Summary and Conclusion:

Phosphorus is predominately stored in mature seeds of cereals and legumes as organic-mineral complex known as phytate (Kumar *et al.* 2010). Phytate-P is poorly available to monogastric animals, though the primary constituents of diets for poultry, fishes and swine are plant-based ingredients which come primarily from the seeds (Lei *et al.* 2003). Phytates are also strongly complexed in soil, representing an important class of organic phosphorous poorly available to plants (Wang *et al.* 2013).

Phytase catalyses the hydrolysis of PA to inorganic monophosphate and lower myo-inositol phosphate, and in some cases to free myo-inositol and thus its supplementation to the plant based animal feed can make phytate P available to the animals (Tan *et al.* 2014). Similarly, unlocking the phytate-P in soil using microbial phytase can provide an eco-friendly solution in agriculture increasing the crop yield.

Currently, the large-scale, commercial production of phytase is mainly based on fungal phytase from *Aspergillus* species, which is suitable for animals with gut pH in acidic range. Although, inclusion of fungal phytase in diets for poultry and swine has resulted in considerable improvement in P retention, it is not much suitable for animals with neutral gut. Efficacy of phytase supplementation is dependent on several factors such as microbial source, catalytic efficiency, temperature and pH optima and other kinetic parameters, specificity to substrate, thermal tolerance during processing of feed and resistance to proteolytic break down by digestive proteinases encountered in the digestive tract. Hence, there is a constant search for a better commercial phytase with activity in acidic to neutral range. Bacterial phytases are an alternative to the fungal enzymes because of their higher specific activity, thermal stability, higher substrate specificity, greater resistance to proteolysis and better catalytic efficiency.

Present study describes the isolation, purification, characterization and gene cloning of bacterial phytases from *Shigella* sp. CD2 and *Bacillus* sp. RS1. Initially, environmental samples were screened for phytase producing bacteria and two different isolates were selected for further studies based on highest phytate clearance zone and phytase activity. Biochemical properties and phylogenetic analysis of 16S rRNA gene sequence identified the isolates as *Shigella* sp. CD2 and *Bacillus* sp. RS1.

The cell bound phytase from *Shigella* sp. CD2 was purified with specific activity and purification fold of 780 U mg^{-1} and 133, respectively. The enzyme was active in the pH range 3.5 to 7.5 and in the temperature range 20 to 80°C with pH and temperature optima of 5.5 and 60°C, respectively. It was highly specific to phytate and retained activity on treatment with proteolytic enzymes. The phytase encoding gene appAs was cloned and sequenced. The gene and encoded protein $AppA_S$ showed highest homology with the $AppA_S$ phytase of *E.coli* and *C.braakii*. The $AppA_S$ amino acid sequence contained the conserved active site motifs, RHGXRXP and HDTN, and five conserved cystein residues, placing it in histidine acid phosphatase (HAP) family of phytase. Moreover, $AppA_S$ contained putative signal peptide of 22 amino acids indicating its periplasmic localization, and three potential sites of N-glycosylation. The calculated molecular mass of the protein with and without the signal sequence were about 47 and 45 kDa, respectively. The $appA_S$ ORF without the signal sequence was PCR amplified, cloned in pPIC9 vector for overexpression in *P.pastoris* GS115. The recombinant phytase, $rAppA_P$ was expressed as extracellular protein with maximum activity level of 62 U ml^{-1} with specific activity of 477 U mg^{-1} protein at 60 h of methanol induction. The protein was glycosylated with molecular mass of 59 and 65 kDa and deglycosylation by Endo H glycosidase resulted in reduction in molecular mass of the enzyme to about 45 kDa. Moreover, $appA_S$ was expressed in *E.coli* to produce $rAppA_E$ in order to compare the biochemical characteristics of the enzyme with that of $rAppA_P$. For this $appA_S$ ORF without the signal sequence was cloned in pET-20b(+) under the control of T7 promoter and recombinant plasmid was transformed into *E.coli* BL21(DE3) to produce $rAppA_E$. The $rAppA_E$ was overexpressed as intracellular protein with maximum activity of 176 U ml^{-1} and specific activity 568 U mg^{-1} protein at 24 h of IPTG induction. The recombinant glycosylated $rAppA_P$ and nonglycosylated $rAppA_E$ were purified with specific activity of 967 and 2982 U mg^{-1}protein, respectively. Thus, $rAppA_P$ represented 50% of the total secreted protein of recombinant *P.pastoris* GS115, whereas $rAppA_E$ was 19 % of the total intracellular protein of recombinant E.coli BL21(DE3). The biochemical properties of the purified phytases were compared. Both the enzymes had more than 50 % activity in the pH range 3.5 to 6.5 with pH optima at 5.5. Both $rAppA_E$ and $rAppA_p$ had temperature optima of 60°C. Compared to $rAppA_p$, $rAppA_E$ had 11 and 18 % greater relative activity at 37 and 50°C, respectively, whereas at higher incubation temperature $rAppA_p$ was more active than $rAppA_E$. Although, the two enzymes didn't differ in their thermostability in the temperature range 10 to 50°C, $rAppA_p$ was more thermotolerant at higher temperature. Both the $rAppA_E$ and $rAppA_P$ were highly specific to sodium phytate as substrate and activity with either of phosphorylated substrates, such as ATP, ADP, pNPP, dSPP, G6P or F6P was negligible. Among the divalent cations, Ca^{2+}, Mg^{2+}, and Mn^{2+} had a stimulatory effect on phytase

activity with 100-130 % relative activity detected in presence of these ions. On the other hand, Cu^{2+}, Fe^{2+}, Zn^{2+} or EDTA showed inhibitory effect toward the recombinant phytases. The $rAppA_E$ and $rAppA_P$ retained 70 and 65 % activity in presence of trypsin, and 55 and 50% of activity in presence of pepsin, respectively, after 1 h of pre-incubation, indicating greater resistance to trypsin.

In the next part of the study, an extracellular, neutral, phytase was isolated from *Bacillus* sp. RS1 and the enzyme was purified and characterized. The native purified enzyme has specific activity and purification fold of 73 U mg^{-1} and 21, respectively, with molecular mass of about 40 kDa. Although the purified phytase shared some enzymatic properties in common with phytases reported from other *Bacillus* species, some differences were also observed. Like other phytases from *Bacillus* species, the RS1 phytase also exhibited optimum activity under neutral pH and strict substrate specificity for the phytate complex. The enzyme showed about 40 % activity in pH range 5.5 to 8.5 with a pH optimum at 6.5 and in the temperature range 10 to 70°C with optimum at 40°C. Unlike other *Bacillus* phytase, the enzyme isolated in present study uniquely retained 65 and 43 % of optimum activity at reaction temperatures 25 and 10°C, respectively and retained more than 30 % of optimum activity at pH 3.5. The putative phytase gene (phy_B) was PCR amplified from *Bacillus* sp. RS1 genome using primers designed from conserved region of other *Bacillus* phytase and then sequenced. The DNA sequence 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*. Alignment of the translation product Phy_B 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], commonly found in phytases belonging to the family β-propeller phytase (BPP) (Kumar et al. 2014). The results suggest that though Phy_B from *Bacillus* sp. RS1 showed lower sequence homology with other *Bacillus* phytases, it encoded a novel phytase belonging to BPP family.

The cost effective production of enzymes requires optimization of fermentation conditions. The optimization of variables for enzyme production is generally carried out using one factor at a time (OFAT) approach, but it does not consider interaction among variables (Gupta et al., 2012). The optimal design of the cultural medium for enzyme production by statistical approaches such as, response surface methodology (RSM) is an alternative strategy that offers minimum number of experiments for a large number of process variables and modelling of interaction among them. Recently, RSM has been utilized successfully to

improve product yield and to reduce development time and cost of biotechnological processes. 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). Using statistical experimental design, phytase production was finally improved to 66 U ml^{-1}, which is in agreement with the predicted response (67.2 U ml^{-1}). The experimental data thus supported the adequacy and applicability of experimental design methods for medium optimization.

Apart from extracellular production of phytase, *Bacillus* sp. RS1 exhibited several PGPR attributes, such as the production of indole acetic acid, siderophore, HCN and ammonia. Hence, the effect of the bacterial inoculants and purified enzyme on germination and growth of chick pea seedling was evaluated. Application of *Bacillus* sp. RS1 as seed/soil inoculant improved the germination and growth of chickpea seedlings with significant increase in root and shoot length as well as total plant biomass, in absence of added inorganic phosphate in soil. In an another experiment chickpea seeds were germinated in low phosphate media containing the purified phytase. The presence of phytase in the medium promoted the germination of chickpea seedling. The results thus indicated that *Bacillus* sp. RS1 has the potential to exploit native organic P to benefit plant nutrition.

In conclusion, phytase producing bacteria, *Shigella* sp. CD2 and *Bacillus* sp. RS1 isolated from environmental samples. The phytase produced by *Shigella* sp. CD2 was cell bound and acidic, whereas *Bacillus* sp. RS1 produced an extracellular and neutral enzyme. The purified phytase from *Shigella* sp. CD2 had several advantageous biochemical properties such as, optimum activity in acidic pH, higher specific activity, protease resistance and substrate specificity. To our knowledge, this is the first report on phytase isolated from *Shigella*. Phytase encoding gene $AppA_S$ was cloned and expressed in *P.pastoris* and *E.coli* to produce $rAppA_E$ and $rAppA_P$, respectively. The purified recombinant enzymes almost retained the biochemical properties of the native phytase, except for thermal stability. The $rAppA_P$ was more thermostable at higher temperature than $rAppA_E$ and native enzyme. Phytase AppA from *Shigella* sp. CD2 displayed 40-70 % activity in the pH range 3.5 to 6.5, which can facilitate phytate degradation in salivary gland (pH 5.0 – 7.0), stomach (fed state pH 6.5, reducing to 3.5 - 4.5 upon stimulation of acid secretion) and upper part of duodenum (pH 4.0 - 6.0). Hence, the enzyme can be used as feed additive for improving the utilization of phytate P by monogastric animals like, swine, poultry and farm animals. However, economical production of $rAPPA_P$ requires improving its expression by optimization of bioprocess and scaling up when the cells are grown in a fermenter. The purified phytase from *Bacillus* sp. RS1 was low-temperature active and had optimal activity at neutral to alkaline

pH, protease resistance and substrate specificity. However, it didn't show thermostability at higher temperature. The gene phy_B encoding phytase (Phy_B) was cloned and sequenced. Although Phy_B had lower homology with other *Bacillus* phytase, it showed the presence of two conserved amino acid sequence motifs of β-propeller phytase indicating novelty. Optimization of phytase production using PB and RSM approaches enhanced the phytase production by 16.53 folds to 66.1 U ml^{-1}. The low temperature active phytase from *Bacillus* sp RS1was effective in improving germination and growth of chickpea seedlings and Pi absorption from soil. Moreover, the potential applications of the enzyme with a relatively high activity at low temperatures, especially at 20-30°C, could be extended to aquaculture and food processing.