CHAPTER 3

Isolation, Characterization and Gene Cloning of phytase from *Bacillus* sp. RS1 and Application in plant growth

3.1. INTRODUCTION

Phosphorous (P) is an essential nutrient for plants and animals. PA (*myo*-inositol 1,2,3,4,5,6-hexakis phosphate, IP6) and its mixed cation salt, phytate, are the chief storage forms of P (60–80%) in plants (Wang *et al.* 2013). Phytates are strongly complexed in soil and constitute 20–50% of total soil organic P that is poorly available for plant absorption (Richardson *et al*. 2009). They also chelate nutritionally important minerals such as, calcium, iron, zinc, magnesium, manganese, copper and molybdenum thereby limiting their bioavailability (Reddy *et al*. 1989, Ramirez and Kloepper 2010). Hence, inorganic phosphate is commonly added to fertilizers for adequate plant growth. While nutritional demand is met, environmental problems arise due to leaching of unabsorbed fertilizer phosphate into water bodies, leading agricultural pollution and eutrophication.

The rhizosphere is the major hot-spot for bacterial activity in soil. A substantial number of bacteria in the rhizosphere may exert a beneficial effect on plant growth. Among them, phosphobacteria, phytate mineralizing bacteria (PMB), and/or phosphate-solubilizing bacteria (PSB), are of great interest for diverse biotechnological applications, and they have been commonly isolated and proposed as inoculants for agricultural improvement (Igual *et al.* 2001, Konietzny and Greiner 2004). In the rhizosphere, the conversion of the insoluble forms of inorganic P to plant accessible form is achieved by the activity of PSB, which release phosphates mainly by secreting organic acids. Phytate mineralizing bacteria produce phytase that hydrolyse phytate to release inorganic phosphate for assimilation by the plants because plants acquire P only in inorganic form (Hayat *et al.* 2010). Studies have indicated that, besides other factors, the ability of some root-colonizing bacteria to make the phytate P in soil available for plant nutrition under phosphate-starvation conditions might contribute to their

plant-growth-promoting activity (Idriss *et al.* 2002, Patel *et al.* 2010, Singh *et al.* 2014). Such researches can contribute in the successful application of phytase-producing microorganisms for making phytate-P available to plants under natural soil conditions. Hence, from both the nutritional and environmental point of view phytase producing rhizospheric microorganisms have been considered benificial in enhancing growth and productivity of plants growing in phytate rich soil (Unno *et al.* 2005, Richardson *et al.* 2009).

Microbial enzymes can be produced in higher quantities by proper fermentation techniques. As enzyme production is closely controlled in microorganisms, hence these controls can be exploited and modified to improve the productivity. Phytase production in bacteria is controlled by a receptor-inducer system; therefore, the composition of the culture medium can affect its production (Dechavez *et al.* 2011). For development of commercial enzymes, high-yielding microbial strains which can secrete the enzyme into their growth medium are always preferred. This is beacause extracellular enzymes are easy to recover which reduces the cost of post-production. Therefore, an elaborate investigation is required to establish the optimum condition for scaling up enzyme production in a fermentation process. The conventional one factor at a time (OFAT) method for medium optimization involves changing one factor while keeping all others constant, which may not determine the effect of interaction of different factors. A number of statistical experimental designs have been used to address such interactions (Lu *et al.* 2007). Among these, full factorial designs provide more complete information, but they require lots of experiments making them impractical for large number of variables. The Plackett-Burman design (Plackett and Burman 1946), a two level fractional factorial design, is especially useful in screening studies by estimating the main effects in limited number of experiments. However, the design does not consider the interaction between variables. The variables screened by Plackett-Burman design may be optimized by using statistical and mathematical optimization tools, such as, Response Surface Methodology (RSM) (Myers and Montgomery 2002). This empirical technique enables to evaluate the relationship between independent variables and to predict the response in an effective experimental design.

Chickpea (*Cicer arietinum*, common name-chana) is an important pulse crop of Leguminosae family. It is highly nutritious pulse and is cultivated throughout the world. Chickpea is valued for its nutritive seeds with high protein (25.3-28.9 %, after dehulling) and carbohydrates (60%) content (Hulse 1991). It is grown in tropical, sub-tropical and temperate regions. India is the largest producer of chickpea contributing to around 70% of the world's total production and is also the largest importer of chana in the world (Abbo *et al.* 2011). The Chickpea plant has finely divided leaves, giving it a feathery appearance. The pods are oblong

(2 to 3 by 1 to 2 cm) and contain one or two beaked seeds which may be white, yellow, red, brown, or nearly black. They do well in a cool, dry climate and are grown in India as a winter crop (Muehlbauer 1996, Abbo *et al.* 2011).

Present investigation aims at isolation, purification and characterization of extracellular phytase from rhizosphere. Further, the production of phytase was optimized by one factor at a time (OFAT) and RSM approaches. Finally, the growth promoting abilities of phytase producing bacteria and phytase were examined in chickpea seedlings.

3.2. MATERIALS AND METHODS

3.2.1. Strains, plasmids and chemicals

Phytic acid (dodecasodium salt) and DEAE-Sephacel were purchased from Sigma, USA. Biogel P-10 and P-100 were from Bio-Rad, USA. The pGEM-T Easy vector system and PCR reagents were purchased from Promega, USA. Restriction enzymes and T4 DNA ligase were from New England Biolabs (Beverly, MA). All other biochemicals and microbiological media were from Promega, USA; Hi Media, India or E.Merck, Germany as described in the section 2.2.1.

3.2.2. Isolation and screening of phytase producers

Isolation of the phytase producing bacteria was done as described in the section 2.2.3. Phytase producing bacteria were isolated from soil samples taken from plant rhizosphere. For detection of phytase producers, bacterial strains were inoculated in phytase screening medium (PSM) containing, glucose, 2.0 %; sodium phytate, 0.2 %; calcium chloride, 0.2 %; ammonium nitrate, 0.5 %; potassium chloride, 0.05 %; magnesium sulfate, 0.05 %; manganese sulphate, 0.001%; ferrous sulphate, 0.001% and agar 1.5 %, pH 7.0 and grown at 37°C. Pure cultures were isolated and stored as described in the section 2.2.3. Each isolate was quantitively tested for phytase production in wheat bran medium (WBM) containing in g l^{-1}: wheat bran, 50; $(NH_4)_2SO_4$, 0.4; $MgSO_4.7\ H_2O$ 0.2 and $CaCl_2$, 2.2; at pH 6.5. Culture samples were withdrawn at different time intervals, centrifuged at 10,000 rpm for 10 min at 4°C. The media supernatant obtained after centrifugation of the bacterial culture was used as extracellular phytase source. Finally, an extracellular phytase producing strain RS1 was chosen for further studies.

3.2.3. Phytase assay and protein estimation

Phytase activity assays were carried out at 37°C for 30 min as described in the section 2.2.5 with slight modifications. The reaction mixture in a final volume of 2 ml contained Tris-HCl buffer (pH 6.5) 100 mM; sodium phytate, 2 mM; $CaCl_2$, 2 mM and 100 µl enzyme preparation. One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 µmol of Pi per minute under the assay conditions. The quantitative estimation of protein was done by method described by Bradford *et al.* (1976) using BSA as standard as described in the section 2.2.6.

3.2.4. Identification of the isolate RS1

Morphological and biochemical characteristics of the isolate were determined by Gram staining, motility, diastase, catalase, oxidase, urease activities, nitrate reduction, gelatin hydrolysis, utilization of carbohydrate and citrate and Indole, methyl red and Voges-Proskauer tests (Smibert and Kreig 1994). The identity of the isolate was confirmed by 16S rDNA analysis.

3.2.4.1. Genomic DNA isolation and agarose gel electrophoresis

The genomic DNA was isolated according to Sambrook and Russel (2001). Bacterial culture was grown in nutrient broth at 37°C for 24 h and then harvested by centrifugation at 6000 rpm for 10 min followed by washing of pellet with NaCl:EDTA (30 mM: 2mM) solution (pH 8.0) thrice. Pellet was resuspended in 100 µl NaCl:EDTA and 100 µl freshly prepared lysozyme (10 mg ml^{-1}) solution and incubated at 37°C for 1 h with intermittent shaking. After making the volume up to 500 µl with NaCl:EDTA solution, 50 µl SDS (10%) and 10 µl proteinase-K (20 mg ml^{-1}) solutions were added. Contents were mixed thoroughly and incubated at 55°C for 1 h. The DNA was extracted with phenol:chloroform protocol followed by precipitation with isopropanol. Finally, the pellet was washed with 70 % ethanol and air-dried and dissolved in 50 µl TE buffer. The DNA sample was checked by TAE-agarose gel electrophoresis as described in the section 2.2.4.2.

3.2.4.2. Amplification of 16S rDNA and sequencing

The 16S rDNA of the strain was PCR amplified from genomic DNA by using universal primer 27F (5'-AGAGTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACT-3') following the same protocol described in the section 2.2.4.2.4. The PCR product (1.4 kb) so obtained was cloned in pGEM-T Easy vector and then sequenced following the same processes described in the section 2.2.4.2.

3.2.4.3. Phylogenetic analysis

The identity of the isolate RS1 was confirmed by phylogenetic analysis of 16S rDNA sequence. The homology analysis of its 16S rDNA sequence was carried out with closely related neighbour sequences retrieved from the GenBank database of the National Center for Biotehnology Information (NCBI), via BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al.* 1990). Phylogenetic relationship was determined by using the software package MEGA 4 (Tamura *et al.* 2007) after obtaining multiple alignments of the data available from public databases by ClustalW (Thompson *et al.* 1994). Bootstrap analysis was

used to evaluate the tree topology of the neighbor-joining data by performing 1,000 replicates (Nei and Kumar 2000).

3.2.5. Optimization of phytase production by *Bacillus* sp. RS1 in submerged fermentation (SmF)

The bacterial inoculum was developed by cultivating the organism in LB broth overnight under shaking (120 rpm) till 0.1 OD_{600} and then inoculated at 2 % v/v into the WBM. Fermentation was allowed to proceed at 37°C under shaking (120 rpm) for 120 h. The fermentation broth was withdrawn at regular intervals and centrifuged at 10,000 rpm for 10 min. The supernatant was considered equivalent to crude enzyme and was used for assaying phytase production.

Phytase production by *Bacillus* sp. RS1 was initially optimized by OFAT approach. For this, the effect of various process variables, such as cultivation time, carbohydrate sources, nitrogen sources, initial medium pH and cultivation temperature, were studied under SmF. Each parameter optimized was incorporated further in the subsequent experiments.

3.2.5.1. Effect of incubation time

To study the phytase activity vis-a`-vis incubation time, fermentation was conducted in the production medium (WBM). Samples withdrawn at different time intervals (24-120 h) were assayed for phytase activity.

3.2.5.2. Effect of carbohydrate source

For investigating effect of additional carbohydrate sources on phytase production, WBM was supplemented with either of the carbohydres, viz. dextrose, sucrose, maltose, galactose, fructose, lactose or starch at 1 % w/v. Fermentation was conducted under shaking (120 rpm) at 37°C. The medium without sugar served as control. Phytase was assayed in the clear culture supernatant after 24 h of incubation. The carbohydrate source showing maximum phytase production was varied further to determine the optimum level.

3.2.5.3. Effect of different nitrogen source

For examining the effect of different nitrogen sources on phytase production, the medium contained either of the following organic or inorganic nitrogen sources, viz. peptone, beef extract, yeast extract, casein acid hydrolysate (0.5 %, w/v) or ammonium nitrate, sodium nitrate, ammonium sulphate (0.05 %, w/v). Fermentation was conducted under shaking

conditions (120 rpm) at 37°C. The nitrogen source showing maximum phytase production was varied to determine optimum level.

3.2.5.4. Effect of incubation temperature and medium pH

The optimum temperature for phytase production was determined by growing the bacteria in different temperatures (4–70°C) for 24 h followed by the enzyme assay with culture supernatant. Similarly, the effect of pH on production was also determined by varying the media pH by using appropriate buffers (0.1 M): glycine-HCl buffer (pH 2 and 3), sodium-acetate buffer (pH 4, 5, 5.5), Tris-acetate buffer (pH 6.0), Tris-HCl buffer (pH 6.5, 7, 8) and glycine-NaOH buffer (pH 9 and 10).

3.2.6 Optimization of phytase production by statistical tools

Plackett–Burman (PB) design was employed to find out the critical medium components or cultural variables that have the most significant influence on phytase production (Plackett and Burman 1946). Eleven variables (wheat bran, sucrose, beef extract, $(NH_4)_2SO_4$, KH_2PO_4, incubation time, incubation temperature, medium pH, inoculums volume, agitation speed and $CaCl_2$) were examined in a total of 12 trials (Table 3.1).

Table 3.1: Variables used in Plackett-Burman design of experiments

Factors in coded form	Factors in actual form	+1	-1
A	Sucrose (%,w/v)	2.0	0.5
B	Beef extract (%, w/v)	1.0	0.5
C	$(NH)_2SO_4$ (%, w/v)	0.04	0.0
D	KH_2PO_4 (%,w/v)	0.05	0.03
E	Incubation time (h)	48	24
F	Incubation temperature (°C)	37	27
G	Medium pH	6.5	5.5
H	Wheat bran (%, w/v)	10	2.5
I	$CaCl_2$ (%, w/v)	0.01	0.00
J	Agitation speed (rpm)	230	100
K	Inoculums volume (ml)	3.5	0.5

In this design, it is assumed that the main factors have no interactions and a first-order multiple regression model is appropriate:

$$Y = \beta_0 + \Sigma\beta_i x_i \ (i = 1, \ldots, k) \qquad \text{Eq.(1)}$$

Where Y is the response function (phytase production) and β_i is the regression coefficient.

All the designed experiments were performed in triplicates under proper conditions and the response/activity was determined in each case, and data represent the mean of three different experiments. The variables that showed the significant effect were determined by the F-test as described by Stanbury *et al.* (1995).

3.2.7. Optimization of major effective variables by using central composite rotatable design (CCRD) based response surface methodology (RSM)

Further optimization of phytase production by RSM using CCRD was based on the study of interaction among three effective parameters selected from the OFAT method i.e. incubation time, incubation temperature and medium pH. CCRD contains a factorial matrix with a centre point and axial points around the centre point that allow the curvature of the model to be established. The distance from the centre point to the factorial point is ± 1 unit for each factor, and the distance space from the centre of the design space to the axial point is $\pm\alpha$, where $\alpha = (2^k)^{1/4}$ [k= number of independent factors]. The variables optimized were incubation time (A), incubation temperature (B) and medium pH (C) with five different coded levels [-α, -1, 0, +1, +α]. For incubation time (A) the coded levels -α, -1, 0, +1, +α are 15.82, 24, 36, 48 and 56.18 h respectively. For incubation temperature (B) the coded levels -α, -1, 0, +1, +α are 23.18, 30, 40, 50 and 56.82°C, respectively, whereas for pH (C) the coded levels -α, -1, 0, +1, +α are 4.32, 5, 6, 7 and 7.68 , respectively (Table 3.2).

Table 3.2: Range of most effective variables used for optimizing phytase production by *Bacillus* sp. RS1 using response surface methodology

Variables	Levels				
	-α	**-1**	**0**	**+1**	**+α**
Incubation time (h)	15.82 (~16)	24	36	48	56.18 (~56)
Incubation temperature (°C)	23.18 (~23)	30	40	50	56.82 (~57)
Medium pH	4.32 (~4)	5.0	6.0	7.0	7.68 (~7.5)

The relation between the coded forms of the input variable and the actual value of the incubation time, incubation temperature and medium pH are described in Eq. (2).

$$X_a = (Z_a - Z_0)/ \Delta Z \quad \text{Eq. (2)}$$

where X_a is a coded value, Z_a is the actual value of the factor, Z_0 is the actual value of the same variable at the centre point and ΔZ the step change of the variable. According to the CCRD model total number of the experimental run is determined by the following equation (Eq. 3).

$$R = 2^k + 2k + n_0 \quad \text{Eq. (3)}$$

where k is the number of independent variable and n_0 is the number of repetitions of the experiments at the centre point. Total number of experimental runs was 20 with 8 factorial, 6 axial and 6 centre point runs. Phytase produced (activity 1U ml^{-1}) from all the experimental run was analyzed by a second order polynomial regression equation (Eq. 4) to better estimate the experimental error.

$$Y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_{11}x_{12} + a_{22}x_2^2 + a_{33}x_3^2 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 \quad \text{Eq. (4)}$$

where Y is the predicted phytase activity, a_0 is the intercept terms, x_i is the independent factors and a_i is the model coefficient parameters. With the help of Eq. (4), all the independent variables were optimized to get a better response.

3.2.7.1. Interpretation and data analysis

The Design Expert Software (Version 8.0.7.1, State-Ease, Minneapolis, MN, USA) was used for the statistical design of the experiments, regression analysis of experimental data, analysis of variance and lack of fit test etc. Prediction of optimal submerged fermentation parameters and creation of response surface plot were also conducted by the same software.

ANOVA through Fisher's test was used to evaluate the effect of independent variables on the response and significant results were identified by a p-value <0.05. Multiple correlation coefficient (R^2) and adjusted R^2 were used as quality indicators to evaluate the fitness of the second order polynomial equation. Contour and three-dimensional surface plots were employed to demonstrate the relationship and interaction between the coded variables and the response. The optimal points were determined by solving the equation derived from the final quadratic model and grid search in RSM plots.

3.2.8. Enzyme purification

The extracellular phytase from the strain RS1 was purified by solvent (ethanol) precipitation, gel filtration and DEAE-Sephacel chromatography.

3.2.8.1. Preparation of Biogel P-2 and P-100

All the preparations were done as described in the section 2.2.8.1 except the buffer used was Tris-HCl buffer, pH 6.5.

3.2.8.2. Preparation of DEAE-Sephacel

The pre-swollen DEAE-Sephacel slurry (Sigma-Aldrich, USA) was treated with 0.5N HCl with slow stirring. The acid treated slurry was washed with distilled water till attaining pH 4.0. After discarding the supernatant, 0.5N NaOH was added to the slurry followed by washing with distilled water till pH 6 to 7. Finally, the ion-exchange material was equilibrated with the Tris-HCl buffer, pH 6.5. About 10 ml of the equilibrated slurry was packed into the column (10x2 cm) for purification of phytase.

3.2.8.3. Purification of phytase from *Bacillus* sp. RS1

Unless otherwise indicated, all operations during extraction and purification of the enzyme were carried out at 4°C and all buffers used for the enzyme purification contained 1 mM $CaCl_2$ because Ca^{2+} ion proved to stabilize the enzyme activity. The bacterial culture grown in 200 ml of wheat bran medium for 24 h under optimized cultural conditions was used for phytase purification. The culture fluid from the production media was collected by centrifugation at 10,000 rpm for 15 min at 4°C. Phytase enzyme from culture supernatant was purified by ethanol precipitation, gel filtration and anion exchange chromatography. To the culture supernatant, three volumes of 95 % cold ethanol was added, and the mixture was maintained on ice for 1 h with agitation. The protein precipitate was harvested by centrifugation at 10,000 rpm for 20 min and resuspended in 0.1 M Tris-HCl buffer (pH 6.5) supplemented with 2 mM $CaCl_2$. The resuspended protein sample was then subjected to the gel filtration chromatography in Biogel P-100 column (Bio Rad, 1x25 cm). The active fractions were pooled and loaded onto DEAE-Sephacel column (2x10 cm) prequlibrated with 0.1 M Tris-HCl buffer (pH 6.5) and eluted with a linear gradient of 0-1M NaCl in the same buffer at a flow rate of 2 ml min^{-1}. The active fractions were pooled and used for subsequent studies.

3.2.9. SDS-PAGE analysis

SDS-PAGE was carried out following the discontinuous method described by Laemmli (1970). The enzyme fractions from various steps of purification were resolved by 12% SDS-PAGE as elaborated in the section 2.2.8.4.

3.2.10. Characterization of phytase

The purified enzyme was characterized for pH optima, temperature optima, thermostability, K_m for phytate, substrate specificity and effects of metal ions and proteases, following the methods described in the section 2.2.10. The phytase activity against the sodium phytate substrate was measured over a pH range of 2.5 to 9.5 in 100 mM of the following buffers: glycine-HCl (pH 2.5 and 3.5), sodium acetate (pH 4.5 and 5.5) and Tris-HCl (pH 6.5–9.5). The optimal temperature for phytase was determined by incubating the purified enzyme at temperatures ranging from 4°C to 90°C at the optimum pH. For thermal stability study, the purified enzyme was preincubated at 20-90°C for 30 min in both presence and absence. K_m value for phytate was determined by Lineweaver-Burk analysis at optimum pH and at 37°C. The substrate specificity of the enzyme was also tested against 2 mM (final concentration) of different phosphate containing compounds: PA, ADP, ATP, p-nitrophenyl phosphate (pNPP), disodium pyrophosphate (dSPP), D-glucose-6-phosphate (G6P), and D-fructose-6-phosphate (F6P). All experimental data were means of triplicate determination. To study the requirement of metal ions for phytase activity, the enzyme assay was performed in presence of 2 mM of Ca^{2+}, Mn^{2+}, Mg^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+} and EDTA after removal of calcium ions in enzyme solution by dialysis. The purified enzyme preparation was poured in a dialysis membrane bag and dialyzed against 0.1M Tris-HCl buffer (pH 6.5) for 4 h. For determining the susceptibility of the phytase to digestive proteases, 50 Units of the purified enzyme was preincubated with 30 Units of pepsin and trypsin at 37°C and phytase activity was monitored 60 min later.

3.2.11. Cloning of phytase gene

The putative phytase gene, phy_B was amplified by PCR using primers phyBF (5'-ATTACGCGATGGTGACAGGG-3') and phyBR (5'-ATTTCCATCTTGTACAGC AAA-3'), which were designed on the basis of the phytase gene sequence of closest organisms identified by 16S rDNA sequence similarity search. A 25 µl PCR reaction mix contained of 5X GoTaq Flexi buffer (Promega), 5.0 µl; 25 mM $MgCl_2$, 2µl; 50 pmol of forward and reverse primers, 1µl each; 10 mM dNTPs, 1µl; 100 ng of template DNA and 2.5 U of Taq DNA polymerase (Promega). Thirty cycles of amplification were carried out at 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min with a 7 min final extension at 72°C. The PCR product was analysed in 1.5 % agarose gel. The PCR product was cloned in pGEM-T Easy vector (Promega, USA) and sequenced followed by homology analysis and phylogenetic studies as described in the section 2.2.10.

3.2.12. Effect of the bacterial inoculation on growth of chick pea

Seeds of chickpea were disinfected with ethanol for 1 min, followed by 1 % sodium hypochlorite for 10 min, and thoroughly washed with sterile distilled water. Then the seeds were germinated for 24 h in an incubator at 25ºC. Surface soil (~15 cm depth) used in the experiment was collected from the university experimental farm, which was dried and sieved through a 2 mm diameter mesh to remove large debris and then autoclaved for 1 h. Sieved soil (200 g) was filled into small plastic pots till 3/4 height.

Bacterial cells were inoculated in 25 ml LB medium in 100 ml conical flask and incubated at 37°C and 120 rpm shaking. After overnight incubation, bacterial inoculum containing approximately 10^8 cfu ml^{-1} was used for seed bacterization. CMC (100 mg) was added to flask containing culture inoculum as adhesive material. Ten gram of germinated seeds was soaked in bacterial suspension for 12 h under 150 rpm shaking. The bacterial suspension was drained off and the seeds were dried overnight aseptically in laminar air flow. Seeds soaked in distilled water amended with CMC served as control. Seeds were placed on the soil surface of the plastic pots and covered with a 1–2 cm layer of the same soil. The plants were grown in a plant growth chamber maintained at 250 µmol m^{-2} s^{-1} photon flux density (12 h/12 h day/night regime), 27±2°C and 70-80 % relative humidity. The plants were sprayed only with sterile distilled water (25 ml) everyday. After 10 days of growth, another batch of bacterial inoculum (~10^8 cfu ml^{-1}) was added to the soil of each pot. For inoculum preparation, the overnight grown bacterial culture was centrifuged at 10,000 rpm for 10 min and the pellet was dissolved in 0.85 % NaCl to add the mixture in soil. Plants were then grown for another 10 days.

3.2.12.1. Estimation of growth parameters and P content

Chick pea seedlings (20 days old) were harvested without root loss, and their fresh weight, root and shoot lengths were recorded. Plants were then dried in an oven (80°C) for 24 h, and the dry weights of whole plants were measured. For analysis of the P content, seedlings were dried in a hot air oven, grounded, and digested in a solution of 15 ml $HClO_4$ and 5 ml HNO_3 and P content was measured spectrophotometrically using vanado-molybdate method (Clesceri *et al*. 1995).

3.2.13. Plant growth promotion by application of purified enzyme

The surface-sterilized chickpea seeds were germinated for 24 h in an incubator at 25ºC. The germinated seeds were grown hydroponically in sterile medium containing phytate or low

phosphate. The aseptically grown seeds were transferred into sterile tubes each containing 10 ml of a low phosphate nutrient solution with the following composition in gl^{-1}: $Ca(NO_3)_2.4H_2O$; 0.88; $NaH_2PO_4.2H_2O$, 0.006; K_2SO_4, 0.39; $MgSO_4.7H_2O$, 0.31; FeEDTA, 0.0031; and 1 ml microelement solution. The pH was adjusted to 6.5 with 2 M NaOH. To each tube, phytate solution (pH 6.5, 1.12 mM final concentration) and 50 Units of purified phytase were added. Chickpea seedlings growing in absence of phytate/phytase served as control. The plants were grown in a plant growth chamber maintained at 250 μmol m^{-2} s^{-1} photon flux density (12 h/12 h day/night regime), 27±2°C and 70-80 % relative humidity. After 7 days, seedlings were compared for observable growth parameter.

3.2.14. Other plant growth promoting capabilities of the strain

Among several plant growth promotion mechanisms exhibited by bacteria, solubilization of insoluble phosphate is the one, which make soil insoluble organic phosphorous available to plant under phosphate-starvation conditions. Besides, other plant growth promoting abilities of *Bacillus* sp. RS1 were also examined. The production of the phytohormone indole 3-acetic acid was also determined by colorimetric measurement at 530 nm using Salkowski's reagent as described by Patten and Glick (2002). Bacterial cells were grown under shaking (120 rpm) for 2 days at 30°C in LB broth at pH 7.0 supplemented with tryptophan (1 mg ml^{-1}) as IAA precursor. After incubation, the cells were centrifuged (3000 rpm for 10 min at 4°C) and 1 ml of supernatant was combined with 2 ml of Salkowski's reagent (150 ml of 95-98 % H_2SO_4, 7.5 ml of 0.5M $FeCl_3.6H_2O$ and 250 ml distilled water) and incubated for 30 min at room temperature. Besides, ammonia production test was performed by growing selected isolates in peptone water for 72 h at 30°C. Change in colour after addition of 1 ml Nessler's reagent (K_2HgI_4; 1.4%) in each tube was observed. The presence of yellow colour indicates ammonia production. For hydrogen cyanide (HCN) production the methodology described by Bakker and Schippers (1987) was used. Isolates were grown on plates of tryptic soy agar (10 %), amended with glycine (4.4 g l^{-1}), and $FeCl_3 \cdot H_2O$ (0.3 mM). A change from yellow to orange, red, brown, or reddish brown was recorded as an indication of weak, moderate, or strongly cyanogenic potential, respectively. Siderophore production was determined by using blue indicator dye and chrome azurol S agar (Schwyn and Neilands 1987). Bacterial isolates exhibiting orange halo zone on chrome azurol S agar after 5 d of incubation at 28°C were considered positive for the production of siderophores.

3.3. RESULTS

3.3.1. Isolation and screening of phytase producing rhizobacteria

Among 25 isolated phytase-positive rhizobacteria, 8 isolates were Gram-positive (Table 3.3) that produced prominent clear zones around the colonies on phytase screening medium (PSM) containing sodium phytate as the selective agent. The reason for selecting Gram positive bacteria was their ability to produce extracellular phytase. Initially, all the phytase producing Gram positive isolates were examined for qualitative and quantitative production of extracellular phytase. Among these isolates, the isolate RS1 predominantly showed highest level of extracellular phytase activity and produced the largest zone of clearance on the phytate-agar plate and hence selected for further studies (Fig 3.1).The bacterial isolate RS1 was morphologically characterized as an aerobic, Gram-positive, rod-shaped bacterium. Homology analysis of 16S rDNA sequence (GenBank accession number KJ879951) by BLAST program revealed highest (99 %) similarity score with *B. megaterium* and *B. subtilis* indicating close relation of RS1 isolate with these two bacteria (Fig. 3.2). However, morphological and biochemical characteristics of RS1 were substantially different from that of *B. subtilis* and *B. megaterium* (Table 3.4). The phylogenetic analysis showed *B. aryabhattai* as the closest strain to the isolate. Hence, the bacterial strain was tentatively named as *Bacillus* sp. RS1.

Table 3.3: Colony characteristics of phytase-producing Gram positive isolates

Sl. No.	Colony morphology
1 **(RS1)**	Irregular, flat, highly spreading type, white, opaque, colonies with a characteristic odour
2	Round, irregular margin, flat surface, matt, opaque, colorless colonies
3	Circular, pin head size, convex, smooth, shiny, opaque, with entire edge, emulsifiable, yellow pigmented colonies
4	Irregular form, lobate margin, raised, rough, opaque, white, larger colonies
5	Irregular form, raised, dull opaque, grayish white, rough colonies
6	Circular, small, translucent, low convex, mucoid colonies
7	Small, irregular margins, flat surface, opaque, white, very sticky, non emulsifiable colonies
8	Large, irregular, opaque, waxy, moist, smooth, whitish colonies

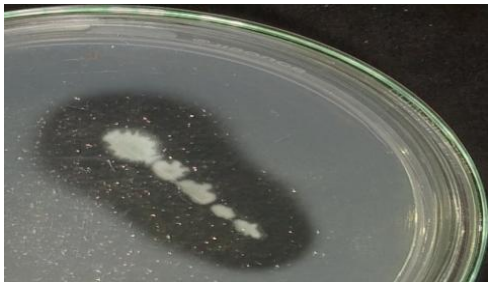
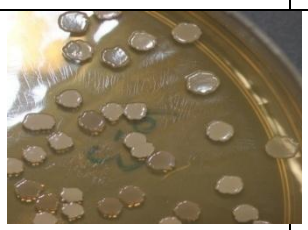


Fig. 3.1: Phytase producing colonies of RS1 on PSM plates

Table 3.4: Comparison of characteristics of *B. subtilis*, *B. megaterium*, *Bacillus* sp. RS1

Tests	**Bacterial Strain RS1**	***Bacillus subtilis***	***B. megaterium***
Motility Test	+	+	+
Catalase Test	+	+	+
Indole Test	-	-	-
Methyl Red Test	+	-	-
Voges Proskauer Test	+	+	-
Citrate Utilization Test	+	+	+
Oxidase Test	+	+	±
Gelatinase Test	-	+	+
Urease Test	-	-	-
Diastase Test	+	+	+
Nitrate Reduction	+	-	±
H_2S Production	-	-	-
Lipase Test	+	+	+
Glucose Fermentation	+ a	+a	+a
Mannitol Fermentation	-	-	+a
Lactose Fermentation	-	+a	+a
Sucrose Fermentation	+a	+a	+a
Xylose Fermentation	-	+a	+a
Colonies on nutrient agar plate			

*+= positive test result, - = negative test result, a= only acid, no gas

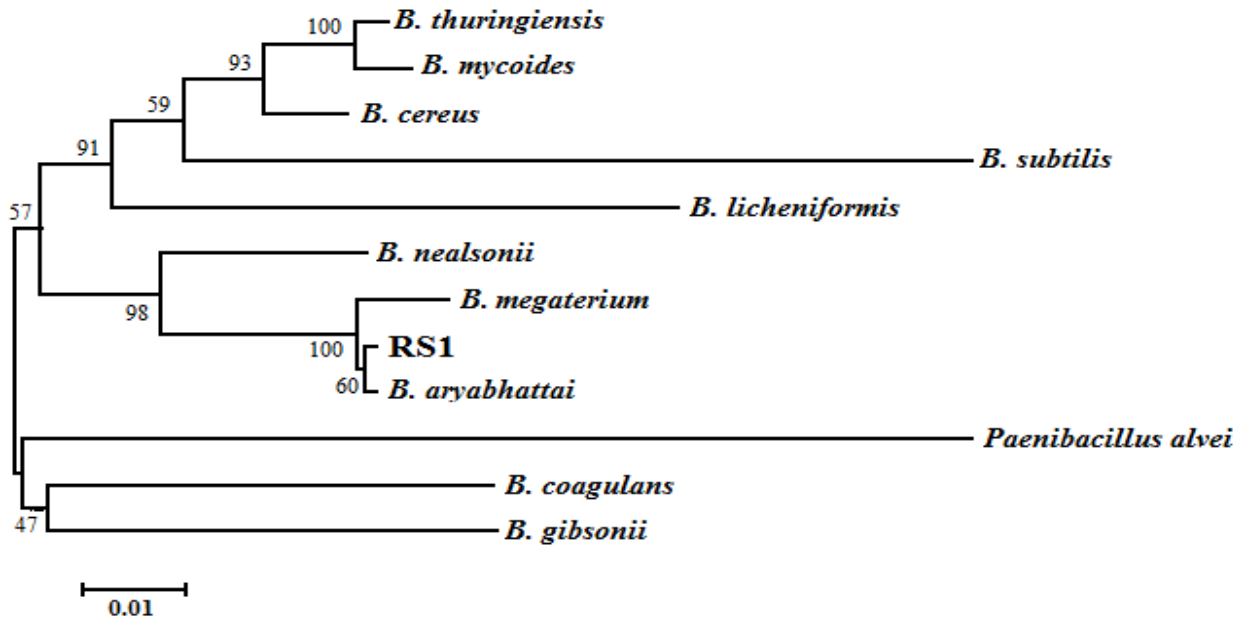


Fig 3.2: Phylogenetic tree showing the relationships of the isolate RS1 to closely related bacteria constructed by Neighbor-Joining method based on 16S rDNA sequence analysis. The numbers at branching points refer to bootstrap values, based on 1000 replicates. The bar represents 1 substitution per 100 amino acids. The source and GenBank Accession Nos. of sequences are: *Bacillus* sp. RS1, KJ879951; *Bacillus subtilis*, AJ277906; *Bacillus megaterium*, DQ408589; *Bacillus cereus*, FJ982661; *Bacillus thuringiensis*, EF206345; *Bacillus mycoides*, AB021192; *Paenibacillus alvei*, D78317; *Bacillus coagulans*, D78313; *Bacillus licheniformis*, FJ447354; *Bacillus gibsonii*, AY737309; *Bacillus aryabhattai*, KF010788; *Bacillus nealsonii*, KF010795.

3.3.2. Optimization of phytase production by *Bacillus* sp. RS1 using one factor at a time (OFAT) approach

The phytase production by *Bacillus* sp. RS1 was optimized with respect to various process variables, such as, incubation time, incubation temperature, medium pH, additional sugar and nitrogen source, in SmF using OFAT approach. As evident from Fig 3.3 a, phytase production increased with incubation time with maximum activity of 15 U ml^{-1} at 24 h of growth and then declined afterward. Besides, medium pH and incubation temperature also affected phytase production. Higher phytase production was observed in the temperature range of 30-50°C with the maximum at 37°C and in the pH range of 5 to 7 with the highest at 6.5 (Fig. 3.3 a & b). Optimization of incubation temperature and medium pH enhanced the phytase production to 19.7 U ml^{-1}. For determination of effect of carbohydrate source on phytase production, the bacterial culture was inoculated in WBM supplemented with either of the sugar (1% w/v), such as, glucose, maltose, lactose, sucrose, galactose and starch and grown for 24 h at 37°C. The supplementation of 1% sucrose supported maximum phytase activity of 28.2 U ml^{-1} (Fig 3.4 b). To optimize the nitrogen source, WBM was prepared with

wheat bran and sucrose as carbon sources and either of organic and inorganic nitrogen sources. The production of phytase was maximum in presence of 1% beef extract (35.1 Uml^{-1}) (Fig 3.4 c, d).

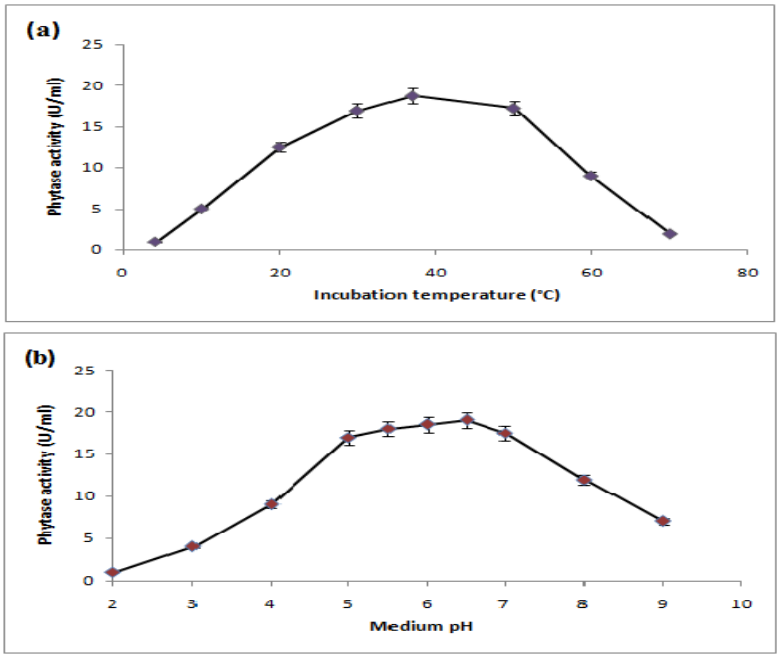


Fig. 3.3: Optimization of phytase production by *Bacillus* sp. RS1. Effect of (a) incubation temperature and (b) medium pH on the phytase production. [1U= 1µmol of Pi released min^{-1} under assay conditions].

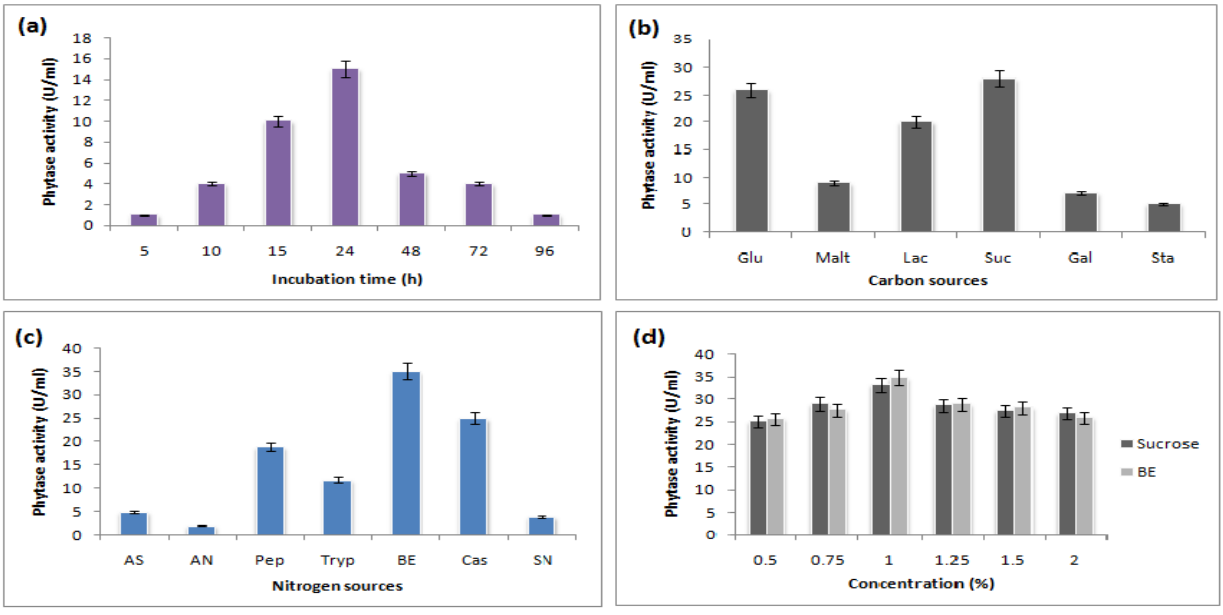


Fig.3.4: Optimization of phytase production by *Bacillus* sp. RS1. Effect of (a) incubation time, (b) carbon sources, (c) nitrogen sources and (d) various concentrations of the optimum carbon & nitrogen sources on the phytase production. [1U= 1µmol of Pi released min^{-1} under assay conditions].

3.3.3. Optimization of phytase production by statistical tools

Identification of most effective cultural variables for high phytase yield was established by PB design. Three variables, i.e., incubation time (A), incubation temperature (B) and medium pH (C) were marked as the most effective ones for their influence on phytase yield (Fig.3.5), which were determined by the F-test (Table 3.6) as described by Stanbury *et al.* (1995). The predicted values and experimental values for phytase production are also presented in Table 3.5. The results of PB design (Table 3.6) also indicated that this model is highly significant.

By using Design Expert, the equation (Eq. 5 and 6) obtained for Plackett-Burman design was as follows:

Final Equation in Terms of Coded Factors:

Activity = +34.49+4.74×A+3.28×C+2.3×D-15.77×E+8.39×F+5.89×G+4.64×H-6.22×K-2.12× L Eq. (5)

Final Equation in Terms of Actual Factors:

Activity = -52.21151+6.32111xSucrose+163.87500× NH_4SO_4+232.91667× KH_2PO_4-1.31451 x Incubation time +1.67883×Incubation temp+11.78167×pH+1.23667×Wheat Bran-0.095628×speed-1.41611×Inoculumvolume Eq. (6)

Table 3.5: Plackett-Burman design matrix with corresponding results (phytase activity, Uml^{-1}) for phytase production by *Bacillus* sp. RS1

Trial/ Run	A	B	C	D	E	F	G	H	I	J	K	Response/ Activity ($U\ ml^{-1}$)	
												Actual	Predicted
1	2.0	1.0	0.0	0.03	24	37	5.5	10	0.0	100	3.5	57.45	60.63
2	2.0	0.5	0.0	0.03	48	27	6.5	10	0.01	230	3.5	14.83	11.65
3	0.5	1.0	0.04	0.05	24	27	5.5	10	0.01	230	3.5	32.38	33.14
4	2.0	0.5	0.04	0.05	24	37	6.5	10	0.01	100	0.5	56.06	57.88
5	2.0	0.5	0.04	0.05	48	27	5.5	2.5	0.0	100	3.5	14.55	14.24
6	0.5	0.5	0.04	0.03	48	37	5.5	10	0.0	230	0.5	18.29	17.98
7	2.0	1.0	0.0	0.05	48	37	5.5	2.5	0.01	230	0.5	15.97	16.29
8	0.5	1.0	0.04	0.03	48	37	6.5	2.5	0.01	100	3.5	28.35	28.67
9	0.5	1.0	0.0	0.05	48	27	6.5	10	0.0	100	0.5	20.32	23.50
10	2.0	1.0	0.04	0.03	24	27	6.5	2.5	0.0	230	0.5	41.54	44.72
11	0.5	0.5	0.0	0.03	24	27	5.5	2.5	0.01	100	0.5	32.52	29.34
12	0.5	0.5	0.0	0.05	24	37	6.5	2.5	0.0	230	3.5	46.20	45.89

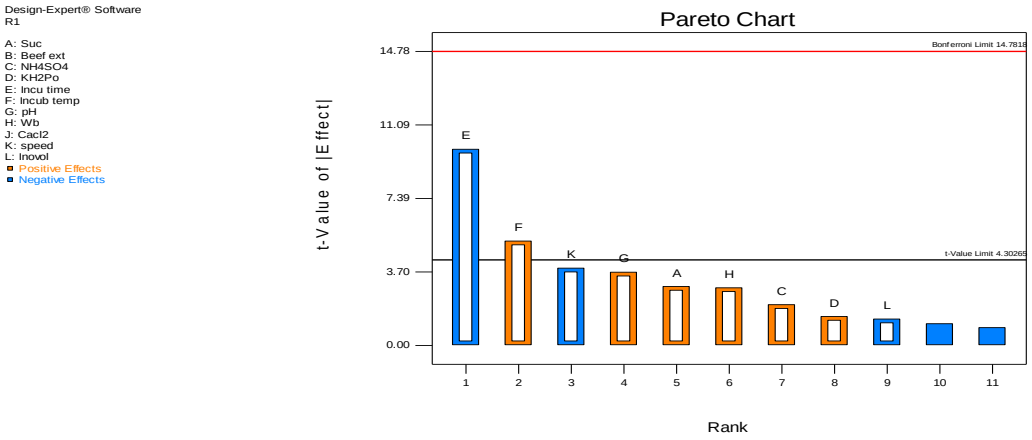


Fig. 3.5: Pareto chart depicting the more significant variables as per the PB design of experiments and determination of most effective variables based upon F-test values are shown in Table 3.1 and 3.2.

Table 3.6: Determination of most effective variables influencing phytase yield by *Bacillus* sp. RS1 from the Plackett-Burman design of experiments

Source	**Sum of squares**	**Df**	**Mean square**	**F Value**	**p-value Prob > F**
Model	5487.43	9	609.71	19.86	0.04 (Significant)
A-Sucrose	269.71	1	269.71	8.79	0.0975
C-$(NH_4)_2SO_4$	128.90	1	128.90	4.20	0.1770
D-KH_2PO_4	65.10	1	65.10	2.12	0.2826
E-Incubation time	2985.89	1	2985.89	97.27	0.0101
F-Incubation temp	845.54	1	845.54	27.54	0.0344
G-Medium pH	416.42	1	416.42	13.56	0.0665
H-Wheat bran	258.08	1	258.08	8.41	0.1012
K- Agitation speed	463.64	1	463.64	15.10	0.0603
L-Inoculum volume	54.15	1	54.15	1.76	0.3154
Residual	61.40	2	30.70		
Cor total	5548.83	11			

It can be seen from both the Eq. 5 and Fig. 3.4 that incubation temperature and medium pH exerted positive effect, while incubation time had negative effect on phytase production by *Bacillus* sp. RS1.

3.3.4. Optimization of phytase production using RSM

The selected three variables, incubation time (A), incubation temperature (B) and medium pH (C), which had maximum influence on phytase yield were further optimized using CCRD of RSM, keeping the other parameters constant. Suitable levels for these three parameters were determined by CCRD of RSM. For phytase production optimization twenty experimental runs were performed using different combinations of the variables as per CCRD. The experimental design and results of CCRD are shown in Table 3.7. The predicted values and experimental values for phytase production are also presented in Table 3.7.

Table 3.7: Response (phytase activity) obtained from CCRD of factors in coded levels

Run no.	**Type**	**Factor1 A: Incubation time**	**Factor2 B: Incubation temperature**	**Factor3 C: Medium pH**	**Phytase activity (*U ml^{-1}) actual**	**Phytase activity (*U ml^{-1}) predicted**
1	Axial	-α	0	0	66.10	66.84
2	Axial	+α	0	0	11.39	10.13
3	Factorial	-1	+1	+1	55.70	55.27
4	Axial	0	-α	0	36.40	35.31
5	Factorial	+1	+1	+1	23.11	23.10
6	Center	0	0	0	35.12	35.13
7	Axial	0	0	-α	31.20	30.83
8	Axial	0	+α	0	35.70	36.27
9	Factorial	+1	-1	-1	14.10	14.89
10	Center	0	0	0	35.12	35.13
11	Factorial	+1	+1	-1	18.20	18.71
12	Center	0	0	0	35.12	35.13
13	Center	0	0	0	35.12	35.13
14	Axial	0	0	+α	41.20	41.06
15	Center	0	0	0	35.12	35.13
16	Center	0	0	0	35.12	35.13
17	Factorial	-1	+1	-1	52.16	51.41
18	Factorial	+1	-1	+1	22.10	23.21
19	Factorial	-1	-1	-1	35.12	35.13
20	Factorial	-1	-1	+1	58.10	57.95

*1U= 1µmol of Pi released min^{-1} under assay conditions

Analysis of variance (ANOVA) was performed to the experimental design. The result of ANOVA shows that model is significant ($p< 0.05$) and can better predict the data. Within the model, B (incubation temperature), C (medium pH), AB (Incubation time×incubation temperature), AC (Incubation time × medium pH), A^2 (Incubation time^2), B^2 (incubation temperature^2), and C^2 (medium pH^2) are the significant ($p< 0.005$) model terms.

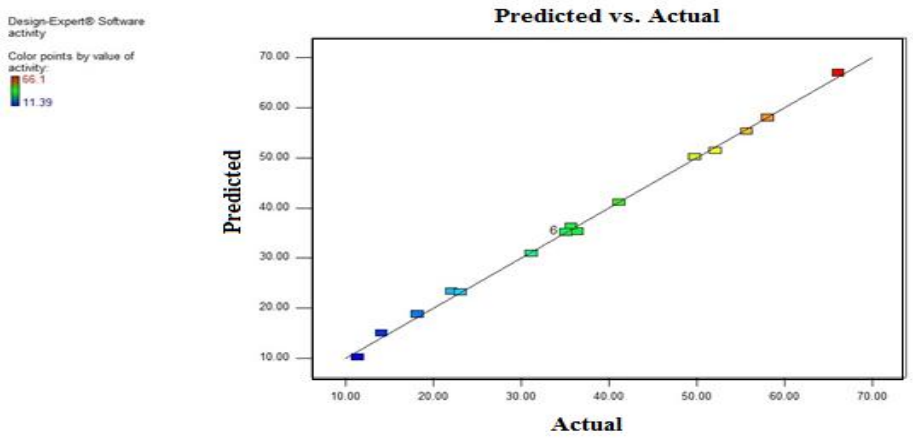


Fig. 3.6: Predicted versus actual (experimental) activity plot for optimized phytase production by *Bacillus* sp. RS1 using response surface methodology design.

The plot (Fig. 3.6) for the observed phytase activity (the response) versus model predicted phytase activity shows that these are very close with each other and data points are split by 45° line indicating a reasonable agreement of the predicted response with the observed ones.

Table 3.8: ANOVA for CCR design

Source	Sum of squares	df	Mean square	F Value	p-value Prob > F
Model	4041.93	9	449.10	658.31	< 0.0001 (significant)
A-time	3882.31	1	3882.31	5690.81	< 0.0001
B-temperature	1.11	1	1.11	1.63	0.2310
C-pH	126.52	1	126.52	185.46	< 0.0001
AB	3.32	1	3.32	4.86	0.0520
AC	0.14	1	0.14	0.21	0.6567
BC	7.70	1	7.70	11.29	0.0072
A^2	20.27	1	20.27	29.71	0.0003
B^2	0.78	1	0.78	1.15	0.3092
C^2	1.18	1	1.18	1.73	0.2178

Table 3.9: Values of different parameters obtained from RSM design applied for optimization of phytase production by *Bacillus* sp. RS1

Sl. No.	Parameter	Value
1.	Standard Deviation	0.83
2.	Mean	36.30
3.	C.V. %	2.28
4.	PRESS	52.34
5.	R^2	0.9983
6.	Adjusted R^2	0.9968
7.	Predicted R^2	0.9871
8.	Adequate Precision	97.102

Using the results of these experiments, second-order polynomial regression equation for phytase yield was obtained which is represented in Eqs. (7) and (8) in coded and actual form, respectively.

Final Equation in Terms of Coded Factors:

Activity =+35.13-16.86 × A+0.29 × B+3.04× C+0.64× A× B+0.13× A× C-0.98× B× C+1.19 × A^2+0.23× B^2+0.29 × C^2 Eq. (7)

Final Equation in Terms of Actual Factors:

Activity =+77.59967-2.27946 × time+0.23765 × temp+3.13389× pH+5.36458E-003× time × temp+0.011146× time × pH-0.098125× temp × pH+8.23560E-003 × $time^2$+2.33100E-003 $temp^2$+0.28613× pH^2 Eq. (8)

The quality of the model can be checked using various statistical parameters. In the present case, squared correlation (R^2), adjusted R^2 and predicted R^2 were taken into consideration (Table 3.9), which were 0.9983, 0.9968 and 0.9871, respectively. Results thus indicate that this model is significant and can explain 99.83 % variability in the response and only less than 1% of the variability is due to noise. Moreover, the similarity between R^2 and adjusted R^2-values shows the adequacy of the model to predict the response. The value of the coefficient of variation (CV% =2.28) also indicates the precision and reliability of the model. Also, the adequate precision of the model, which is an indicator of signal to noise ratio, was found to be 97.102.

The response surface plots and their contour plots described by second-order polynomial equation were generated in order to investigate the interactions among variables and optimal

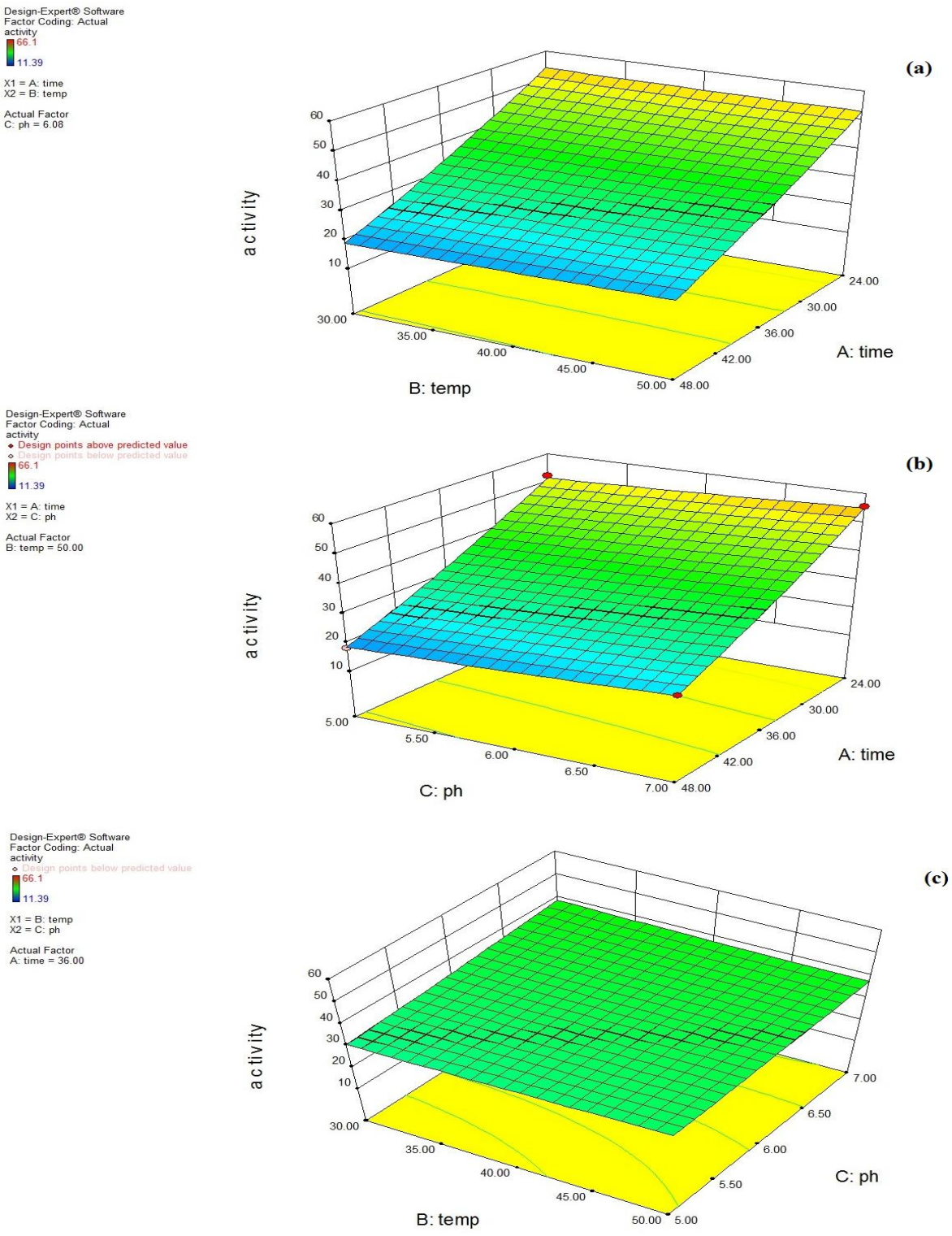


Fig. 3.7: Response surface curves of phytase production from *Bacillus* sp. RS1, showing interaction between (a) incubation time and temperature; (b) incubation time and medium pH; and (c) incubation temperature and medium pH.

levels of variables for phytase production (Fig. 3.7). From the plots it is evident that phytase production varied significantly with change in incubation time (Fig. 3.7 a & b). But it was not much affected by change in incubation temperature and medium pH and phytase activity remained almost unaltered in the temperature range of 30°-50°C (Fig. 3.7 a & b) and pH range of 5-7 (Fig. 3.7 b & c). Though these variables independently had significant effect on phytase production, no high level interaction was observed between them. Through solving Equation 5 and analyzing response surface plots, the optimized condition of phytase production was found to be 16 h of incubation at 40°C temperature and medium pH 6.0. In order to validate the experimental model, five verification experiments were performed using the statistically optimized medium. The condition led to enhancement of phytase production to 66.1 U ml^{-1}, which is comparable to the model predicted value (67.2 U ml^{-1}). The perfect agreement between the observed and predicted values confirms the validity and precision of the model. Hence, the CCRD based RSM models was considered as accurate and reliable for predicting the production of phytase by *Bacillus* sp. RS1.

3.3.5. Purification of phytase from *Bacillus* sp. RS1

The extracellular phytase from *Bacillus* sp. RS1 was purified to apparent homogeneity from cell-free supernatant using ethanol precipitation, ion exchange, and gel filtration chromatography. The enzyme was purified to 20.84 fold with specific activity of 72.97 and 31% recovery (Table 3.10). SDS-PAGE analysis of active fraction at each step of purification revealed the presence of one prominent band of molecular weight approximately of 40 kDa at gel filtration step (Fig. 3.8).

Table 3.10: Purification of phytase from *Bacillus* sp. RS1

Purification step	Total activity (U*)	Total protein (mg)	Specific activity U mg^{-1} protein	Purification fold	Yield (%)
Crude	87.50	25.00	3.50	1.00	100
Ethanol precipitation	56.50	8.00	7.00	2.00	65
Biogel P-100	33.00	1.04	31.50	9.00	38
DEAE-Sephacel	27.00	0.37	72.97	20.84	31

*1U = 1µmole of Pi released min^{-1}

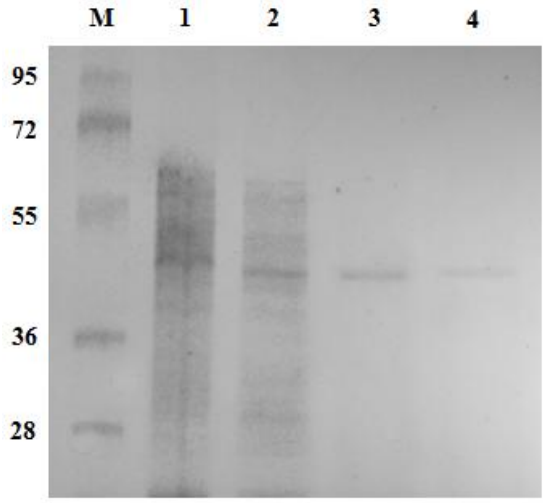


Fig. 3.8: Purification of phytase from *Bacillus* sp. RS1. SDS-PAGE analysis of Crude extract (Lane 1), ethanol precipitation fraction (Lane 2), Biogel P100 eluate (Lane 3), DEAE-Sephacel eluate (Lane 4). Lane M- Protein molecular weight marker.

3.3.6. Characterization of the phytase

The optimum temperature of *Bacillus* sp. RS1 phytase was determined by measuring enzymatic activity at the temperature range 4 to 90°C. The enzyme had optimum activity at 40°C and a decline in activity was noted on incubating the reaction above and below the optimum temperature (Fig. 3.9a). Notably, the enzyme exhibited markedly high activity at low temperature with retention of 65 and 43 % of the optimum activity at reaction temperature 25 and 10°C, respectively. The pH profile for phytase activity was evaluated at pH 2.5 to 9.5. The enzyme showed activity in the pH range 3.5 to 9.5 with pH optimum at 6.5 (Fig. 3.9b). To determine the thermal stability, the enzyme was pre-incubated for 30 min at the temperatures, 20, 30, 40, 50, 60, 70, 80, 90°C. Phytase from *Bacillus* sp. RS1 was relatively more thermostable in presence of $CaCl_2$ and thus maintained almost 100 % activity on pre-incubation of the enzyme at the temperature range 20-50°C and retained about 63 % of activity at 70°C, followed by a sharp decline in thermostability. However in absence of $CaCl_2$, a drastic reduction in activity was noted at pre-incubation temperature above 40°C (Fig. 3.9c).

The phytase activity increased in presence of Ca^{2+} and Mg^{2+} ions, while other metal ions, viz, Zn^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+}, and EDTA had inhibitory effect (Table 3.11). The enzyme showed 100% activity with PA and no considerable activity was detected with other phosphorylated substrates like ATP, ADP, dSPP, pNPP, G6P and F6P (Table 3.11). The

enzyme retained 54 and 35% activity in presence of 30 Units of trypsin and pepsin for 60 min, respectively.

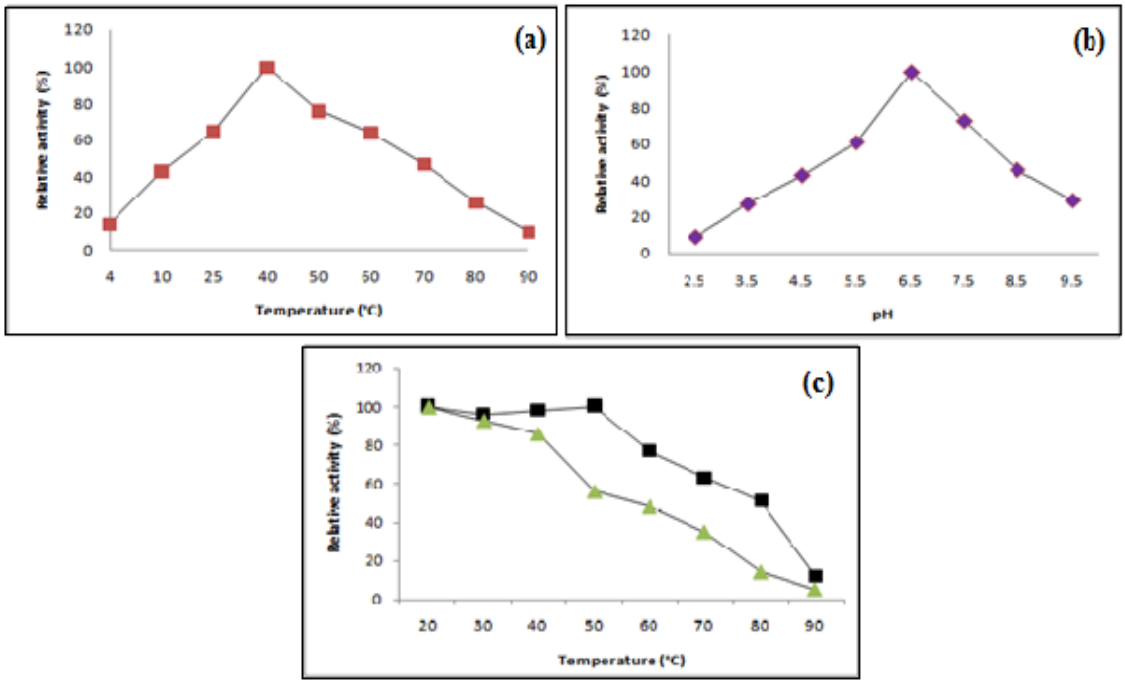


Fig. 3.9: Effect of pH and temperature on phytase purified from *Bacillus* sp. RS1. The 100 % relative activity was 72.97 Umg^{-1} (a) Effect of temperature on phytase activity; (b) Effect of pH on phytase activity. (c) Effect of temperature on stability of phytase in presence and absence of $CaCl_2$. The effect of pH was determined in 100 mM glycine-HCl (pH 2.5 & 3.5), 100 mM sodium acetate (pH 4.5 & pH 5.5), 100 mM Tris-HCl (pH 6.5- 9.5) buffer at 37°C; Purified enzyme preparation was incubated at indicated temperature for 30 min and enzyme activity was determined under standard conditions as described in Materials and method section.

Table 3.11: Properties of phytase from *Bacillus* sp. RS1

Properties	**Results**
pH optimum	6.5
Temperature optimum (°C)	40
*Substrate specificity (Sodium phytate)	100
$^{\$}$Thermal stability (%)	100
Specific activity (U/mg, 37°C)	72.97
Activity in presence of trypsin (1 h)	54 %
Activity in presence of pepsin (1 h)	35 %
Activity in presence of 2mM metal ions (%) Ca^{2+} Mg^{2+}	 117 132

***Activity in presence of ATP, ADP, pNPP, dSPP, G6P, F6P and also in presence of Mn^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}, EDTA was negligible.**

 # The enzyme (50 U) was pre-incubated with pepsin or trypsin for 30 min followed by determination of phytase activity.

$^{\$}$Activity after pre-incubation of enzyme at 50 °C for 30min.

3.3.7. Cloning of the phytase gene and homology analysis

The partial phytase gene from *Bacillus* sp. RS1 was amplified by PCR using primers designed from the conserved region of phytase sequences from other *Bacillus* species available in the database. The PCR product (816 bp) was cloned in pGEM-T Easy vector (Promega) and sequenced. The nucleotide sequence was then translated into protein sequence (Fig. 3.10) by ExPASy Translate (http://expasy.org/tools/dna.html) before comparing with other phytases protein sequences available in NCBI (Fig. 3.11) (Gasteiger *et al.* 2003). The BLAST search using both the nucleotide and deduced amino acid sequence identified the protein as 3-phytase with low sequence homology with phytases of other *Bacillus* sp. In BLAST search, the nucleotide sequence of putative phytase from *Bacillus* sp. RS1 showed 70 % homology with *B. subtilis* alkaline phytase gene. Amino acid sequence of RS1 phytase showed 36 %, 34 % and 32 % homology with 3-phytase of *B. subtilis, B. atrophaeus* and *B. amyloliquefaciens*, respectively, indicating its novelty. Alignment of *Bacillus* sp. RS1 phytase and other related phytases by MultAlin program (Corpet 1988) indicated the presence of two conserved motifs, namely, "D-A-[A/T/E]-D-D-P-A-[I/L/V]-W" and "N-N-[V/I]-D-[I/L/V]-R-[Y/D/Q]", which are reported to be conserved in 66 different β-propelar phytase (BPPhy) sequences (Huang *et al.* 2009).

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                816 nucleotides, 272 amino acids
   1 GGCGTTACATCACTG TCATTCTTCTTCGAT GATGCGGCGGATGAT CCGGCGATCTGGGTT CATGAAAAACATCCG
   1  G  V  T  S  L   S  F  F  F  D  D  A  A  D  D   P  A  I  W  V   H  E  K  H  P
  76 GCGAAAAAATCAATC GTTGATTATGATAAA AAAGAAGTTCGCGCG CGCCGCGTTCTGTTC TCAACAGAAAATTCA
  26  A  K  K  S  I   V  D  Y  D  K   K  E  V  R  A   R  R  V  L  F   S  T  E  N  S
 151 TTCATCCTGATGTCA CTGTGGCAGGCGCAG GTTAATAATGTTGAT GTTCGCTATTCAATC GAACTGAATGGCAAA
  51  F  I  L  M  S   L  W  Q  A  Q   V  N  N  V  D   V  R  Y  S  I   E  L  N  G  K
 226 AAAGTTTGTATGCCG CATCCGCAGCCGGTT CGCCGCTCAAAAAAT ACAATCGAAGTTTAT GCGATCGATTCACGC
  76  K  V  C  M  P   H  P  Q  P  V   R  R  S  K  N   T  I  E  V  Y   A  I  D  S  R
 301 GGCAAACGCAAAATC GAAAAACAGCAGTTC CATACAGATCCGAAA CATCCGATCTGTCTG CCGCCGATCTTCCTG
 101  G  K  R  K  I   E  K  Q  Q  F   H  T  D  P  K   H  P  I  C  L   P  P  I  F  L
 376 CGCTTCATGGATTCA GCGTGTATCACAGCG CGCAAACAGGAACAT TTCACACATGAATGG AAACAGGCGAATAAA
 126  R  F  M  D  S   A  C  I  T  A   R  K  Q  E  H   F  T  H  E  W   K  Q  A  N  K
 451 GGCAATCTGTCATCA CTGAAATTCCTGTAT CCGCGCTTCTGGCAT ATCCTGGGCAAAGGC TATGTTCAGCAGACA
 151  G  N  L  S  S   L  K  F  L  Y   P  R  F  W  H   I  L  G  K  G   Y  V  Q  Q  T
 526 GGCAAACGCTGTGTT AATTTCTTCGCGATC CTGCGCCCGAAAGAA AATCTGGTTGCGGAT GATGAATATGGCGCG
 176  G  K  R  C  V   N  F  F  A  I   L  R  P  K  E   N  L  V  A  D   D  E  Y  G  A
 601 ACATATACACAGCAG CGCAAAATGCGCCCG TCAGGCAATCTGATG ATGTCACCGGCGGAA GATCAGCGCGGCCGC
 201  T  Y  T  Q  Q   R  K  M  R  P   S  G  N  L  M   M  S  P  A  E   D  Q  R  G  R
 676 CTGCTGGCGGCGGAT CGCCGCTCAGTTGAT TCAAAAGGCGAAGTT CCGACAATCTATAAT CGCATCGCGCAGAAT
 226  L  L  A  A  D   R  R  S  V  D   S  K  G  E  V   P  T  I  Y  N   R  I  A  Q  N
 751 CCGCCGGGCTATGAT ACATGTAAATATCGC ATCTGTCATGGCTAT CGCACAGCGTGGGAA AAATCA
 251  P  P  G  Y  D   T  C  K  Y  R   I  C  H  G  Y   R  T  A  W  E   K  S
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Fig. 3.10: Partial nucleotide (816 bases) sequence and deduced amino acid sequence (272 amino acids) of the putative phytase gene from *Bacillus* sp. RS1; conserved sequences are boxed.

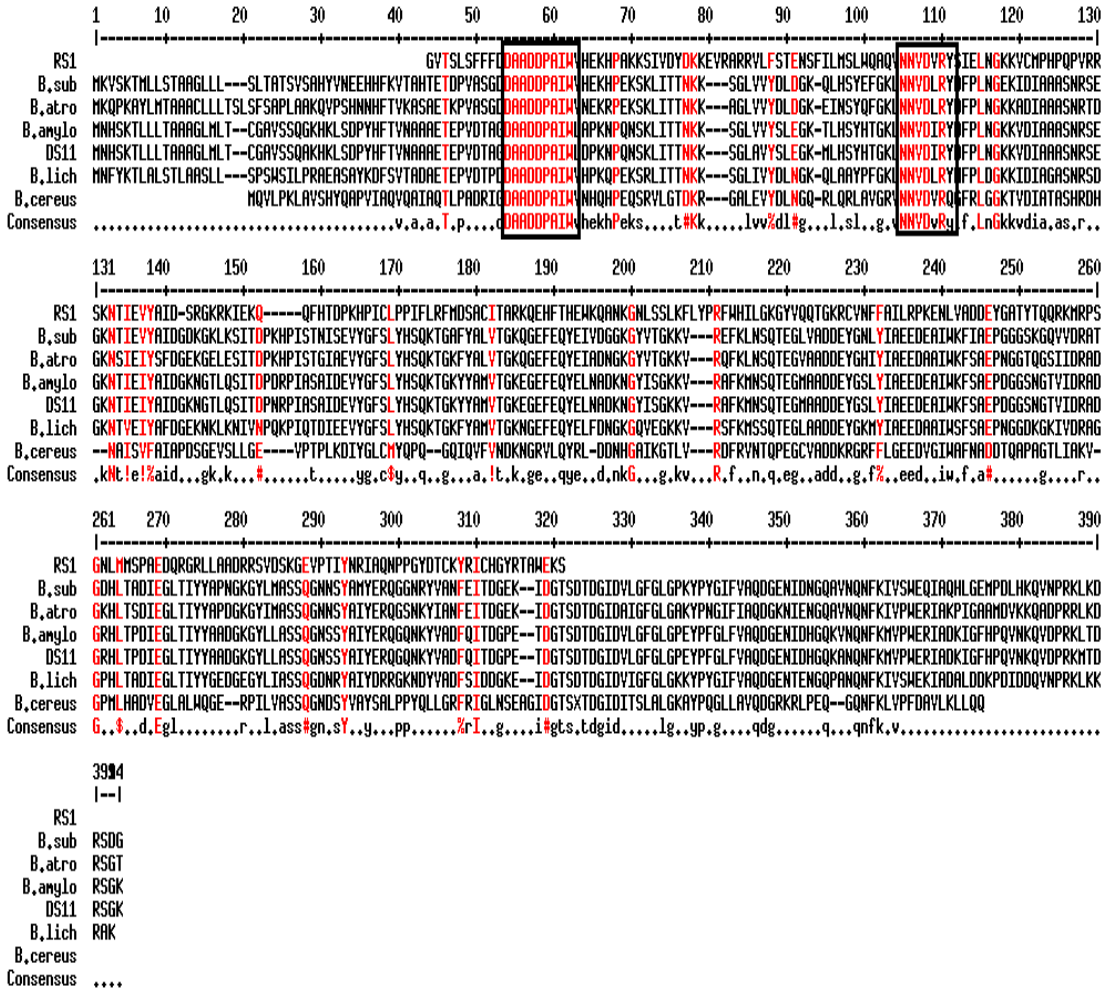

Fig. 3.11: Multiple alignment of homologs of the *Bacillus* sp. RS1 phytase. Conserved active site motifs are boxed. The source and GenBank Accession Nos. of proteins are: *Bacillus subtilis*, WP_043857689; *Bacillus amyloliquefaciens*, AAL25193; *Bacillus licheniformis*, AAU22048; *Bacillus* sp. DS11, AAC38573; *Bacillus cereus*, AHM26864; *Bacillus atrophaeus*, WP_010788969.

Furthermore, the absence of the conserved motifs, RHGXRXP and HD rules it out as a member of HAPhy family. The results thus revealed novelty of the identified phytase. A phylogenetic tree was constructed based on the alignment using the neighbour joining method. The topology of the phylogram also suggests that the phytase from *Bacillus* sp. RS1 is different from other known phytases (Figure 3.12).

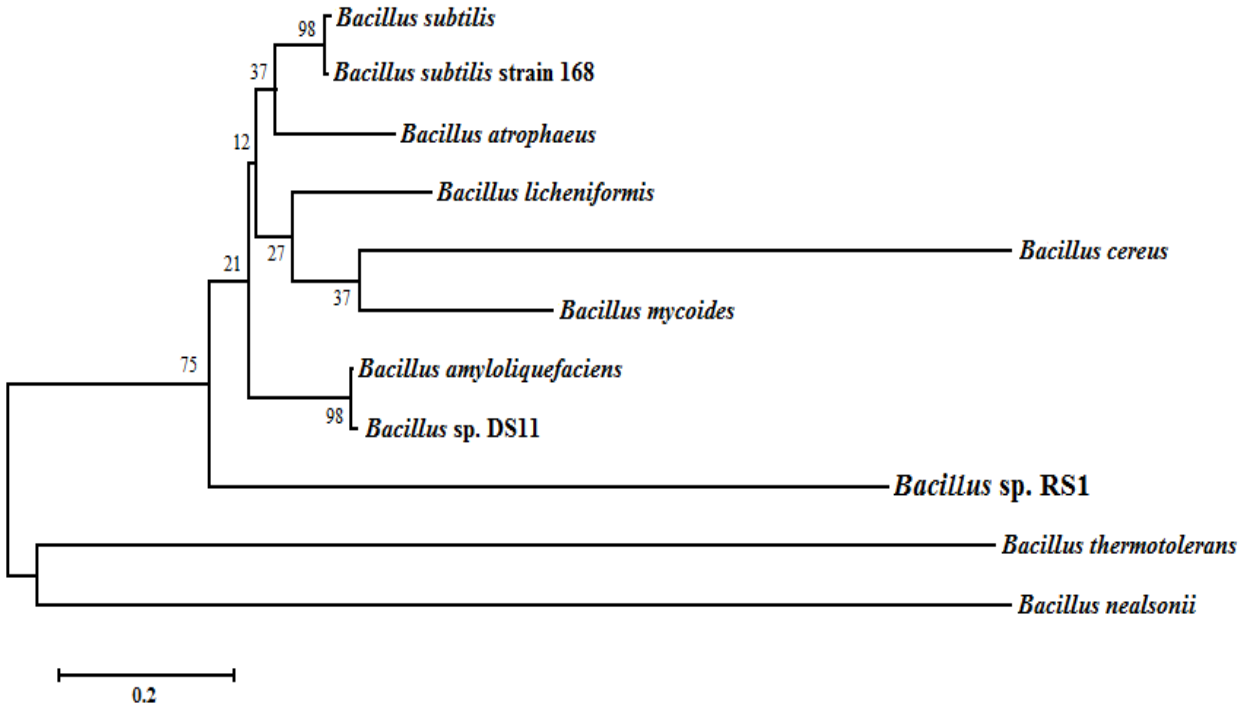

Fig 3.12: Phylogenetic tree of Phy_B with other related phytase protein sequences constructed by Neighbor-Joining method. The numbers at branching points refer to bootstrap values, based on 1000 replicates. The bar represents 2 substitutions per 10 amino acids. The source and GenBank Accession Nos. of sequences are: *Bacillus subtilis* 3-phytase, WP_043857689; *Bacillus amyloliquefaciens* phytase, AAL25193; *Bacillus licheniformis* phytase, AAU22048; *Bacillus subtilis* subsp. *subtilis* str. 168 phytase, CAB13871; *Bacillus* sp. DS11 phytase, AAC38573; *Bacillus cereus* phytase, AHM26864; *Bacillus atrophaeus* phytase Phy, WP_010788969; *Bacillus thermotolerans* phytase, KKB36193; *Bacillus nealsonii* phytase, ADX99261; *Bacillus mycoides* 3-phytase, EEM02603.

3.3.8. Effect of *Bacillus* sp. RS1 inoculum on growth of Chickpea plants

For monitoring the plant growth promoting ability of *Bacillus* sp. RS1, the bacterial inoculum was applied to chickpea seeds before germination and to the 10 days old seedlings and grown for further 10 days. The 20 days old seedlings were analyzed for various growth parameters. The treated plants exhibited higher shoot length, root length, fresh weight, and dry weight in comparison to control plants. Average shoot and root lengths of treated plants were 72 and 38 % greater than that of the control plants. Similarly, the dry weight of total biomass of bacterial inoculum treated chick pea seedlings was about two fold greater than that of control plants (Table 3.12, Fig 3.13). The chick pea seedlings were analyzed for phosphate content. Plants treated with *Bacillus* sp. RS1 had 23 % higher phosphate content as compared to that of the control plants. There was no significant change in pH of soil samples collected from different seedlings.

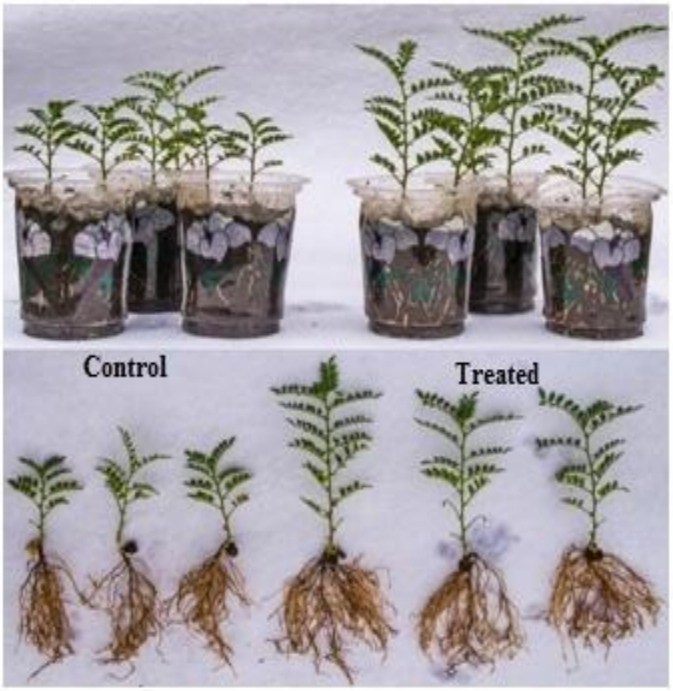

Fig 3.13: Effect of *Bacillus* sp. RS1 in growth of Chick pea plant

Table 3.12: Effect of *Bacillus* sp.RS1 in growth and P content of Chickpea plant

Parameters	Control	Bacteria treated sample
Plant fresh weight (g)	0.426± 0.013	1.935±0.029
Plant dry weight (g)	0.186±0.012	0.378±0.006
Root length (cm)	7.50±0.05	10.42±0.092
Shoot length (cm)	7.17±0.028	12.33±0.066
P content (mg/g)	132.0±2.12	163.0±4.01

3.3.9. Plant growth promotion by application of purified enzyme

Supplementation of the purified phytase-enzyme to the germination medium containing phytate stimulated the seedling growth. Results in Fig. 3.13 revealed that seedlings growing in presence of phytase (Fig. 3.14 a and b) were more than twice in size in comparison to that growing in absence of phytase (Fig. 3.14 c and d).

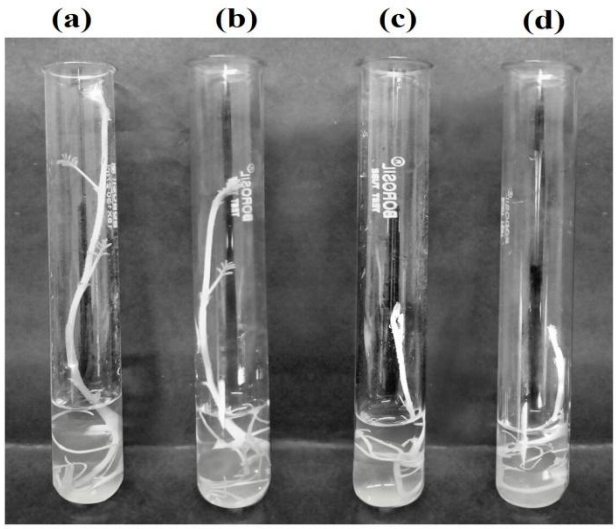

Fig. 3.14: Germination of Chickpea seedlings in presence (a,b) and absence of purified phytase (c,d)

3.3.10. Other plant growth promoting capabilities

The strain showed positive result for IAA production indicated by observed pink colour in the culture flask. The isolate released greater quantities of IAA in the presence of a physiological precursor, tryptophan in culture medium. The isolate was also found positive for HCN, siderophore and ammonia production.

3.3. DISCUSSION

Phytase producing bacteria were screened and isolated from rhizospheric soil. Eight gram positive, phytase producing bacteria obtained in initial screening were further screened for phytase production by SmF and an isolate RS1 with extracellular phytase activity of 4 U ml^{-1} was selected for further studies**.** The bacterium was identified as *Bacillus* based on morphological, biochemical and 16S rDNA sequence analysis and hence named as *Bacillus* sp. RS1. The 16S rDNA sequence of the bacterium showed close homology with *B. subtilis* and *B. megaterium*, however, substantial morphological and biochemical differences with both the organisms were also noted. The bacterium produced maximum extracellular phytase after 24 h of incubation, and thereafter enzyme production suffered sharp decline. As observed, *B. laevolacticus* produced maximum phytase after 24 h of incubation (Gulati *et al.* 2007).

The large-scale commercial applications of microbial enzymes require a cost effective production process, which in turn demands proper optimization of production. Here, optimization of phytase production by OFAT approach showed an influence of nutrient sources and their concentrations on bacterial growth and enzyme production. The maximum phytase production was noted in a medium (pH 6.5) supplemented with 1 % sucrose and 1 % beef extract as carbohydrate and nitrogen sources, respectively, at 37°C and 24 h of fermentation. The optimization of cultivation temperature and pH of phytase production by OFAT approach indicates mesophilic and neutrophilic nature of the bacterium. The optimization of phytase production by OFAT approach enhanced the phytase activity from 4 U ml^{-1} (unoptimized level) to 35 U ml^{-1} (optimized level).

Statistical tools, like Plackett–Burman (PB) design and response surface methodology (RSM) have variously been applied for optimization of phytase production from fungal, yeast and bacterial sources (Bajaj and Wani 2012). In the present study, the cultural condition for phytase production by *Bacillus* sp. RS1 was optimized by two step approach. First, the effect of several bacterial growth parameters on phytase production was studied using the Plackett-Burman design. In the second step, response surface methodology was used to determine the optimum values. In this study, the PB design was used to determine the most important factors influencing phytase production by *Bacillus* sp. RS1. The results of PB design showed that among eleven tested components, incubation time, incubation temperature and medium pH exhibited statistically significant effect on enzyme production. The incubation temperature and medium pH exerted positive effect, while incubation time had negative effect on phytase production by *Bacillus* sp. RS1. There are several reports describing the use of these methods

in medium optimization for production of phytase and other enzymes by microorganisms (Tasharrofi *et al.* 2011). Medium pH constitutes an important variable for fermentative enzyme production (Chadha *et al.* 2004). Besides, the other variables studied in the present investigation have also been analysed by a number of workers (Gulati *et al.* 2007, Bajaj and Wani 2012, Farhat-Khemakhem *et al.* 2012, Saha and Ghosh 2014). Singh and Satyanarayana (2008) examined 11 variables on the basis of PB designs and reported that magnesium sulphate, incubation period, ammonium sulphate and Tween-80 as the major effective variables influencing phytase production by *Sporotrichum thermophile* on cane molasses-based medium. In the second step, phytase production parameters with significant effect i.e. incubation temperature, incubation time and medium pH, were optimized using RSM. This method has been used in similar studies including optimization of culture conditions for phytase production by *Nocardia* sp. MB 36 (Bajaj and Wani 2012) and *B. subtilis* 168 (Farhat-Khemakhem *et al.* 2012). Using statistical experimental design, phytase production by *Bacillus* sp. RS1 was enhanced to 66.1 U ml^{-1} which was in agreement with the predicted response (67.2 U ml^{-1}). The experimental data indicates the adequacy and applicability of the experimental design for medium optimization. The RSM-mediated optimization of three most effective variables resulted in overall enhancement of phytase yield by 16.53-fold from the unoptimized level. Phytase production from *S. thermophile* was improved by 3.73-fold upon optimization by RSM (Singh and Satyanarayana 2008).

The extracellular phytase from *Bacillus* sp. RS1 was purified to near homogeneity by ethanol precipitation, gel filtration and ion exchange chromatography techniques with specific activity and purification fold of 72.97 U mg^{-1} and 20.97, respectively. Phytase has been purified from several *Bacillus* species including, *B. nealsonii* (Yu and Chen 2013), *B. subtilis* C43 (Sreedevi and Reddy 2013), *Bacillus* sp. KHU-10 (Choi *et al.* 2001), *Bacillus subtilis* (Kerovuo *et al.* 1998). Phytase from *Bacillus nealsonii* ZJ0702 was purified to homogeneity with yield of 5.7% and purification fold 44 (Yu and Chen 2013). Choi and coworkers (2001) reported the purification of phytase from *Bacillus* sp. KHU-10 by acetone precipitation, DEAE-Sephacel and phenyl-Sepharose column chromatographies with specific activity 36 U mg^{-1} and purification fold 105. The apparent subunit molecular mass of the purified phytase was approximately 40 kDa as determined by SDS-polyacryamide gel electrophoresis. The obtained molecular weight is closer to those of phytase from *B. amyloliquefaciens* (44 kDa), *B. licheniformis* (47 kDa) and *B. nealsonii* (43 kDa) (Kim *et al.* 1998, Tye *et al.* 2002, Yu and Chen 2013), while phytase from *B. subtilis* (natto) (Shimizu 1992) and *B. subtilis* (Powar and Jagannathan 1982) had a lower molecular weight of 38 kDa and 36 kDa, respectively. Phytase from *Bacillus* sp. RS1 was found to be active in broader pH range of 3.5 to 9.5 with optimum

activity at pH 6.5. As in the present study, phytases from several *Bacillus* species like *B. subtilis, Bacillus* sp KHU-10, *B. licheniformis, B. amyloliquefaciens, B. laevolacticus, B. nealsonii* are more active in the pH range 5.5 to 8.5. (Powar and Jagannathan 1982, Kerovuo *et al.* 1998, Kim *et al.* 1998, Choi *et al.* 2001, Idriss *et al.* 2002, Tye *et al.* 2002, Gulati *et al.* 2007, Tran *et al.* 2011, Yu and Chen 2013). The contrasting feature of the isolated phytase from other *Bacillus* phytases is the retention of substantial activity also in acidic pH indicating its wide range of applications. The optimum temperature of *Bacillus* sp. RS1 phytase in absence of Ca^{2+} was 40°C as reported earlier for phytase from *Bacillus* sp. KHU-10 (Choi *et al.* 2001) and *Bacillus* sp. T4 (Park *et al.* 2012). Phytase from other *Bacillus* species had greater temperature optima of 60°C for *B. subtilis* (natto) (Shimizu 1992), 70°C for *B. laevolacticus* (Gulati *et al.* 2007) and 55°C for *B. nealsonii* (Yu and Chen 2013). However, unlike other *Bacillus* phytases, the RS1 phytase exhibited marked (>40 %) activity at lower temperature of even 10°C. *Bacillus* phytases are quite active at the high temperature of 80-95°C, but have very low activity under cold conditions (Fu *et al.* 2008). Generally low temperature leads to reduction in enzymatic activity, driven by increase in hydration energy of the nonpolar groups. A significant activity of *Bacillus* sp. RS1 phytase at low temperature might be due to its lower inherent hydrophobicity at low temperature. For determining the thermostability property, the activity of RS1 phytase was measured after pre-incubation at various temperatures for 30 min. The enzyme was found to be more thermostable in presence of Ca^{2+} ions. Consequently, on preincubation at 50°C, it showed 100 and 50 % activity in presence and absence of Ca^{2+}, respectively. The results thus indicate the stabilizing effect of Ca^{2+} ions on the enzyme against thermal denaturation. Previous studies have already reported that alkaline phytases are highly specific for the calcium-phytate complex and require Ca^{2+} ion for activity (Zeng *et al.* 2011). As observed in present work, *Bacillus* sp. T4 phytase was found to be stable up to 40°C, but at 50°C it retained 10 and 70 % of the initial activity in absence and presence of Ca^{2+}, respectively (Park *et al.* 2012). Similarly, a new alkaline β-propeller phytase (PhyA115) characterized from the insect symbiotic bacterium, *Janthinobacterium* sp. TN115, was unstable without Ca^{2+}, losing about 90% of the activity after incubation at 55°C for 4 min, however, preincubation of the enzyme at the same temperature for 30 min in presence of Ca^{2+} caused only 30 % loss of activity (Zhang *et al.* 2011). Moreover, Choi *et al.* (2001) reported the requirement of Ca^{2+} ion for both activity and stability of the phytase from *Bacillus* sp. KHU-10. The three-dimensional structure of the *B. amyloliquefaciens* phytase showed that the enzyme possesses six calcium-binding sites, three of which are involved in enzyme activity, and the other three of which are responsible for stabilizing the enzyme (Ha *et al.* 2000).

Most *Bacillus* phytases are reported to have a high specificity for sodium phytate (Kim *et al.* 1998) and so do the *Bacillus* sp. RS1 phytase. Similar to RS1 phytase, the phytases from *B. subtilis* (Powar and Jagannathan 1982), *Bacillus* sp. DS11 (Kim *et al.* 1998), *Bacillus* sp. KHU-10 (Choi *et al.* 2001) and *B. nealsonii* (Yu and Chen 2013) exhibited absolutely strict substrate specificity for the phytate. In contrast, phytases from *B. laevolacticus* (Gulati *et al.* 2007) and *Bacillus* sp. T4 (Park *et al.* 2012) exhibited little activities towards a few phosphorylated substrates other than phytate. Generally, phytases with broad substrate specificity inherently have rather low specific activity for PA, whereas phytases with narrow substrate specificity have high specific activities (Wyss *et al.* 1999). But *Bacillus* phytases do not fit into this classification as they appear to be very specific for PA, but have apparently low specific activity (Kerovuo 2000). The enzymatic activity of phytase from *Bacillus* sp. RS1 was enhanced by Mg^{2+} and Ca^{2+}, however, Zn^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+} and EDTA inhibited the enzyme. Similarly, the phytase activity of *B. subtilis* MJA was inhibited by Cu^{2+} or Fe^{2+} in a dose dependent manner while, Mg^{2+} or Ca^{2+} had stimulatory effect (El-Toukhy *et al.* 2013). Furthermore, the extracellular phytase from *Enterobacter* sp.4 and *B. subtilis* (natto) was greatly inhibited by Zn^{2+}, Ba^{2+}, Cu^{2+} and Al^{3+} (Shimizu 1992, Yoon *et al.* 1996). The inhibitory effect of EDTA on phytase activity indicates absolute requirement of metal ion (calcium) for the activity as reported earlier by Zeng *et al.* (2011).

Attempts were made to clone the phytase encoding gene phy_B from *Bacillus* sp. RS1 by PCR using primers designed from the conserved region of phytases of *Bacillus* species, available in the database. The PCR product (816 bp) was cloned and sequenced. Homology analysis of nucleotide sequence by BLAST tool showed 70 % homology with *B. subtilis* alkaline phytase gene. Futhermore, BLASTP analysis of the translation product of phy_B revealed 36, 34 and 32 homology with 3-phytase of *B. subtilis*, *B. atrophaeus* and *B. amyloliquefaciens*, respectively. Alignment of the phy_B translation product with six known phytase sequence using MULTALIN revealed the presence of two conserved amino acid sequences D-A-[A/T/E]-D-D-P-A-[I/L/V]-W and N-N-[V/I]-D-[I/L/V]-R-[Y/D/Q] which are commonly found in phytases belonging to the family β-propeller phytase sequences (Kumar *et al.* 2014). Further, *Bacillus* sp. RS1 phytase did not show the highly conserved active site motifs RHGXRXP and HD of histidine acid phosphatase and thus ruling out the possibility of it as a member histidine acid phosphatase (HAP) family. Commonly, the *Bacillus* phytases do not share sequence homology with other phytases and do not possess the RHGXRXP active-site motif characteristic of acid phosphomonoesterases and phytases of fungal or plant origin (Idriss *et al.* 2002). The results suggest that not only both phy_B and Phy_B from *Bacillus* sp. RS1 showed lower sequence homology with other *Bacillus* phytase genes and proteins, but

also the enzyme exhibited some novel properties. Hence it can be concluded that the bacterium encoded a novel phytase belonging to the β-propeller phytase family.

Phytase enzyme preparations have a wide range of applications in animal and human nutrition. Phytase produced by microorganisms in the digestive tract can be very efficient in degrading phytate as demonstrated by the almost complete availability of vegetable P to ruminants (Rodehutscord 2001). However, the microbial ecosystem in monogastric animals is mainly located in the large intestines and it can be assumed that most of the phosphate released from phytate is not absorbed, but excreted out after release by microorganisms. The released phosphate contributes to P pollution in the area of intensive livestock production. Although some cereals such as rye, triticale, wheat, and barley are rich in intrinsic phytase, the use of plant phytase in animal feed is limited, because its content is highly variable even within one feedstuff (Greiner and Konietzny 2006). Moreover, the bioefficacy of cereal phytases was only 40 % compared to microbial phytases and pelleting of feed at temperatures higher than 70°C results in partial inactivation (Pointillart 1988, Cao *et al.* 2007). Phytase application can reduce P excretion by up to 50% that would contribute significantly toward environmental protection. Phytase can also be implicated in plant growth and development by improving the P nutrition by 'mobilization' of P fixed as insoluble polyphosphate and/or phytate, which accounts for 20-50 % of total soil organic P (Wang *et al.* 2013). Although plants have developed numerous mechanisms to increase the availability of soil P, utilization of phytate P from the soil is very limited due to their lack of extracellular phytase activity (Idriss et al. 2002). Hence, the microbial phytase produced by PGPR can support plant growth under P limiting condition in the soil (Podile and Kishor 2006).

The extracellular phytase produced by *Bacillus* sp. RS1 possessed properties such as substrate specificity, activity in wide range of pH, optimum activity in physiological temperature and thermal stability in presence of Ca^{2+} ion. These unique properties make this phytase suitable for feed industry. The enzyme showed significantly high activity at lower temperature and thus can be used as liquid enzyme formulations postpelleting on the cooled feedstuff pellets. *Bacillus* sp. RS1 was also found to produce IAA, ammonia, HCN and siderophore, in addition to low temperature active extracellular phytase and thus could have potential applications in agriculture, especially for promoting the growth and productivity of plants requiring 20-30°C environmental temperature. Being gram positive in nature, the bacterium offers a biological solution to the formulation problem due to their ability to form heat and desiccation-resistant spores that can be formulated into stable products (Idriss *et al.* 2002). In the present study application of *Bacillus* sp. RS1 as seed/soil inoculant improved the germination and growth of chick pea seedlings with significant increase in root and shoot

length as well as total plant biomass, in absence of added inorganic phosphate in soil. The results thus indicates that *Bacillus* sp. RS1 have potential to exploit native organic P to benefit plant nutrition.

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