ABSTRACT

Phytic acid (myo-inositol 1,2,3,4,5,6-hexakis phosphate, IP6) and its cation salt, phytate are the storage form of phosphorous (P) in cereals and legumes. Phytates are hydrolysed by enzyme phytase to release inorganic phosphate (Pi) for utilization by animals and plants. Phytate phosphorus is poorly available to monogastric animals like poultry, fishes and swine, due to the lack or low activity of phytase in their gastrointestinal tract. Phytic acid is also present in soil as complexes, representing an important class of organic P, which is unavailable for plant nutrition. Hence, improved P nutrition is achievable by hydrolysis of phytate using supplemental phytase in both the feed and the field. Although the commercial production of phytase has focused on the fungus *Aspergillus*, studies have suggested bacterial phytases are more promising as feed supplement. Present study describes the isolation, purification, characterization and gene cloning of bacterial phytases from *Shigella* sp. CD2 and *Bacillus* sp. RS1. Further, the appAs gene encoding $AppA_S$ phytase from *Shigella* sp. CD2 was expressed in *Pichia pastoris*. The characteristic properties of the enzyme were compared with that expressed in *E. coli* strain BL21 (DE3).

In preliminary experiment environmental samples were screened for phytase-producing bacteria and two bacterial strains isolated from rhizosphere, namely, *Shigella* sp. CD2 and *Bacillus* sp. RS1 were selected for further studies, based on highest phytate clearance zone and phytase activity. The cell bound, acidic phytase from *Shigella* sp. CD2 was purified by ammonium sulphate precipitation, gel filtration and ion exchange chromatography. The purified enzyme had several advantageous biochemical properties such as, high specific activity (760 U mg^{-1}), acidic pH optimum (5.5), thermostability, protease resistance and substrate specificity. To our knowledge, this is the first report on phytase from *Shigella*. Phytase encoding gene, $appA_S$ was isolated from *Shigella* sp. CD2, which appeared as a member of histidine acid phosphatase (HAP) family. The $appA_S$ was expressed in *E. coli* and *Pichia pastoris* to produce recombinant phytases, $rAppA_E$ and $rAppA_P$, respectively. The $rAppA_P$ was expressed as extracellular, glycosylated protein with maximum activity of 62 U ml^{-1} (specific activity of 477 U mg^{-1}) at 60 h of methanol induction, whereas $rAppA_E$ was expressed as nonglycosylated, intracellular protein with maximum activity of 176 U ml^{-1} (specific activity 568 U mg^{-1}) at 24 h of IPTG induction. The glycosylated $rAppA_P$ had molecular mass of 59 and 65 kDa and deglycosylation of the enzyme by Endo H glycosidase reduced the molecular mass to about 45 kDa, similar to that of $rAppA_E$. The recombinant

phytase $rAppA_P$ and $rAppA_E$ were purified with specific activity of 967 and 2982 U mg^{-1}, respectively. The purified recombinant enzymes retained the biochemical properties of the native phytase, except for thermal stability. The $rAppA_P$ was more thermostable than $rAppA_E$ at higher temperatures.

The extracellular phytase from *Bacillus* sp. RS1 was purified by solvent precipitation and chromatographic techniques, as a 40 kDa protein with specific activity and purification fold of 73 U mg^{-1} and 21, respectively. The optimum pH and temperature of the enzyme were 6.5 and 40°C, respectively. The enzyme exhibited strict substrate specificity for phytate. Unlike other *Bacillus* phytases, the purified phytase from *Bacillus* sp. RS1 was found to be active at low temperature and uniquely retained 65 and 43 % of the optimum activity at reaction temperatures 25 and 10°C, respectively. Optimization of phytase production by one factor at a time (OFAT) and response surface methodology (RSM) approaches enhanced the phytase production. The enzyme production (66 U ml^{-1}) was closer to the model diagnosed value of 67 U ml^{-1} and was about 16.53 fold higher than that in unoptimized basal medium. The partial gene (phy_B) encoding phytase was cloned and sequenced. The translation product Phy_B revealed 36, 34 and 32 % homology with 3-phytase of *B. subtilis*, *B. atrophaeus* and *B. amyloliquefaciens*, respectively. Alignment of the translation product Phy_B with known *Bacillus* phytase sequences 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] that are commonly found in phytases belonging to the beta propeller phytase (BPP) family. The results thus indicate novelty of the phytase isolated from *Bacillus* sp. RS1.

As *Shigella* sp. CD2 phytase showed optimal activity in the acidic pH range (3.5-6.5), the enzyme expressed extracellularly in *P.pastoris* ($rAPPA_P$) can be considered as a good candidate as feed additive for improving the utilization of phytate phosphorus by monogastric 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. On the other hand, application of *Bacillus* sp. RS1 as seed/soil inoculant improved the germination and growth of chickpea seedlings and the purified enzyme was also found effective in improving germination and growth of chickpea seedlings. Moreover, the potential applications of the enzyme which had a relatively high activity at low temperatures, especially at 20-30°C, could be extended to aquaculture and food processing.