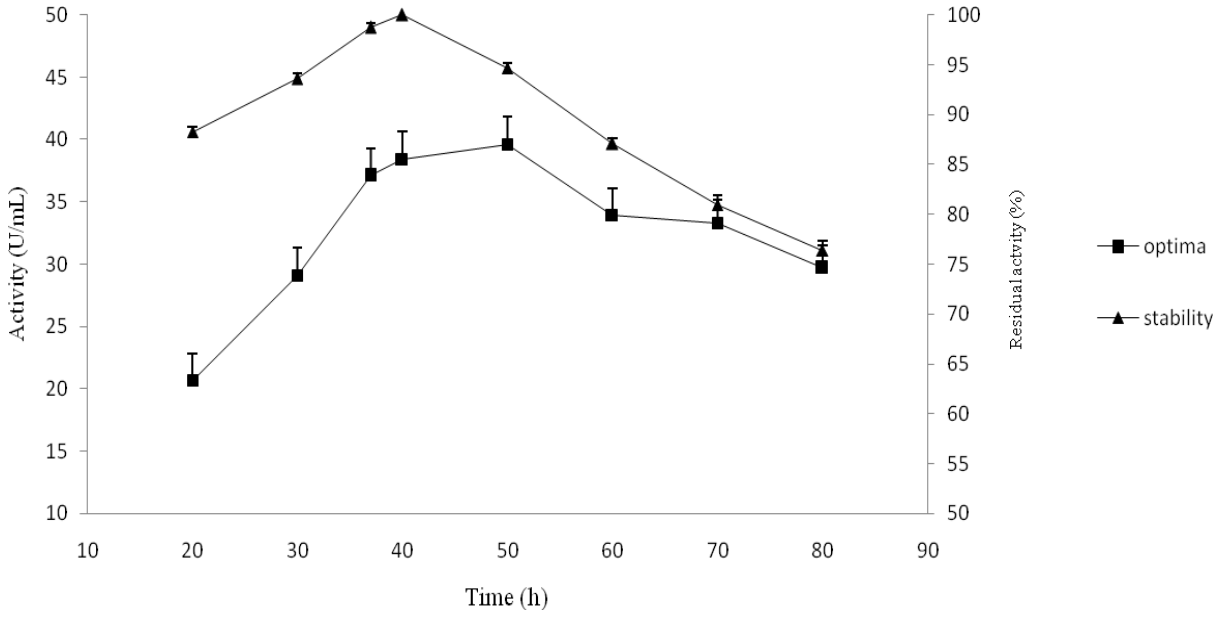
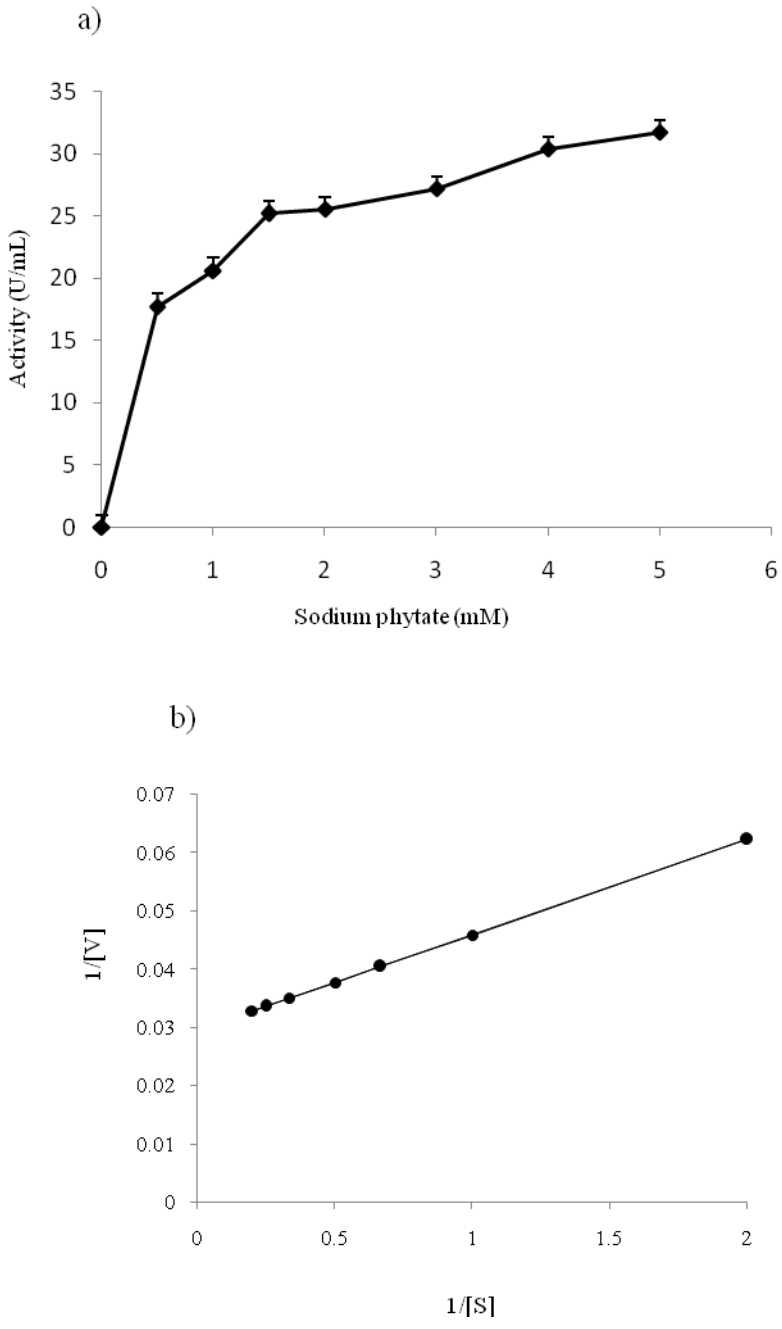


Fig.8 Kinetic parameter of enzyme phytase:
a) Michaelis-Menten curve; b) Lineweaver-Burk plot



Temperature optima and stability

The optimum temperature for purified phytase activity was found to be 40°C (Fig. 7) and the enzyme retained an activity of over 76% at a temperature range of 20 to 80°C. The enzyme was stable at temperature below 50°C with a relative activity of more than 90%. However, at temperature above 50°C partial loss of activity was observed.

This observation is in accordance to previous studies where scientist have also found that the optimum temperature for most phytase produced from *Bacillus* sp., *Pseudomonas*, *Shigella* sp was in the range of 40-50°C (Patki *et al.,* 2015). As thermal stability of an enzyme is important for its application in feed industry and phytases produced from our isolate may be a good alternative to available enzymes due to its stability at high temperatures (Saribuga *et al.,* 2014).

Effect of metal ions on purified phytase enzyme activity

Effect of various metal ions such as Cu^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, Ca^{2+} and Mn^{2+} on the purified phytase activity was examined using calcium phytate as the substrate.

The purified phytase enzyme activity was inhibited by these cations at both the concentration of 0.5mM and 1mM. (Table 5) and corroborates results with earlier studies (Kim *et al.,* 2015).

However, Shimizu (1992) have found that activity of extracellular phytase from *B. subtilis* (natto) N-77 strain was inhibited by the addition of EDTA, Fe^{2+}, Zn^{2+}, Ca^{2+}, Ba^{2+}, Cu^{2+},and Al^{3} suggesting that metal ions are not required for the functioning of enzyme as different organisms require different metal ions (Roy *et al.,* 2012).

Substrate specificity and Kinetic parameters

The purified phytase has very narrow substrate specificity as it hydrolysed only phytic acid salts and showed highest activity towards sodium phytate. Phytase produced from *E. coli* has been reported to be highly specific for phytate whereas phytase from *Aspergilus fumigates, Klebsiella pneumonia* and *V. volvacea* were found to have broad specificity for phosphoryated substrate but relatively low specificity to phytate (Menezes-Blackburn, 2015). The results on varying substrate concentration revealed that the enzyme activity follows normal Michaelis-Menten curve. K_m and V_{max} values from the Lineweavers-Burk Reciprocal plot were found to be 0.5455mM and 33.927µmol/min (Fig 9). The lower K_m values suggests higher affinity of substrate whereas higher V_{max} suggests a higher efficiency, although the K_m of phytases from different organisms was reported to be in the range of 0.08 to 10mM showing variable affinity for different phosphorylated substrates (Parhamfar *et al.,* 2015). Interstingly, phytase from *P acidilactici* BNS5B has lower K_m for sodium phytate suggesting its higher substrate affinity.

The data of present study indicated that by optimizing various physic-chemical parameters for the phytase production using OVAT followed by Plackett–Burman and Response surface methodology statistical methods enhanced the production of phytase from 0.59U/mL to 55.53U/mL leading to approximately 94 fold increase. Moreover, the purified enzyme showed high specific activity, substrate specificity, good pH profile and thermostability which could promote its industrial application as well as in human beings to enhance the bioavailability of minerals, but needs to be evaluated either clinically or experimentally.

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