

Phytase Production from a Novel *Klebsiella* sp. on Wheat Bran for Animal Feed Digestion

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Abstract

Background. The present study was aimed to isolate phytase producing bacteria and optimize the physico-chemical parameters of their phytase production.

Materials and methods. Four bacterial isolates (Phs4, Phs5, Phs6, and Phs8), based on clear zone formation on phytase screening medium, were selected and tested for finding out the highest phytase producing strain. The production of phytase was then optimized and its biochemical properties were determined to judge the applicability of phytase as a digestive aid in animal feed. **Results.** The 4 bacterial isolates (Phs4, Phs5, Phs6 and Phs8) were identified by morphological, cultural, biochemical and molecular characterization as *Burkholderia cepacia*, *Escherichia coli*, *Klebsiella pneumoniae* and *Klebsiella* sp. respectively. Of these isolates, Phs8 (*Klebsiella* sp.) was found to produce maximum phytase in shake culture in a basal medium containing Na-phytate at 37°C and pH 5.5 after 72 hours of incubation. The omission of Na-phytate from the medium almost completely abolished the phytase production capacity of the isolate and thus signified its important role as an inducer. Among the different complex carbon sources, viz., glucose, wheat bran, rice bran and chickpea, maximum phytase production (94 unit/ml) was obtained with wheat bran under comparable cultivation conditions. The phytase works best at a

temperature of 37°C and pH of 4.0 with a wide temperature stability (more than 80% activity up to 80°C) and wide pH stability (more than 80% activity within a range of pH 3-8). Although Zn²⁺, Co²⁺, and Fe²⁺ slightly increased the phytase activity Cu²⁺ and Mg²⁺ strongly inhibited the enzyme. **Conclusion.** The present findings will be very useful for the development of a bioprocess of the enzyme for its large-scale production at the pilot and finally at the commercial level.

Keywords: Phytase; animal feed; *Klebsiella* sp.; enzyme activity.

Abbreviations: PSM, phytase screening medium; PPM, phytase production medium; LB, Luria-Bertani; RPM, rotation per minute; PCR, polymerase chain reaction; dNTPs, deoxyribonucleotides; EDTA, ethylenediaminetetraacetic acid; EtBr, ethidium bromide; KIA, Kligler iron agar; MIU, motility indole urease test; VP, Voges-Proskauer test.

1. Introduction

Phosphorus is one of the most important minerals in animal nutrition and essential constituent of life (Hegsted 1968; Maathuis, 2009). Although phosphorus can be obtained from many sources, phytic acid is the major storage form typically found in cereals, legumes and oil seeds- the main components of animal feed. In fact, most of plant origin food contain 50- 80% of their phosphorus in the form of phytic acid (Harland & Morris, 1995). Phytic acid has widely been known for its anti-nutritional activities which chelate the micronutrients like calcium, magnesium, manganese, sodium, zinc, iron etc., certain proteins and few enzymes and prevents their bioavailability for monogastric animals as they lack the enzyme needed for digestion of phytic acid (Badone, Amelotti, Cassani, & Pilu, 2012; Gupta, Gangoliya,

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Significance | Economic production of bacterial phytase as a poultry feed supplement

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& Singh, 2015; J Kerovuo & Tynkkynen, 2000; Selle, Cowieson, Cowieson, & Ravindran, 2012).

Phytases are a special class of phosphatases that catalyze the formation of less phosphorylated myo-inositol derivatives and inorganic phosphate by sequential hydrolysis of myo-inositol-(1,2,3,4,5,6)-hexakisphosphate or phytic acid (InsP6) (Haros, Bielecka, Honke, & Sanz, 2007). Monogastric animals including poultry lack adequate level of phytase enzyme in their digestive tracts and thus can't hydrolyze the phytic acid present in the feed (Singh, Kunze, & Satyanarayana, 2011). As a result, almost 70% of phosphate is released in their excreta as manure and subsequent runoff can cause severe eutrophication that may become a threat to the aquatic lives (Jorquera, MARTÍNEZ, Maruyama, Marschner, & de la Luz Mora, 2008). Therefore, inorganic phosphate is supplemented with the feed to ensure the proper growth of the animals. However, this doesn't diminish the anti-nutritive value of the phytic acid present in the feed and also increase the overall cost of feed production. Thus, as alternative, supplementation of phytase in animal feed has long been practiced and found effective both to enhance the nutritional value of plant material by liberating the phosphate from proper digestion of phytic acid and reduce the phosphate pollution (Mullaney & Ullah, 2003; Musapuor, Afsharmanesh, & Shahrabak, 2006). Microbial phytase is considered most promising for commercial production, though phytase has also been derived from several other sources like plant and animals (Gontia, Tantwai, Rajput, & Tiwari, 2012; Haros et al., 2007; Hill, Kysela, & Elimelech, 2007; Pandey, Szakacs, Soccol, Rodriguez-Leon, & Soccol, 2001). Most of the scientific work has been done on those originating from filamentous fungi such as *Aspergillus niger* (Soni & Khire, 2007), *Rhizopus oligosporus* and *Cladosporium* species (Quan, Tian, Fan, & Kikuchi, 2004). Other than these species, phytase from bacterial sources such as *Escherichia coli* (Rt Greiner, Konietzny, & Jany, 1993), *Pseudomonas* sp. (Cho et al., 2003), *Klebsiella* sp. (Ralf Greiner & Carlsson, 2006) and *Bacillus* sp. (Janne Kerovuo, Lauraeus, Nurminen, Kalkkinen, & Apajalahti, 1998) were also identified. Recent studies suggest that bacterial phytases have a high potential for use as feed additives due to their biochemical properties like stability at low pH and high temperature, increase affinity for phytate etc. (Elkhalil, Männer, Borriss, & Simon, 2007; Jones, 2013).

The present study is aimed to isolate the phytase producing bacteria from different sources like paddy field, wheat field, corn field, poultry soil, poultry feces and rhizosphere soil and optimize the cultural conditions of the most potential isolate, i.e., *Klebsiella* sp. for enhanced production of phytase.

2. Materials and Methods

2.1 Screening and identification of phytase producing bacteria

Soil samples were collected from different prominent habitats of

phytase producing bacteria such as wheat field, corn field, rice field, poultry farm and rhizosphere soil. From the each collected sample, 1g of soil was suspended in 0.85% saline and diluted. Diluted samples (10^3 or 10^4) were then spread onto the PSM plates (Glucose 15g/L, $(\text{NH}_4)_2\text{SO}_4$ 5g/L, KCl 0.5g/L, MgSO_4 0.1g/L, NaCl 0.1g/L, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1g/L, FeSO_4 0.01g/L, MnSO_4 0.01g/L, Na-Phytate 5g/L, Agar 15g/L) and incubated at 37°C for 1-4 days and observed for the clear zones of hydrolysis around the colonies which gave an indication of extracellular phytase production (Bae, Yanke, Cheng, & Selinger, 1999). Then selected colonies were grown in 50 ml liquid medium (Phytase production medium-glucose 15.0g/L, tryptone 1.0g/L, Na-phytate 1.0g/L, CaCl_2 0.3g/L, MgSO_4 0.5g/L, MnSO_4 0.04g/L, and FeSO_4 0.0025g/L at pH 6.5) (Aziz et al., 2015). Isolates were first inoculated in LB broth for overnight and then re-inoculated (3% inoculum) in the phytase production media. Culture was incubated at 37°C for 1-3 days at 150 rpm. Crude enzyme was harvested by centrifugation at 6000 rpm for 10 minutes at 4°C and the clear supernatant was used as the source of extracellular phytases and for enzymatic assay. Bacterial isolates were subjected to a series of biochemical tests (Cappuccino & Sherman, 2008) and 16s rDNA sequencing for identification.

For the molecular identification of the isolates, amplification of 16s rDNA conserved sequence (amplicon size 1465 bp) by PCR was done using 27 forward (5'-AGAGTTTGATCMTGGCT-CAG-3') and 1492 reverse (5'-CGGTTACCTGTGTACGACTT-3') primers under standard conditions. The PCR reaction contain 10 µl master mix (Taq polymerase, dNTPs, MgCl_2 , 10X buffer and PCR grade water), 7 µl PCR grade water, 1 µl of template and 1 µl of each primer in 20 µl reaction volume. The PCR products were checked by horizontal electrophoresis in 1.5% agarose slab gel in Tris-borate EDTA (TBE) buffer and the EtBr stained DNA bands were then observed on a UV transilluminator (Gel Doc, BioRad, USA).

The purified PCR products were sequenced by chain termination method in DNA Solution Laboratory, Dhaka, Bangladesh. The partial 16S rDNA gene sequences of studied bacteria were then analyzed with nucleotide BLAST search in GenBank (NCBI) to generate a table of similar organisms for each test bacterium. FASTA format of the sequences obtained from these organisms were checked for multiple sequence alignment. The phylogenetic trees for each bacterium were generated using the Neighbor-Joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The rate

variation among sites was modeled with a gamma distribution (shape parameter = 0.05). Evolutionary analyses were conducted in MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018).

2.2 Enzymatic assay

The phytase activity was determined by modified Bea et al. (Bae et al., 1999) method. 300 μ l of the enzyme solution was incubated with 1.2 ml of substrate solution [0.2%(w/v) sodium phytate (Sigma, St Louis, MO, USA) in 0.1 M sodium acetate buffer, pH 5.0] for 30 minutes at 37°C and then 1.5 ml of 10% (w/v) trichloroacetic acid was added to stop the reaction. From this mixture, 1.5 ml was transferred to a new test tube and mixed with 1.5 ml ammonium molybdate ferrous sulfate mixture, which had been prepared by mixing 4 volumes of 1.5% (w/v) ammonium molybdate (Merck, Germany) in 5.5% sulfuric acid (Merck, Germany) with 1 volume of 2.7% (w/v) ferrous sulfate solution. The inorganic phosphate, liberated from Na-phytate by the action of phytase, reacts with ammonium molybdate ferrous sulfate mixture to produce a colored compound phosphomolybdate which was measured spectrophotometrically at 700 nm. One enzyme unit (IU) was defined as the amount of enzyme liberating 1 μ mol of inorganic phosphate in 1 minute under the assay conditions.

2.3 Effect of different carbon sources on phytase production

Effect of different carbon sources on phytase production was observed by preparing the production medium with different carbon source. Wheat bran, rice bran or chickpea (1% w/v) was used as carbon sole source as alternative to glucose (0.3% w/v) with the original basal medium. The flasks were incubated at 37°C at 150 rpm for 72 hours. The phytase activity was measured at 24 hours of interval.

2.4 Effect of Na-phytate concentrations on phytase production

Two different concentrations of Na-phytate (0.03g/L and 0.05g/L) were used with wheat bran, as carbon source, and other mineral salts. Conical flasks with 50ml medium were inoculated with actively growing culture and were incubated at 37°C at 150 rpm for 72 hours.

2.5 Effect of pH on phytase production

The effect of pH in culture medium containing wheat bran (best carbon source) on the production of phytase was studied at pH value of 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5. pH was adjusted in production medium by adding 0.1M NaOH/0.1M HCl. The culture medium in the flasks were inoculated with actively growing culture and incubated at 37°C at 150 rpm for 72 hours.

2.6 Effect of temperatures on phytase production

The production of phytase was carried out with the different temperature at 30, 37, 40, 45 and 50°C by keeping the agitator (revolutionary speed) at 150 rpm, and pH at 5.5 for 72 hours. The

crude enzyme extract was separated by centrifugation at 6,000 rpm for 10 minutes at 4°C. The supernatant was then used to assay phytase activity.

2.7 Biochemical characterization of phytase

Effect of temperature and pH on phytase assay

The activity of crude enzyme was determined by incubating the enzyme with 0.2% (w/v) Na-phytate as a substrate at various temperatures ranged from 25 to 55°C for 30 minutes and optimal pH of crude phytase was determined by incubating in different buffer solutions (Glycine-HCl buffer pH 2~3, Na-acetate-acetate buffer pH 4~6 and Tris-HCl, pH 7.0~9;) at 37°C for 30 minutes.

Stability of enzyme at different pH and temperatures

Stability of the enzyme was studied at different pH and temperatures by pre-incubation (with or without 5mM CaCl₂) with buffer. The enzyme was maintained at different pH (2~9) and different temperatures from 40 to 100°C in water bath for 1 hour. The residual phytase activity in the samples at different intervals was assayed using sodium phytate as substrate.

Effect of various metal ions on phytase activity

Impact of various metal ions (Ca²⁺, Co²⁺, Cu²⁺, Mg²⁺, Fe²⁺, Na⁺ and Zn²⁺) was investigated by pre-incubating the phytase enzyme at room temperature in a specified ion (5mM final concentration) containing buffer solution. After 1 hour of incubation, substrate (0.2% Na-phytate) was added and residual activity of the enzyme was measured under standard assay conditions (untreated enzyme was taken as control).

2.8 Statistical analysis

All enzymatic assay (Unit/ml) was carried out in triplicate and data presented as Mean \pm SD.

3. Results

Four phytase producing bacterial isolates were isolated from soil samples. Among them, *Klebsiella* sp. had revealed as the highest phytate hydrolyzer on wheat bran under comparable conditions employed. The effect of different carbon sources, temperatures and pH for maximum production of enzyme were investigated. The properties of crude enzyme were also characterized.

3.1 Isolation and identification of the phytase producing bacteria

Each soil sample was diluted suitably and plated on phytase screening medium. After 72 hours of incubation at 37°C, nine isolates were selected based on the clear zone formed upon Na-phytate hydrolyzation around the colonies. Of them, four isolates (named as Phs4, Phs5, Phs6 and Phs8) were found producing larger clear zone (1.4mm, 1.1mm, 1.6mm and 2.5mm) and identified as *Burkholderia cepacia*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Klebsiella* sp. respectively by microscopic, cultural and biochemical tests (Table 1) and 16S rDNA gene sequence analysis (result shown for *Klebsiella* sp. in Figure 1). Both solid and liquid screening processes were employed to deter-

Table 1 | Summary of different biochemical tests.

Biochemical Tests		Isolate 1 (Phs4)	Isolate 2 (Phs5)	Isolate 3 (Phs6)	Isolate 4 (Phs8)
Oxidase test		+	-	-	-
Catalase test		+	+	+	+
Indole test		-	+	-	-
Citrate test		+	+	+	+
KIA		+	+	+	+
MIU		+	-	+	+
Methyl red		-	+	+	+
VP		-	-	-	-
Nitrate reduction		-	+	+	+
Starch Hydrolysis		-	-	-	-
Acid Fermentation	Dextrose	A ⁺ , G ⁺			
	Sucrose	A ⁻ , G ⁻	A ⁺ , G ⁺	A ⁺ , G ⁺	A ⁺ , G ⁺
	Lactose	A ⁻ , G ⁻	A ⁺ , G ⁺	A ⁺ , G ⁺	A ⁺ , G ⁺

‘+’= Positive; ‘-’= Negative; ‘A+’= Acid produced; ‘A-’= Acid not produced; ‘G+’= Gas produced; ‘G-’=Gas not produced.

mine the ability of the isolated strains to produce phytase. Among the four isolated bacterial strains, *Klebsiella* sp. was found to produce maximum phytase on phytase production medium and thus selected for further analysis (Figure 2).

3.2 Effect of different carbon sources on phytase production by *Klebsiella* sp.

Result showed that highest phytase activity (94 unit/ml) was found in medium containing wheat bran as a carbon source after 72 hours of incubation (Figure 3). Other two carbon sources, rice bran and chickpea, showed enzyme activity of 58 and 14 unit/ml respectively. Whereas, 44 unit/ml activity was found using glucose as a carbon source. Hence, the following studies were done using basal medium containing wheat bran as a carbon source. On the other hand, ability of the Na-phytate as inducer of phytase production by the bacterium became prominent from the

fact that, the enzyme activity in all carbon sources was almost abolished without Na-phytate.

3.3 Optimization of Na-phytate concentration for phytase production by *Klebsiella* sp.

Two different concentrations of Na-phytate was used for phytase production in phytase production medium containing wheat bran as carbon source under different cultivation hours. Among them, 0.5% Na-phytate showed better activity (94 unit/ml) as compared that of 0.3% (32 unit/ml) after 72 hours of incubation (Figure 4). Further increase of Na-phytate might increase the enzyme activity but needs investigation.

3.4 Effect of pH on phytase production

Phytase production was assayed in a wide range of pH (from 4.5 to 7.5) to determine the effect of pH on the enzyme production. Figure 5 showed that the optimum pH of phytase production for

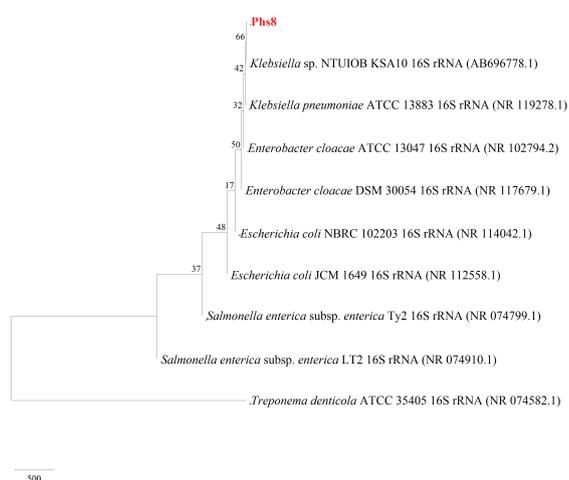


Figure 1 | Phylogenetic tree analysis of Phs8 isolate with aligned sequences.

Klebsiella sp. was 5.5 after 72 hours of incubation at 37°C and 150 rpm. The decrease in enzyme production was observed by both increasing and decreasing the pH value from 5.5. The relative enzyme activity was found at pH 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 as 74, 81, 96, 64, 54, and 44 % as compared to the highest activity at pH 5.5. Activity at pH 5.5 was taken as 100%.

3.5 Effect of temperatures on phytase production

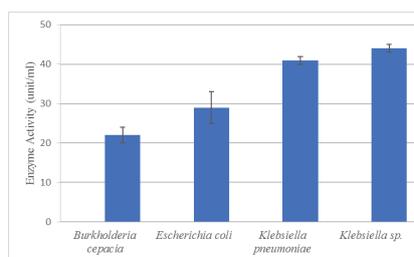


Figure 2 | Quantitative screening of the isolated bacteria for phytase activity.

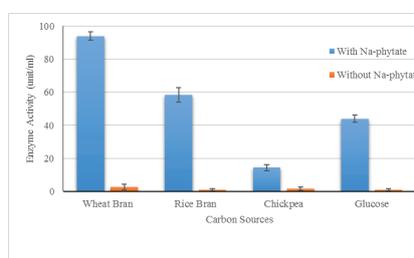


Figure 3 | Effect of different carbon sources on phytase production by *Klebsiella* sp. (Phs8).

The wheat bran with basal component medium was inoculated with 3% inoculum and incubated at various temperatures ranging from 30-50°C at 150 rpm for 72 hours. It is evident from the results that maximum phytase production was observed at 37°C after 72 hours of incubation. As the temperature increased above 37°C, the relative enzyme activity decreased gradually to 94, 83 and 75 % at 40, 45 and 50°C respectively. Similar result found with

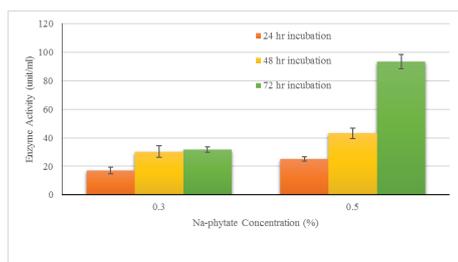


Figure 4 | **Optimization of Na-phytate concentrations for phytase production.**

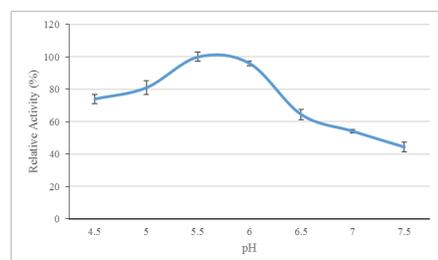


Figure 5 | **Effect of pH on production of phytase by *Klebsiella* sp.**

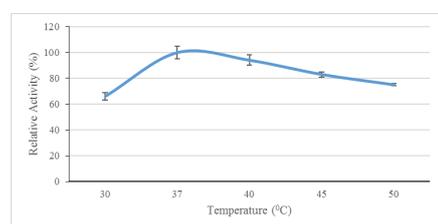


Figure 6 | **Effect of temperatures on phytase production by *Klebsiella* sp.**

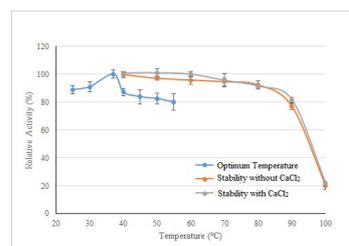


Figure 7 | **Optimum temperature and thermostability of phytase from *Klebsiella* sp.**

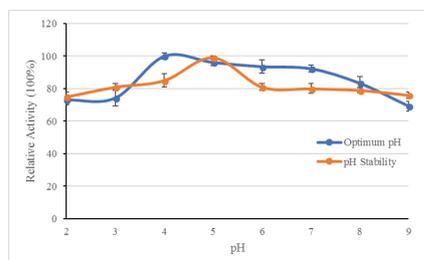


Figure 8 | **Optimum pH and pH stability of phytase from *Klebsiella* sp.**

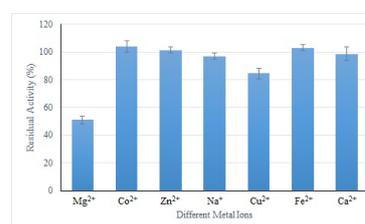


Figure 9 | **Effect of different metal ions on enzyme activity.**

decrease in temperature from the optimum value to 30°C with relative activity 66% (Figure 6). The enzyme activity at optimum temperature i.e., at 37°C was taken as 100%.

3.6 Biochemical characterization of phytase

Optimum temperature for phytase activity

The phytase activities were assayed at different temperatures ranging from 25–55°C to examine the effect of temperature on its function. Enzyme activity increased within temperature range of 25–37°C and a maximum increase was found at 37°C for crude enzyme, then it started to decrease from 40°C onward and became least at 55°C (Figure 7). The enzyme however retained 80% of its highest activity at 55°C. The enzyme activity at optimum temperature was taken as 100%.

Thermo-stability of the enzyme produced by *Klebsiella* sp.

Presence of Ca²⁺ ion may enhance the enzyme stability due to their ability to act as a co-factor for the enzyme. The reaction mixtures of crude enzyme were incubated at various temperatures ranging from 40–100°C for 1 hour in absence and presence of 5mM of CaCl₂. After incubation, the residual activity was measured under standard assay conditions. Activity of control, without any temperature incubation, was taken as 100%. Although at 40–80°C no or slight decrease in enzyme activity was found in absence of CaCl₂ a marked decrease in activity was found with further increase in temperature from 90–100°C with residual activities as 77 and 20% respectively as compared to the control. Presence of CaCl₂, however, resulted in non-significant higher residual activities at each respective temperature value (Figure 7).

Optimum pH and stability of phytase activity

A broad pH range of 2–9 was examined to study the effect of pH on the enzyme activity (Figure 8). The enzyme was found to be stable over broad pH range with optimum activity at pH 4 which indicates the acidic nature of the enzyme. A decrease in the enzyme activity was found in further acidic pH. On the other hand, a gradual but slight decrease in activity was found with increase in pH from 4.0 to pH 8.0 suggesting a broad stability range (80 to 100% residual activity) of the enzyme. The stability of the enzyme on different pH was checked by incubating it at various buffers of pH range 2–9 for 1 hour. After incubation, the residual activity was assayed and the results revealed the maximum residual activity as 99% at pH 5.0 as compared to the control and marked increase was found by increasing the pH from 2–5 with residual activities as 75, 81, 85 and 99% of its original activity respectively. Whereas, marked drop was found at pH 6–9 as 81, 80, 79 and 76% of its original activity, respectively (Figure 8). Such result indicated the stability of the phytase over wide pH range particularly in the range of pH 3–8.

Effect of different metal ions on phytase activity

The effect of various metal ions at 5mM final concentration was studied following pre-incubation with crude enzyme for 1 hour at room temperature and the residual activity was assayed. Most of the metal ions tested had a stimulatory effect like Zn²⁺, Co²⁺ and Fe²⁺ with relative activities of 101, 104 and 104% respectively, whereas Cu²⁺ was found moderately inhibitory (85%).

Ca²⁺(99%) and Na⁺(97%) had almost no effect on the enzyme activity. While Mg²⁺ had exerted almost 50% inhibitory effect on enzyme as compared to the control (Figure 9).

4. Discussion

In the present study, samples were collected from habitat like paddy field, wheat field, corn field, poultry soil, poultry feces and rhizosphere soil, which are believed to be rich source of phytase producing bacteria, to isolate the phytate degrading bacteria. Although nine isolates, from 19 soil samples, were found to have phytase activity only four isolates showed significant results. These four isolates were named as Phs4, Phs5, Phs6 & Phs8. The isolates Phs4, Phs5, Phs6, and Phs8 were identified as *Burkholderia cepacia*, *Escherichia coli*, *Klebsiella pneumoniae* and *Klebsiella* sp. respectively by morphological, cultural, biochemical characteristics, and 16s rDNA PCR followed by phylogenetic tree analysis. Among these four-bacterial strains, *Klebsiella* sp. (Phs8) was found to produce maximum enzyme activity (44 unit/ml) in phytase producing medium. Several researchers including Aziz et al., Wang et al., Escobin-Moopera et al., and Unno et al. found the phytase activity from similar bacteria in different studies (Aziz et al., 2015; Escobin-Mopera et al., 2012; Unno, Okubo, Wasaki, Shinano, & Osaki, 2005; Wang et al., 2004).

For an organism, to grow and to produce desired metabolites there is the need of energy and physical support which provided by the substrates (Pandey et al., 2001; Spier et al., 2008). The primarily selected medium (PPM) was taken as a basal medium and different parameters of culture such as carbon source, temperature and pH were studied (Aziz et al., 2015). Several carbon sources including glucose, wheat bran, rice bran and chickpea were used in basal medium separately, keeping the other component unchanged, to check their ability in phytase production. Use of alternative carbon sources like wheat bran, rice bran and chickpea not only reduced the cost of production but also increased the enzyme activity, though not by all carbon sources. Particularly, the phytase activity with wheat bran was found more than two times higher than that of with glucose. This is due to the fact that wheat bran contain phytic acid (3-6% of the dry weight) complexed with many minerals and serve as additional phytate source and thereby more phytase activity (Bilgiçli & İbanoğlu, 2007). This study was in harmony with Mittal et al. (Mittal, Singh, Goyal, Yadav, & Aggarwal, 2012) who found wheat bran and orange peel flour as the highest phytase producing carbon sources by *Klebsiella* sp. Addition of Na-phytate (purest form) have an inducing effect on production of phytase. The importance of Na-phytate became clear from the fact that, absence of Na-phytate from the basal medium nearly abolished the phytase activity for all carbon sources used in our experiment.

While the optimal temperature for production of phytases from various sources varies from 30 to 80°C (Wang et al., 2004), the optimum temperature of phytase production was observed at

37°C from *Klebsiella* sp. (isolate Phs8). The maximum production of phytase by *Klebsiella* sp. at 37°C temperature has also been reported by several other researchers (Aziz et al., 2015; Wang et al., 2004). Another most important factor is pH of medium for enzyme production. The maximum phytase production was observed at pH 5.5. And further increase in pH reduced the growth and enzyme production. Maximum phytase production by *Klebsiella pneumoniae* SCTb2 was reported at pH 6.0 by Das et al. (Das, Bandyopadhyay, & Sen, 2013).

The biochemical properties of *Klebsiella* sp. phytase have much similarities with other bacterial and fungal phytases. The cell free supernatant of culture has the phytase activity that exhibited temperature optima at 37°C (Wang et al., 2004) and stable at wide range (up to 80°C) of temperature. Phytase from some other microbes has also been found to function best at 37°C (Aly, Tork, Al-Garni, & Kabli, 2015; El-Toukhy, Youssef, & Mikhail, 2013). As the main intended application of phytase is the digestive aid in animal feed, it needs to be thermotolerant, so that the enzyme can withstand high temperature during pelleting process of food especially for monogastric animals (Arpana et al., 2011). At least 80% residual activity of phytase was found over broad pH range (pH 3-8) with highest activity at pH 4.0 which indicates its acidic nature. Thus, the temperature and pH stability profile of the *Klebsiella* sp. phytase, which is similar to the conditions in the stomach of monogastric animals, made this an ideal candidate to be used as digestive aid in the animal feed of monogastric animals (Elkhalil et al., 2007; Escobin-Mopera et al., 2012; Sajidan et al., 2004). The catalytic activity of the phytase was not significantly affected by Ca²⁺ and Na⁺ at 5mM concentration. This implied that even in the presence of Ca²⁺ ion or Ca-phytate complex, the phytase retained its almost full activity (99%) (Escobin-Mopera et al., 2012). Other divalent cations like Zn²⁺, Co²⁺ and Fe²⁺ had a stimulatory effect on phytase. Phytase from *Klebsiella pneumoniae* subsp. *pneumoniae* XY-5 was found to be stimulated in presence of Zn²⁺ and Co²⁺ (Wang et al., 2004). On the other hand, Cu²⁺ showed moderate and Mg²⁺ showed strong inhibition profile to *Klebsiella* sp. phytase with reduction of 15% and 50% of the enzyme's initial activity respectively (Wang et al., 2004). Bacterial phytase, from *Klebsiella* sp. and as well from other bacterial species, was found to be strongly inhibited by Mg²⁺ (Escobin-Mopera et al., 2012; Hong, Chu, & Chung, 2011; Kalsi, Singh, Dhaliwal, & Kumar, 2016; Zhang et al., 2011).

5. Conclusion

Among the different environmental isolates, *Klebsiella* sp. (Phs8) exhibited the highest potentiality to produce the phytase enzyme. Based on the results, it has been found that *Klebsiella* sp. (Phs8) was able to enhance phytase production in cultures medium containing wheat bran as the carbon source, thus making the

production process cheaper. In addition, the acido-thermo-stability nature of the enzyme implied its potential applicability in feed industry as a digestive aid.

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Competing financial interest

The author(s) declare no competing financial interests.

Author contributions

DA designed and performed the experiments, MMK prepared the draft and final version of the manuscript, MMM collected the samples, SNK and MMH supervised the whole work and critically reviewed the manuscript.

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