Original article

Isolation and biochemical characterization of acido-thermophilic extracellular phytase producing bacterial strain for potential application in poultry feed

Arpana Mittal, MSc[1]
Gulab Singh, MSc[1]
Varsha Goyal, MSc[1]
Anita Yadav, PhD[2]
Kamal Rai Aneja, PhD[1]
Sanjeev Kumar Gautam, PhD[2]
Neeraj Kumar Aggarwal, PhD[1]

[1]Department of Microbiology, Kurukshetra University Kurukshetra, Haryana, India
[2]Department of Biotechnology, Kurukshetra University Kurukshetra, Haryana, India

Address for correspondence:
Dr. Neeraj Kumar Aggarwal, Department of Microbiology, Kurukshetra University Kurukshetra, Haryana 136119, India
Tel: +91 9416975023
Fax: +91 1744 238035
Email: neerajkuk26@rediffmail.com

How to cite this article:
Mittal A, Singh G, Goyal V, Yadav A, Aneja KR, Gautam SK, Aggarwal NK. Isolation and biochemical characterization of acido-thermophilic extracellular phytase producing bacterial strain for potential application in poultry feed. Jundishapur J Microbiol. 2011; 4(4): 273-282.

Received: December 2010
Accepted: May 2011

Abstract

Introduction and objective: Phytic acid, which is the main constituent of animal diet, is not digested by monogastric animals and hence, create problem in the availability of phosphorus in their diet. It also causes environmental pollution by extra supplemented phosphorus in animal's diet. Hence, acido-thermophilic phytase producer bacterial strain has been isolated in this study for its potential use in poultry feedings.

Material and methods: Samples for the screening of phytase producers were collected from different habitats. 1g of soil was inoculated in culture broth containing 2% (w/w) phytate organic substrate. The qualitative screening for phytase production was performed by agar plate containing sodium phytate followed by quantitative screening using shaking flask method. Phytase activity was determined and the selected isolate was biochemically characterized using the standard biochemical techniques.

Results: AR58 bacterial isolate, isolated from poultry field soil, showing significant extracellular phytase production was selected. After qualitative and quantitative screening, AR58 showed a hydrolytic zone of 42 mm diameter and 395 IU/ml phytase activity. AR58 was identified as *Klebsiella* sp. The enzyme had maximum activity at 55°C and pH range from 3.5 to 5.5.

Conclusion: Our finding suggests that acido-thermophilic phytase from *Klebsiella* sp. could have great potential for feed industries.

Significance and impact of the study: In the present study, phytase accumulation by the strain of *Klebsiella* sp. has significant values. Hence, this enzyme could find application in the animal feed industry for improving the nutritional status of feed as well as combating environmental pollution.

Keywords: Phytase; Acido-thermophilic; Monogastric animals; Qualitative screening; Quantitative screening

Introduction

Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakisdihydrogenphosphate) and mixed cation salts of phytic acid, designated as phytate, are a group of organic phosphorus (P) compounds found widely in nature especially in legumes, cereals, and oilseed crops which serve as a major source of nutrients for the animals. These crops have an important constituent of phytic acid whose salt form, phytate, is an anhydrous storage form of more than 80% of the total phosphorus in cereals and legumes. In terrestrial ecosystems, they are synthesized by plants, accumulate in seeds during the ripening period and are regarded as the primary storage form of myo-inositol (an important growth factor) [1] and phosphorus in grains [2] and in pollen [3].

The ruminants digest phytic acid with the help of phytases produced by their anaerobic ruminal micro flora. However, simple-stomached animals such as pig, poultry and fish are deficient in gastrointestinal tract phytases. So, in the context of human and animal nutrition, the following two aspects of phytic acid are critically important [4]. Monogastric animals have only low levels of phytate-degrading enzymes in their digestive tracts, and since phytic acid itself is not resorbed, feed for animals is supplemented with inorganic phosphorus to meet phosphorous requirement. Phytic acid is an antinutrient constituent in plant-derived food and feed, since it form complexes with proteins, amino acids [5] and a variety of metal ions such as calcium, magnesium, iron and zinc.

It forms complexes with these minerals because it posses a high phosphate content, which results in a high negative charge over a wide pH range. So, it chelates with positively charged divalent cations (Fig. 1), rendering a poor absorption of the bound metals in small intestine [6]. This is partially attributed to the wide-spreading human nutritional deficiencies of calcium, iron and zinc in developing countries where the staple foods are plant origin [7]. Phytases have been studied most intensively in the seeds of plants [8]. But they are also found in plants, microorganisms, and in some animal tissues [9]. The first commercial phytase products were launched into market in 1991 [10].

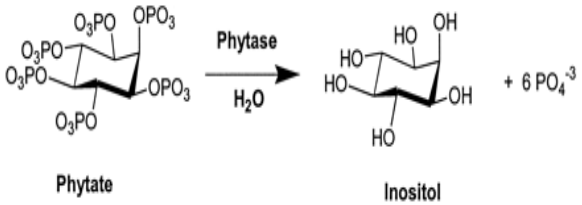

Fig. 1: Mechanism of phytate hydrolysis by phytase

Because of these problems, there is considerable interest in phytate degrading enzyme. A diverse class of enzymes, phosphatases, catalyzes the cleavage of monophosphoester bonds in various organo-phosphate compounds, but these enzymes are not capable of hydrolyzing the monophosphoester bonds in phytic acid. As the hydrolysis of phytic acid has great importance, a special class of enzymes hydrolyzing phytic acid has evolved the phytases (myo-inositol hexakisphosphate phosphohydrolases) which hydrolyze phytic acid to less phosphorylated myo-inositol derivatives (in some cases to free myo-inositol), releasing inorganic phosphate and other divalent elements (Fig. 1).

Shieh and Ware [11] first reported the isolation of phytase producing microorganisms using selective phytase screening medium. They prepared insoluble calcium phytate containing turbid screening media, which turned transparent, owing to solubilization, by diffused phytases from the isolates. After that, culture enrichment technique was used to isolate phytase-producing microorganisms. Phytases have

been detected in different bacteria (Gram-positive, Gram-negative rods and cocci) viz. *Aerobacter aerogenes* [12], *Escherichia coli* [13], *Klebsiella aerogenes* [14] and *Pseudomonas* sp. [15], although, phytase activity is detected in various fungi [16]. But various studies have confirmed *A. niger* strains to be the producers of extracellular phytase [17] and is most commonly employed for industrial purposes. Similarly, many species of yeast were reported as phytase producers i.e. *Saccharomyces cerevisiae, Candida tropicalis, Torulopsis candida, Debaryomyces castelli, Kluyveromyces fragilis, Schwanniomyces castelii, Pichia anamola, Arxula adeninivorans* [18-23].

Therefore, phytases are considered to be potential candidate for use as an enzyme that have great value in enhancing the nutritional quality of phytate-rich foods and feed [24,25]. Phytases are not only used as animal feed additive in diets for monogastric animals but there is great potential for the use of this class of enzymes in processing and manufacturing of food for human consumption [26]. In addition, phytase would be an eco-friendly product, reducing the amount of phosphorus entering the environment or problems resulted by eutrophication and constant chelating of nutrient factors from soil, as supplementation of phytase in the diets for monogastric animals reduces the fecal phosphate excretion up to 50%.

As stomach has acidic environment and feed pelleting process requires higher temperature i.e. 50°C. So, our study is based on the isolation of acido-thermophilus phytase producer bacterial strain with high phytase activity so that it can work with maximum potential in animal feed and in future can be exploited for direct inclusion in poultry feed.

Materials and methods

Qualitative screening of phytase producing bacterial strains

Samples for the screening of phytase producers were collected from different habitats such as the soils of poultry fields, cattle sheds, dairy wastes, garden soil, rotten fruits and vegetables, rhizospheric soil, cereals and pulses fields. For the enrichment of phytase producing microorganisms, 1g soil was suspended in 25mL sterile deionized water, pH 7.5 in three flasks containing different substrates @ 2% (w/v) (wheat bran, powdered orange peel, sugarcane bagasse) and incubated under stationary conditions for 72h at 50°C. Isolation of phytase producers was performed by the agar plate method of Quan *et al.* [27].

For primary screening, 100µl suspension of these flasks was plated onto a phytase screening turbid agar media plates (PSM) containing 1.5% glucose, 0.1% Na-phytate (Hi media, India), 0.2% NH_4NO_3, 0.05% KCl, 0.05% $MgSO_4.7H_2O$, 0.03% $MnSO_4$, 0.03% $FeSO_4.7H_2O$ and 2.0% agar (pH 7.5) and incubated at 50°C for 2-5 days. Bacterial isolates, capable of hydrolyzing Na-phytate which can be recognized by their surrounding clear halo, were selected and repeatedly streaked on nutrient agar plates. Hydrolytic zone was calculated by subtracting the diameter of zone of growth from diameter of total halo area [28].

Quantitative screening of phytase producing microorganisms

Each of the bacterial isolates was grown in 50ml of liquid medium containing 0.1 % sodium phytate, 1% peptone, 0.2% $(NH_4)_2SO_4$, 0.05% KCl, 0.05% $MgSO_4.7H_2O$, 0.03% $MnSO_4$, 0.03% $FeSO_4.7H_2O$, pH 7.5 in a 250ml flask and incubated at 50°C for three days on a rotary shaker (NSW- 256) at 200rpm. Crude

enzyme was harvested by centrifugation at 10,000g for 10mins at 4°C and the clear supernatant was used as the source of extracellular phytases. Sodium phytate was used as substrate for assaying the activity of phytase. Phytase activity was determined by measuring the amount of liberated inorganic phosphate. The reaction mixture consisted of sodium phytate (Sigma; 0.5% w/v) prepared in sodium acetate buffer (0.2 M, pH 5.5) and 0.2ml of supernatant. After incubation at 50°C for 30mins, the reaction was stopped by adding an equal volume of 15% trichloroacetic acid.

The liberated phosphate ions were quantified by mixing 100µl of assay mixture with 900µl of 1.0 M H_2SO_4- 10% ascorbic acid- 2.5% ammonium molybdate (3:1:0.1) (v/v) [29]. After 20mins of incubation at 50°C, absorbance was measured at 820nm. Control for the enzyme assay was run simultaneously that contained all the reagents but the reaction was terminated prior to the addition of heat inactivated enzyme [30]. One enzyme unit (IU) was defined as the amount of enzyme liberating 1µmol of inorganic phosphate in 1min under the assay conditions.

Biochemical characterization of selected culture

The bacterial isolates showing maximum extracellular phytase activity was selected for biochemical characterization following standard biochemical techniques [31-32]. Biochemical characterization is done under the two headings, morphological characterization and physiological characterization. Under the morphological characteristics, colony configuration, Gram's reaction and study of spore were followed while under the physiological test, growth of culture under different temperatures and pH requirement were studied. The selected isolate was further characterized biochemically following various biochemical test including indole production test, methyl red test, Voges proskauer test, citrate utilization, casein hydrolysis, gelatin hydrolysis, starch hydrolysis, urea hydrolysis, catalase test, oxidase test, Tween 20 hydrolysis, Tween 40 hydrolysis, Tween 80 hydrolysis, amylase production, hydrogen sulphide production and fermentation of carbohydrates (glucose, starch, lactose).

Results and discussion

This study was taken up in search of novel microbial phytases. The survey of recent literature suggests that many new thermophilic fungal sources of phytases e.g. *Thermoascus aurantiacus* and *Sporotrichum thermophile* [33,30] have been reported. Though, *A. niger* strains are known to be the best producers of extracellular phytases [17] and are most commonly employed for industrial purposes. Many phytase producing bacteria have also been isolated, screened and reported for their ability to produce phytases e.g. *Pseudomonas* sp. [34], *Bacillus* sp. [35], *E. coli* [36] and anaerobic rumen bacteria, particularly in *Mitsuokella jalaludinii* [37].

Traditionally the phytase producing strains were screened on a medium containing calcium phytate/sodium phytate. The medium is opaque and phytase producing colonies are picked on the basis of the clearing zones around the colonies. However, such producers also result in selection of false positive isolates. The clearing zones can also be formed by shift of pH towards acidic [17]. Therefore, secondary screening on liquid medium was employed to choose only the potential phytase producers [38].

This study reports the isolation and screening of novel phytase producing thermophilic/thermo tolerant strains. Total 70 bacterial putative extracellular phytase

producer isolated from different habitats soil were selected on the basis of clear halo zone on PSM and were tabulated (Table 1) on the basis of their habitat, zone of hydrolysis and enzyme activity in liquid medium. We totally studied thirteen habitats in our study and cattle shed or poultry field soil were found to be the most preferred habitat for isolation of phytase producing bacterial strain (Fig. 2).

Table 1: Qualitative and quantitative screening of different phytase producing isolates from different habitats

Isolates	Habitats	Diameter (mm) of zone of substrate hydrolysis	Enzyme activity (IU/ml)[a]
AR1	Cattle shed	21±1	155±1
AR2	Dairy wastes	10±1	69±1
AR3	Rotten orange	17±1	91±1
AR4	Rhizospheric soil	21±1	146±1
AR5	Poultry field	28±2	163±1
AR6	Wheat field	28±1	156±1
AR7	Rice field	19±1	132±1
AR8	Jowar field	13±1	84±1
AR9	Bajra field	12±1	78±1
AR10	Wheat field	31±1	170±1
AR11	Rotten pomegranate	28±1	160±1
AR12	Cattle shed	29±1	161±1
AR13	Dairy wastes	15±2	77±1
AR14	Rotten apples	23±1	152±1
AR15	Dairy wastes	19±1	100±1
AR16	Poultry fields	32±1	174±1
AR17	Garden soil	27±1	158±1
AR18	Garden soil	29±1	165±1
AR19	Cattle shed	34±1	183±1
AR20	Dairy waste	16±1	80±1
AR21	Bajra field	18±1	87±1
AR22	Poultry field	26±1	154±1
AR23	Rotten orange	25±1	149±1
AR24	Rhizospheric soil	23±1	134±1
AR25	Rice field	28±1	161±1
AR26	Jowar field	16±1	81±1
AR27	Garden soil	19±1	99±1
AR28	Rotten pomegranate	27±1	155±1
AR29	Poultry field	37±1	319±1
AR30	Cattle shed	31±1	281±1
AR31	Rhizospheric soil	38±1	321±1
AR32	Garden soil	30±2	277±1
AR33	Rice field	13±0.5	86±1
AR34	Wheat field	27±3	187±1
AR35	Poultry field	28±1	189±1
AR36	Cattle shed	20±1	108±1
AR37	Dairy waste	19±1	106±1

Table continued.

AR38	Chana field	17±1	98±1
AR39	Rhizospheric soil	26±1	181±1
AR40	Wheat field	35±1	311±1
AR41	Garden soil	22±1	168±1
AR42	Rhizospheric soil	25±1	181±1
AR43	Rice field	18±1	99±1
AR44	Chana field	15±1	95±1
AR45	Poultry field	30±1	278±1
AR46	Cattle shed	24±1	176±1
AR47	Bajra field	14±1	92±1
AR48	Dairy waste	11±3	81±1
AR49	Poultry field	40±1	390±1
AR50	Jowar field	10±1	80±1
AR51	Garden soil	09±1	76±1
AR52	Cattle shed	21±1	195±1
AR53	Chana field	08±1	75±1
AR54	Rhizospheric soil	23±0.25	173±1
AR55	Rice field	19±1	171±1
AR56	Cattle shed	21±1	193±1
AR57	Wheat field	31±1	298±1
AR58	**Poultry field**	**42±1**	**395±1**
AR59	Dairy waste	13±1	90±1
AR60	Garden soil	23±2	170±1
AR61	Rice field	27±1	185±1
AR62	Poultry field	35±1	310±1
AR63	Wheat field	37±1	316±1
AR64	Dairy waste	19±1	169±1
AR65	Cattle shed	29±1	276±1
AR66	Garden soil	20±1	102±1
AR67	Rice field	21±1	102±1
AR68	Rhizospheric soil	22±1	104±1
AR69	Wheat field	37±1	312±1
AR70	Poultry field	39±1	318±1

Results presented are the mean of three independent experiments with standard deviation values. [a] Under unoptimized conditions.

Out of seventy strains, eight strains i.e. AR29, AR31, AR40, AR58, AR62, AR63, AR69 and AR70 forming clear peripheral hydrolytic zones having a diameter of 37, 38, 35, 42, 35, 37, 37, 39 mm on turbid agar plates, were selected after the quantitative screening of isolates in phytate containing culture broth. The study of qualitative and quantitative screening for phytase activity clearly reflects that phytase activity is directly related to zone of hydrolysis i.e. more the zone more will be the enzyme activity. AR58 from poultry field having a hydrolytic zone of 42mm (Fig. 3) and showing phytase activity of 395 IU/ml was selected for biochemical characterization with the aim to exploit this strain for animal feed in poultry in future.

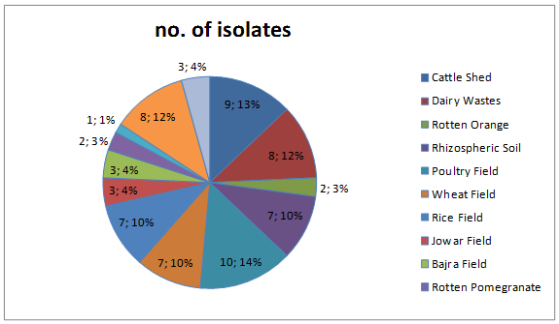

Fig. 2: Distribution of phytase producing bacterial strains in different habitats

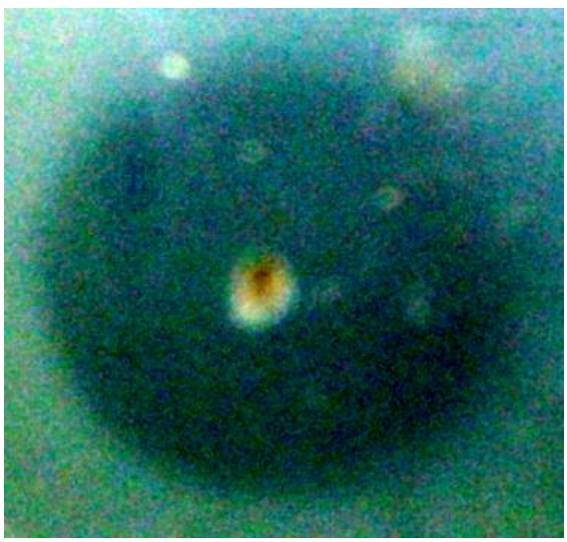

Fig. 3: Hydrolytic zone by AR58 on phytase screening turbid agar plate

One advantage of this strain is that it can produce maximum enzyme within a short period of cultivation (72h) as compared to reported different bacterial and fungal strains. This feature makes the strain a promising candidate for the production of phytase on a commercial scale. Temperature affects various metabolic processes such as protein denaturation, enzymatic inhibition, promotion or inhibition on production of a particular metabolite, cell death, etc. But for food digestion in stomach, enzymes have to be thermo tolerant so that it can withstand high temperature during pelleting process of food especially in monogastric animals. Similarly, the pH of the production medium also plays a significant role in the production of metabolites. *Klebsiella* sp. produces phytase in acidic medium at high temperature of 55°C. This feature makes the strain to be active in thermo-acidic environment occurring in stomach having acidic pH. Therefore, this enzyme can find application in animal feed industry for improving the nutritional status of feed.

Morphological characterization showed that AR58 was Gram's negative rods having gummy surface with entire margin. The results of biochemical analysis of the isolate AR58 was tabulated in Tables 2-4. AR58 was identified as *Klebsiella* sp. after biochemical characterization. So, in the present study, strain of *Klebsiella* sp. is capable of producing acido-thermophilic phytase which can be exploited for the feed of monogastric animals especially in poultry feedings to satisfy the phosphorus nutrition and combating the problem of environmental pollution.

Table 2: Morphological characteristics of bacterial phytase producing isolate AR58

Colony morphology	
Configuration	Circular
Margin	Convex with entire
Elevation	Slightly raised
Surface	Smooth, gummy
Pigment	Creamish
Opacity	Opaque
Gram's reaction- negative	
Cell shape	Rod
Size(µm)	1.5-3.0
Arrangement	Single & pairs
Spore(s)	
Endospore	+
Position	Terminal
Shape	Round
Motility	-

Table 3: Physiological characteristics of bacterial phytase producing isolate AR58

Growth at temperature	
Tests	Results
10°C	-
20°C	-
30°C	+
40°C	++
50°C	+++
55°C	+
60°C	-
65°C	-
70°C	-
Growth at pH	
pH 4.0	-
pH 4.5	-
pH 5.0	-
pH 5.5	+
pH 6.0	+
pH 6.5	++
pH 7.0	+++
pH 7.5	+
pH 8.0	-
Growth on NaCl (%)	
2.0	+
4.0	+
6.0	-
Anaerobic Growth	-

Table 4: Biochemical characteristics of bacterial phytase producing isolate AR58

Biochemical characteristics	
Tests	Results
Indole test	-
Methyl red test	-
Voges proskauer test	+
Citrate utilization	+
Casein hydrolysis	+
Gelatin hydrolysis	-
Starch hydrolysis	-
Urea hydrolysis	+
Catalase test	+
Oxidase test	-
Tween 20 hydrolysis	+
Tween 40 hydrolysis	+
Tween 80 hydrolysis	-
Amylase	-
H_2S production	-
Fermentation of carbohydrates	
Acid from glucose	+
Acid from lactose	+
Acid from sucrose	+
Gas from glucose	+
Gas from lactose	-
Gas from sucrose	+

Conclusion

Our finding suggests that phytase accumulation by the acido-thermophilic strain of *Klebsiella* sp. has significant values which can be exploited for industrial production of phytase. Moreover, this enzyme can be used in the animal feed industry for improving the nutritional status of feed and in combating environmental pollution.

Acknowledgement

Authors gratefully acknowledge the University Research Scholarship awarded to Arpana Mittal by Kurukshetra University, Kurukshetra.

Sources of funding: University Research Scholarship (URS) awarded to Arpana Mittal by Kurukshetra University, Kurukshetra.

References

1) Reddy NR, Sathe SK, Salunkhe DK. Phytates in legumes and cereals. *Adv Food Res*. 1982; 28: 1-92. PMID: 6299067
2) Turner BL, Paphazy MJ, Haygarth PM, Mckelvie ID. Inositol phosphates in the environment. *Philos T Roy Soc B.* 2002; 357: 449-69. PMID: 12028785
3) Hara A, Ebina S, Kondo A, Funaguma T. A new type of phytase from pollen of *Typha latifolia* L. *Agric Biol Chem*. 1985; 49: 3539-44.
4) Wodzinski RJ, Ullah AHJ. Phytase. *Appl Microbiol Biotechnol*. 1996; 42: 263-302. PMID: 8865587
5) Pallauf J, Rimbach G. Nutritional significance of phytic acid and phytase.

Arch Tierernahr. 1997; 50: 301-19. PMID: 9345595
6) Cheryan M. Phytic acid interactions in food systems. *CRC Crit Rev Food Sci Nutr*. 1980; 13: 297- 336. PMID: 7002470
7) Manary MJ, Krebs NF, Gibson RS, Broadhead RL, Hambridge KM. Community-based dietary phytate reduction and its effect on iron status in Malawian children. *Amm Trop Paediatr*. 2002; 22: 133-36. PMID: 12070948
8) Greiner R. Purification and characterization of three phytases from germinated lupine seeds (*Lupinus albus* var. Amiga). *J Agric Food Chem.* 2002; 50: 6858-64. PMID: 12405788
9) Konietzny U, Greiner R. Molecular and catalytic properties of phytate-degrading enzymes (phytases). *Int J Food Sci Technol*. 2002; 37: 791-812.
10) Haefner S, Knietsch A, Scholten E, Braun J, Lohscheidt M, Zelder O. Biotechnological production and applications of phytases. *Appl Microbiol Biotechnol*. 2005; 68: 588-97. PMID: 16041577
11) Shieh TR, Ware JH. Survey of microorganisms for the production of extracellular phytase. *Appl Environ Microbiol*. 1968; 16: 1348-51. PMID: 4300171
12) Yoon SJ, Choi YJ, Min HK, *et al*. Isolation and identification of phytase-producing bacterium, *Enterobacte*r sp. 4, and enzymatic properties of phytase enzyme. *Enz Microb Technol.* 1996; 18: 449-54.
13) Greiner R. Properties of phytate degrading enzymes from germinated lupine seeds (*Lupinus albus* var. Amiga.). Proceedings of the 4th European Conference on grain legumes. Cracow, Poland, 2000; 398-9. Paris, France: AEP.
14) Sajidan A, Farouk A, Greiner R, Jungblut P, Muller EC, Borriss R. Molecular and phycological characterization of a3-phytase from the bacterium *Klebsiella pneumonia* ASR1. *Appl Microbiol Biotechnol*. 2004; 65: 110-8. PMID: 14727093
15) Kim HW, Kim YO, Lee JH, Kim KK, Kim YJ. Isolation and characterization of a phytase with improved properties from *Citrobacter braakii*. *Biotechnol Lett.* 2003; 25: 1231-4. PMID: 14514072
16) Liu BL, Rafiq A, Tzeng YM, Rob A. The induction and characterization of phytase and beyond. *Enz Microb Technol*. 1998; 22: 415-24.
17) Gargova S, Sariyska M. Effect of culture conditions on the biosynthesis of *Aspergillus niger* phytase and acid phosphatases. *Enz Microb Technol*. 2003; 32: 231-5.
18) Graf E. Phytic acid: Chemistry and applications. Minneapolis, Pitlatus Press, 1986; 101-10.
19) Lambrechts C, Boze H, Moulin G, Galzy P. Utilization of phytate by some yeasts. *Biotechnol Lett*. 1992; 14: 61-6.
20) Segueilha L, Lambrechts CM, Boze H, Moulin G, Galzy P. Purification and properties of the phytase from *Schwanniomyces castellii*. *J Ferment Bioeng*. 1992; 74: 7-11.
21) Mochizuki D, Takahashi H. Method for producing phytase, Patent: EP 0931837-A, 28 July, 1999.
22) Sano K, Fukuhara H, Nakamura Y. Phytase of the yeast *Arxula adeninivorans*. *Biotechnol Lett*. 1999; 21: 33-8.
23) Vohra A, Satyanarayana T. Purification and characterization of a thermostable and acid-stable phytase from *Pichia anomala*. *World J Microbiol Biotechnol*. 2002; 18: 687-91.
24) Aloconda TM, Matinez MJ. Purification and characterization of a β-glucosidase from the phytopathogenic fungus *Fusarium oxysporum* sp. melonis. *Lett Appl Microbiol*. 1996; 22: 106-1.
25) Ugwuanyi JO, McNeil B, Harvey LM. Production of protein-enriched feed using agro-industrial residues as substrates. *Biotechnology for agro-industrial residues utilization.* 2009; 77-103.
26) Greiner R, Koneitzny U. Phytase for food application. *Food Technol Biotechnol*. 2006; 44: 125-40.
27) Quan C, Zhang L, Wang Y, Ohta Y. Production of phytase in a low phosphate medium by a novel yeast *Candida krusei*. *J Biosci Bioeng*. 2001; 92: 154-60. PMID: 16233076

28) Chaiharn M, Lumyong S. Phosphate solubilization potential and stress tolerance of rhizobacteria from rice soil in Northern Thailand. *World J Microbial Biotechnol*. 2009; 25: 305-14.
29) Gulati HK, Chadha BS, Saini HS. Production and characterization of thermostable alkaline phytase from *Bacillus laevolaticus* isolated from rhizospheric soil. *J Ind Microbiol Biotechnol.* 2007; 34: 91-8. PMID: 16967265
30) Singh B, Satyanarayana T. A marked enhancement in phytase production by a thermophilic mould *Sporotrichum* thermophile using statistical designs in a cost-effective cane molasses medium. *J Appl microbial.* 2006; 101: 344-52. PMID: 16882141
31) Aneja KR. Experiments in microbiology plant pathology and biotechnology. 4 ed., New Delhi, New Age International Publishers, 2005; 245-75.
32) Benson HJ. Microbiological application: laboratory manual in general microbiology. USA, McGraw Hill Publication, 2004; 470-91.
33) Nampoothiri KM, Tomes GJ, Roopesh K, *et al*. Thermostable phytase production by *Thermoascus aurantiacus* in submerged fermentation. *Appl Biochem Biotechnol.* 2004; 118: 205-14. PMID: 15304750
34) Richardson AE, Hadobas PA. Soil isolates of *Pseudomonas* spp. that utilize inositol phosphates. *Can J Microbiol*. 1997; 43: 509-16. PMID: 9226870
35) Choi YM, Suh HJ, Kim JM. Purification and properties of extracellular phytase from *Bacillus* sp. KHU-10. *J Prot Chem*. 2001; 20: 287-92. PMID: 11594462
36) Greiner R, Konietzny U, Jany KD. Purification and characterization of two phytases from *Escherichia coli*. *Arch Biochem Biophys*. 1993; 303: 107-13. PMID: 8387749
37) Lan GQ, Abdullah N, Jalaludin S, Ho YW. Culture conditions influencing phytase production of *Mitsuokella jalaludinii*, a new bacterial species from the rumen of cattle. *J Appl Microbiol*. 2002; 93: 668-74. PMID: 12234350
38) Singh B, Satyanarayana T. Applications of phytase of thermophilic mould, *Sporotrichum thermophile*: A review. *J Sci Ind Res*. 2010; 69: 411-4.