CHAPTER 2

Isolation, Characterization, Gene Cloning and Expression of phytase from *Shigella* sp. CD2

2.1. INTRODUCTION

Phytic acid (myo-inositol 1,2,3,4,5,6-hexakis phosphate, IP6) and its mixed cation salt, phytate are a group of organic phosphorous (P) compounds found widely in nature. In terrestrial ecosystem, they are mainly synthesized by plants. Phytate accumulates in large amount during ripening of cereal and legume seeds accounting for 60-80 % of the total P and thus can serve as an important source of P nutrition (Ariza *et al.* 2013). Although phytates are very important in many physiological functions, it is considered solely as antinutrients because of the binding with starch and protein and their strong chelating ability with divalent minerals, such as Ca^{2+}, Mg^{2+}, Zn^{2+}, and Fe^{2+}. The formation of insoluble mineral-phytate complexes at physiological pH values is considered to be the major reason for poor mineral bioavailability, because these complexes are hardly absorbed in the gastrointestinal tract (Elhadi *et al*. 2011, Tai *et al*. 2013). They are also strongly complexed in soil, representing an important class of organic P, which is poorly available to plants (Wang *et al.* 2013).

Phytic acid is hydrolysed by a special group of enzymes called phytases (myo-inositol hexakisphosphate hydrolases) that can convert it to less phosphorylated myo-inositol derivatives and inorganic phosphate (Pi) (Olazaran *et al.* 2010). The enzyme has already been reported from a good array of microorganisms, plants and some animal tissues. Phytases from these sources exhibit variations in structure and catalytic mechanism and consequently, have been categorized into cystein phytases (CPs), histidine acid phosphatases (HAPs), β-propeller phytases (BPPs) and purple acid phosphatases (PAPs) (Maldonado *et al.* 2014). Moreover, the ExPASy enzyme database (http://www.expasy.ch/enzyme/) classifies phytases into three different groups: 3-phytase (alternative name, 1-phytase; EC 3.1.3.8), 4-phytase (alternative name, 6-phytase; EC 3.1.3.26), and 5-phytase (EC 3.1.3.72) based on the position of

specificity of the initial hydrolysis of phytate (Vats and Banerjee 2004, Olazaran *et al.* 2010). To date, phytases have been reported from many bacteria such as *Escherichia coli* (Griener *et al.* 1993, Yao *et al.* 2013, Tai *et al.* 2013), *Bacillus* sp. (Olazaran *et al.* 2010), *Klebsiella* sp. (Sajidan *et al.* 2004), *Yersinia intermedia* (Huang *et al.* 2006a), *Erwinia carotovora* (Huang *et al.* 2009), *Dickeya paradisiaca* (Gu *et al.* 2009); most of them belong to the family enterobacteriaceae.

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. However, the gastrointestinal microbial flora 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 after release by microorganisms (Haefner *et al.* 2005). Due to unavailability of phytate P for plant and animal nutrition, it is a common practice to add inorganic P as plant fertilizer and as an animal feed supplement. However, the inefficient utilization of phytate consequently contributes to P pollution problems in the areas of intensive crop and livestock production. The released PA and phytate contribute to eutrophication and algal bloom (Jorquera *et al.* 2008). Thus, from both environmental and economic point of view, release of phytate P for animal and plant nutrition is essential and thus attracting significant research as well as industrial interest.

Addition of phytases to the feeds of simple-stomach animals like pigs and poultry makes them able to digest and extract P nutrient from plant feed, thereby rendering the traditional supplementary phosphate feeding unnecessary. Similarly, phytase producing microorganisms can be utilized for increasing the availability of soil P to plants for their growth and development. Consequently, the reduced release of phytate leads to minimization of environmental phosphate load.

The commercialization of phytases requires not only a practical use and delivery system of the enzyme but also the capability to produce the enzyme economically. Till date, large-scale production of cheap phytases mainly for animal feed has been solved through fermentation of genetically modified microorganisms (Heafner *et al.* 2005, Tran *et al.* 2010). Phytases belonging to class histidine acid phosphatase have been used successfully as a feed additive. The commercial production of phytase is currently focused mainly on the fungal histidine acid phytase from *Aspergillus* species (Cao *et al.* 2007). However, fungal phytases have lower specific activity and substrate specificity, and reduced resistance to proteolysis. Consequently, a wave of research on bacterial phytases has led to the development of a new

generation of phytases, which in many aspects are superior and more promising to the first generation of fungal phytases as a feed additive (Lei *et al.* 2013). Bacterial phytases are reported to be thermostable, substrate specific, resistant to proteases and have better catalytic efficiency (Olazaran *et al.* 2010). The substrate specificity property of the enzyme is highly desirable to prevent hydrolysis of other phosphate compounds so that they remain available for animal uptake.

A number of bacterial phytases have been isolated and corresponding genes have been cloned and several expression systems have been examined in the hopes of producing phytases best suited for animal feed industry (Kerovuo and Tynkkynen 2000, Huang *et al.* 2006, Yao *et al.* 2013). There is enormous importance in finding economically competitive expression system for low-cost production of phytases through recombinant techniques. Among the prokaryotic expression systems *E. coli* has been used as homologous as well as heterologous expression system for effective production of industrially relevant recombinant enzymes, including phytases. Enzyme production cost can be reduced if the enzyme can be secreted by the host. *E.coli* BL21, a popular expression host, can produce and secrete bioactive heterologoous proteins from both prokaryotic and eukaryotic sources. Alternatively, the methylotrophic yeast *Pichia pastoris* has been successfully used as a host for heterologous gene expression, producing high levels of recombinant proteins, including phytases (Maifiah *et al.* 2011). The use of *Pichia* as expression system has several advantages. It can grow in simple defined media, reach a very high cell density, and accumulate extremely high concentrations of intra- or extracellular protein under the control of the *AOX1* promoter (Daly and Hearn 2005). A coding sequence cloned under the control of the *AOX1* promoter is highly expressed when methanol is used as the sole carbon source and is repressed by most other carbon sources (Daly and Hearn 2005). In addition, *P. pastoris*, as a eukaryotic expression system, can carry out protein processing, folding, and posttranslational modifications (Xiong *et al.* 2006).

Present study describes purification and characterization of phytase from newly isolated bacterial strain *Shigella* sp. CD2. The putative phytase gene was isolated and sequenced and expressed in *P. pastoris.* The characteristic properties of *P. pastoris* expressed enzyme were compared with that expressed in *E.coli*.

2.2. MATERIALS AND METHODS

2.2.1. Strains, Vectors and Chemicals

Phytic acid-dodecasodium salt and CM-cellulose were purchased from Sigma, USA. Biogel P-2 and P-100 were from BioRad, USA. Ni-Sepharose Fast Flow column was from GE Healthcare, UK. Molecular biology kits were purchased from Promega, USA; QIAGEN, Germany and Invitrogen, USA. The bacterial strain used in this study *Shigella* sp.CD2 (Accession no. FR 745402) was isolated from wheat rhizospheric soil. The pGEM-T vector system and PCR reagents were purchased from Promega, USA. Restriction enzymes and T4 DNA ligase were from New England Biolabs (Beverly, MA). *Escherichia coli* BL21(DE3) and pET-20b(+) vector (Novagen, Madison, WI) were used for bacterial expression. MagicMedia™ *E.coli* Expression Medium was purchased from Invitrogen, San Diego, CA. The expression medium has two components, (a) Ready to use Medium and (b) IPTG solution. For expression in eukaryotic system, *P. pastoris* G115(*his4*) and pPIC9 expression vector were purchased from Invitrogen, San Diego, CA. Plasmid pPIC9 contains the promoter and terminator of the *P. pastoris AOX1* gene, the α-mating factor prepro-secretion signal from *S. cerevisiae* and the *HIS4* auxotrophic selection marker for transforming *P. pastoris* GS115. Regeneration dextrose base (RDB), buffered glycerol-complex (BMGY), and buffered methanol-complex (BMMY) media were prepared according to the manual of the *Pichia* Expression kit (Invitrogen, San Diego, CA). All other biochemicals and microbiological media were from Sigma-Aldrich, USA; E. Merck, Germany and HiMedia Laboratory, India.

2.2.2. Sample collection

For screening and selection of phytase-producing bacteria, a wide range of environmental samples, such as rhizospheric soil, dung, rotten wood log, waste water etc, were collected. The samples were collected in sterile containers and stored under refrigerated condition until use.

2.2.3. Isolation and screening of phytase producing bacteria

All the samples were serially diluted (10^{-1} to $10^{-7)}$ followed by spread-plating of each dilution on nutrient agar plates. The plates were incubated at 37°C overnight. The representative individual colonies were selected based on their morphology. After obtaining pure cultures, all the isolated strains were qualitatively screened for phytase production. For

this, bacterial strains were inoculated onto 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. The individual colonies were picked, resuspended in sterile LB broth and grown at 37°C overnight to obtain pure cultures. Equal volumes of each grown culture and glycerol (80 %) were mixed and stored at -20°C, until further use. The cultures from the glycerol stocks were propagated for two generations before any experiment could be performed.

All phytase producing bacterial strains were quantitatively screened for phytase production in the phytase production medium [PPM, containing glucose, 0.1 %; sodium phytate, 1 %; calcium chloride, 0.01 %; ammonium sulfate, 0.1 %; potassium chloride, 0.07 %; magnesium sulfate, 0.01 %; trace element solution 0.1 ml, D-mannose 0.1 %, pH 5.0] at 37°C under shake flask culture. Two millilitre culture was withdrawn at 24, 48, 72, 96 and 120 h of incubation period and analyzed for intracellular, extracellular and periplasmic phytase activity. The bacterial culture was centrifuged at 8000 rpm for 10 min at 4°C; supernatant and cell pellet fractions were used as source of extracellular and intracellular phytase, respectively. For intracellular activity, bacterial pellet was resuspended in 100 mM sodium-acetate (pH 5.5) and Tris-HCl (pH 7.5) buffer followed by sonication. The sonicated extract was centrifuged at 10,000 rpm for 10 min and phytase activity was determined in the supernatant. The periplasmic activity was quantified following the method described by Tran *et al.* (2010). The cell pellet was treated with the ice-cold extraction reagent containing 500 mM sucrose, 1 mM EDTA, 100 mM Tris-HCl (pH 8.0) and 100 μg ml^{-1} lysozyme followed by repeated centrifugation to remove the sphaeroplast. Finally, a phytase-producing strain CD2 was chosen for further studies based on highest qualitative and quantitative production of phytase.

2.2.4. Identification of the isolate CD2

2.2.4.1. Morphological and biochemical characterization

Morphological and biochemical characteristics of the bacterial 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).

2.2.4.2. Phylogenetic analysis of isolate CD2 based on 16S rDNA sequence

2.2.4.2.1. Isolation of genomic DNA

Genomic DNA was isolated from logarithmic phase culture by Marmur's method (Yates *et al.* 1987). The bacterial culture grown in LB broth [tryptone, 1 %; yeast extract, 0.5 %; NaCl, 1%; pH 7.5] overnight at 37°C was harvested by centrifugation at 8,000 rpm for 10 min at 4°C and cell pellet was washed with 0.1M EDTA: 0.15M NaCl solution (1:1) followed by centrifugation at 10,000 rpm for 5 min. The bacterilal pellet was suspended in 2-3 ml of 0.1 M EDTA: 0.15 M NaCl solution and was stored at -20°C for at least 4 h. Then cells were thawed at 55°C till dissolution and 67 µg ml^{-1} solution of lysozyme was added and incubated at 37°C for 30 min. To the lysate SDS was added and incubated at 55°C for 15 min, which was followed by treatment with Proteinase-K (4 µg ml^{-1}) at 55°C for 30 min. The DNA preparation was then sequentially extracted with equal volume of Tris-saturated phenol (pH 8), 1:1 mixture of Tris saturated-phenol: chloroform and chloroform. DNA was precipitated from the aqueous phase by adding two volume ethanol followed by centrifugation at 10,000 rpm for 10 min at 4°C. The DNA pellet was washed with 75 % chilled ethanol, air dried and dissolved in TE buffer [10 mM Tris and 1 mM EDTA (pH 8.0)].

2.2.4.2.2. Agarose gel electrophoresis of DNA

1 % agarose in 1X Tris-acetate EDTA (TAE) buffer [1 litre of 50X solution contains 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA] was melted and then cooled to 50-60°C. It was then supplemented with 5µg ml^{-1} ethidium bromide (EtBr). The melted agarose was then poured in a casting tray fitted with a teflon comb forming wells. DNA sample was mixed with DNA loading dye (1X) and loaded onto the wells. Electrophoresis was performed in a horizontal electrophoresis tank using 1X TAE buffer. DNA band was visualized on a UV-transilluminator.

2.2.4.2.3. Preparation of competent *E.coli* JM109

The *E.coli* strain JM109 (Promega) was made competent by chemical method using $CaCl_2$. The bacterial culture was grown in LB medium at 37°C under shaking. The overnight grown culture was inoculated in fresh LB medium and grown till optical density of 0.4 to 0.5. The bacterial cells were pelleted by centrifugation at 4000 rpm for 10 min followed by washing with 100 mM $CaCl_2$. The cells were resuspended in the same solution and used for transformation.

2.2.4.2.4. PCR amplification and cloning 16S rDNA

16S rDNA was amplified by PCR using universal primer 27F (5'-AGAGTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') designed from conserved regions of the gene and genomic DNA as template. The reaction mixture in a final volume of 25µl contained: 10X reaction buffer, 1.5 µl; 50 mM $MgCl_2$, 1 µl; 10 mM dNTP mix, 2µl; and 7 picomole forward and reverse primers, 1µl each; template, 100 ng; Taq polymerase, 2.5 Units. PCR was performed for 30 cycles with initial denaturation at 94°C for 5min followed by denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 1 min for every individual cycle and then a final extension at 72°C for 7 min. The PCR product was then separated on 1% agarose TAE gel. The PCR amplicon was cloned into pGEM-T Easy vector (Promega, USA) (Fig.2.1) following manufacturer's instruction. For cloning, the PCR product was extracted from the agarose gel by using Gel Extraction Kit (QIAGEN, Germany) by manufacturer's protocol.

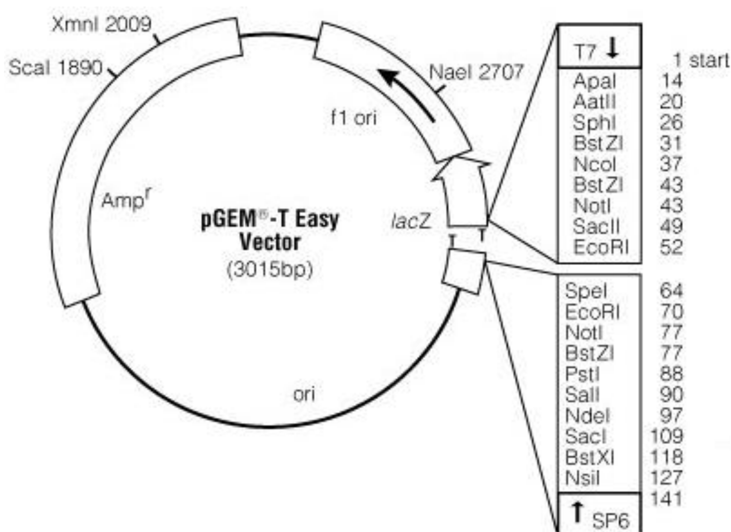

Fig.2.1: The map of pGEM-T Easy vector DNA: a double-stranded closed circular plasmid of 3015 base pairs; Amp^R, ampicillin resistance gene; lacZ, enzyme beta-galactosidase gene; MCS (multiple cloning site) with a series of unique restriction sites; Ori, *E. coli* origin of replication and f1origin of replication.

The ligation reaction mixture in a total volume of 10 µl contained: 2X ligation buffer, 5µl; pGEM-T Easy vector, 50 ng (1µl); PCR product, 2 µl; T4 DNA ligase (3 Unit μl^{-1}), 1 µl; and 1 µl H_2O. The reaction mixture incubated overnight at 4°C was transformed into competent *E. coli* JM109 cells by heat shock at 42°C for 90 sec and immediately incubated

on ice for 10 min. The transformed cells were revived in SOC medium [2 % tryptone, 0.5 % yeast extract, 0.05 % NaCl in distilled water, in which 1ml of 1 M filter-sterilized $MgCl_2$ and $MgSO_4$ solutions, and 1 ml of 2 M filter-sterilized glucose solution added prior to use] for 1 h at 37°C with shaking at 150 rpm. The transformants were then selected on ampicillin (50 µg ml^{-1}) agar plate supplemented with 20 % IPTG and 2 % (w/v) X-gal by incubating overnight at 37°C.

2.2.4.2.5. Isolation of plasmid from transformed *E.coli* JM109 and restriction digestion

Plasmid was isolated from the recombinant white colonies by alkaline lysis method (Birnboim and Dolly 1979). Bacteria containing the desired plasmid were harvested from overnight grown culture in nutrient broth (NB) by centrifugation at 8,000 rpm for 5 min at 4°C. Bacterial pellet was suspended in ice-cold Alkaline lysis solution-I [25 mM Tris-HCl (pH 8.0), 50 mM glucose and 10 mM EDTA] by vigorous shaking. In the next step, the bacterial suspension was treated with freshly prepared Alkaline lysis solution-II [10 % sodium dodecyl sulfate (SDS) and 10 N NaOH]. After mixing the content by inverting the tube several times, the lysate was stored on ice for 5 min. Then ice-cold Alkaline lysis solution-III [5M potassium acetate (pH 5.2)] was added followed by storage on ice for 5 min. The cell lyasate was centrifuged at 10,000 rpm for 10 min at 4°C. The clear supernatant was transferred to a fresh tube and extracted sequentially with an equal volume of 1:1 phenol:chloroform and with chloroform. Plasmid DNA was finally precipitated from the aqueous phase by addition double volumes of absolute alcohol followed by centrifugation at 10,000 rpm for 10 min at 4°C. The precipitated plasmid was washed with 75 % ethanol, air dried and suspended in TE buffer. The recombinant plasmid was analyzed for the presence of DNA insert by restriction digestion with *Eco*RI. The digestion mixture of a final volume of 50 µl contained: 10X buffer, 5 µl; *Eco*RI, 2 Units (1 µl); plasmid, 5 µl and 39 µl double distilled water. The mixture was incubated for 16 h at 37°C. The digested plasmid was then checked by TAE-agarose gel electrophoresis. The recombinant plasmid was then used for sequencing of the inserts using vector specific T7 and SP6 universal primers.

2.2.4.2.6. Phylogenetic analysis

The phylogenetic relationship of the isolate CD2 was determined by comparing its 16S rDNA sequence 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 analysis was performed 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).

2.2.5. Phytase assay

Phytase activity was determined by the method described by Shimizu (1992) based on the formation of colored complex between inorganic P (Pi) released from phytate and ammonium molybdate. The reaction mixture in a final volume of 2 ml contained 100 mM acetate buffer (pH 5.5), 2 mM sodium phytate, and 100 µl enzyme preparation. The reaction was carried out at 37°C for 30 min followed by termination of reaction by adding 2 ml of 10 % trichloroacetic acid (TCA). The reaction mixture was centrifuged at 6,000 rpm for 10 min. The released Pi was measured in the supernatant by adding 2 ml of Pi-reagent [0.5 % ammonium molybdate, 5N sulphuric acid and 2 % ascorbic acid], incubating the preparation for 20 min at 37 °C and measuring absorbance at 610 nm. The amount of Pi released from phytate was determined by using Pi standard curve (Fig.2.2). One unit (U) of phytase activity represents 1µmol of Pi released min^{-1} under assay conditions.

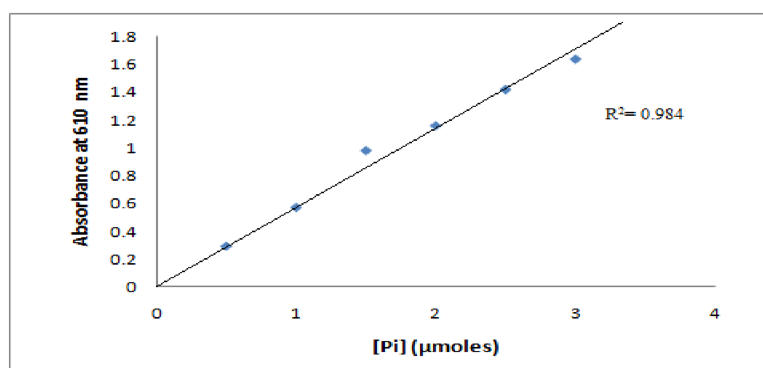

Fig. 2.2: Standard curve for inorganic phosphate (Pi)

2.2.6. Protein estimation

Quantitative estimation of protein was done by the method described by Bradford *et al.* (1976), using BSA as standard (Fig.2.3). To 100 µl of protein sample, 3.0 ml of Bradford reagent [100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 ml 95% ethanol; to the solution 100 ml 85% (w/v) phosphoric acid was added and total volume was made 1 litre with distilled water] was added. The reaction mixture was incubated in room temperature for 10 min followed by determination of absorbance at 595 nm.

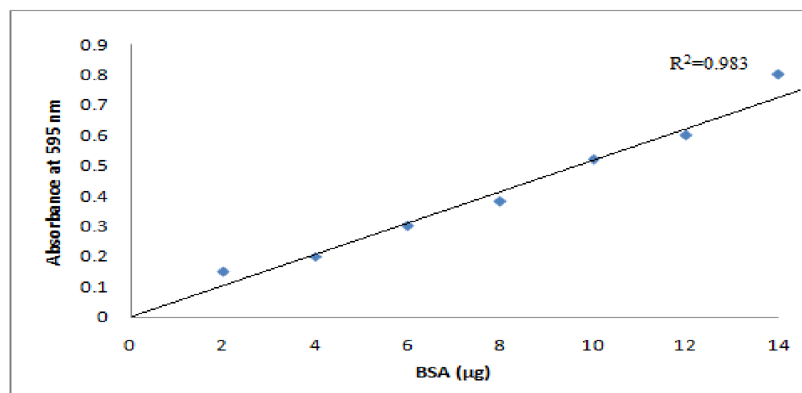

Fig. 2.3: Standard curve for Bovine Serum Albumin (BSA)

2.2.7. Optimization of phytase production

The phytase production by the strain CD2 was optimized with respect to carbon (C) and nitrogen (N) sources, and incubation time. Each parameter optimized was incorporated further in the subsequent experiments. To determine the time course of phytase production, the bacterial strain was grown in PPM (pH 5.0), at 37°C under shaking. Samples were withdrawn at 24 h intervals for a period of 120 h and intracellular phytase activity was monitored. For determination of effect of C source, PPM was supplemented with either of the carbon source (1 % w/v) i.e. glucose, glucose+mannose, mannose, sucrose, lactose, maltose and starch. The culture was grown under shaking at 37°C for 72 h followed by determination of phytase activity. Similarly, to determine the effect of N source, different organic and inorganic N compounds such as ammonium sulphate, ammonium nitrate, sodium nitrate, peptone, tryptone, beef extract, yeast extract, casein were used at 1 % (w/v) each, in the production medium containing glucose as C source. The culture was grown under shaking at 37°C for 72 h followed by determination of phytase activity. Further, effect of different concentration of the selected carbon and nitrogen sources on enzyme production was also investigated.

2.2.8. Phytase purification

Phytase was purified by using ammonium sulphate precipitation, ion-exchange chromatography and gel filtration chromatography.

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

The hydrated and degassed slurry of Biogel P (BioRad, USA) was equilibrated with 100 mM sodium acetate buffer, pH 5.0 then packed into glass column.

2.2.8.2. Preparation of CM-Cellulose

The pre-swollen CM-cellulose slurry (Sigma-Aldrich, USA) was suspended in 0.1M NaOH containing 0.5 M NaCl for 10 min with intermittent stirring. The ion-exchanger was then allowed to settle and excess of solution was decanted. The process was repeated with only 0.5 M NaCl and then with 0.1 M HCl containing 0.5 M NaCl. The slurry was then repeatedly washed with distilled water till the pH became about neutral. Finally, the charged CM-cellulose was suspended overnight in 100 mM sodium acetate buffer, pH 5.0 and then packed into glass column.

2.2.8.3. Purification of phytase

Unless otherwise indicated, all operations during extraction and purification of the enzyme were carried out at 4°C. The bacterial culture was grown in 100 ml PPM for 72 h at 37°C under optimized cultural conditions. Cells were harvested by centrifugation at 8000 rpm for 10 min at 4°C. The cell pellet was initially washed with 50 mM acetate buffer, pH 5.0 (Buffer A) by centrifugation at 8000 rpm for 10 min at 4°C. The cell pellet was suspended in 5 ml of Buffer A, lysed by ultrasonication followed by centrifugation of cell lysate at 10,000 rpm for 20 min and total volume of the supernatant was increased to 50 ml using Buffer A. To the supernatant solid ammonium sulphate was added gradually with continuous stirring to bring the final concentration to 30 % saturation. After 30 min, the precipitated proteins were removed by centrifugation at 10,000 rpm for 20 min. The supernatant was decanted and further additions of solid ammonium sulphate were made to it to achieve 70 % saturation. After allowing to stand for 30 min, it was centrifuged at 10,000 rpm for 20 min. The pellet was dissolved in 2 ml of Buffer A and desalted in a P-2 column (Bio Rad, 2x 10 cm). The desalted enzyme was subjected to cation-exchange chromatography on a CM-Cellulose column (2x 15 cm) pre-equilibrated with Buffer A and eluted with the linear gradient of 0-0.50 M NaCl in the same buffer. The active fractions were pooled, concentrated and subjected to gel filtration chromatography on a P-100 column (Bio Rad, 1x25 cm) equilibrated with Buffer A. Fractions of 1.5 ml each were collected and analysed for protein and phytase activity. The active fractions were pooled and stored at 4°C for further studies.

2.2.8.4. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

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. Resolving gel solution [30 % acrylamide, 4 ml; 1.5 M Tris-HCl (pH 8.8), 2.50 ml; 10 % SDS, 0.10 ml; 10 % APS, 0.10 ml; TEMED 0.004 ml and 3.30 ml water] of 5 cm length

was poured between two glass plates which were clamped together but held apart by plastic spacers and was allowed to set. The stacking gel mixture (0.80 cm) [1M Tris-HCl (pH 6.8), 0.38 ml; 10 % SDS, 0.03 ml; 10 % APS, 0.03 ml; TEMED, 0.003 ml; 2.10 ml water] was poured on the top of resolving gel and a plastic comb was placed on the stacking gel. After polymerization the comb was carefully removed to provide loading wells. Glass plate assembly with the gel was placed in vertical electrophoresis system with running buffer tank containing the running buffer [25 mM Tris-HCl (pH 8.0), 250 mM glycine, 0.10% (w/v) SDS]. The protein sample was mixed with SDS gel loading buffer [50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2 % (w/v) SDS, 0.10 % bromophenol blue, 10% glycerol] and heated in a boiling water bath for 10 min. Protein samples and pre-stained protein molecular weight markers (Fermentas, Vilnius, Lithuania) were loaded in the wells and electric field was applied. When the dye reached at the bottom of the tank, power was turned off. Gel was removed carefully from the glass plates and stained with CBB R-250 reagent [0.1 % Coomassie Brilliant Blue R-250 in 10 % acetic acid and 40 % methanol] for overnight, destained to remove unbound stains and visualized under transilluminator.

2.2.9. Characterization of phytase

The purified enzyme was characterized for molecular weight, pH optima, temperature optima, K_m for phytate, thermostability, cation requirement, substrate specificity and protease resistance.

2.2.9.1. Molecular weight

The purified phytase enzyme was fractionated by using 12 % SDS-PAGE by the method of Laemmli (1970) as mentioned in previous section 2.2.8.4. The protein molecular weight markers used were: 210, 107, 75, 47, 32, 25, 15 kDa.

2.2.9.2. pH optimum, temperature optimum and thermostability

The pH optimum of phytase was determined by measuring the enzyme activity at pH 2.5- 7.5 in the following buffers: 100 mM glycine (pH 2.5 and 3.5), 100 mM sodium acetate (pH 4.5 and 5.5), and 100 mM Tris-HCl (pH 6.5 and 7.5). The normal assay procedure was followed except varying the buffer solution. The optimum temperature for phytase activity was determined at optimum pH and at temperature ranging from 15° to 80°C. The incubation temperature was varied for the standard assay procedure. Thermostability of the enzyme was determined by preincubating the enzyme at 15°-80°C for 30 min followed by measuring activity under standard conditions.

2.2.9.3. Determination of K_m

Phytase enzyme preparation was incubated with various substrate concentrations (0.1−20 mM) in 50 mM sodium acetate buffer (pH 5.5) at 37°C. K_m for phytate was determined by Lineweaver-Burk plot.

2.2.9.4. Effect of metal ions

The effect of metal ions and chemical reagents on phytase was determined by measuring enzymatic activity in presence of 2 mM Ca^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, K^{+}, Na^{+}, SDS, EDTA, β-ME or cystein-HCl. Activity without addition of ions or reagents was used as control. Data is shown as mean of three independent experiments.

2.2.9.5. Substrate specificity

Substrate specificity of the enzyme was determined by replacing PA in the standard assay mixture with an equal concentration (2 mM) of the following phosphorylated compounds: adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), p-nitrophenyl phosphate (pNPP), disodium pyrophosphate (dSPP), glucose 6-phosphate (G6-P) and fructose 6-phosphate (F6-P).

2.2.9.6. Effect of proteases

For determining the susceptibility of the *Shigella* sp. CD2 phytase to digestive proteases, 50 Units of purified enzyme was preincubated with 30 Units pepsin and trypsin at 37°C and phytase activity was monitored 60 min later.

2.2.10. Cloning of the phytase gene and nucleotide sequence analysis

The phytase gene was amplified from genomic DNA by using internal primers, FPS (5’-GCC<u>CATATG</u>AAAGCGATCTTGATCCCATT-3´ with 5’ *Nde*I restriction site) and RPS (5’-AT<u>AAGCTT</u>CAAACTGCACGCCGGTATGCG-3’ with 5’*Hind*III restriction site). PCR amplification was performed in 25µl reaction volume. The reaction mixture contained 5X reaction buffer, 5 µl; 25 mM $MgCl_2$, 2 µl; 10 mM dNTP mix, 1µl; 50 picomole forward and reverse primers, 1µl each; 50 ng genomic DNA construct as template, 2µl; Taq polymerase (5 Units μl^{-1}), 0.25 µl. PCR was performed for 30 cycles with initial denaturation at 94°C for 3 min, followed by a strand denaturation at 94°C for 30 sec, primer annealing at 55°C for 1 min, strand extension at 72°C for 1 min, for every individual cycle and a final extension period at 72°C for 7 min. The PCR product was resolved by 1% agarose TAE gel electrophoresis and then extracted from the gel by using Gel Extraction kit (QIAGEN, Germany) following

manufacturer's instruction. The PCR product was then cloned in pGEM-T Easy vector following manufacturer's instruction as described in section 2.2.4.2. The recombinant plasmid was analyzed for the presence of DNA insert by restriction digestion with *Eco*RI and the cloned PCR product was sequenced using T7 and SP6 universal primers. Homology search in GenBank was done using the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al.* 1990). The amino acid sequence of the cloned gene was deduced and protein sequence was aligned by ClustalW program (http://www.ebi.ac.uk/clustalW) (Thompson *et al.* 1997). The phylogenetic analysis of the protein was performed by neighbour joining method using MEGA 4 (Tamura *et al.* 2007). Bootstrap analysis was used to evaluate the tree topology of the neighbour joining data by performing 500 replicates. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

2.2.11. Expression of phytase AppA$_S$ in *Pichia pastoris* GS115

2.2.11.1. Construction of *P. pastoris* expression plasmid

Mature phytase gene without the signal sequence (appA$_S$) was amplified from genomic DNA by using internal primers, PhyF (5'-ATGAATTCGCTCAGAGTGAGCCGGAG-3´ with 5' *Eco*RI restriction site) and PhyR (5'-GATGCGGCCGCCAAACTGCACGCC GGTATG-3' with 5' *Not*I restriction site) and cloned in pGEM-T Easy as detailed in section 2.2.4.2. The recombinant pGEM-T Easy vector harbouring the phytase gene was named pGEMT-appA$_S$. For expression in *P. pastoris*, the pGEMT-appA$_S$ plasmid was cut with *EcoR*I and *Not*I. The 1.2 kb fragment released from the pGEMT-appA$_S$ plasmid was ligated into *P. pastoris* expression vector pPIC9 (Fig.2.4) digested with the same enzymes. The reaction mix for *Eco*RI/*Not*I digestion reaction of pGEMT-appA$_S$ or pPIC9 in a final volume of 50 µl contained: 10 X Buffer, 5 µl; plasmid, 5 µl; and restriction enzyme 2 Units. The reaction mixture was incubated for 2h at 37°C. The *Eco*RI/*Not*I digested 1.2 kb DNA fragment and pPIC9 vector were purified by phenol-chloroform extraction followed by ethanol precipitation.

Ligation of 1.2 kb DNA fragment into pPIC9 was carried out in a reaction containing 10X T4 DNA ligase buffer, 1 µl; prepared pPIC9 vector, 1µl; prepared 1.2 kb DNA insert, 3 µl; and T4 DNA ligase (4 Units $µl^{-1}$), 1 µl. The ligation reaction was incubated for a period of 16 h at 4°C and then transformed into competent *E. coli* JM109 by heat shock method as described in the section 2.2.4.2. Transformants were selected on LB-agar plate containing ampicillin (50 µg ml^{-1}) and incubated overnight at 37°C. Plasmids was isolated from

ampicillin-resistant colonies by using Miniprep kit (Fermentas) following manufacturer's instruction and checked by TAE-agarose gel electrophoresis. The presence of 1.2 kb insert in the plasmid was ascertained by restriction endonuclease digestion. The recombinant pPIC9 harbouring the phytase gene was named pPIC9-appA$_S$.

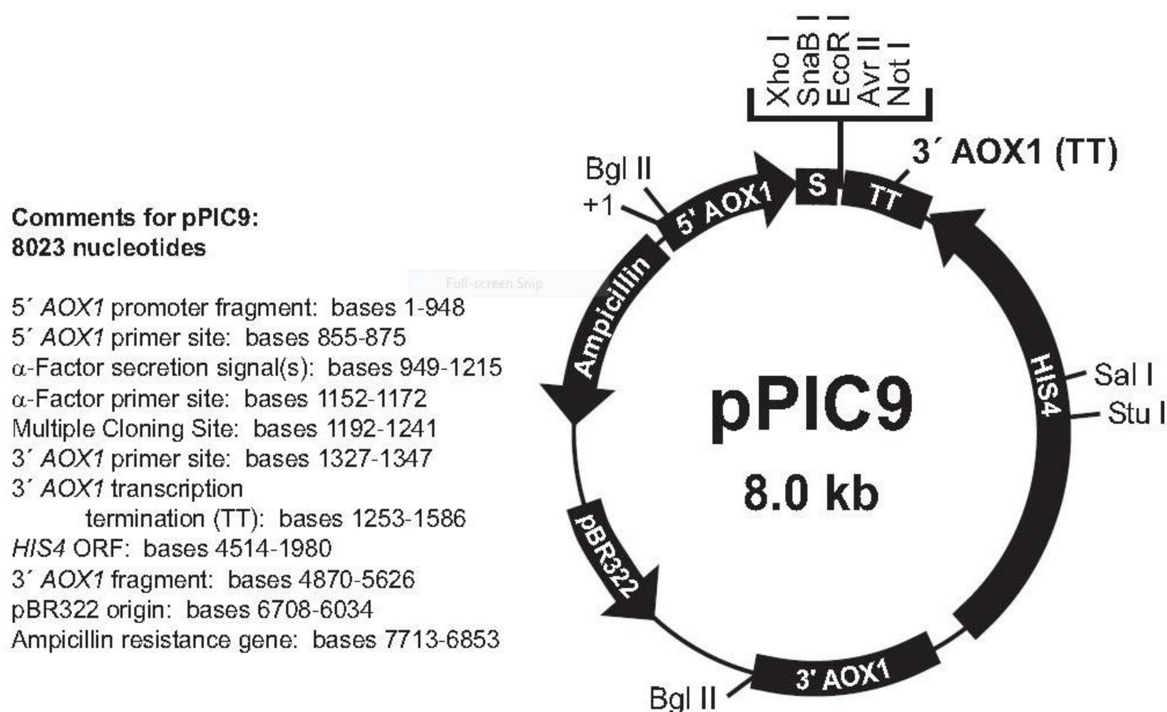

Fig.2.4: The map of pPIC9 plasmid vector for extracellular expression in *Pichia pastoris*. 5'*AOX1*, *AOX1* promoter; 3'*AOX1* TT, *AOX1* transcriptional terminator; 3'*AOX1*, *AOX1* downstream region; AmpR, ampicillin resistance gene; *HIS4*, *P. pastoris* wild-type gene coding for histidinol dehydrogenase; pBR322 origin of replication.

2.2.11.2. Preparation of competent *P. pastoris* GS115 and transformation of pPIC9-appA$_S$

Competent *P. pastoris* GS115 cells were prepared by the Lithium chloride transformation protocol according to the manual of the *Pichia* Expression kit (Invitrogen, San Diego, CA). *P. pastoris* culture was grown in 50 ml of YPD [1 % yeast extract, 2 % peptone and 2 % dextrose] at 30°C with shaking, to an OD_{600} of 0.8 to 1.0 (approximately 10^8 cells ml^{-1}). The cells were harvested and washed with 25 ml of sterile distilled water and centrifuged at 1,500 rpm for 10 min at room temperature. The cell pellet was then suspended in 1 ml of 100 mM LiCl and transferred to a 1.5 ml microcentrifuge tube. The cells were again pelleted down at maximum speed for 15 sec and the LiCl supernatant was removed carefully with a pipette.

The pellet was resuspended in 400 μl of 100 mM LiCl and dispensed into 50 μl aliquots in 1.5 ml microcentrifuge tubes for immediate use.

For transformation, pPIC9-appA$_S$ was linearized with *Bsp*EI and then transformed into competent *P. pastoris* GS115 cells. The competent LiCl-cell solution was centrifuged to remove the LiCl and reagents were added to the cells in the following order: 50 % PEG, 240 μl; 1 M LiCl, 36 μl; 12 mg ml^{-1} single-stranded DNA, 25 μl; plasmid DNA or *Bsp*E1 linearized pPIC9-appA$_S$ (5-10 μg) in 50 μl sterile water. PEG shields the cells from the detrimental effects of the high concentration of LiCl. Each tube was vortexed vigorously until the cell pellet was completely mixed (~1 min) followed by incubation at 30°C for 30 min without shaking. Heat shock was given to the cells in a water bath at 42°C for 20-25 min. The tubes were centrifuged at 6,000 to 8,000 rpm and the transformation solution was removed with a pipette. The pellet was gently resuspended in 1 ml of sterile YPD and incubated at 30°C with shaking. After 1 h, 25-100 μl of the cell suspension was plated on selective media and incubated at 30°C for 2-3 days. Transformants were selected for ability to grow on histidine-deficient medium. The His$^+$ transformants were screened for Mut$^+$ and Mut^S phenotypes. The integration of expression cassette into the genome of *P. pastoris* GS115 was ascertained by PCR using the 5' *AOX1* and 3' *AOX1* primers.

2.2.11.3. Screening of *Pichia* transformants for Mut$^+$ and Mut^S phenotypes

After colonies of His$^+$ GS115 transformants appeared on plates, the His$^+$ colonies were further screened for the Mut$^+$ and Mut^S phenotypes. Because Mut^S transformants do not produce alcohol oxidase (the product of the *AOX1* gene), they cannot efficiently metabolize methanol as a carbon source; therefore, they grow poorly on minimal methanol (MM) medium. This slow growth on methanol was used to distinguish His$^+$ transformants, in which the *AOX1* gene has been disrupted (His$^+$ Mut^S), from His$^+$ transformants with an intact *AOX1* gene (His$^+$ Mut$^+$).

2.2.11.4. Expression of appA$_S$ in *P. pastoris* GS115

The Mut$^+$ and Mut^S *P. pastoris* GS115 cells transformed with pPIC9-appA$_S$ was inoculated into 10 ml of YPD and incubated at 30°C overnight with vigorous shaking. One milliliter of starter culture was transferred to 100 ml of BMGY [1 % yeast extract, 2 % peptone, 100 mM potassium phosphate (pH 6.0), 1.34 % YNB [yeast nitrogen base with ammonium sulfate without amino acids, 4 × 10^{-5} % biotin and 1 % glycerol)] and was grown at 30°C and 300 rpm shaking until cultures reached an OD$_{600}$ of 1. Cells were subsequently harvested by centrifugation at 1500 rpm for 5 min and used to inoculate 100 ml of BMMY [1

% yeast extract, 2 % peptone, 100 mM potassium phosphate (pH 6.0), 1.34 % YNB (yeast nitrogen base with ammonium sulfate without amino acids), 4 × 10^{-5} % biotin and 0.5 % methanol] containing methanol as inducer and the culture was incubated with vigorous shaking at 30°C for 96 h. The induction was maintained by adding 0.5 % (v/v) methanol every 24 h. The culture was analyzed for extracellular and periplasmic phytase activity at 12 h intervals. For isolation of extracellular fraction, the culture was centrifuged at 1500 rpm for 5 min and the cell free medium was concentrated and diafiltered by Vivaspin-20 (30 kDa cutoff) sample concentrator (GE Healthcare, UK). For periplasmic fraction isolation, cell pellet suspended in 100 mM sodium acetate buffer (pH 5.5) containing zymolyase (3 mg ml^{-1}) was incubated at 37°C for 50 min and then centrifuged at 10,000 rpm for 10 min. The supernatant obtained after centrifugation served as periplasmic fraction. Induction of phytase overexpression was determined by 12 % SDS-PAGE. The transformants with pPIC9 plasmid was used as control. Recombinant protein produced by $appA_S$ in *P. pastoris* was named $rAppA_P$.

2.2.12. Expression of phytase $AppA_S$ in *Escherichia coli*

2.2.12.1. Construction of *E. coli* expression plasmid and its transformation into competent *E. coli* BL21(DE3)

For *E.coli* expression, the pGEMT-$appA_S$ plasmid was cut with *EcoR*I and *Not*I. The resulting 1.2 kb DNA fragment was ligated into *Eco*RI and *Not*I sites of pET-20b(+) (Fig.2.5). The ligation reaction mixture in a total volume of 10µl contained: 10X ligation buffer, 1µl; prepared pET-20b (+) vector, 1µl (0.1 µg μl^{-1}); prepared insert, 2 µl; T4 DNA ligase (4 Units μl^{-1}), 1 µl and 4 µl ddH_2O. The reaction mixture was incubated overnight at 4°C and was transformed into competent *E.coli* JM109 cells by heat shock at 42°C for 90 sec, immediately followed by incubation on ice for 10 min. The transformed cells were revived in SOC medium [2 % tryptone, 0.5 % yeast extract, 0.05 % NaCl in distilled water, in which 1ml of 1 M filter-sterilized $MgCl_2$ and $MgSO_4$ solutions, and 1 ml of 2 M filter-sterilized glucose solution added prior to use] for 1 h at 37°C with shaking at 150 rpm. The transformants were selected in LB-agar plates containing ampicillin (50 µg ml^{-1}) as selection agent, by incubating overnight at 37°C. Plasmids was isolated from ampicillin-resistant colonies by using Miniprep kit (Fermentas, Lithuania) following manufacturer's instruction and checked by TAE-agarose gel electrophoresis. The presence of 1.2 kb insert in the plasmid was ascertained by restriction endonuclease digestion. The recombinant pET-20b(+) harbouring the phytase gene was named pET-20b(+)-$appA_S$, which was transformed into competent *E. coli* BL21(DE3) cells following the methods described in the section 2.2.11.1.

Fig. 2.5: The map of pET-20b(+) plasmid vector: The expression vector includes f1 and pBR322 origin of replication; T7 promoter, T7 transcription start, T7 terminator sequences; AmpR, ampicillin resistance gene; Multiple cloning site (MCS); an N-terminal pelB signal sequence for potential periplasmic localization and an optional C-terminal His•Tag sequence.

2.2.12.2. Expression of appA$_S$ in *E. coli* BL21(DE3)

The expression of appA$_S$ in *E. coli* BL21(DE3) was analysed by using MagicMedia^{TM} *E. coli* Expression Medium (Invitrogen, San Diego, CA) following manufacturer's instruction. *E. coli* BL21 (DE3) cells transformed with pET-20b(+)-appA$_S$ was grown overnight in LB medium at 37°C and 200 rpm shaking. The culture at 1 % (v/v) was inoculated into the MagicMedia (19: 1, Ready to use medium: IPTG solution) and grown overnight at 37°C and 300 rpm shaking. The cells were then harvested by centrifugation at 10,000 rpm for 10 min. The cell pellet was suspended in 50 mM acetate buffer (pH 5.5), lysed by sonication and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant, and the pellet dissolved in 50 mM acetate buffer (pH 5.5) served as soluble and pellet fractions, respectively. Induction of appA$_S$ expression in both the fractions was determined by 12 % SDS-PAGE. Proteins of molecular weights 28, 36, 55, 72, 95 kDa, were used as marker. Both the fractions were also checked for phytase activity. *E. coli* BL21 (DE3) transformed with pET-20b(+) vector was used as control. Recombinant protein produced by appA$_S$ in *E.coli* BL21 (DE3) was named rAppA$_E$.

2.2.13. Purification of rAppA$_E$ and rAppA$_P$

For purification of rAppA$_E$, the IPTG induced culture of *E.coli* BL21(DE3) transformed with pET-20b(+)-appA$_S$ was harvested by centrifugation at 10,000 rpm for 10 min. The cell pellet was suspended in 50 mM sodium acetate buffer (pH 5.5) and lysed by sonication. The supernatant obtained after centrifugation of the sonicated cell lysate was loaded onto a Ni-Sepharose Fast Flow column (2 x 5 cm, GE Healthcare, UK) pre-equilibrated with 50 mM sodium acetate (pH 5.5) containing 10 mM imidazole. The column was washed with the same buffer and then bound proteins were eluted with an elution buffer containing 50 mM sodium acetate (pH 5.5) and 100 mM imidazole. Phytase activity was determined in each fraction and active fractions were pooled for subsequent studies. The rAppA$_P$ was purified from the cell free medium of pPIC9-appA$_S$ transformed *P. pastoris* GS115 culture induced with methanol for 48 h. The concentrated and diafiltered cell free medium was loaded on to CM-cellulose column and bound protein was eluted by acetate buffer (50 mM, pH 5.5) with linear gradient of 0-0.5 M NaCl. The active fractions were pooled and used for subsequent analysis.

2.2.14. Characterization of rAppA$_E$ and rAppA$_P$

The pH optima was determined by measuring the enzyme activity at pH 3.5-8.5 in the following buffers (100 mM): glycine-HCl (pH 3.5), sodium acetate (pH 4.5 and 5.5), and Tris-HCl (pH 6.5, 7.5 and 8.5). The optimum temperature for activity was determined at optimum pH and at temperature ranging from 10 to 80°C. Thermostability of the enzyme was determined by preincubating the enzyme at 10 to 80°C for 30 min followed by measuring activity under standard conditions. To study the effect of metal ions and salts (2 mM), phytase activity was monitored in presence of Ca^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+} and EDTA. For determining the susceptibility to digestive protease, the 50 Units of purified rAppA$_E$ or rAppA$_P$ was preincubated with pepsin and trypsin (30 Units, Sigma) at 37°C and phytase activity was monitored 60 min later. Substrate specificity of the enzyme was determined by replacing sodium phytate in the standard reaction mixture with an equal concentration (2 mM) of phosphorylated compounds, such as p-nitrophenyl phosphate (pNPP), ATP, ADP, disodium pyrophosphate (dSPP), D-glucose-6-phosphate (G6P), and D-fructose-6-phosphate (F6P).

2.2.15. Deglycosylation

The deglycosylation of rAppA$_P$ was carried out using Endo H glycosidase (Endo H, New England Biolabs) following manufacturer's instruction. The reaction mixture containing,

50 Units of purified rAppA$_P$, 600 µl of 50 mM Tris buffer (pH 7.0) and 10 Units of Endo H, was incubated at 37°C for 2 h. N-glycosylation was determined by assessing the migration shift of Endo H treated rAppA$_P$ in 12 % SDS-PAGE.

2.2.16. Western blot analysis

For immunoblot analysis, purified rAppA$_E$ and deglycosylated rAppA$_P$ proteins separated by 12 % SDS-PAGE, were transferred to polyvinylidene difluoride (PVDF) membrane by semi-dry method using Electroblotting apparatus (Atto, Japan). A purified rabbit antibody raised against *E.coli* phytase, diluted 1:1000 prior to application, was the primary antibody. The reacted polypeptide was visualised with a secondary antibody, goat anti-rabbit IgG-alkaline phosphatase conjugate using colorimetric based nitroblue tetrazolium chloride and 5-bromo chloro-3-indolylphosphate-p-toluidine (NBT/BCIP) detection kit (Invitrogen, USA). Broad range pre-stained standards were used as markers.

2.3. RESULTS

2.3.1. Isolation and screening of phytase producers

Phytase producing bacteria were isolated from the environmental samples by dilution plate method. Abundant colonies were obtained on nutrient agar plates after incubation, which were selected based on their colony morphology. The screening of microbial phytase producer was based on formation of clear zone on the solidified PSM containing sodium phytate as the selective agent. Out of total 108 isolates, 17 formed prominent clearance zone and thus considered as phytase producers (Table 2.1).

2.3.2. Screening and identification of *Shigella* sp.CD2

All phytase producing bacteria were qualitatively and quantitatively screened for phytase production in PPM broth. The bacterial isolate CD2 was chosen for further studies because it appeared to be the best producer of cell bound phytase, both qualitatively and quantitatively. The bacterial isolate was morphologically characterized as a gram negative motile, rod shaped bacterium. The colonies were white, circular with smooth margin and convex on nutrient agar plate (Fig 2.6). The bacteria was characterized biochemically as negative in citrate utilization, gelatin hydrolysis, diastase and Voges-Proskauer tests, oxidation and fermentation of glucose, and positive in indole, catalase and methyl-red test (Table 2.2). Phylogenetic analysis based on 16S rRNA gene sequence comparisons showed that the isolate CD2 (GenBank accession number FR745402) belong to the branch encompassing members of genus *Shigella* (Fig. 2.7) and was most closely related to *E.coli* and *Shigella dysenteriae* with 100 % 16S rDNA sequence similarity in BLAST search. The strain was further confirmed as *Shigella*, by its growth on McConkey's agar plates with white colonies and inability to produce gas during carbohydrate fermentation and formation of red colonies on XLD agar (Brenner 1984). Hence, the bacterial isolate was tentatively named as *Shigella* sp. CD2.

2.3.3. Production of phytase by *Shigella* sp.CD2

The localization of phytase production by *Shigella* sp. CD2 was determined by monitoring phytase activity in intracellullar and periplasmic fraction and in extracellular medium. A significant phytase activity was observed in the intracellullar and periplasmic fractions but not in extracellular fraction. From the results in Fig 2.8 it is evident that a notable phytase activity was detected from the exponential phase, at 24 h of growth and the

Table 2.1: Colony characteristics of phytase-positive isolates on nutrient agar plates

Environmental Source	Sl. No	Colony Morphology
Waste water	1	Irregular, concentric, raised, umbonate elevation, rough, opaque, colorless colonies
	2	Large, irregular margin, flat and smooth surface, opaque, colorless colonies
	3	Circular, entire edge, convex, smooth, shiny, opaque, yellow pigmented, emulsifiable colonies
	4	Irregular, wavy, raised, rough, opaque, white colonies with dry texture
Dung	5	Larger, round, circular, smooth, convex, opaque, colorless colonies
	6	Circular, entire edge, flat surface, non-mucoid, translucent colonies
	7	Round, circular, raised, pulvinate margin, smooth, opaque colonies
	8	Round, irregular margin, raised, smooth, opaque colonies
	9	Irregular, concentric, raised, smooth, opaque, white colonies
Rhizospheric soil	10	Circular, entire, convex, smooth, opaque, mucoid colonies
	11	Irregular, wrinkled, wavy, raised, opaque, dirty white colonies
	12	Very small colonies, round, entire edge, convex, smooth, opaque, colorless, emulsifiable
	13 **(CD2)**	Small, circular, entire edge, convex, smooth, translucent, colorless colonies
	14	Filamentous margin, flat surface, round to spreading, opaque, greyish colony
	15	Circular, entire edge, weakly umbonate elevation, smooth surface, opaque and red pigmented colonies
Rotten wood log	16	Large, round to spreading, irregular margin, flat surface, rough, opaque, pale colonies
	17	Round to irregular shape, undulate to fimbriate margin, rough surface, attached strongly to agar, opaque, whitish colonies

Table 2.2: Biochemical characteristics of the isolate CD2

Biochemical Tests	Bacteria CD2
Gram Reaction	-
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	-
KCN Test	-
Glucose Fermentation	+a
Mannitol Fermentation	-
Lactose Fermentation	-
Sucrose Fermentation	-
Xylose Fermentation	-

+ = Positive, - = Negative result. +a=Only acid, no gas

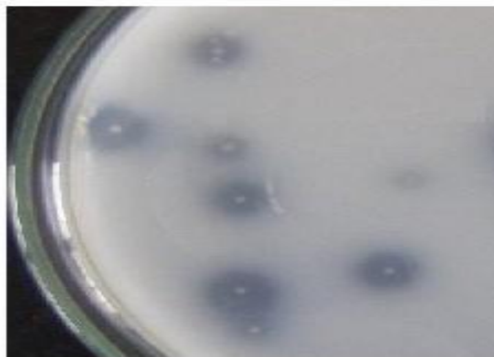

Fig.2.6: Phytase producing colonies of CD2 with a clear halo around them on PSM plates.

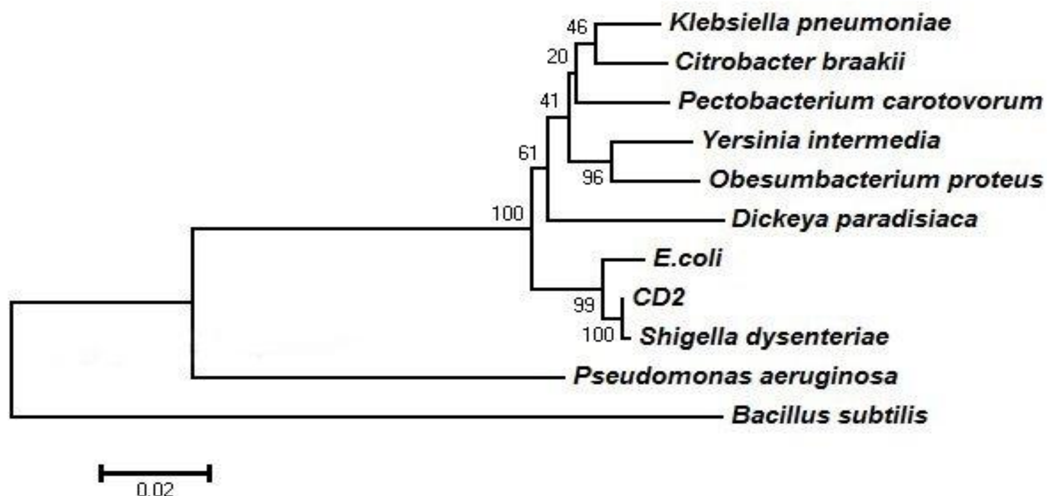

Fig. 2.7: Phylogenetic tree showing the relationships of the isolate CD2 to other closely related bacteria. The numbers at branching points refer to bootstrap values, based on 1000 replicates. The bar represents 2 substitutions per 100 amino acids. The source and GenBank Accession Nos. of sequences are: *Shigella* sp. CD2, FR745402; *Shigella dysenteriae* (ATCC 13313T), X96966; *Escherichia coli* (ATCC 11775T), X80725; *Dickeya paradisiaca* (ATCC 33242T), Z96096; *Klebsiella pneumoniae* (ATCC 13883T), X87276; *Yersinia intermedia* (ATCC 29909T), AF366380; *Citrobacter braakii* (ATCC51113T), AF025368; *Obesumbacterium proteus* (ATCC 12841T), AJ233422; *Pectobacterium carotovorum* (ATCC15713T), AJ233411; *Pseudomonas aeruginosa* (ATCC 10145T), HE978271; *Bacillus subtilis* (ATCC 6051T), AJ276351.

activity increased sharply afterward with maximum during the post-exponential phase at 72 h, followed by decline in activity till 120 h. Further, phytase production was optimized with respect to C and N sources. The maximum production was found, in PPM supplemented with 1 % glucose and 1 % $(NH_4)_2SO_4$ as C and N-source, respectively, at 37°C and at 72 h of growth (Fig.2.9).

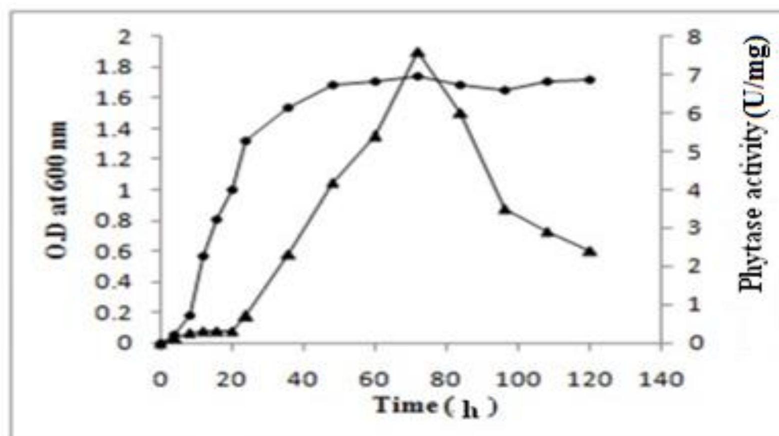

Fig.2.8: Kinetics of phytase production by *Shigella* sp. CD2 in PPM broth at 37°C. Bacterial growth curve (circle) and phytase activity (triangle). Results represent the mean of three independent experiments.

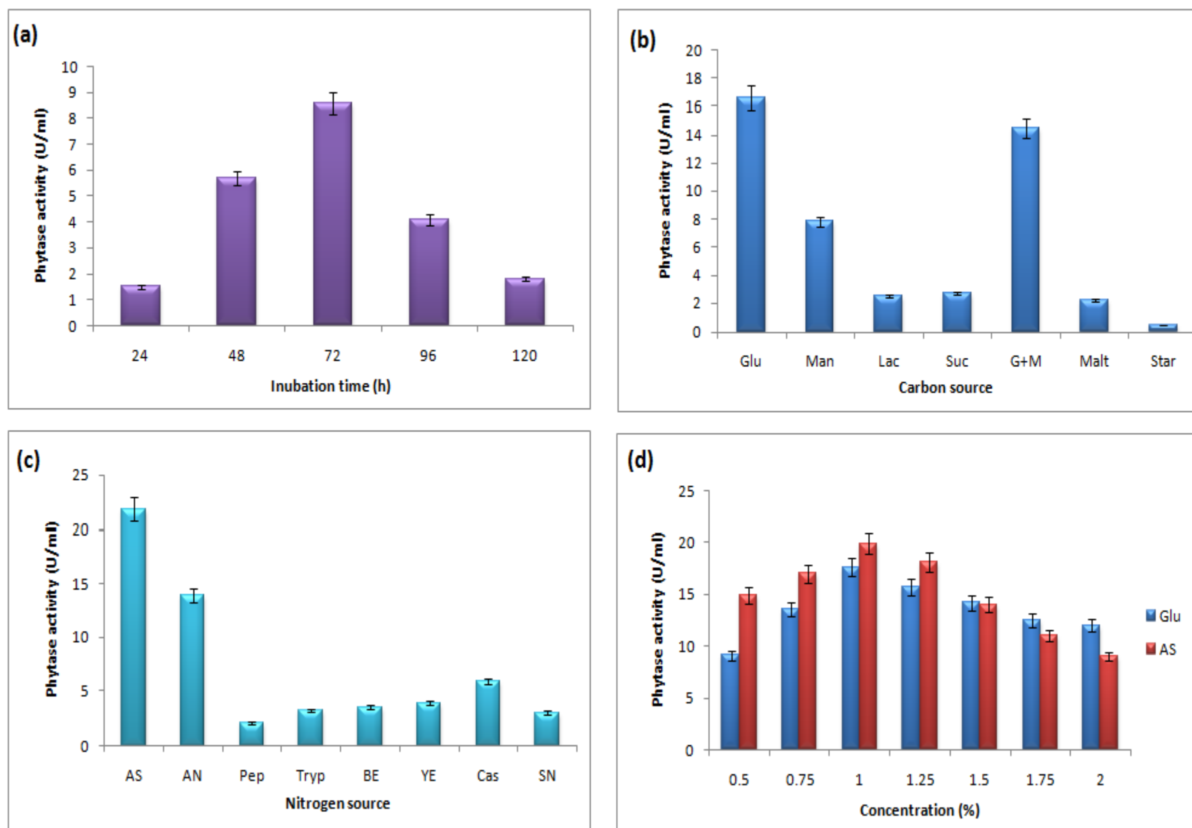

Fig.2.9: Optimization of phytase production by *Shigella* sp. CD2. Effect of (a) incubation time, (b) carbon sources, (c) nitrogen sources and (d) various concentrations of C- and N-sources on the phytase production. [1U= 1μmol of Pi released min^{-1} under assay conditions].

2.3.4. Purification of phytase from *Shigella* sp. CD2

Phytase from CD2 was purified from 72 h grown culture by $(NH_4)_2SO_4$ precipitation, cation-exchange chromatography and gel filtration and the results of enzyme purification are shown in Table 2.3. The bacterial culture, grown at 37°C for 72 h in PPM, was harvested by centrifugation. The cells were then suspended in buffer followed by lysis using ultrasonication. The supernatant obtained after centrifugation was subjected to $(NH_4)_2SO_4$ precipitation and about 68 % of the enzyme was recovered in 30-70 % saturated $(NH_4)_2SO_4$ fraction. The desalted enzyme preparation was subjected to CM-cellulose chromatography, which resulted in removal of substantial amount of proteins and enhanced the specific activity to about 162 U mg^{-1}. The active fractions were pooled, concentrated and subjected to gel filtration chromatography. The enzyme was finally purified 133 folds with specific activity of 780 U mg^{-1} protein and recovery of the enzyme was 41 %. The purified protein appeared as a single band on the SDS-PAGE with an apparent molecular mass of about 45 kDa (Fig.2.10).

Table 2.3: Purification of phytase from *Shigella* sp.CD2

Purification Step	Total Protein (mg)	Total Phytase activity (U*)	Sp. Activity (U mg^{-1})	Fold Purification	% Yield
Crude	19.2	112.00	5.83	1.00	100.00
Ammonium sulphate (30-70%)	4.4	76.00	17.27	2.96	67.80
CM-cellulose	0.4	65.00	162.00	27.87	58.00
Biogel P-100	0.06	46.00	780.00	133.00	41.00

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

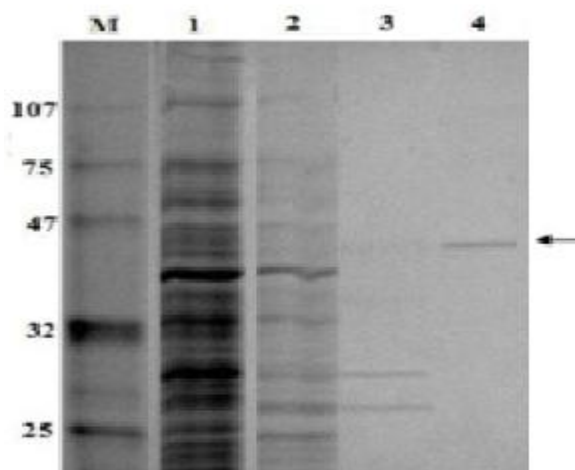

Fig. 2.10: Purification of phytase from *Shigella* sp. CD2. SDS-PAGE analysis of Crude extract (Lane 1), 30-70% ammonium sulphate fraction (Lane 2), CM-Cellulose eluate (Lane 3), Biogel P100 eluate (Lane 4). Lane M-Protein molecular weight marker.

2.3.5. Characterization of purified phytase

The purified enzyme was characterized for molecular weight, pH optima, temperature optima, K_m for phytate, thermostability, cation requirement, substrate specificity and protease resistance and the results are summarized in Table 2.4.

2.3.5.1. Determination of pH optimum, temperature optimum and thermostability

The pH optimum of phytase was determined by measuring the enzymatic activity using buffer in the pH range 2.5-7.5. Phytase displayed significant activity in the pH range 3.5 to

7.5 with optimum pH at 5.5. The enzyme retained only 10 % and 20 % activity at pH 2.5 and 7.5, respectively (Fig. 2.11a). To investigate the effect of temperature, phytase activity was determined at various incubation temperatures ranging 20-80°C under standard assay conditions. Phytase activity increased sharply with increasing the incubation temperature with optimum activity at 60°C (Fig. 2.11b). Though the enzyme showed a rapid loss of activity above the T_{opt}, it still retained about 60 and 30 % of the maximum activity at 70° and 80°C, respectively. The thermal stability of the enzyme was determined by pre-incubating the purified enzyme preparation from 20° to 80°C for 30 min and then assaying under standard conditions. As evident from the results in Fig. 2.11c, that the enzyme showed thermostablity up to preincubation temperature of 50°C for 30 min as it retained almost 90 % activity. Interestingly, the activity initially declined by about 25 % on preincubation at 60°C followed by a significant increase in activity till 80°C.

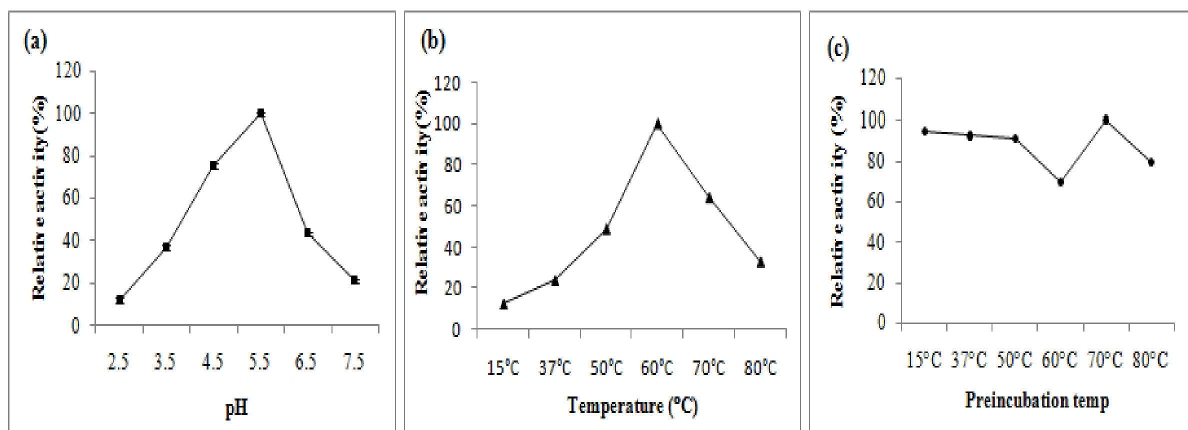

Fig.2.11: Effect of pH and temperature on activity of purified CD2 phytase. The 100 % relative activity was 780 U mg^{-1} (a) Effect of pH on phytase activity. The effect of pH was determined in 100 mM glycine (pH 2.5 & 3.5), 100 mM sodium acetate (pH 4.5 & pH 5.5), 100 mM Tris-HCl (pH 6.5 & 7.5) buffer at 37°C; (b) Effect of temperature on phytase activity; (c) Effect of temperature on stability of phytase. 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.

2.3.5.2. K_m for phytate and substrate specificity

In order to determine the K_m for phytate, phytase activity was measured in presence of increasing concentration of sodium phytate. The enzyme showed hyperbolic response to increasing concentrations of phytate. Apparent K_m value for phytate as determined by Lineweaver-Burk plot was 0.25 mM. The specificity of the enzyme to its substrate was

examined by replacing sodium phytate in the reaction mixture with equal concentration (2 mM) of various other phosphorylated compounds such as, ATP, ADP, para-nitro phenyl phosphate (pNPP), disodium pyrophosphate (dSPP), glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P). The enzyme was remarkably specific to its substrate phytate and its activity in presence of other substrates was almost negligible (Fig.2.12).

2.3.5.3. Effect of protease on phytase activity

To determine the susceptibility to digestive protease, the 50 Units of purified $AppA_s$ was preincubated with 30 Units of pepsin and trypsin at 37°C and phytase activity was monitored 60 min later. The enzyme showed resistance to proteases, with about 80 % of activity remaining after treatment with pepsin and trypsin (Table 2.4)

Table 2.4: Properties of phytase from *Shigella* sp. CD2

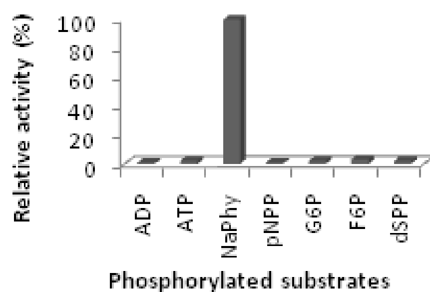

Fig.2.12. Substrate specificity of phytase from Shigella sp CD2

Properties	Results
pH optimum	5.5
Temperature optimum (°C)	60
*Substrate specificity (Sodium phytate)	100 %
K_m Phytate (mM)	0.25
Specific activity (U mg^{-1}, 37°C)	780
Activity in presence of trypsin (1 h)	80 %
Activity in presence of pepsin (1 h)	80 %

*Activity in presence of ATP, ADP, dSPP, P-NPP, G6P, F6P was negligible

2.3.5.4. Effect of divalent cations and chemicals on phytase activity

The effect of different metal ions (divalent cations) and chemical reagents on phytase was determined by measuring enzymatic activity in presence of 2 mM Ca^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, SDS, EDTA, β-ME or Cys-HCl. Activity without addition of ions or reagents used as control. Phytase activity was inhibited by 65%, 72% and 87% in the presence of Fe^{2+}, Zn^{2+} and Cu^{2+}, respectively, whereas, the addition of Mg^{2+}, Co^{2+}, Mn^{2+} and Ca^{2+} led to increase in activity. Moreover, SDS showed a significant inhibitory effect on the enzyme; other metal ions or chemicals, mentionably EDTA, had little or no effect (Table 2.5).

Table 2.5: Effect of divalent cations and chemicals on the phytase activity of *Shigella* sp. CD2

Ions (2mM)	Relative activity (%)	Chemicals (2mM)	Relative activity (%)
*Control	100	*Control	100
Ca^{2+}	119	EDTA	96
Mn^{2+}	121	SDS	04
Mg^{2+}	135	β-ME	100
Cu^{2+}	13	Cys-HCl	108
Zn^{2+}	28		
Co^{2+}	121		
Fe^{2+}	35		

2.3.6. Cloning and sequencing of phytase gene and phylogenetic analysis

The phytase gene from *Shigella* sp. CD2 was amplified by PCR using primers designed from the phytase sequences from related bacterial genus in the database. The PCR product was cloned in pGEM-T Easy vector and sequenced using vector specific T7 and SP6 universal primers. The sequence analysis of the insert indicated presence of an ORF of 1299 bp, encoding protein of 432 amino acids with a stop codon (Fig.2.13). The nucleotide sequence was deposited in the GenBank database under accession number FR865899. Homology analysis of deduced amino acid sequence by BLAST program revealed 98 and 62% similarity with AppA phytase of *E.coli* and *Citrobacter braakii*, respectively. Hence, *Shigella* sp. CD2 phytase ORF was named as $appA_S$ and encoded protein as $AppA_S$. An alignment of the amino acid sequence with enteric bacterial phytases from the GenBank using ClustalW program showed the presence of N-terminal RHGXRXP motif and the C-terminal HDTN motif at amino acid 38-44 and 325-326, respectively, which are common in phytases belonging to HAP family (Yao *et al.* 2011). Moreover, five cysteine residues were also conserved among these phytases as shown in the Fig. 2.14. *E. coli* AppA and *Shigella* sp. CD2 AppAs differed in sequence at six positions. The amino acids S, R, K, E, M, A in *E. coli* AppA were substituted by P, Q, N, K, K, T in $AppA_S$ in the amino acid positions 102, 190, 202, 208, 298, 299, respectively (Fig.2.14). *Shigella* sp. CD2 phytase ORF was named as $appA_S$ and encoded protein as $AppA_S$. A phylogenetic tree then constructed based on the alignment using the neighbor joining method. The topology of the phylogram also confirmed that $AppA_s$ was closely related to AppA phytase of *E. coli* and *Citrobacter braakii* (Fig.2.15).

```
M  K  A  I  L  I  P  F  L  S  L  L  I  P  L  T  P  Q  S  A      20
atgaaagcgatcttgatcccattttatctcttctgattccgttaaccccgcaatctgca    60
F  A  Q  S  E  P  E  L  K  L  E  S  V  V  I  V  S  R  H  G      40
ttcgctcagagtgagccggagctgaagctggaaagtgtggtgattgtcagtcgtcatggt   120
V  R  A  P  T  K  A  T  Q  L  M  Q  D  V  T  P  D  A  W  P      60
gtgcgtgctccaaccaaggccacgcaactgatgcaggatgtcaccccagacgcatggcca   180
T  W  P  V  K  L  G  W  L  T  P  R  G  G  E  L  I  A  Y  L      80
acctggccggtaaaactgggttggctgacaccgcgcggtggtgagctaatcgcttatctt   240
G  H  Y  Q  R  Q  R  L  V  A  D  G  L  L  A  K  K  G  C  P      100
ggacattaccaacgccagcgtctggtggctgacggattgctggcgaaaaagggctgcccg   300
Q  P  G  Q  V  A  I  I  A  D  V  D  E  R  T  R  K  T  G  E      120
cagcctggtcaggtcgcgattattgctgatgtcgacgagcgtacccgtaaaacaggcgaa   360
A  F  A  A  G  L  A  P  D  C  A  I  T  V  H  T  Q  A  D  T      140
gccttcgccgccggctggcacctgactgtgcaataaccgtacatacccaggcagatacg   420
S  S  P  D  P  L  F  N  P  L  K  T  G  V  C  Q  L  D  N  A      160
tccagtcccgatccgttatttaatcccctaaaaactggcgtttgccaactggataatgcg   480
N  V  T  D  A  I  L  S  R  A  G  G  S  I  A  D  F  T  G  H      180
aacgtgactgacgcgatcctcagcagggcaggagggtcaattgctgactttaccggcat    540
R  Q  T  A  F  R  E  L  E  Q  V  L  N  F  P  Q  S  N  L  C      200
cggcaaacggcgtttcgcgaactggaacaggtgcttaatttcccacaatcaaacttgtgc   600
L  N  R  E  K  Q  D  K  S  C  S  L  T  Q  A  L  P  S  E  L      220
cttaaccgtgagaaacaggacaaaagttgttcattaacgcaggcattaccatcggaactc   660
K  V  S  A  D  N  V  S  L  T  G  A  V  S  L  A  S  M  L  T      240
aaggtgagcgccgacaatgtctcattaaccggtgcggtaagcctcgcatcaatgctgacg   720
E  I  F  L  L  Q  Q  A  Q  G  M  P  E  P  G  W  G  R  I  T      260
gagatatttctcctgcaacaagcacagggaatgccggagccggggtggggaaggatcacc   780
D  S  H  Q  W  N  T  L  L  S  L  H  N  A  Q  F  Y  L  L  Q      280
gattcacaccagtggaacaccttgctaagtttgcataacgcgcaatttatttgctacaa    840
R  T  P  E  V  A  R  S  R  A  T  P  L  L  D  L  I  K  T  A      300
cgcacgccagaggttgcccgcagccgcgccaccccgttattagatttgatcaagacagcg   900
L  T  P  H  P  P  Q  K  Q  A  Y  G  V  T  L  P  T  S  V  L      320
ttgacgccccatccaccgcaaaaacaggcgtatggtgtgacattacccacttcagtgctg   960
F  I  A  G  H  D  T  N  L  A  N  L  G  G  A  L  E  L  N  W      340
tttatcgccggacacgatactaatctggcaaatctcggcggcgcactggagctcaactgg   1020
T  L  P  G  Q  P  D  N  T  P  P  G  G  E  L  V  F  E  R  W      360
acgcttccaggtcagccggataacacgccgccaggtggtgaactggtgtttgaacgctgg   1080
R  R  L  S  D  N  S  Q  W  I  Q  V  S  L  V  F  Q  T  L  Q      380
cgtcggctaagcgataacagccagtggattcaggtttcgctggtcttccagactttacag   1140
Q  M  R  D  K  T  P  L  S  L  N  T  P  P  G  E  V  K  L  T      400
cagatgcgtgataaaacgccgctgtcattaaatacgccgcccggagaggtgaaactgacc   1200
L  A  G  C  E  E  R  N  A  Q  G  M  C  S  L  A  G  F  T  Q      420
ctggcaggatgtgaagagcgaaatgcgcagggcatgtgttcgttggccggttttacgcaa   1299
I  V  N  E  A  R  I  P  A  C  S  L  *                           433
atcgtgaatgaagcacgcataccggcgtgcagtttgtaa
```

Fig.2.13: Nucleotide (1-1299) and deduced amino acid sequences (432) of the putative phytase gene appAs, from *Shigella* sp. CD2. The conserved histidine acid phosphatase family active site motifs are underlined. The stop codon is shown by asterisk.

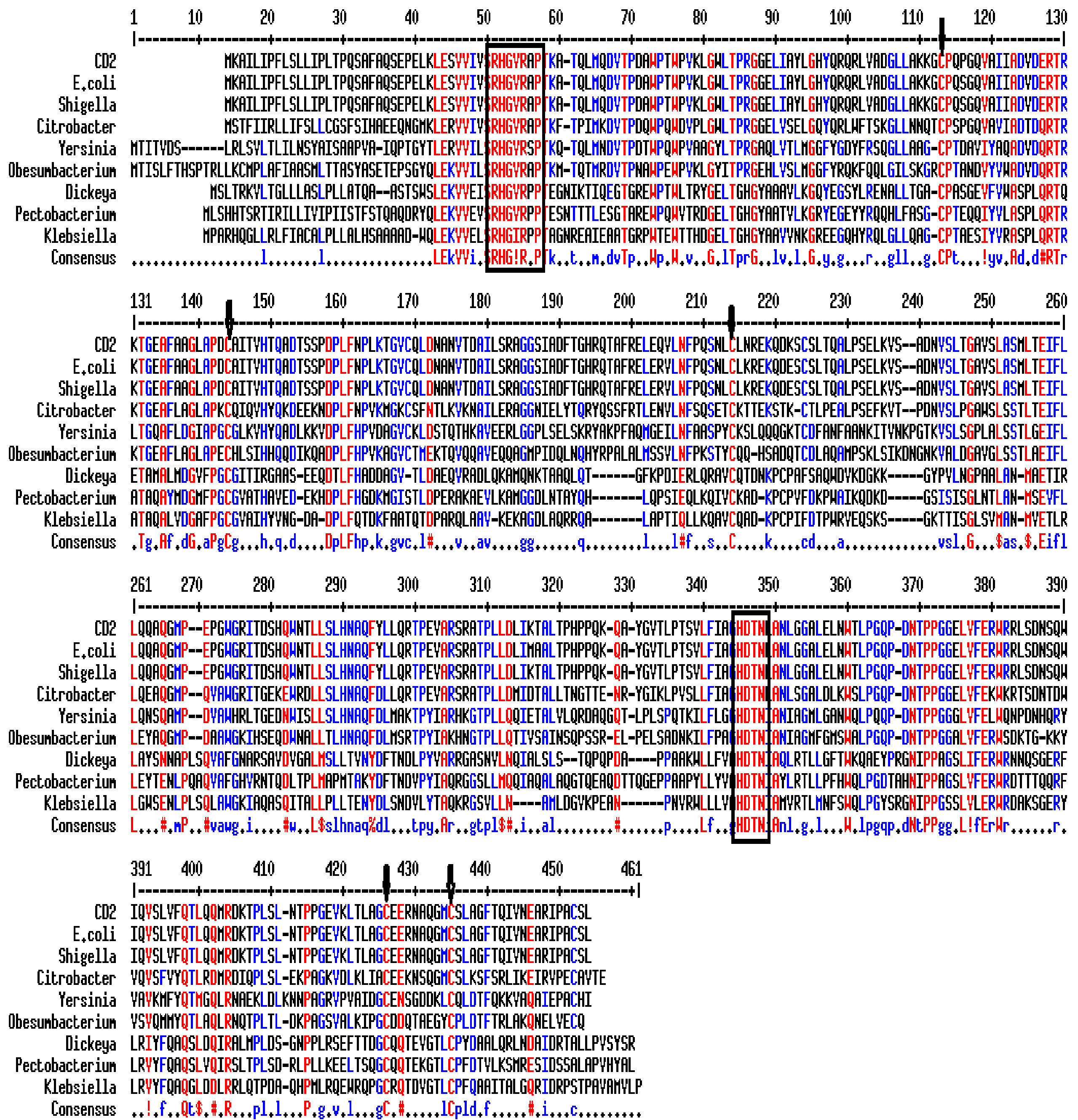

Fig.2.14: Multiple alignment of homologs of the *Shigella* sp. CD2 phytase $AppA_S$. Conserved active site motifs are boxed and conserved cystein residues are shown by arrows. The source and GenBank Accession Nos. of proteins are: *Shigella* sp. CD2, CCA94903; *E.coli* AppA, EDX38944; Putative phytase sequence deduced from *Shigella sonnei* complete genome sequence, CP000038; *Dickeya paradisiaca*, ABW76125; *Klebsiella pneumoniae* AAM23271; *Yersinia intermedia* ABI95370.1; *Citrobacter braakii* AAS45884; *Obesumbacterium proteus* AAQ90419; *Pectobacterium carotovorum* subsp. *carotovorum* ABY76184.

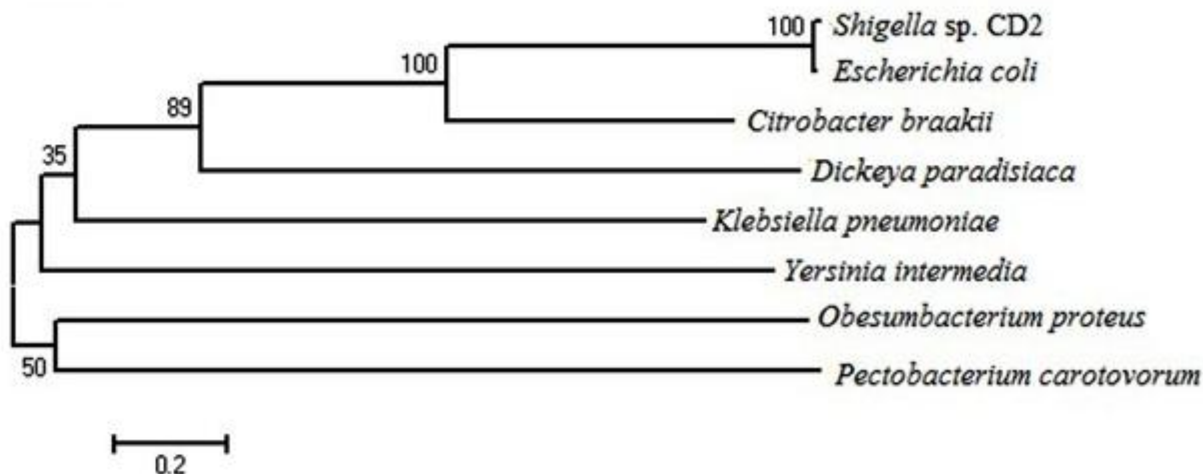

Fig.2.15: Phylogenetic tree of homologs of the *Shigella* sp. CD2 phtase $AppA_S$. Bootstrap values (%) from analysis of 500 bootstrap replicates are given at the respective nodes. The bar represents 2 substitutions per 10 amino acids. For GenBank Accession Nos. of proteins, see the Fig. 2 ligand.

The signal peptide cleavage site was identified using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) (Petersen *et al.* 2011). The $AppA_S$ contained putative signal peptide of 22 amino acids, and three potential sites of N-glycosylation. The calculated molecular mass of the protein with and without signal sequence were about 47 and 45 kDa, respectively and a theoretical pI of 5.67.

2.3.7. Expression of $appA_S$ in *P. pastoris* G115

The expression of enzymes as secreted protein is one of the useful and important characteristics for their economical production in industry. *P. pastoris* has been successfully used as host organism for extracellular production of recombinant proteins at high level, including phytases (Cereghino and Cregg 2000). The $appA_S$ was cloned in *P. pastoris* expression vector pPIC9. The recombinant plasmid pPIC9-$appA_S$ carried the $appA_S$ expression cassette consisting of 1.2 kb $appA_S$ gene in frame with *Sacharomyces cerevisiae* α-factor secretion signal, flanked by *AOX1* promoter and terminator sequences. Transformation of pPIC9-$appA_S$ into *P.pastoris* GS115 gave about 20 His^+ transformants. The integration of appAs-expression cassette into the genome of *P. pastoris* GS115 transformants was ascertained by PCR using 5’ and 3’ *AOX1* primers. PCR amplification products of about 0.5 kb and 1.7 kb in pPIC9 transformed and pPIC9-$appA_S$ transformed *P. pastoris* GS115, respectively, indicated the integration of $appA_S$-expression cassette into the genome of the later (Fig.2.16).

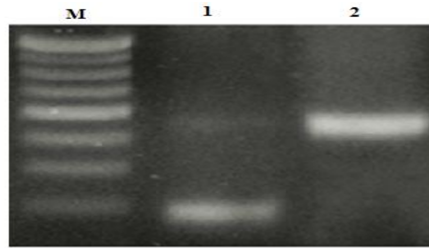

Fig.2.16: PCR products of pPIC9 transformed (Lane 1) and pPIC9-$appA_S$ (Lane 2) transformed *P. pastoris* GS115 DNA with 500 bp DNA ladder (Lane M)

The pPIC9-$appA_S$ transformed *P. pastoris* GS115 colonies were screened for Mut^+ and Mut^S phenotypes. Both Mut^+ and Mut^S transformants were analysed for extracellular and periplasmic phytase expression. The transformant was initially grown in BMGY medium until culture reached an OD_{600} value of 1 and then the culture was transferred to BMMY medium containing 0.5% methanol as inducer and grown for 96 h. Extracellular and periplasmic phytase activity and pH of the medium were determined at every 24 h intervals. A Mut^+ colony with highest extracellular phytase activity was selected for shake flask expression. The selected transformant showed maximum extracellular recombinant phytase ($rAppA_P$) activity of 62 U ml^{-1}, with specific activity 477 U mg^{-1}, at 60 h of methanol induction and an extracellular protein concentration of 0.13 mg ml^{-1}. The SDS-PAGE analysis of concentrated and diafiltered cell free extract showed two protein bands of approximate molecular mass 59 and 65 kDa (Fig.2.17a).

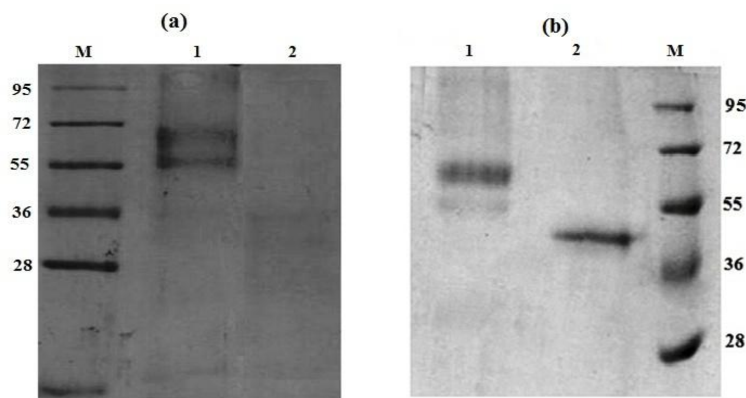

Fig. 2.17: Expression of *Shigella* sp. CD2 phytase $appA_S$ in *P. pastoris* GS115 cells **(a)** SDS-PAGE analysis of recombinant phytase ($rAppA_P$). Lane, M- Molecular Weight markers in kDa, 1- extracellular fraction of *P.pastoris* GS115 transformed with pPIC9-$appA_S$, 2- extracellular fraction of *P.pastoris* GS115 transformed with pPIC9. Cultures were induced by 0.5 % methanol for 48 h and diafiltered culture supernatant containing 50 µg protein was loaded in each lane of a 12 % SDS-PAGE gel. **(b)** SDS-PAGE analysis of glycosylated and deglycosylated $rAppA_P$. Lane, M- Molecular Weight markers in kDa, 1- glycosylated $rAppA_P$, 2- deglycosylated $rAppA_P$. The $rAppA_P$ (50 U) was incubated with Endo H (10 U) for 2h. The protein was analyzed and compared with untreated enzymes.

The enhanced molecular mass of rAppA$_P$ might be due to glycosylation that normally occurs for yeast secreted proteins. The deglycosylation treatment to rAppA$_P$ with Endo H resulted in single band with apparent molecular mass of 45 kDa, similar to that of *E. coli* expressed rAppA$_E$ (Fig.2.17b).

2.3.8. Expression of appA$_S$ in *E.coli* BL21(DE3)

The mature appA$_S$ (without the signal sequence) was cloned into *E. coli* expression vector pET-20b (+) and the recombinant plasmid pET-20b (+) appA$_S$ was transformed into *E. coli* BL21(DE3). The transformant was induced in Magic Media supplemented with IPTG and cells were disrupted by sonication after overnight induction. Recombinant phytase (rAppA$_E$) overexpression in the soluble and pellet fractions of sonicated cells was analyzed by SDS-PAGE. As shown in the results of Fig.2.18, the soluble fraction of the induced cell exhibited protein overexpression band of approximately 45 kDa, which agrees with the predicted molecular weight deduced from the amino acid sequence of AppA$_S$. The soluble and pellet fraction of IPTG induced *E. coli* BL21(DE3) transformant was also analyzed for phytase activity. Phytase activity level in the soluble fraction was 176 U ml^{-1} (specific activity 568 U mg^{-1}), whereas negligible activity was detected in the pellet fraction. The recombinant phytase activity was about 101 folds greater than that of native phytase (5.60 U mg^{-1}) from *Shigella* sp. CD2. The result thus indicates a correlation of rAppA$_E$ overexpression with phytase activity.

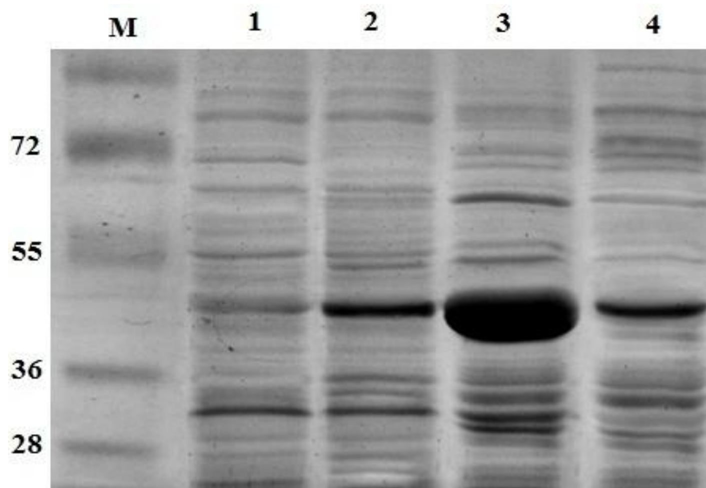

Fig. 2.18: Bacterial expression of phytase appA$_S$ of *Shigella* sp. CD2 in *E.coli* BL21(DE3): SDS-PAGE analysis of recombinant phytase (rAppA$_E$). Lane, M- Molecular weight markers in kDa, **1**- soluble fraction of induced BL21 (DE3) transformed with pET20b (+) vector, **2**-pellet fraction of induced BL21 (DE3) transformed with pET20b (+) vector, **3**-soluble fraction of induced BL21 (DE3) transformed with pET20b(+) -appA$_S$, **4**- pellet fraction of induced BL21 (DE3) transformed with pET20b(+) -appA$_S$. Approx. 25 µg protein from the soluble fraction was loaded in each lane of 12 % SDS-PAGE gel.

2.3.9. Purification and properties of $rAppA_E$ and $rAppA_P$

The $rAppA_E$ was purified from the soluble fraction of *E.coli* BL21 (DE3) using Ni-Sepharose Fast Flow affinity chromatography and $rAppA_P$ was purified by cation exchange chromatography of diafiltered extracellular fraction of pPIC9-$appA_S$ transformed *P. pastoris* GS115. The specific activities of purified $rAppA_P$ and $rAppA_E$ were 967 and 2982 U mg^{-1}, respectively. Western blot analysis of purified $rAppA_E$ and purified deglycosylated rAppAp using rabbit polyclonal antibody against *E.coli* phytase further demonstrated that the specific band with apparant molecular mass of 45 kDa was recombinant phytase (Fig.2.19).

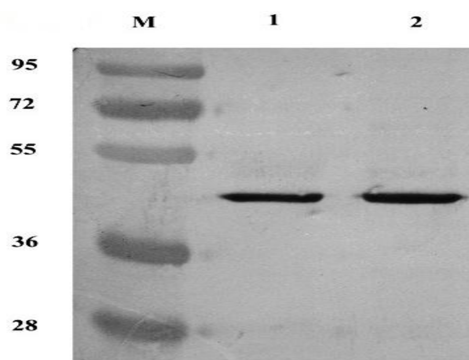

Fig.2.19: Western blot analysis. Lane, M- Molecular weight marker, 1-purified $rAppA_E$, 2-purified and deglycosylated $rAppA_P$.

Compared with the glycosylated $rAppA_P$, the non-glycosylated $rAppA_E$ was more active at pH 3.5- 7.5. Both the enzymes had more than 50 % activity in the pH range 3.5 to 6.5 with pH optima at 5.5 (Fig 2.20 a) and also had temperature optima of 60°C. Compared to $rAppA_P$,

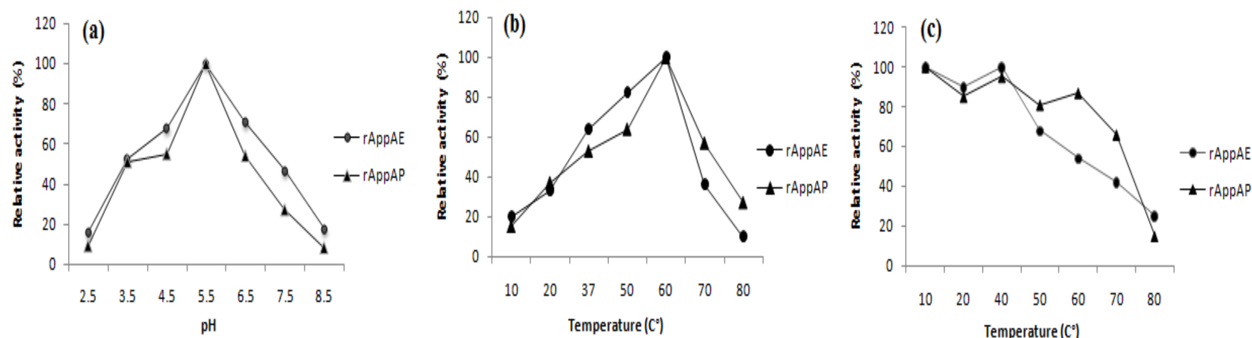

Fig.2.20: Characterization of purified recombinant phytases: **(a)** pH profile of $rAppA_E$ and $rAppA_P$. The effect of pH was determined in following buffers (100 mM): glycine-HCl (pH 2.5 and 3.5), sodium acetate (pH 4.5 and pH 5.5), and Tris-HCl (pH 6.5, 7.5 and 8.5). **(b)** Temperature profile of $rAppA_E$ and $rAppA_P$ at optimum pH and 10 to 80°C. **(c)** Thermal stability of $rAppA_E$ and $rAppA_P$. Purified enzyme preparation was pre-incubated at indicated temperature for 30 min followed by determination of enzymatic activity as described in 'Materials and Methods' section.

$rAppA_E$ had 11 and 18% greater relative activity at 37 and 50°C, respectively, whereas at higher incubation temperature $rAppA_P$ was more active than $rAppA_E$ (Fig 2.20 b). For determination of thermal stability, the purified $rAppA_E$ or $rAppA_p$ were pre-incubated at 10 to 80°C for 30 min and then assayed for enzymatic activity. 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. Consequently at 60 and 70° C, phytase activity of $rAppA_p$ was 33 and 24 % higher than that of $rAppA_E$, respectively (Fig. 2.20 c).

Table 2.6: Properties of phytase AppAs expressed in *E.coli* ($rAppA_E$) and *P. pastoris* ($rAppA_P$) listed along with that of the native enzyme from *Shigella* sp. CD2

Properties	Results		
	$rAppA_E$	$rAppA_P$	Native (CD2)
*Substrate specificity (Sodium phytate)	100%	100%	100%
K_m Phytate (mM)	0.22	0.18	0.25
Specific activity of purified enzyme (U mg^{-1} protein, 37°C)	2982	967	780
$Thermostability (%)	100	100	95
#Activity in presence of trypsin	70%	65%	80%
#Activity in presence of pepsin	55%	50%	80%
Activity in presence of metal ions (20 mM):			
Ca^{2+}	130%	105%	119%
Mg^{2+}	125%	110%	135%
Mn^{2+}	109%	102%	121%

*Activity in presence of ATP, ADP, pNPP, dSPP, G6P, F6P was negligible.

$Activity after pre-incubation of enzyme at 40ºC for 30 min.

Recombinant enzyme (50 U) was pre-incubated with pepsin or trypsin for 60 min followed by determination of phytase activity.

Both the $rAppA_E$ and $rAppA_P$ showed high specificity to the substrate, exhibiting 100% relative activity with sodium phytate. Activity with other phosphorylate substrates, such as ATP, ADP, p-NPP, G6-P or F6-P was almost negligible. K_m values for phytate as

determined by Lineweaver- Burk plot were 0.22 and 0.18 mM for the $rAppA_E$ and $rAppA_P$, respectively (Table 2.6). Analysis of the effect of various metal ions on phytase activities revealed that Ca^{2+}, Mg^{2+}, and Mn^{2+} had a stimulatory effect, as higher activity (100–130 % relative phytase activity) was detected from the enzymes incubated in the presence of these ions. On the other hand, Cu^{2+}, Fe^{2+}, Zn^{2+} or EDTA showed inhibitory effect toward the recombinant phytases. To determine the protease resistance the purified recombinant phytases (50 Units) were pre-incubated separately with 30 Units of either pepsin or trypsin at 37°C. 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. (Table 2.6)

2.4. DISCUSSION

Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis phosphate, IP6) is mainly synthesized by plants. It is the major storage form of phosphorous (P) in cereals and legumes, that is poorly available to monogastric animals. Although PA and its cation salts are very important in many physiological functions, it is considered solely as antinutrients because they bind with starch and protein and strongly chelate with divalent minerals, such as Ca^{2+}, Mg^{2+}, Zn^{2+}, and Fe^{2+} affecting their bioavailability (Tai *et al.* 2013). Due to unavailability of phytate P for animal nutrition, it is a common practice to add inorganic P as an animal feed supplement. However, the inefficient utilization of phytate consequently contributes to P pollution problems in the areas of intensive crop and livestock production. The released PA and phytate contribute to significant environmental pollution (Jorquera *et al.* 2008). Thus, from both environmental and economic point of view, release of phytate P for animal and plant nutrition is essential.

Phytases catalyze the hydrolysis of phosphate moieties from PA, thereby inhibiting the metal ion chelating and protein/carbohydrate binding abilities of PA. The supplementation of animal feed with phytases may improve bioavailability of P, protein and carbohydrate to monogastric animal besides reducing P pollution. A number of microorganisms, mainly of bacterial and fungal origin, are capable of synthesizing phytase (Li *et al.* 2008). At present, all authorized phytase preparations as feed additives are produced by recombinant strains of filamentous fungi (Olazaran *et al.* 2010). The exploitation of phytase in the feed industry has been hampered by high productivity cost, low stability, low specific activity and poor substrate specificity. The characteristics of phytase important in industrial applications are high specific activity, pH optima corresponding to various parts of digestive tract, resistance to stomach proteases and high temperature (Promdonkoy *et al.* 2009). Bacterial phytases are promising as an alternative to fungal enzymes because of their higher specific activity, pH profile, substrate specificity, protease resistance and higher thermal stability.

Present study describes purification, characterization, gene cloning, sequence analysis and expression studies of phytase from *Shigella* sp. CD2. Initial experiments were carried out to isolate novel phytase-producing bacteria from various environmental samples. Previous studies have reported isolation of phytase-producing bacteria from different sources such as, rhizospheric soil (Gulati *et al.* 2006, Jorquera *et al.* 2011), poultry farm soil and cattle shed soil (Singh *et al.* 2013), cattle dung (Roy *et al.* 2014), rotten wood log, digestive tracts of fishes (Khan and Ghosh 2012), compost materials (Eida *et al.* 2013) etc. Among the isolated bacteria, strain CD2 was selected for further studies because of the production of highest level of cell-bound phytase. The conventional taxonomic analysis and the phylogenetic analysis of

16S rDNA sequence identified the isolate CD2 as a species of the genus *Shigella* and hence, given the name *Shigella* sp. CD2, belonging to the γ-proteobacteria group. The γ-proteobacteria is the highest-reported group to carry phytase-like genes (Lei et al. 2013). Many representatives of this group have already been well studied with respect to phytase production such as, *E. coli* (Greiner *et al.* 1993, Yao *et al.* 2013), *Enterobacter* sp. (Yoon *et al.* 1996), *Xanthomonas* sp. (Chatterjee *et al.* 2003), *Citrobacter* sp. (Kim *et al.* 2003), *Klebsiella* sp. (Sajidan *et al.* 2004), *Obesumbacterium* sp. (Zinin *et al.* 2004), *Yersinia* sp. (Huang *et al.* 2006a), *Dickeya* sp. (Gu *et al.* 2009), *Erwinia* sp. (Huang *et al.* 2009), *Pseudomonas* sp. (Hosseinkhani *et al.* 2009) etc. Although, the putative phytase genes from *Shigella* sp. (*S. boydii*, *S. dysenteriae* and *S. flexneri*) have been identified previously by whole genome sequence analysis (Lim *et al.* 2007), studies on the phytase enzyme from *Shigella* sp. are lacking and to our knowledge, this is the first report with detailed study on a phytase isolated from *Shigella*.

Shigella sp. CD2 produced a cell-bound phytase which exhibited activity in both periplasmic and intracellular fractions as reported earlier for *E. coli* phytase (Greiner et al. 1993). Most of the phytase-producing strains of Enterobacteriaceae family were found to produce intracellular phytases; the only bacterium of Enterobacteriaceae family showing extracellular phytase activity was *Enterobacter* sp. (Yoon *et al.* 1996, Oh *et al.* 2004). In *Shigella* sp. CD2, a significant phytase production was noted during the end of exponential phase at 24 h, with maximum activity during the stationary phase at 72 h of bacterial growth. The increased activity during stationary phase suggests that phytase is not much required for balanced growth and induced by either nutrient or energy limitation known to occur in the stationary phase. Previous studies showed that phytase synthesis could be stimulated by anaerobiosis (Greiner *et al.* 1993) or limitation of phosphate (Greiner *et al.* 1997). The *Shigella* sp. CD2 produced phytase only in presence of phytate in the medium as reported for the enzyme from *Klebsiella terrigena* (Greiner *et al.* 1997). In contrast, phytate had no influence on the synthesis of phytate-degrading enzyme in *Escherichia coli* (Greiner *et al.*1997). Phytase from *Shigella* sp. CD2 was purified from 72 h grown culture by $(NH_4)_2SO_4$ precipitation, cation-exchange chromatography and gel filtration. The 133 fold purified enzyme had specific activity of 780 U mg^{-1} protein. The enzyme has already been purified from few genera of Enterobacteriaceae and they differ in their specific activity. The specific activity of phytase from *Shigella* sp. CD2 was much higher than that of *Klebsiella* sp. ASR1 (Sajidan *et al.* 2004) and *Bacillus* phytases (Yao *et al.* 2011) and quite lower than that of *Citrobacter braakii* (3457 U mg^{-1}) (Kim *et al.* 2003). SDS-PAGE analysis of the purified enzyme indicated an apparent molecular mass of about 45 kDa indicating that the enzyme is a

single chain or homomultimeric protein. The molecular weight of phytase in this study is quite close to that of other bacterial phytases, which have molecular mass between 37 to 45 kDa (Lei *et al.* 2013). The *Shigella* phytase has a molecular weight lower than that of *C. braakii* (49 kDa); same as that of *E. coli* (Yao *et al.* 2013), *E. carotovora* (Huang *et al.* 2009) and *Y. intermedia* (Huang *et al.* 2006a); and higher than that of *Bacillus* sp. (Olazaran *et al.* 2010). Previously, all the microbial phytases hitherto characterized as monomeric enzymes, whether fungal or bacterial in origin (Mukhametzyanova *et al.* 2012).

The purified phytase $AppA_S$ had pH optimum of 5.5. It retained more than 40 % of maximal activity in the pH range 3.5-6.5. The optimum pH of most of the reported microbial phytases are in the range 4.0 to 5.5, while a higher pH optimum in the neutral to alkaline range was noted for *Bacillus* species (Lei *et al.* 2013). Phytase AppA from *E.coli* (Yao *et al.* 2013), *O. proteus* (Zinin *et al.* 2004), *C. braakii* (Kim *et al.* 2006), *Y. intermedia* (Huang *et al.* 2006a), and *E. carotovora* (Huang *et al.* 2009) had pH optima of 4.5, 4.9, 5.0, 4.5 and 5.5, respectively. There are some microbial phytases that differ from others by having two pH optima, for example, *Citrobacter freundii* phytase at pH 2.5 and 4.5 (Zhao *et al.* 2010), and *A. niger* phytase at 2.5 and 5.5 (Han and Lei 1999) and *Dickeya paradisiaca* recombinant phytase at pH 4.5 and 5.5 (Gu *et al.* 2009). Interestingly, phytase from *Klebsiella pneumoniae* subsp. *pneumoniae* strain XY-5 exhibited two pH optima (3.7 and 5.5) only when assayed at two different temperatures, i.e., 37°C and 55°C (Wang *et al.* 2004). In contrast to most other phytases, the enzyme from *A. fumigatus* had a broad pH optimum and at least 80% of the maximal activity was reported at pH values between 4.0 and 7.3 (Wyss *et al.* 1999).

To investigate the effect of temperature, phytase activity was determined at different temperatures ranging 20-80°C. The optimum *in vitro* temperature was 60°C and a drastic decrease in the enzyme activity was noted at temperatures above and below the optimum value. Most of the phytases reported earlier had optimal temperature of 44-60°C. As in the present study, phytase from *B. subtilis*, *E. coli*, *Klebsiella aerogenes*, *Enterobacter* sp.4, *K. oxytoca* MO-3 and *Selenomonas ruminantium* were optimally active in the temperature range 50° and 60°C, whereas phytase from *Bacillus* sp. DS11 was optimally active at 70°C (Rao *et al.* 2009, Yao *et al.* 2011). Two *E. coli* phytases characterized previously had optimum temperature of 60°C (Stahl *et al.* 2003, Tai *et al.* 2013). When compared with its mesophilic counterparts, the *E. carotovora* phytase had a relatively lower optimum temperature (40°C). Thermostability of phytases is desirable for their application in the animal feed industry because feed pelleting involves a step of 80 to 85°C for few seconds. As evident from the results that the CD2 phytase was thermostable upto 50°C retaining 90 % of its activity,

followed by a drop at 60°C. Interestingly, the enzyme regained its activity at higher temperatures showing 100 % and 75 % activity at 70°C and 80°C, respectively. The observed property is strikingly different from phytases reported in other Enterobacteriaceae genera, like *E. coli* and *Klebsiella*, which showed a drastic loss in phytase activity on incubation of enzyme above 60°C (Greiner *et al.* 1993, Sajidan *et al.* 2004). But similar observation has been previously reported in case of *Aspergillus fumigatus* phytase, which after heat denaturation upto 90°C refolds completely into a native-like fully active conformation, thereby regaining its activity (Pasamontes *et al.* 1997); though a later report did not confirm these results (Ullah *et al.* 2000). Phytases from most of the bacteria belonging to enterobacteriaceae family showed moderate thermostability. Phytase from *C. braakii* maintained 100 % activity till incubation temperature 50°C and the activity declined to 50 and 20 % at incubation temperature 60 and 70°C, respectively (Kim *et al.* 2006). Similarly, *E.coli* AppA phytase showed 55 % and 20 % activity, when preincubated at 60° and 70°C, respectively (Yao *et al.* 2013). Among bacteria, phytase from *Bacillus* sp. MD2 and *B. laevolacticus* exhibited very high thermostability retaining activity upto 80-100°C (Tran *et al.* 2010). Apparent K_{m} value for phytate as determined by Lineweaver-Burk plot was 0.25 mM. The observed K_{m} value is in tune with that of *E. carotovora* phytase with K_m of 0.25 mM (Huang *et al.* 2009) and *Klebsiella pneumonia* phytase with K_m of 0.28 mM (Sajidan *et al.* 2004), while *E.coli* phytase showed higher K_m (0.55 mM) (Luo *et al.* 2004) and so did *C. braakii* phytase (0.46 mM) (Kim *et al.* 2003).

To determine the substrate specificity of the *Shigella* phytase, the hydrolysis of various phosphorylated compounds by the purified enzyme was studied. *Shigella* phytase $AppA_S$ showed narrow substrate specificity, exhibiting 100 % relative activity with PA and negligible activity when the substrate was ADP, ATP, p-NPP, G6-P or F6-P, as reported earlier for *E.coli* (Greiner *et al.* 1993, Tai *et al.* 2013) and *O. proteus* (Zinin *et al.* 2004). In contrast, phytases from *K. pneumoniae, V. volvacea* and *A. fumigatus* have shown a broad specificity for other phosphorylated substrates while showing relatively low specificity to phytate (Xu *et al*. 2012).

The phytase $AppA_S$ retained about 80% of activity on treatment with pepsin and trypsin for 1 h indicating good resistance of the enzyme to proteases. The property of resistance to protease is important in application of the enzyme as animal feed supplement for hydrolysis of indigestible phytate for increasing phosphate bioavailability. Phytases from *C. braakii* (Kim *et al.* 2003) *K. pneumoniae* (Sajidan *et al*. 2004) and *Y. intermedia* (Huang *et al.* 2006a) have also shown higher protease resistance. *Yersinia* AppA phytase retained more than 80 %

activity, when pre-treated with pepsin or trypsin for 2 h (Huang *et al.* 2006). However, *E.coli* phytase AppA lost 70% activity on treatment with pepsin for 2h, whereas it retained about 65 % activity on treatment with trypsin for same time period (Tai *et al.* 2013). In the present study, phytase activity was inhibited 65 % by Fe^{2+}, 72% by Zn^{2+} and 87% by Cu^{2+}, whereas, presence of Mg^{2+}, Ca^{2+}, $Mn^{2+}$and Co^{2+} in the reaction enhanced the enzymatic activity. SDS severely inhibited phytase activity indicating its denaturing effect on the enzyme. Besides that, insensitivity of the enzymatic activity to EDTA indicated that functioning of enzyme do not have absolute requirement of metal ions. However, it is difficult to determine whether the inhibitory effect of various metals is due to direct binding to the enzyme, or whether the metal ions form poorly soluble complexes with PA and therefore decrease the active substrate concentration. As in the present study, phytase AppA from *Yersinia intermedia* was strongly inhibited by Zn^{2+}, Fe^{2+} and SDS (Huang *et al.* 2006a). Phytase from *Enterobacter* sp. 4 was greatly inhibited by Zn^{2+}, Ba^{2+}, Cu^{2+} and Al^{3+} (Yoon *et al*. 1996). In another stydy, Wyss *et al*. (1999) reported considerable depression in phytase activity of *E. nidulans* and *A. terrus* by Cu^{2+}, and of *A. fumigatus* by several metal ions.

Phytases belonging to the family histidine acid phosphatase are classified into three different groups on the basis of amino acid sequence and biochemical properties i.e. PhyA, PhyB and PhyC (Lei *et al.* 2013). The most of the biochemical properties of the novel phytase from *Shigella* sp. CD2 resembled that of histidine acid phytase of group PhyC. This group includes acid phytases which are mostly intracellular, monomeric, nonglycosylated proteins composed of 354–439 amino acids with a molecular mass of ~42-45 kDa (Wyss *et al.* 1999) and they usually have a single optimal pH (~5.0–6.0) and exhibit an optimal temperature at 40-60°C (Oh *et al*. 2004).

The phytase encoding gene, appAs was cloned and sequenced; it encoded a protein ($AppA_S$) of 432 amino acids with a signal peptide of 22 amino acids. Homology analysis of deduced amino acid sequence by BLAST program revealed 98 and 62 % similarity with AppA phytase of *E.coli* and *C. braakii*, respectively. Alignment of the $AppA_S$ with other enteric bacterial phytases in the Gen Bank showed presence of N-terminal RHGXRXP and C-terminal HDTN motif, which are common in phytases belonging to HAP family (Kumar *et al.* 2012). To date, seven other genera of Enterobacteriaceae have been reported to produce phytase, and relevant genes have been cloned and all of them belong to HAP family (Yao *et al.* 2011). As in the present study phytase AppA from *C. braakii* was more closely related to the *E.coli* AppA than to other HAPs (Kim *et al.* 2006). On the other hand, phytase gene from *O. proteus* showed maximum identity of 53 % and 47 % to phosphoanhydride phosphatase from *Yersinia pestis* and *E.coli* AppA, respectively (Zinin *et al.* 2004). The high identity of

the sequences, position of the active center and the cystein residues allows us to suggest that the *E. coli* phytase and *Shigella* sp. CD2 phytase proteins may have similar three-dimensional structure and a similar mechanism of the enzyme action.

The $AppA_S$ from *Shigella* sp. CD2 was expressed in *E.coli* and *P. pastoris*. Since the yield and properties of any given protein or enzyme are significantly affected by the expression capability of hosts (Muller *et al*. 1998) and their post-translational modifications, such as glycosylation (Stahl *et al.* 2003), both the prokaryotic and eukaryotic expression systems were employed to gain a comparable overview towards improving phytase function and production. In case of *Pichia* expression, recombinant His^+ *P. pastoris* GS115 cells were screened for Mut^+ and Mut^S phenotypes. Phytase activity was found significantly higher in a Mut^+ strain which was thus selected for further studies. The selected transformant showed maximum extracellular recombinant phytase ($rAppA_P$) activity of 62 U ml^{-1}, with specific activity 477 U mg^{-1}, at 60 h of methanol induction. The $rAppA_P$ activity was higher than that of PhyC gene encoding neutral phytase expressed in *P. pastoris* (12. 5 U ml^{-1}) (Zou *et al.* 2006). However, the yield is lower than AppA phytase of *C. braakii* (197 U ml^{-1}) and *E.coli* (112.50 U ml^{-1}) (Kim *et al.* 2006, Tai *et al.* 2013). The lower activity of $rAppA_P$ might be due to observed increase in the medium pH during cultivation of *P. pastoris*. The expression level and activity of $rAppA_P$ could be increased by further optimization of bioprocess and control of medium pH at <7. Moreover, reduced phytase activity could also be due to the variation in codon usage between *E.coli* and *P. pastoris*. Previous studies have shown the effect of codon bias on expression and activity of recombinant phytase and other enzymes (Wang *et al.* 2005, Zou *et al.* 2006). The extracellular expression of *phy*C gene from *B. subtilis* WHNB02 in *P. pastoris* yielded 2.40 U ml^{-1} phytase. Synthesis of *phy*C according to *P. pastoris* codon usage without altering the protein sequence enhanced activity by about 8 folds to 18.50 U ml^{-1} (Zou *et al.* 2006). Similarly, Xiong *et al.* (2006) used *P. pastoris* preferred codons and modified signal sequences to improve the expression of heterologous phytase from *Peniophora lycii* by 13.6 fold. SDS-PAGE analysis of $rAppA_P$ showed two protein bands of approximate molecular mass 59 and 65 kDa. The yeast *Saccharomyces cerevisiae* and *P. pastoris* are reported to introduce several post translational modifications to proteins destined for extracellular secretion (Stahl *et al.* 2003, Kim *et al.* 2006). The enhanced molecular mass of $rAppA_P$ was found to be due to glycosylation because deglycosylation of $rAppA_P$ with Endo H resulted in single band with apparent molecular mass of 45 kDa, similar to that of *E.coli* expressed $rAppA_E$. As in the present study, recombinant AppA from *E. coli* expressed in *P. pastoris* appeared as diffused band of molecular size 55 kDa in SDS-PAGE. However, the sharp single band was clearly observed after the purified phytase was deglycosylated (Stahl *et*

al. 2003). Similarly, AppA from *C. braakii* expressed in *E. coli* had molecular size of 49 kDa, whereas same protein expressed in *Saccharomyces cerevisiae*, migrated as a broad diffusion band (110-160 kDa) in SDS-PAGE gel due to extensive N-linked glycosylation (Kim *et al.* 2006).

The phytase appAs was also expressed in *E.coli* in order to compare the biochemical characteristics of the enzyme with that expressed in *P. pastoris*. The mature $appA_S$ (without the periplasmic signal sequence) was cloned into *E. coli* expression vector pET-20b (+) and the recombinant plasmid pET-20b(+)-$appA_S$ was transformed into *E. coli* BL21(DE3). The periplasmic signal sequence was removed for targeting the enzyme to the intracellular space as *E.coli* itself contains two different forms of AppA phytase in periplasm (Rodriguez *et al.* 2000). *E. coli* BL21(DE3) harbouring pET-20b(+)-$appA_S$ expressed AppAs in the soluble fraction as 45 kDa protein ($rAppA_E$), which agrees with the predicted molecular weight deduced from the amino acid sequence of $AppA_S$. Contrary to the present work, most of the studies on expression of recombinant phytase in *E. coli* have shown accumulation of phytase as inclusion body in the cell. AppA phytase from *C. braakii* was expressed in *E.coli* as inclusion body and expression of the protein even at temperature less than 30°C could not get a good yield in soluble fraction (Kim *et al.* 2006). Similarly, expression of phytase gene from *Bacillus* (Rao *et al.* 2008) in *E.coli* resulted in formation of inclusion bodies which required downstream processing to recover the active enzyme. Phytase activity level of $rAppA_E$ in the soluble fraction was 176 U ml^{-1} (specific activity 568 U mg^{-1}). The recombinant phytase activity was about 101 folds greater than that of native phytase (5.60 U mg^{-1}) from *Shigella* sp. CD2 (Roy *et al.* 2012). The results thus indicate a correlation of $rAppA_E$ overexpression with phytase activity.

The $rAppA_P$ and $rAppA_E$ were purified and their characteristic properties were compared. The specific activities of purified $rAppA_P$ and $rAppA_E$ were 967 and 2982 U mg^{-1}, respectively. The difference in glycosylation between the two enzymes partially affected their biochemical properties. Both the recombinant enzymes had pH optima of 5.5 with greater than 50 % of their relative activity maintained between pH 3.5 to 6.5. The pH optimum of most of the enterobacterial phytase AppA is in the range of 4.5 to 5.5. Phytase AppA from *E. coli*, *O. proteus*, *Y. intermedia*, *C. braakii*, and *E. carotovora* have pH optima value of 4.5, 4.9, 5.0, 4.5 and 5.5, respectively (Zinin *et al.* 2004, Huang *et al.* 2006a, Huang *et al.* 2006b, Huang *et al.* 2009, Tai *et al.* 2013). Both $rAppA_E$ and $rAppA_p$ had temperature optima of 60°C, but the latter was more active at 70 and 80ºC. The temperature optima of other reported bacterial phytases are similar in the similar range. Phytases from *O. proteus*, *E. carotovora*, *E.*

coli and *Y. intermedia* were optimally active at 40-50, 40, 65, and 55°C, respectively. Although, rAppA$_E$ and rAppA$_p$ didn't differ in their thermostability in the temperature range 10 to 50°C, rAppA$_P$ was more thermotolerant at higher temperature. The activity of rAppA$_P$ was 33 and 24 % higher than that of rAppA$_E$ at 60 and 70°C, respectively. The increased thermotolerance of rAppA$_P$ may be due to its glycosylaion. Glycosylaion is one of the most important post-translational modification affecting protein function and properties (Maldonado *et al.* 2014). N-glycosylation is reported to influence biochemical properties of proteins, such as molecular mass, isoelectric point, surface charge distribution and thermotolerance (Guo *et al.* 2008). Hyperglcosylated phytase from *A. fumigatus* expressed in *P. pastoris* retained 40 and 30% of activities at 80 and 90°C for 10 min. However, thermostability property of deglycosylated phytase reduced significantly upon *in vitro* deglycosylation of the protein (Stahl *et al.* 2003). Similarly, phytase from *C. braakii* expressed in *S. cerevisiae* retained 50% higher activity upon heat treatment at 70°C for 30 min as compared to *E.coli* expressed protein (Kim *et al.* 2006). K_m values for phytate were 0.18 and 0.22 mM for the rAppA$_E$ and rAppA$_P$, respectively. The phytase AppA$_S$ identified in this study has K_m value for phytate less than that of phytase from *E. carotovora* (0.25 mM), *E. coli* (0.55 mM), *K. pneumoniae* (0.28 mM) and *O. proteus* (0.34 mM) but higher than that of phytase from *Y. intermedia* (0.125 mM) (Sajidan *et al.* 2004, Luo *et al.* 2004, Zinin *et al.* 2004, Huang *et al.* 2006a, Huang *et al.* 2009). The difference in K_m for phytate of rAppA$_E$ and rAppA$_P$ might be due to glycosylation of the later, however, there are very few reports on effect of glycosylation on K_m. Recently, Yao *et al.* (2013) reported an alteration in K_m of recombinant *E.coli* AppA phytase on enhancement of glycosylation. The K_m values for WT, Q258N mutant and Q258N/Q349N mutant were 0.48, 0.53 and 0.43 mM, respectively. Both the rAppA$_E$ and rAppA$_P$ were highly specific to the substrate, sodium phytate. Activity with either of phosphorylated substrates, such as ATP, ADP, pNPP, G6P or F6P was almost negligible. As in the present study, phytase AppA from *E. carotovora* and *Y. intermedia* was highly specific to phytate, whereas enzyme from *E.coli* and *O. proteus* also cleaved P-containing organic compounds other than phytate at a slower rate (Luo *et al.* 2004, Zinin *et al.* 2004, Huang *et al.* 2006a, Huang *et al.* 2009). In contrast, phytases from *A. fumigatus* and *K. pneumoniae* showed a broad specificity for phosphorylated substrates but relatively low specificity to phytate (Ullah *et al.* 2000, Xu *et al.* 2012). Analysis of the effect of various metal ions on phytase activities revealed that Ca^{2+}, Mg^{2+}, and Mn^{2+} had a stimulatory effect, as higher relative phytase activity (100-130 %) was detected in presence of these ions. On the other hand, Cu^{2+}, Fe^{2+}, Zn^{2+} showed inhibitory effect toward the recombinant phytases. The stimulatory effect of Ca^{2+}, Mg^{2+} and inhibitory effect of Fe^{2+} and Zn^{2+} have been reported earlier in case of AppA phytase from *Y. intermedia* and *E. coli* (Huang *et al.* 2006a, Tai *et al.*

2013). Typically $MgSO_4$ has been used as stabilizer of phytase in feed industry (Heafner *et al.* 2005). 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 incubation. The purified recombinant *E. coli* phytase was resistant to trypsin as most of the phytase activity remained even after 2 h of incubation at 37°C (Tai *et al.* 2013). On the contrary, the enzymes from *A. japonicas* and *A. niger* were found to be sensitive to trypsin, but were resistant to pepsin (Promdonkoy *et al.* 2009).

Glycosylation is a major factor of concern in case of expression in a eukaryotic system like *P. pastoris*. In eukaryotes, most of extracellular and membrane-associated proteins are glycosylated to some extent, though the role of the glycosylation is still remained unclear. Vasudevan *et al.* (2011) attempted expression of *A. niger* phytase *PhyA* in *E. coli* system where the inactive recombinant *phyA* expressed in *E. coli* cytosol supported the fact that glycosylation and proper disulfide bonds are mandatory for producing a functionally active fungal phytase. Han and Lei (1999) showed that glycosylation had considerable effect on the biosynthesis of *A. niger* phytase expressed in *P. pastoris* and also on thermostability of the expressed enzyme, as deglycosylation of the secreted phytase resulted in reduction in the size from 95 to 55 kDa and in thermostability by 34 %. In another study, Casey *et al*. (2003) showed the importance of glycosylation in preventing proteolytic inactivation of extracellular phytase produced by *A. niger*. Glycosylation was also found to improve the resistance of phytase to SDS mediated denaturation (Bagger *et al*. 2007). Researchers have also created *E. coli* AppA phytase mutants with elevated glycosylation with improved thermostability as compared with the wild-type enzyme (Yao *et al*. 2013). However, few studies have also indicated ineffectiveness of glycosylation on improving the thermostability, protein folding, or specific activity among phytases expressed in *A. niger*, *Hansenula polymorpha*, and *Saccharomyces cerevisiae* (Wyss *et al.* 1999). Furthermore, increased levels of glycosylation have been even shown to reduce thermostability of phytase (Fierobe *et al.* 1997). Thus, effects of glycosylation on phytase thermostability cannot be simply generalized.

To be used as an effective monogastric feed additive, a phytase needs to be stable at acidic pH and physiological temperature, which prevail in the gut of swine and poultry; for example, temperature of monogastric gut generally ranges from 39 to 44°C (for pig and turkey 39-40°C, and for chicken 40-44°C) (Vasudevan *et al.* 2011). 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) (Golovan *et al.* 2001). Hence, the study indicates suitability of *Shigella* sp. CD2 phytase as

feed additive for monogastric animals with acidic pH and physiological temperature prevailing in their digestive tract. The two recombinant enzymes showed similar molecular mass, pH optimum and profile, temperature optimum and profile, and phytate-phosphate hydrolysis efficiency. The lack of significant impact of the expression systems on phytase property offers flexibility for the selection of phytase fermentation systems. But the higher thermostability of the hyperglycosylated form of the recombinant enzyme has a clear industrial advantage over its non-glycosylated counterpart produced in the bacterial host. Besides, this study also showed distinct paths for future applications of the $appA_S$ gene as it could be used for economical production of an extracellular phytase with high activity under optimized conditions. It can also be used to compute the modifications of the enzyme in order to enhance its industrial potential using rational or semi-rational sequence comparison methods.

2.4. REFERENCES

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