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Enhancement of Phytase Production from a New Probiotic Strain *Bacillus subtilis* P6

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A B S T R A C T

An integrated classical and statistical optimization approach involving the combination of Placket–Burman design (PBD) and Central Composite Design (CCD) was employed for increasing phytase yield. PBD was used to evaluate the effect of 9 variables related to phytase production from probiotic strain *B. subtilis*, and three statistically significant variables, namely, glucose, beef extract and potassium phosphate were selected for further optimization studies. The levels of five variables for maximum phytase production were determined by a CCD. The optimum values for the factors were determined via response surface methodology (RSM) as: 6.59 gl^{-1} of glucose, 6 gl^{-1} of beef extract and 2.75 gl^{-1} of potassium phosphate respectively. Phytase production improved from 2.74 $EUml^{-1}$ to 46.76 $EUml^{-1}$ indicating 17-fold increase in activity after optimization.

Introduction

The growing awareness of the diet and health has prompted an increasing demand of food products that can support health apart from providing basic nutrition (Haros *et al.*, 2009). Since last six decades, a lot of antibiotics have been incorporated in animal feed to improve their growth, efficiency and to protect them from pathogenic microorganisms.

But, antibiotic resistance has become a major public health concern today (Sharma *et al.*, 2014). The gastrointestinal microflora plays beneficial role in the health and nutrition of animals, and probiotics, live microorganisms help in the maintenance of gut microflora. Probiotics have been strongly recommended as alternatives to antibiotics for food animals (Reid and Friendship, 2002). Recently, a great deal of attention has been devoted to the genuine value of bacterial species as multifunctional probiotics, which secrete various extracellular enzymes for enhancing feed digestibility as well as many antimicrobial compounds for improving animal performance (Lee *et al.*, 2012); among them phytase holds the key position. Phytate is a natural phosphate reservoir in plant based animal feed and acts as an anti-nutritional factor in gut of animals and humans by restricting absorption of proteins, carbohydrates, amino acids and metals viz. Zn^{2+}, Fe^{3+}, Ca^{2+} and Mg^{2+}. However, in order to meet the phosphorus requirement, diet of monogastric animals are supplemented with

inorganic phosphorus which causes the excretion of large amount of phosphorus in the excreta resulting in environmental pollution and several human health problems (Jorquera *et al.,* 2011). Phytase (*myo*-inositol hexakisphosphate phosphohydrolase, E.C. 3.1.3.8 or E.C. 3.1.3.26) are group of enzymes that hydrolyze phytate to inositol phosphate, *myo*-inositol and inorganic phosphate. Today, phytase extracted from fungi (Nautophos®, Allzyme® SSF, Finase® P/L) is commercialized as feed supplement; on the other hand several bacterial species have been used as probiotics.

But till date, phytase producing probiotic strains have not been commercialized. However, a probiotic strain with phytase activity can perform double function and enhance productivity of animals manifolds. Simultaneously, they can also reduce the serious environmental problems caused by undigested phytate, which is main byproduct of human and animal excreta. Hence, from last few years, this area has become a major public health concern and is drawing the interest of health and research professionals all around the world.

One of the major cornerstones in biotechnology today is the optimization of the cultivation conditions for enhancing the productivity. Screening and evaluation of nutritional requirements of microorganisms is an important step in any bioprocess development.

Optimization studies involving one factor-at-a-time approach is tedious, tends to overlook the interaction among the factors and might lead to misinterpretation of results. In contrast, statistical strategies are preferred and more advantageous and mitigate the error in determining the effects of parameters in an economical manner (Sharma and Satyanarayana, 2006). The empirical technique is a traditional optimization method employing one-factor-at-a-time strategies which is simple, easy and explains the individual effect of different components. Unfortunately, it is tedious and fails to explain the interactions among the factors (Awad *et al.,* 2011). Statistical optimization is a proven tool for overcoming the limitations of the "one-factor at a time' method. It is more efficient technique since it can provide statistical data with a relatively small number of experiments.

In our previous endeavor, we isolated phytase producing potential probiotic strain *Bacillus subtilis* P6 from exotic environment (Sharma and Trivedi, 2015). Considering the high potential of phytase for use as feed supplement and its future prospective in animal feed and human nutrition, the present appraisal therefore aims at achieving the multifold improvement in the phytase production through sequential classical and statistical optimization strategy from probiotic strain *Bacillus subtilis* P6 so as to verify whether it can become a new kind of feed additive for food and animal feeding in the future.

Materials and Methods

Chemicals

Phytic acid as a dodecasodium salt was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and media were products of Merck (Darmstadt, Germany). All reagents were analytical grade.

Bacterial strain and inoculum preparation

The phytase producing probiotic bacterial strain *Bacillus subtilis* P6 (NCBI Accession no. KJ872821) was procured from Bacterial Culture Collection Centre, (BGCC # 2393) Rani Durgavati University, Jabalpur (M.P.),

which was originally isolated from poultry soil and identified. The strain was maintained on Luria Bertani (LB) agar slant (pH 7) and stored at 4 °C. The seed inoculum was prepared by adding a single colony of 8 h old bacterial culture by transferring aseptically in 20 ml pre-autoclaved phytase screening medium (PSM) broth containing gL^{-1}:10g glucose; 2g $CaCl_2$; 5g NH_4NO_3; 0.5g KCl; 0.5g $MgSO_4.7H_2O$, 0.01g $FeSO_4.7H_2O$, 0.01g $MnSO_4.H_2O$, 4.0g sodium phytate (pH 6.0) in Erlenmeyer flask, incubated at 37 °C in a rotary shaking incubator for 20 h at 150 rpm. The 2.5% inoculum (A_{600} = 0.6-0.8) of this culture used as primary inoculum.

Optimization of fermentation parameters by one factor-at-a time approach

Optimization of Physical parameters

Effect of batch time

To investigate the effect of batch time, 2.4% inoculum was transferred in 100 ml PSM broth and after an interval of 12, 16, 20, 24, 28, 32, 36, 40, 44, 48 and 52 h enzyme activity was estimated.

Effect of inoculum age and size

The effect of different inoculum age (4, 8, 12, 16, 20, 24, 28, 32 and 36 h) and inoculum size (0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4% v/v) on phytase production was investigated.

Effect of pH and temperature

Effect of pH on phytase production was studied by adjusting the pH of PSM in the range of 3-7. To study the effect of temperature on phytase production, the test strain was allowed to grow at different temperature (20, 25, 30, 35, 40, 45, 50 and 55 °C) in PSM broth set at optimum pH for optimum period and phytase activity was investigated.

Optimization of Nutritional parameters

Effect of carbon sources and nitrogen sources

Effect of various carbon sources on phytase production was assessed by substituting maltose, sucrose, lactose, xylose, rhamnose and glycerol (1%) separately in place of glucose (control) in the minimal medium. Further, the Effect of various nitrogen sources on phytase production was investigated under optimal pH, temperature and carbon source by substituting ammonium nitrate (0.5%) with malt extract, beef extract, yeast extract and ammonium sulphate separately in PSM broth.

Effect of inorganic phosphate

The effect of different phosphate salts (0.4 %) viz. calcium phytate, potassium phosphate, sodium di hydrogen phosphate and potassium di hydrogen phosphate on phytase production was studied.

Statistical Optimization

Screening of significant variables by Plackett-Burman Design (PBD)

Based on single-factor experiment for the phytase production, one variable each of incubation period, pH and temperature and two each of carbon, nitrogen and phosphate source found to have positive impact on phytase production were screened statistically to indentify the critical parameters for increasing phytase production using PBD. Tables 1 and 2 illustrate the variables and their levels used in experimental design constructed by using Design Expert® software version 9.0.2 (Stat -Ease, Inc., Minneapolis, USA). Each variable was studied at two different levels, a high level (+1) and a low level (-1). All the experiments were performed in triplicates and the average of phytase production was taken as response.

The data obtained from Plackett Burman Design (PBD) on phytase production were subjected to analysis of variance (ANOVA) and the statistical software "Design Expert® 9.0.2" Stat -Ease, Inc., Minneapolis, USA was used to analyze the experimental design.

Optimization by Response Surface Methodology (RSM)

On the basis of PBD results analysis, three variables viz. glucose, beef extract and potassium phosphate was chosen for further optimization by the response surface methodology using Central Composite Design (CCD). Each factor in the design was studied at five different levels (-α, -1, 0, +1, + α) (Table 3). A set of 20 experiments were carried out.

All variables were taken at a central coded value considered as zero (Table 4). The response value (Y) in each experiment was the average of the phytase activity in triplicates. A second order polynomial equation was then fitted to the data by a multiple regression procedure. The experimental results of RSM were fitted via the response surface regression procedure, using the following second order quadratic polynomial equation:

$$Y_i = \beta o + \sum i\ \beta_i X_i + \sum ii\ \beta_{ii} X_i^2 + \sum ij\ \beta_{ij} X_i X_j$$

Where, Y_i is the predicted response, $X_i X_j$ are the independent variables, β_o is the intercept, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the interaction factors.

Validation of the experimental model

The statistical model was validated taking phytase production under the optimum conditions predicted by the model in shake flasks level and phytase activity was determined.

Phytase assay

Phytase activity was assayed under acidic condition according to the method of Greiner (2004). One unit of phytase (EU) activity is defined as the amount of enzyme that releases 1 µM of inorganic phosphate per min under standard assay conditions.

Results and Discussion

Optimization using classical approach

Today, the researchers around the world have paid a great attention to development of antimicrobial resistance and transferring of antibiotic resistance genes from animal to human microbiota (Mathur and Singh, 2005). On the other hand, increasing of pollution in fresh water bodies such as algal bloom and eutrophication are the matter of great attention and discussion. In this context, phytase producing probiotic strains could be a possible solution to above mentioned problems. In the present communication, we report, optimization of phytase production from probiotic strain *Bacillus subtilis* P6.

The results of 'one factor at a time' approach for optimization of phytase production revealed that phytase production occurred after 44 h of incubation (Figure 1). Decline in phytase production after 44 h could be owing to the increased biomass production which might have resulted in the depletion of nutrients or production of toxic metabolites, affecting enzyme synthesis.

In previous reports maximum phytase yield recorded in 56-72 h from *Bacillus* sp. (Demirkan *et al.,* 2014), *Pseudomonas* sp. (Hosseinkhani *et al.,* 2009), *Klebsiella* sp. (Mittal *et al.,* 2012). This indicates that *B. subtilis* P6 can produce large amount of phytase within a short period. This feature makes *B. subtilis* P6 a promising candidate

for production of phytase at commercial scale. The maximum phytase production was observed with 20 h of inoculum (2.5% v/v) (Figures 2 and 3), but declined with the increase in the age and size of inoculum, which might be due to increased competition for nutrient uptake and exhaustion of nutrients creating nutrient imbalance (Roopesh *et al.*, 2006) while lower concentrations may not be sufficient for maximum enzyme production (Sabu *et al.*, 2002).

In the present study, the optimum phytase production by *B. subtilis* P6 was at pH 5.5 (Figure 4). A pH beyond the optimum level may interfere with the amino acid composition of the enzyme decreasing its activity (Esakkiraj *et al.*, 2009). Largely, bacteria prefer pH around 5.0-7.0 for best growth and phytase production (Vohra and Satyanarayana, 2003). Activity at slightly acidic to alkaline pH values makes the *Bacillus* phytases suitable as feed additives for monogastric animals having stomach pH values of 5.5-7.0.

The maximal phytase production was observed at 37 °C and the enzyme production decreased with further increase in temperature (Figure 5). Optimal temperature for production of most phytases varies from 30 to 80 °C (Wang *et al.*, 2004). Given these findings, it may be safe to speculate that the *Bacillus* enzyme in this study may be able to perform optimal phytate degrading activities at the body temperature of monogastric animals like swine, poultry, fish etc.

The maximum phytase production was recorded in presence of glucose (12.23 IU/ml) and yeast extract (16.78 IU/ml) (Figures 6 and 7). This might be due to the fact that glucose acts as a good energy and membrane stabilizing agent and yeast extract is best source of vitamin which is required for growth and development of cell. Glucose is known to stabilize lysozomal membranes; thereby reducing protease release (Wilson and Walker, 2000). In recent past glucose and sucrose for *B. subtilis* DR6 (Singh *et al.*, 2013), sucrose for *B. laevolacticus* (Gulati *et al.*, 2007) and wheat bran for *Bacillus amyloliquefaciens* FZB45 (Idriss *et al.*, 2002) were reported as best carbon source while $NH_4H_2PO_4$ (Gulati *et al.*, 2007; Mittal *et al.*, 2012), yeast extract (Sasirekha *et al.*, 2012) as best nitrogen sources for the production of phytase. Therefore, in comparison to earlier observations, phytase from *B. subtilis* P6 can be produced at low cost.

The result of the present study revealed that the expression of phytase by *B. subtilis* P6 could be stimulated by the presence of potassium phosphate (Figure 8) although addition of inorganic potassium phosphate in the medium does not affected phytase production by *B. laevolacticus* (Gulati *et al.*, 2007). The buffering capacity of phosphate may have a positive effect on phytase synthesis (Lan *et al.*, 2002).

Statistical Optimization

The factorial approach for process optimization is convenient and can yield several-fold improvement in process as demonstrated in many cases (Dash *et al.*, 2007). In the present study, a PBD was employed for screening the most significant medium components and culture conditions influencing the phytase production. Table 5 illustrates the PBD for 9 selected variables and the corresponding response (phytase production). The Pareto chart illustrates the order of significance of the variables affecting phytase production (Figure 9). Table 6 shows the influence of each variable along with the related coefficient, *P*-value and t-value. Based on regression analysis, it was evidenced that glucose, beef extract and potassium phosphate, were positive signal factors;

whereas, pH, temperature, incubation period, maltose, yeast extract and KH_2PO_4 affected the response at a significant negative level. A *P*-value less than 0.05 indicate that the model terms are significant. The F-test for ANOVA indicated that glucose (0.0009), beef extract (0.0003) and potassium phosphate (<0.0001) were the factors that significantly affected the enzyme production for *B. subtilis* P6 PBD. Final Equation in terms of coded factors for *B. subtilis* P6 phytase obtained through PBD is represented as:

Table.1 Experimental variables at different levels for phytase production using Plackett-Burman design

Variable	Units	Symbol	Coded levels	
			Low (-1)	High (+1)
pH	pH	A	5.5	6.0
Temperature	^oC	B	37	45
Incubation period	h	C	72	96
Glucose	gl^{-1}	D	10	15
Maltose	gl^{-1}	E	10	15
Yeast extract	gl^{-1}	F	2	5
Beef extract	gl^{-1}	G	2	5
Potassium di hydrogen phosphate	gl^{-1}	H	1	4
Potassium phosphate	gl^{-1}	J	1	4

Table.2 Plackett-Burman Design Matrix of 12 run for phytase production

Run Order	Experimental values										
	A	B	C	D	E	F	G	H	J	K	L
1	-1.000	-0.231	-1.000	1.000	1.000	-1.000	1.000	1.000	1.000	-1.000	-1.000
2	-1.000	-0.231	1.000	-1.000	1.000	1.000	1.000	-1.000	-1.000	-1.000	1.000
3	1.000	-0.231	1.000	-1.000	-1.000	-1.000	1.000	-1.000	1.000	1.000	-1.000
4	1.000	-1.000	-1.000	-1.000	1.000	-1.000	1.000	1.000	-1.000	1.000	1.000
5	-1.000	-0.231	1.000	1.000	-1.000	-1.000	-1.000	1.000	-1.000	1.000	1.000
6	-1.000	-1.000	-1.000	-1.000	-1.000	-1.000	-1.000	-1.000	-1.000	-1.000	-1.000
7	1.000	-0.231	-1.000	1.000	1.000	1.000	-1.000	-1.000	-1.000	1.000	-1.000
8	1.000	-1.000	1.000	1.000	1.000	-1.000	-1.000	-1.000	1.000	-1.000	1.000
9	1.000	-1.000	1.000	1.000	-1.000	1.000	1.000	1.000	-1.000	-1.000	-1.000
10	-1.000	-1.000	-1.000	1.000	-1.000	1.000	1.000	-1.000	1.000	1.000	1.000
11	-1.000	-1.000	1.000	-1.000	1.000	1.000	-1.000	1.000	1.000	1.000	-1.000
12	1.000	-0.231	-1.000	-1.000	-1.000	1.000	-1.000	1.000	1.000	-1.000	1.000

Table.3 Experimental variables at different levels for phytase production using Central Composite design

Variables	Units	Symbol	Coded values				
			-1.682	-1	0	+1	+1.682
Glucose	g/l	A	6.59104	10	15	15	23.409
Beef extract	g/l	B	-0.727171	2	6	10	12.7272
Potassium phosphate	g/l	C	-1.03403	0.5	2.75	5	6.53403

Table.4 Central composite design matrix of 20 run for phytase production

Run order	Experimental values		
	A	B	C
1	0.000	0.000	0.000
2	0.000	-1.682	0.000
3	-1.682	0.000	0.000
4	0.000	0.000	0.000
5	0.000	0.000	1.682
6	-1.000	-1.000	-1.000
7	-1.000	1.000	1.000
8	0.000	0.000	-1.682
9	1.000	-1.000	-1.000
10	0.000	0.000	0.000
11	-1.000	1.000	-1.000
12	0.000	0.000	0.000
13	1.000	1.000	-1.000
14	-1.000	-1.000	1.000
15	0.000	0.000	0.000
16	0.000	1.682	0.000
17	1.682	0.000	0.000
18	1.000	1.000	1.000
19	1.000	-1.000	1.000
20	0.000	0.000	0.000

Table.5 Plackett-Burman design for optimization of phytase production

Run Order	Experimental values											Phytase activity (EU ml^{-1})	
	A	B	C	D	E	F	G	H	I	J	K	$Y_{Experimental}$	$Y_{Predicted}$
1	-1	-0.231	-1	1	1	-1	1	1	1	-1	-1	35.59	35.69
2	-1	-0.231	1	-1	-1	-1	-1	1	-1	-1	1	22.06	21.98
3	1	-0.231	1	-1	1	1	1	1	1	1	1	32.27	32.29
4	1	-1	-1	-1	1	-1	1	1	-1	1	1	20.59	20.99
5	-1	-0.231	1	1	-1	-1	-1	1	-1	1	1	27.36	28.36
6	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	26.01	24.95
7	1	-0.231	-1	1	1	1	-1	-1	-1	1	-1	29.56	29.35
8	1	-1	1	1	1	-1	-1	-1	1	-1	1	40.11	39.66
9	1	-1	1	1	-1	1	1	1	-1	-1	-1	25.43	25.38
10	-1	1	-1	1	-1	1	1	-1	1	1	1	37.07	36.68
11	-1	-1	1	-1	1	1	-1	1	1	1	-1	38.46	37.25
12	1	-0.231	-1	-1	-1	1	-1	1	1	-1	1	35.31	37.25

A: pH; B: Temperature; C: Incubation period; D: Glucose; E: Maltose; F: Yeast extract; G: Beef extract; H: Potassium di hydrogen phosphate; I: Potassium phosphate; J&K: Dummy variables

Table.6 Statistical analysis of culture conditions for phytase production by Plackett-Burman design

Source	Sum of Squares	Df	Mean Square	Coefficient	Standard error	F-value	P>F (P-value)
Model	467.98	4	117.00	30.82	0.31	103.56	< 0.0001
D-Glucose	34.75	1	34.75	1.70	0.31	30.76	0.0009
F-Yeast extract	2.96	1	2.96	0.50	0.31	2.62	0.1495
G-Beef extract	47.20	1	47.20	-1.98	0.31	41.78	0.0003
J- Potassium phosphate	383.07	1	383.07	5.65	0.31	339.08	< 0.0001
Residual	7.91	7	1.13				
Cor Total	475.89	11					

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case D, G and J are significant model terms. The Model F-value of 103.56 implies the model is significant.

Table.7 Central composite design (CCD) of factors in coded levels with Phytase activity as response

Run no.	Glucose (A) gl^{-1}	Beef extract (B) gl^{-1}	Potassium phosphate (C) g/l	Phytase activity (EU ml^{-1})	
				$Y_{Experimental}$	$Y_{Predicted}$
1	0.000	0.000	0.000	34.09	35.47
2	0.000	-1.682	0.000	36.85	35.93
3	-1.682	0.000	0.000	21.67	21.40
4	0.000	0.000	0.000	36.94	35.47
5	0.000	0.000	1.682	29.93	29.50
6	-1.000	-1.000	-1.000	33.68	34.28
7	-1.000	1.000	1.000	24.67	24.85
8	0.000	0.000	-1.682	35.89	35.92
9	1.000	-1.000	-1.000	42.68	42.78
10	0.000	0.000	0.000	35.98	35.47
11	-1.000	1.000	-1.000	28.71	28.26
12	0.000	0.000	0.000	35.78	35.47
13	1.000	1.000	-1.000	40.03	39.96
14	-1.000	-1.000	1.000	19.89	20.24
15	0.000	0.000	0.000	34.85	35.47
16	0.000	1.682	0.000	36.91	37.43
17	1.682	0.000	0.000	46.76	46.63
18	1.000	1.000	1.000	46.67	46.35
19	1.000	-1.000	1.000	37.81	38.54
20	0.000	0.000	0.000	35.13	35.47

Table.8 Analysis of Variance (ANOVA) for response surface quadratic model for the Production of phytase

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	951.46	9	105.72	137.39	< 0.0001	Significant
A-Glucose	768.34	1	768.34	998.55	< 0.0001	
B-Beef extract	2.74	1	2.74	3.57	0.0883	
C-Potassium phosphate	49.82	1	49.82	64.74	< 0.0001	
AB	5.12	1	5.12	6.65	0.0274	
AC	48.02	1	48.02	62.41	< 0.0001	
BC	56.50	1	56.50	73.43	< 0.0001	
A^2	3.83	1	3.83	4.97	0.0499	
B^2	2.63	1	2.63	3.42	0.0943	
C^2	13.74	1	13.74	17.86	0.0018	
Residual	7.69	10	0.77			
Lack of Fit	2.77	5	0.55	0.56	0.7278	not significant
Pure Error	4.92	5	0.98			
Cor Total	959.16	19				

df: degree of freedom; R^2: 0.9920; Adj R^2: 0.9848; Adeq precision: 42.544; C.V. %: 2.52; AB, AC and BC represents the interaction effects of variables A, B and C; A^2, B^2 and C^2 are the squared effects of the variables.

Fig.1 Effect of incubation time on phytase production

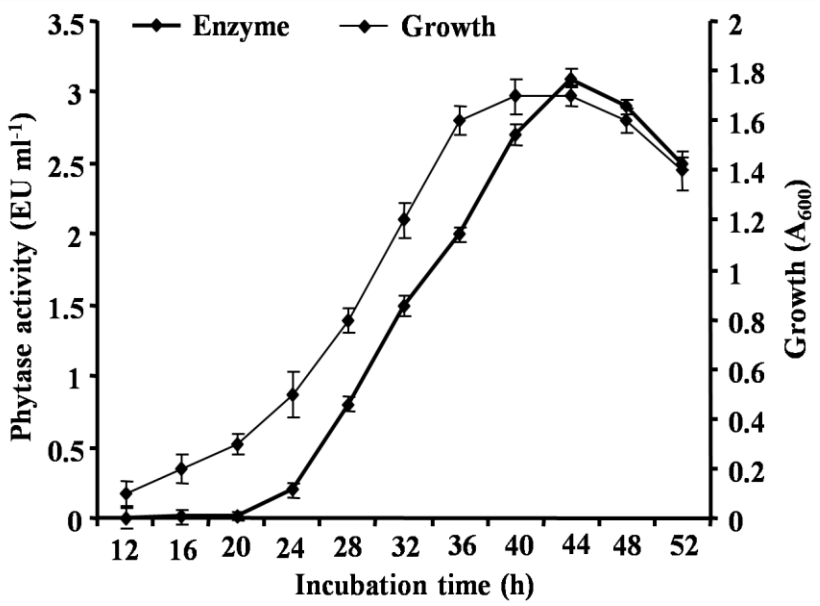


Fig.2 Effect of inoculum age on phytase production

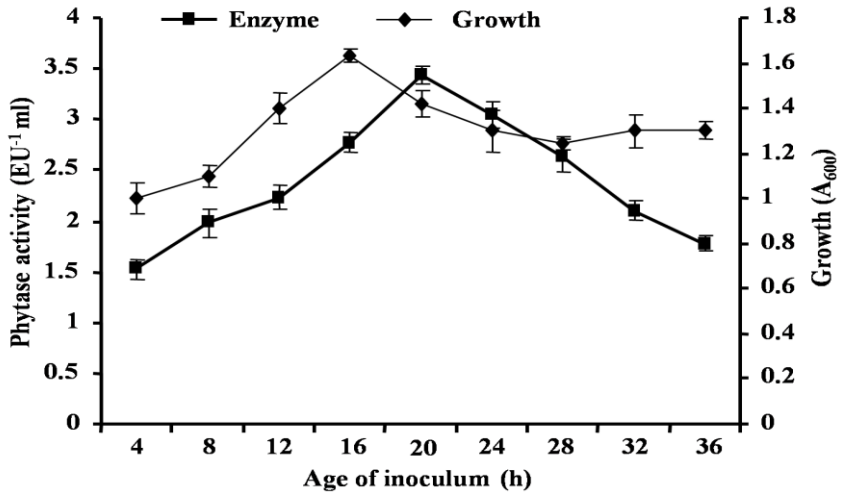


Fig.3 Effect of inoculum concentration on phytase production

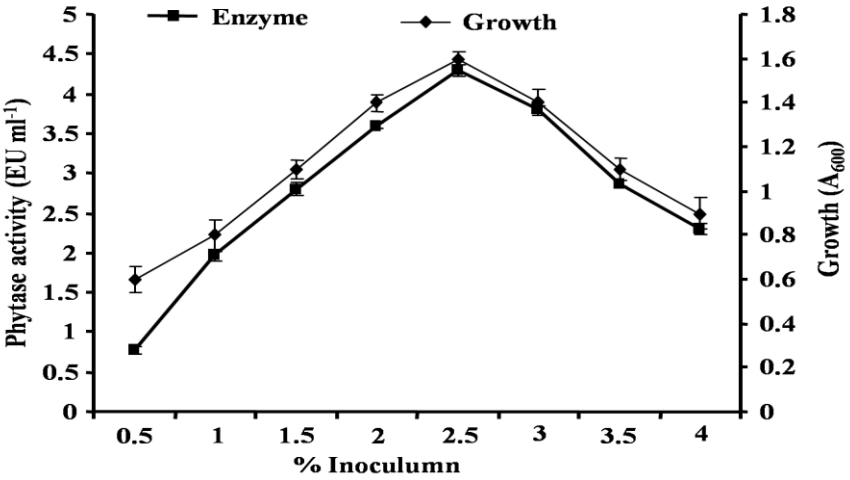


Fig.4 Effect of pH on phytase production

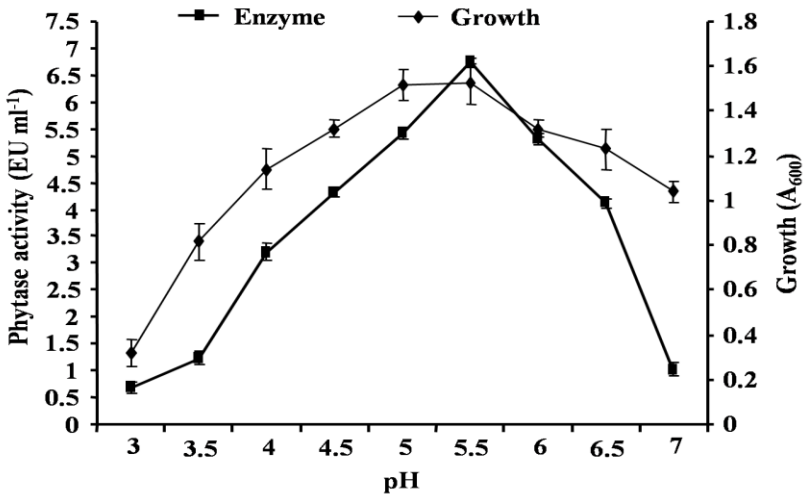


Fig.5 Effect of temperature on phytase production

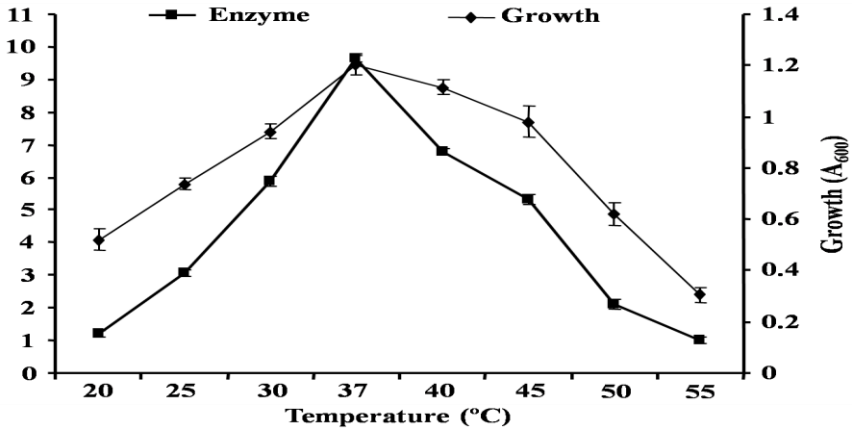


Fig.6 Effect of carbon sources on phytase production

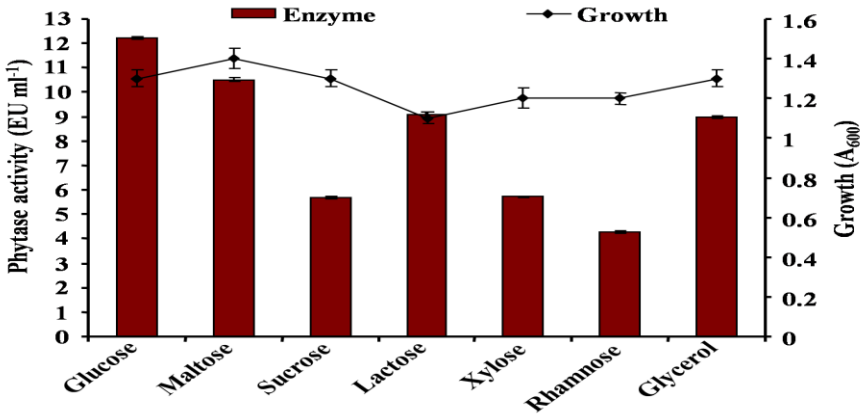


Fig.7 Effect of nitrogen sources on phytase production

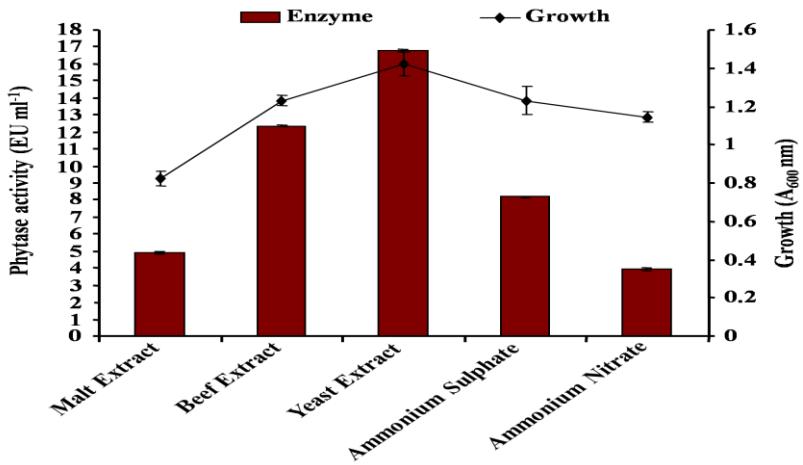


Fig.8 Effect of inorganic phosphates on phytase production

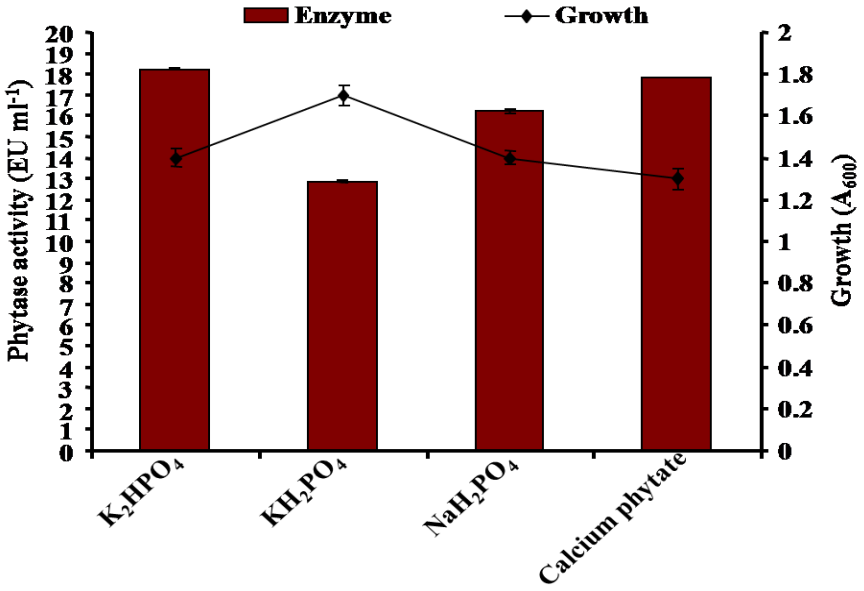


Fig.9 Pareto chart representing the significant factors that influenced Phytase production from *B. subtilis* P6

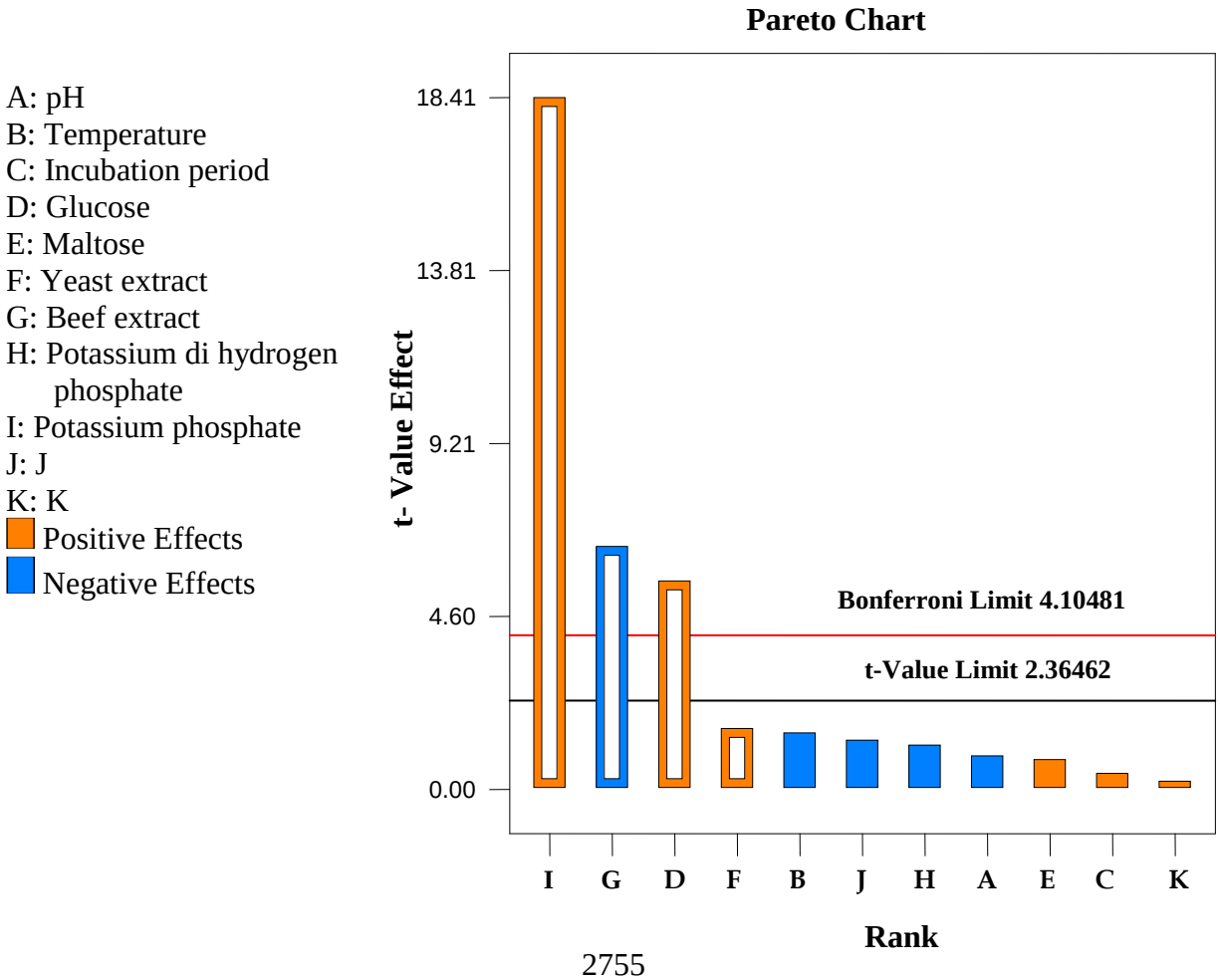


Fig.10 Response Surface Curve showing the effect of beef extract and potassium phosphate on Phytase production from *B. subtilis* P6

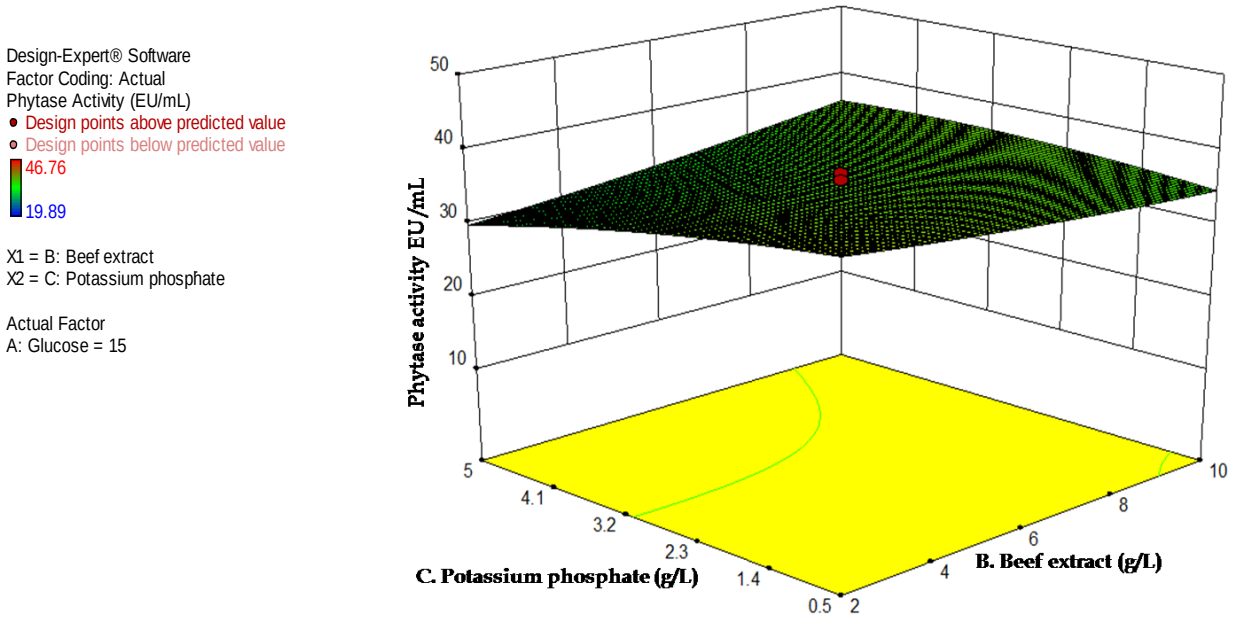


Fig.11 Response Surface Curve showing the effect of beef extract and glucose on Phytase production from *B. subtilis* P6

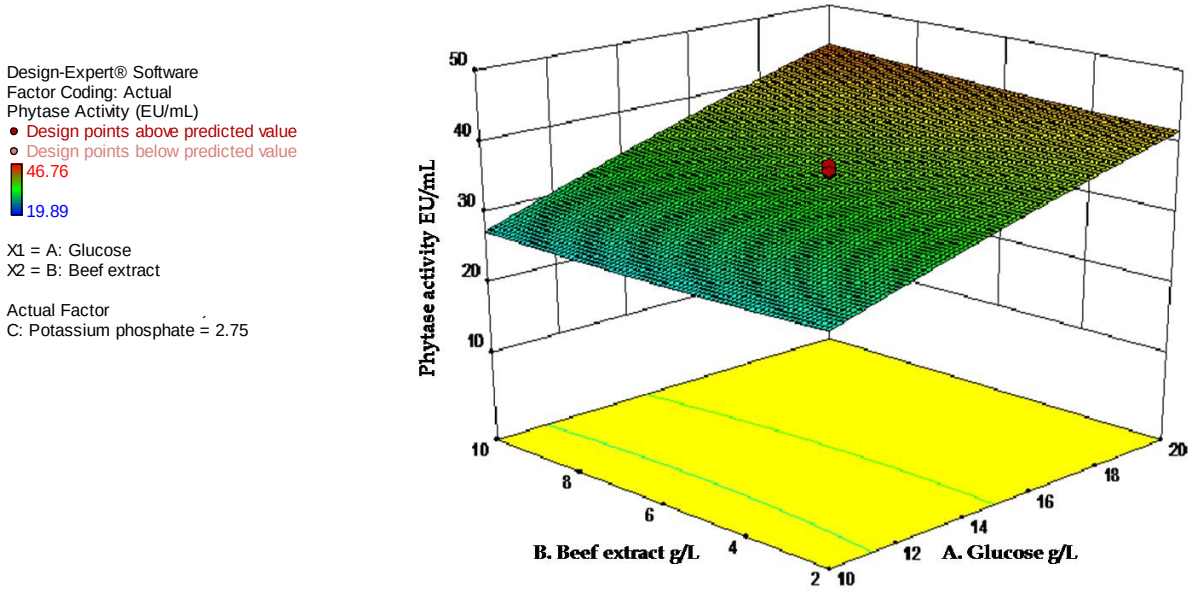
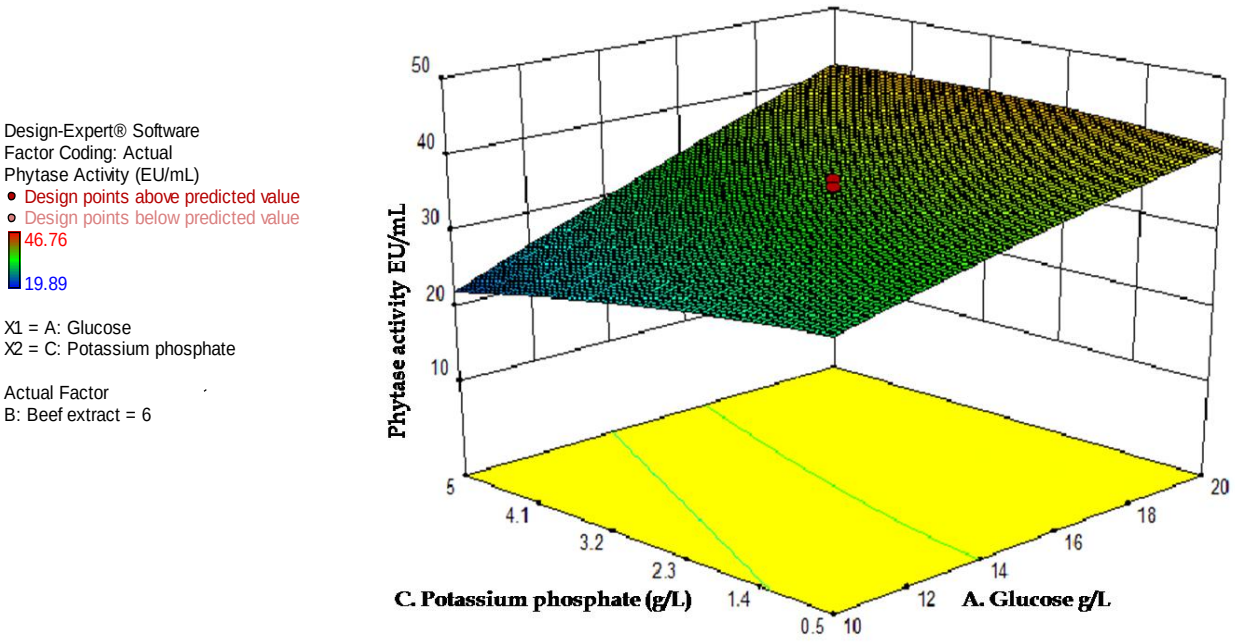


Fig.12 Response Surface Curve showing the effect of potassium phosphate and glucose on Phytase production from *B. subtilis* P6



$Y_{\textit{B. subtilis}\ \text{phytase}} = + 30.82 + 1.70 * D + 0.50 * F - 1.98 * G + 5.65 * J$

Where, D: glucose; F: Yeast extract; G: beef extract; J: potassium phosphate.

Optimization by Response Surface Methodology

The Central Composite Design experiment was found to be very efficient indicating the optimal concentrations of individual factors among different factors. The mean predicted and observed responses are illustrated in table 7. The regression equation coefficients were calculated (Table 8) and the experimental data were fitted to a second-order polynomial equation. Significant interaction was found between glucose and beef extract; potassium phosphate and glucose (Figures 10–12) revealing its importance in the phytase production. The surface plot suggested that increase in beef extract and potassium phosphate concentration beyond the central value resulted in constant increase in enzyme production; however, phytase production increased continuously with increase in glucose concentration. For *B. subtilis* P6, glucose and beef extract were found to be more effective than potassium phosphate. The results obtained from the central composite design experiments were fitted to a second order polynomial equation to explain the dependence of phytase production by *B. subtilis* P6 on the medium components:

$Y_{\textit{B. subtilis}\ \text{P6 phytase}} = + 35.47 + 7.50 * A + 0.45 * B - 1.91 * C + 0.80 * AB + 2.45 * AC + 2.66 * BC - 0.52 * A^2 + 0.43 * B^2 - 0.98 * C^2$

Where, A: glucose; B: beef extract; C: potassium phosphate.

The model predicted a 17-fold increase in enzyme production over un-optimized medium with maximum phytase production of 46.76 U ml^{-1} after 44 h of incubation. An

interesting finding observed in the present study was the phytase production at an overall high concentration of phosphorus. It might be due to the fact that presence of K_2HPO_4 served as a buffer for pH of the media (Rani *et al.,* 2013).

Validation of the model

To confirm the validity of the model, three assays were done under the conditions predicted by RSM software as glucose (23.4 g/l), beef extract (6 g/l) and potassium phosphate (2.5 g/l). The estimated phytase value was 46.63 g/l and the experimental phytase value was 46.76 g/l indicating the efficacy of the model for prediction of phytase production.

We report the optimization of phytase production from probiotic strain *B. subtilis* P6. The *B. subtilis* P6 with phytase activity could replace commercial phytase addition, and its probiotic function can regulate gut microflora and can contribute to replace antibiotics application. As a result, nutrient digestibility, immunity and animal production can be increased in a cost effective manner. Since, optimization of process parameters resulted in a considerable increase in phytase production. Response Surface Optimization using CCD yielded a 17 fold increase in phytase production as compared to un-optimized medium. Thus, *B. subtilis* P6 can be used as an ideal supplement in food and animal feed; however, to harness its potential nutritional and physiological role, further investigations are required.

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References

Dash, S.S., Gummadi, S.N. 2007. Enhanced biodegradation of caffeine by *Pseudomonas* sp. using response surface methodology. *Biochem. Eng. J*. 36: 288-293.

Demirkan, E., Baygın, E., Usta, A. 2014. Screening of phytate hydrolysis *Bacillus* sp. isolated from soil and optimization of the certain nutritional and physical parameters on the production of phytase. *Turk. J. Biochem*. 39(2): 206–214.

Esakkiraj, P., Immanuel, G., Sowmya, S.M., Iyapparaj, P. and Palavesam A. 2009. Evaluation of protease producing ability of fish gut isolate *Bacillus cereus* for aqua feed. *J. Food Biopro. Technol*. 2: 383-390.

Haros, M., Carlsson, N.G., Almgren, A., Larsson-Alminger, M., Sandberg, A.S., Andlid T. 2009. Phytate degradation by human gut isolated *Bifidobacterium pseudocatenulatum* ATCC27919 and its probiotic potential. *Int. J. Food Microbiol*. 135: 7–14.

Hosseinkhani, B., Emtiazi, G., Nahvi, I. 2009. Analysis of phytase producing bacteria (*Pseudomonas* sp.) from poultry feces and optimization of this enzyme production. *Afr. J. Biotechnol*. 8(17): 4229-4232.

Idriss, E.E., Makarewicz, M., Farouk, A., Rosner, K., Greiner, R., Bochow, H., Richter, T. and Borriss, R. 2002. Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant-growth-promoting effect. *Microbiol*. 148: 2097–2109.

Jorquera, M.A., Crowley, D.E., Maschener, P., Greiner, R., Fernandez, M.T., Romero, D., Menezes-Blackburn, D., de la Mora, M. 2011. Identification of beta – propeller phytase-encoding genes in culturable *Paenibacillus* Spp., from rhizosphere of pasture plants on volcanic soils. *FEMS Microbiol. Ecol*. 75: 163–172.

Kammoun, R., Farhat, A., Chouayekh, H., Bouchaala, K., Bejar, S. 2012. Phytase

production by *Bacillus subtilis* US417 in submerged and solid state fermentations. *Annals. Microbiol*. 62: 155-164.

Lan, G.Q., Abdullah, N., Jalaludin, S., Ho, Y.W. 2002. Culture conditions influencing phytase production of *Mitsuokella jalaludinii*, a new bacterial species from the rumen of cattle. *J. Appl. Microbiol*. 93: 668-674.

Lee, J., Park, I., Choi, Y. and Cho, J. 2012. *Bacillus* strains as feed additives: *in vitro* evaluation of its potential probiotic properties. *Revista Colombiana de Ciencias Pecuarias*, 25: 577–585.

Mittal, A., Singh, G., Goyal, V., Yadav, A., Aggarwal, N.K. 2012. Production of phytase by acido-thermophilic strain of *Klebsiella* sp. using orange peel flour under submerged fermentation. *Inno. Romanian Food Biotechnol*. 10: 18-27.

Rani, R., Arora, S., Kumar, S., Ghosh, S. 2013. Optimization of medium components for the production of phytase by *R. oryzae* using statistical approaches. *J. Bioremed. Biodeg*. S: 18: 1-6.

Reid, G., Friendship, R. 2002. Alternatives to antibiotic use; probiotics for the gut. *Anim. Biotechnol*. 13: 97-112.

Roopesh, K., Ramachandran, S., Nampoothiri, K.M., Szakacs, G., Pandey, A. 2006. Comparison of phytase production on wheat bran and oil cakes in solid-state fermentation by *Mucor racemosus*. *Biores. Technol*. 97: 506-511.

Sabu, A., Sarita, S., Pandey, A., Bogar, B., Szakacs, G., Soccol, C.R. 2002. Solid-State fermentation for production of phytase by *Rhizopus oligosporus*. *Appl. Biochem. Biotechnol*. 102-103.

Sasirekha, B., Bedashree, T., Champa, K.L. 2012. Optimization and partial purification of extracellular phytase from *Pseudomonas aeruginosa* p6. *European J. Exp. Biol*. 2(1): 95-104.

Shamna, K.S., Rajamanikandan, K.C.P., Mukesh Kumar, D.J., Balakumaran, M.D., Kalaichelvan, P.T. 2012. Extracellular production of phytase by a native *Bacillus subtilis* strain. *Ann. Biol. Res*. 3(2): 979-987.

Sharma, A. Trivedi, S. 2015. Evaluation of *in vitro* probiotic potential of phytase producing bacterial strain as a new probiotic candidate. *Int. J. Food Sci. Technol*. 50(2): 507-514.

Sharma, P., Tomar, S.K., Goswami, P., Sangwan, V., Singh, R. 2014. Antibiotic resistance among commercially available probiotics. *Food Res Int*. 57: 176–195.

Singh, N.K., Joshi, D.K., Gupta, R.K. 2013. Isolation of phytase producing bacteria and optimization of phytase producing parameters. *J. Microbiol*. 6(5): 1-6.

Vohra, A., Satyanarayana, T. 2003. Phytases: Microbial sources, production, purification, and potential biotechnological applications. *Crit. Rev. Biotechnol*. 23: 29-60.

Wang, X., Upatham, S. Panbangred, W.A., Isaramgkul, D., Summpunn, P., Wiyakrutta, S., Vootisom, V.M. 2004. Purification, characterization, gene cloning and sequence analysis of a phytase from Klebsiella *pneumoniae*, sub spp. *Pneumoniae* XY5. *Sci. Asia* 30: 383-390.

Wilson, K., Walker, J. 2000. Practical Biochemistry, Principles and techniques, 5th edition. Cambridge University Press, UK.

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