International Journal of Molecular Medicine and Advance Sciences, 1 (2): 116-120, 2005

Phytase Gene Expression in *Pichia pastoris* and Analyses of its Biochemical Characteristics

Q.Q.Yin, Q. H. Zheng and X. T. Kang
Department of Animal Science, Henan Agricultural University, Zhengzhou 450002, P. R. China

Abstract: Phytase gene from *A. niger* was amplified by PCR and inserted into two plasmids (pPICZá A and pGAPZá A) to form two shuffle vector cassettes (Vppi and Vpga). *P. pastoris* were transformed by electroporation. Phytase gene was expressed successfully in the host. After 4 d incubation in liquid media, phyatse activity in the supernatant reached the peak, 39.00 μ/mL^{-1} for *P. pastoris* with Vppi and 28.46 μ/mL^{-1} for *P. pastoris* with Vpga. They were increased by 51 folds for Vppi phytase and 37 folds for Vpga phytase, compared with the original *A. niger* (0.76 μ/mL^{-1}). The analyses of biochemical characteristics of phytases showed that the optimum temperature and pH range were 30-50°C and 2.0-5.5, respectively. The optimum phytase concentrations for the inorganic phosphorous (P) released from soybean were 0.25 μ/g^{-1} soybean for Vppi phytase, 0.50-1.00 μ/g^{-1} for Vpga phytase and wild-type phytase. The expressed phytase from Vppi was better for thermostability and for P released at 0.25, 0.50 and 1.00 μ/g^{-1} soybean levels than Vpga phytase and wild-type phytase.

Key words: Phytase, gene expression, *Pichia pastoris*, biochemical characteristics

INTRODUCTION

Phytic acid exists in the diets of non-ruminant animals (0.5-1.4%), where it serves as a P reservoir. It is a so powerful chelating agent that the solubility and digestibility of many nutrients are reduced by the formation of phytate complexes including amino acids (lysine, histidine and arginine, etc.) and starch complexes as well as mineral complexes [1]. Phytase came to its commercial use in 1994 since its first discovering in 1907. Phytase can catalyze the hydrolysis of phytate and release inorganic P[2] and phytate-bound calcium (Ca), iron (Fe), Zinc (Zn) and Magnesium (Mg) minerals[3]. Because there is little phytase activity in the digestive tracts of the non-ruminant animals[4], these animals can't use these minerals effectively, specially the phytate-bound P[5]. P is an important mineral in animal nutrition, but it is always deficient in the natural diets of the non-ruminant animals. The addition of phytase in animal diet can reduce P excretion by 30-50%[1] as well as reduce the supplementation of inorganic P in animal diets.

Although bean, wheat and corn contain phytase, it is not enough to digest phytate-bound minerals effectively. At present, only a little commercial phytase is applied in animal production due to its high production cost. How to reduce cost and improve phytase production and activity becomes more and more important. It was reported that microorganisms might be a more feasible source of the enzyme[6]. The first microbial phytase was from *A. niger*[7]. Because the original fungi or bacteria produce low level of phytase, phytase gene transformation in *A. niger* was first used to improve its production for commercial use[8]. Although phytase gene has been expressed in tabacco seeds and soybean cells, plant phytase increased apparent P digestibility by 31%, while microbial phytase increased apparent P digestibility by 54% in the (Fig. 1). This is why more and more researches focus on the microbial phytase production. Phytase genes isolated from *A. niger*[8], *E. coli*[9] and Bacillus sp [10] have been over-expressed in *A. niger*[8] and other microorganisms. The activity and pH of phytase have an important effect on phytase availability, while the current phytases often have low activity and un-optimum pH range. The objectives of this study were: i) Isolated bacteria from pig gut to produce one kind of phytase with a broad pH range, which can be active in animal stomach; ii) Compared phytase production with two different plasmids; iii) Studied the biochemical characteristics of the expressed and wild-type phytases.

MATERIALS AND METHODS

Isolation of bacteria with phytase activity Took 5 g samples from an adult pig stomach, mixed with 45 ml anaerobic solution[11], diluted 10^5-10^6 folds and cultured for 3 days anaerobically in a medium containing calcium phytate as an indicator for indicating phytase activity (pH 3.0). The positive colonies were selected according to the diameter of the bright haloes around the colony. The

bacterium with phytase activity was classified as *A. niger* according to its 16S rDNA sequence (100% identification) with Yin and Zheng's protocol[12].

A. niger phytase was prepared as follows: Medium 10 (M10) was prepared according to Caldwell and Bryant's protocol[11], modified by adding 10% (v/v) pig stomach liquid. *A. niger* was incubated in Hungate tubes with M10 medium at 37°C for 5 days. One milli litter was taken daily to estimate phytase activity. The culture was centrifuged at 12000xg for 5 min. Phytase activity was determined in the supernatant of the culture according to the following protocol 0.5 mL of the diluted sample (diluted with 0.2 M sodium citrate-citric acid buffer, pH 5.5) was pre-warmed at 37 °C water bath for 5 min, mixed with 0.5 mL 1% sodium phytate (Dissolved in 0.2M sodium citrate-citric acid buffer, pH 5.5), incubated at 37°C for 15 min and then added with 1 mL of 15% trichloroacetic acid (TCA) to stop the reaction. The blank samples were prepared by adding 0.5 mL diluted sample and 1.0 mL TCA and 15 min later 0.5 mL sodium phytate was added. The absorbance against that of sample blank at 820 nm was measured. One phytase unit was defined as the activity that released 1 μ mol of inorganic phosphorous from sodium phytate per minute.

Shuffle vector cassettes: pPICZá A (3.6kb) and pGAPZá A (2.9kb) (Invitrogen, San Diego, USA) plasmids were used in this study. Phytase genes (1.25kb)[9] were amplified from genomic DNA of *A. niger* by PCR with the following primers: Upstream, 5'-GGCGAATTCATGTGGTATTTCC; downstream, 5'-GCCTCTAGACAAACTGCACGCC. The PCR reaction mixtures contained 250 ng of genomic DNA as template, 100 p mole of each primer, 5 U of AmpliTaq DNA polymerase, 12.5 mM $MgCl_2$, 200 um each dNTP and the volume was adjusted to 50 μL with de-ionized water. The reaction was performed by the GeneAmp PCR system 2400 (Perkin Elmer, Norwalk, CT. USA). The thermal program included 1 cycle at 94°C (3 min), 25 cycles of 94°C for 0.8 min, 56°C for 1 min and 72°C for 2 min, 1 cycle of 72°C for 10 min and stored at 4°C. The plasmids (pPICZá A and pGAPZá A) and phytase gene were partially digested with EcoRI and XbaI separately and ligated together to form the Vppi and Vpga victors. The phytase genes were led by a á-factor signal peptide and 5'-AOX1 promoter in pPICZá A and a pGAP signal peptide and GAP promoter in pGAPZá A. The antibiotic for selection was zeocin. Before electroporation, Vppi and Vpga were linearized by restriction enzyme SacI and BspHI, respectively.

Electroporation: The competent cell of *P. pastoris* (X33) was prepared as the following: One fresh colony was selected and incubated in a 1 Litter flask with 200 mL of YPD medium (1% yeast extract, 2% peptone and 2% dextrose) at 30 °C and 200 rpm until OD_{600}=1.3-1.5. The cell was centrifuged (1500¡Ág, 5 min, 4 °C) and washed with ice cold sterile de-ionized water twice and with 1 M sorbitol once and then resuspended in 500 μ L ice cold 1 M sorbitol and kept in ice and used within one day. Mixed 80 μ L of the cell with 5-10 μ g of linearized vector and transferred to an ice-cold 0.2 cm electroporation cuvette. Pulsed the cell according to the parameters for yeast (1.5 kv and 5 msec).

Incubation and growth: After electroporation, immediately added 1 mL of 1M ice-cold sorbitol to the cuvette, transferred the mixture into a sterile test tube and incubated 1-2 h without shaking at 30°C. Spread the cell on YPDS plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar, 1 M sorbitol and 100 $\mu g/mL^{-1}$ zeocin) and incubated for 2-3 days. Picked the colonies and incubated in YPD medium for *P. pastoris* with Vpga and BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 100 mM potassium phosphate, pH 6.0, 0.4 ppm biotin and 1% glycerol) for *P. pastoris* with Vppi containing 20 μ g/mL^{-1} zeocin at 30°C and 200 rpm. The OD_{600} values were determined after 24 h incubation. The cells were centrifuged and kept on incubating after the OD values were adjusted to 1 with YPD medium for *P. pastoris* with Vpga and BMMY medium (The 1% glycerol in BMGY medium was replaced by 1% methanol, methanol was used to induce enzyme gene expression) for *P. pastoris* with Vppi. Five hundreds micro litters of the sample was taken daily for phytase activity analysis and then 0.5 mL methanol (10%, v/v) was added daily in BMMY medium to keep 1% concentration of methanol.

Enzyme activity and property: The culture was centrifuged at 12000xg for 5 min. Phytase in the supernatant was extracted and purified with Rodriguez's protocol[13]. The thermostability of the phytases was estimated according to the following protocol: The enzyme concentrations were adjusted to the same levels (μ/mL^{-1}). Two hundreds micro litters of the diluted sample was added into the pre-warmed (30 min) test tubes containing 9.8 mL 0.2 M sodium citrate-citric acid buffer (pH 5.5) and mixed well. The temperature levels were: 30, 40, 50, 60, 70, 80, 90 and 100 °C. The reaction was stopped by putting the tubes into ice immediately after 15 min reaction. The resident phytase activity was estimated with the above protocol.

The optimum pH was determined with the buffers of 0.2 M glycine-HCl (pH 1.0, 2.0, 3.0), 0.2 M sodium citrate-citric acid (pH 3.5, 4.0, 4.5, 5.0, 5.5) and 0.2 M Tris-HCl (pH

Table 1: The phytase activity during incubation (μ/mL^{-1} medium)

Days	1	2	3	4	5	6	7	8
Vpga	5.47±3.01^{A}	22.52±1.88^{Ba}	25.39±1.74^{B}	27.02±2.49^{B}	26.65±1.36^{Ba}	17.45±4.30^{B}	22.65±2.90^{B}	28.46±2.09^{Bb}
Vppi I	9.28±0.67^{A}	16.05±0.76^{B}	21.67±1.17^{Ca}	26.42±1.91^{D}	27.51±1.94^{D}	24.57±1.60^{DE}	25.28±1.32^{DEb}	23.00±4.87^{E}
Vppi II	13.25±0.79^{A}	24.15±0.86^{B}	32.56±1.86^{C}	39.00±1.33^{D*}	37.17±1.65^{D}	---	---	---
E	0.34±0.09^{Aa}	0.53± 0.12^{b}	0.76± 0.18^{Bc}	0.59± 0.11^{b}	---	---	---	---

Note: Means (n=5) in a row lacking a common superscript letter differ (p<0.01 orpP<0.05). A, B, C, D and E: p<0.01, a, b and c: P<0.05. "Vpga" and "Vppi" indicated that the phytase from plasmids (pPICZá A and pGAPZá A) respectively; "E" indicated the phytase from original *A. niger*. "*" means phyatse activity from Vppi II was significant higher than that from Vpga and Vppi I in the 4th day incubation (P<0.01).

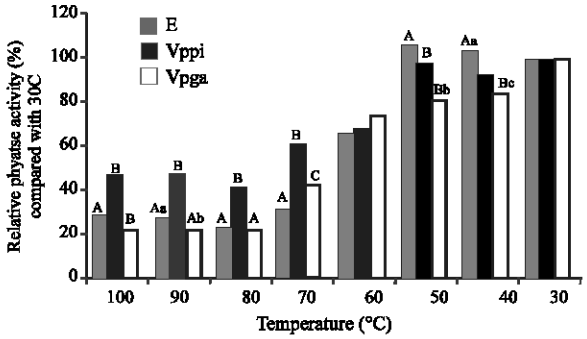

Fig. 1: The thermostability of phytase. The phytase activity at 30°C was used as the control (100%), the other groups were divided by this activity and then timed by 100. Means (n=5) lacking a common superscript letter differ (p<0.01 or p<0.05). A, B and C: p<0.01, a, b and c: p<0.05

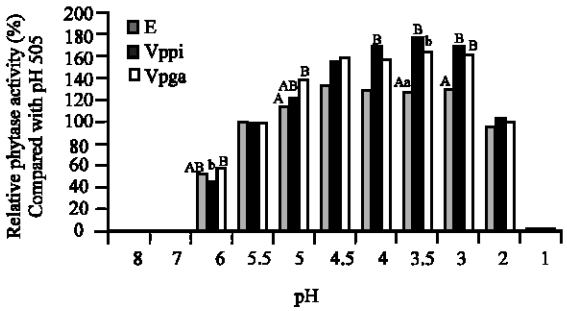

Fig. 2: Optimum pH of the expressed and original phytase. The phytase activity at pH 5.5 was used as the control (100%), the other groups were divided by this activity and then timed by 100. Means (n=5) lacking a common superscript letter differ (p<0.01 or p<0.05). A and B means p<0.01, a and b means P<0.05

6.0, 7.0, 8.0). The corresponding 1% sodium phytates at the different pH levels were prepared separately.

P released from soybean by phytase: Mixed 1.5 g soybean (Passed through 60 screen meshes per square inch) with 15 mL of 0.2 M sodium citrate-citric acid (pH 5.5) in 50 mL flasks. The added phytase levels were 0.0, 0.25, 0.5 and 1.0 μ/g^{-1} soybean. The mixture was incubated at 37°C for 3 h with shaking at 130 rpm and the reaction was stopped by putting the samples into ice immediately. The samples were centrifuged at 800¡Ág for 15 min (4 °C). The supernatant was used for inorganic P analysis with the following protocol: (i) P standard preparation: Weighed exactly pre-dried (80 °C, 2 h) 4.3533 g ammonium phosphate and dissolved it into a 1 L flask to make the P concentration to 10 mg/mL and adjusted P concentration to 0.00, 0.04, 0.08, 0.12, 0.16, 0.20, 0.24, 0.28, 0.32, 0.36 and 0.40 mg/mL. (ii) Samples prepared: One milli Litters sample was deproteinized by mixing with 4 mL of 12.5% TCA, kept at room temperature for 15 min, centrifuged for 15 min at 350¡Ág. (iii) P estimated: Five hundreds micro litters of the supernatant and standard solution were mixed with 2.5 mL of MS solution (1% sodium molybdate and 14 mL/l concentrated H_2SO_4) and 0.25 mL of Elon solution (3% sodium bisulfate and 1% p-methylamino phenol sulfate), incubated at room temperature for at least 60 min and then read the absorbance values at 700 nm.

Statistic analysis: Experimental data were expressed as the means and standard errors. The data were analysed using the ANOVA procedures of Statistical analysis Systems Institute. Duncan's multiple range test was used to compare treatment means. Difference were considered statistically significant at p<0.05.

RESULTS

Phytase gene expression: Table 1 showed that the phytase gene was expressed in *P. pastoris* successfully. Nine colonies from *P. pastoris* with Vpga and 26 colonies from *P. pastoris* with Vppi were collected and incubated. Five out of 9 and 5 out of 26 colonies had the higher phytase activity. Phytase activity of *P. pastoris* with Vppi reached the peaks (27.51-39.00 μmL^{-1}, p<0.01) at the 4-5th day incubation and then slipped down to 23.00 μ/mL^{-1} (p<0.05) at the 8th d. Phytase activity of *P. pastoris* with Vpga reached the peaks (27.02 μ/mL^{-1}, p<0.05) at the 3-4th d incubation and slipped down at the 5-7th day and then increased to the second peak with 28.46 μ/mL^{-1} (p>0.05) at the 8th day. In addition, phytase activity from the second-time transformation of *P. pastoris* with Vppi (Vppi II, 5 colonies for 4 d incubation) was significant higher than that of *P. pastoris* with Vpga and Vppi I (39.00 vs. 27.02 and 27.51 μmL^{-1}, p<0.01). The protocol from Invitrogen

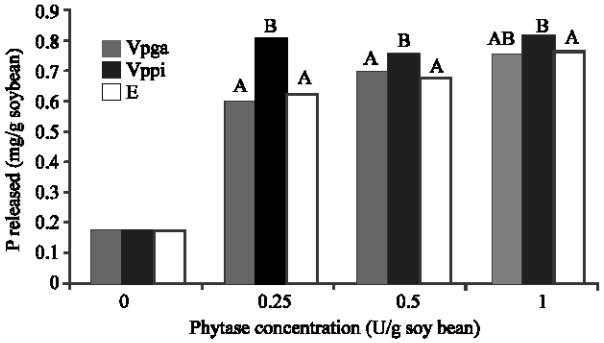

Fig. 3: P released from soybean with the expressed phytase (mg/g soybean). Means (n=8) lacking a common superscript letter differ ($p<0.01$ orp$P<0.05$). A and B means $p<0.01$, a and b means $p<0.05$. The released P increased when phytase concentration increased from 0.0 to 1.0 μg^{-1} ($p<0.01$) except for Vppi phytase

company has recommend increasing zeocin concentration in the selected media in order to get the colony to produce high phytase activity. But when the concentration was increased from the recommended amount (100 μg^{-1}mL) to 200-500 μg^{-1}mL, no colony appeared.

The thermostability and optimum pH range of phytase: Phytase activity decreased with temperature increasing. The optimum temperature to keep more than 60% of the original phytase was 60°C. It appeared that phytase activity was reduced quickly at 70°C. When the temperature is above 80°C, the residual phytase activity was about 20% of the activity at 30°C for Vpga phytase and wild-type phytase and 40% for Vppi phytase (Fig.1).

Figure. 2 indicated that the optimum pH range of phytase was 2.0-5.5. The expressed phytase had higher activity than the wild-type phytase ($P<0.05$ or $P>0.05$). At pH points of 1, 7 and 8, the relative phytase activity is only 1-8% of that at pH 5.5.

Phosphorus released from soybean by the phytases: Figure. 3 showed that the phytase could increase P released from soybean significantly when it was added into soybean from 0 to 1.0 μg^{-1} soybean ($p<0.05$ and $p<0.01$). The maximum P released was obtained at 1.0 μg^{-1} soybean for all three phytases. The most economic phytase concentration for P released was 0.25 μg^{-1} for Vppi phytase and 0.50-1.00 μg^{-1} for Vpga and wild-type phytase. Vppi phytase was better than Vpga and wild-type phytase to release P from soybean at 0.25, 0.50 and 1.0 μg^{-1} levels ($p<0.01$). There was no significant difference between wild-type and Vpga phytase ($p>0.05$).

DISCUSSION

The transformed *P. pastoris* produced higher phytase activity than the original *A. niger* (0.76 μ/mL^{-1}). The expressed phytase activity was 37 folds for Vpga phytase and 51 folds for Vppi phytase more than that of the original *A. niger*. *P. pastoris* has been used to express high levels of heterologous protein and phytase with vector (pPIC9), in which the phytase activity was increased by 13 folds[14]. The highly expressed phytase in the transformed *P. pastoris* is due to the inserted plasmids containing the phytase gene to secrete phytase. The more plasmids inserted or reproduced in host, the more phytase secreted[15]. This study shows that Vppi phytase activity is 44% (39 vs. 27 μmL^{-1}) more than Vpga phytase. The reason may be that the inducer (methanol) and the different signal peptide and promoter used in Vppi phytase production induced phytase secretion. In commercial production of phytase, one more day incubation will increase the cost significantly. The incubation time can be shorten to 2 days for *P. pastoris* with Vpga ($p>0.05$) and 4 days ($p>0.05$) for *P. pastoris* with Vppi according to the required phytase activity and the ratio of input and output.

The former research showed that the wild-type phytase retained 40% of its activity after it was heated at 68°C for 10 min[16] and the expressed phytase activity in soybean cells decreased rapidly when the temperature was above 63°C[17]. This research agreed with the former results to some extents. The deceasing phytase activity at high temperature must be considered when the phytase is added in the pelleted process. Because the general temperature for pellet is about 65-80°C, the recommending pelleted temperature should be below 65°C according the resident phytase activity in this study.

Figure 2 showed that the optimum pH range was 2.0-5.5 for the expressed and wild-type phytase, which had a more broad pH optimum than the former report[13, 18]. The broad pH range of this phytase makes it more available for feed additive than the other kind of phytase. At pH points of 1, 7 and 8 the relative phytase activity was below 8%. This was due to the protein structure of phytase changed under the strong basic or acidic conditions to make phytase lose its activity. The pH ranges in the stomach and small intestine are 2-3 and 6-7 for the non-ruminant animals[19], so the two expressed phytases can help to digest minerals in the stomach early and reduce the anti-nutrients of phytate effectively. The optimum pH ranges of phytases from plant, fungi and *A. niger* were 5.0-6.5 (1)£¬4.5-6.0[8] and 3.0-5.0[18], respectively. In the future, it is suggested that the different phytases from the different sources whose optimum pH is 6-7 or 2-5 can be used together to make the phytate digested in intestine as well as stomach.

Phytase could increase P released from feedstuffs significantly by incubation with 0.2 M sodium acetate-

acetic acid (pH 5.5) buffer[1]. From this result, the amount of phytase added in the commercial diets was 250 μKg^{-1} for Vppi phytase and 500-1000 μKg^{-1} for Vpga phytase and wild-type phytase. This is a fast and economical method to determine phytase availability.

The results also showed that the Vppi expressed phytases had the better thermostability and ability to release boundary P than the Vpga expressed and wild-type phytase. The reason may be that the different signal peptide and promoter affect the biochemical characteristics of phytase.

REFERENCES

1. Selle, P.H., 1997. The potential of microbial phytase for the sustainable production of pigs and poultry: An Australian perspective, Korean society of animal nutrition and feedstuffs. 7th Short Course on Feed Techonology. 3 rd April, Ansung, Korea, pp: 1-39.
2. Wodzinski, R. J. and A. H. J.Ullah, 1996. Phytase. Adv. Appl. Microbiol., 42: 263-302.
3. Murry, A.C., R.D. Lewis and H.E. Amos, 1997. The effect of microbial phytase in a pearl millet-soybean meal diet on apparent digestibility and retention of nutrients, serum mineral concentration and bone mineral density of nursery pigs. J. Anim. Sci., 75: 1284-1291.
4. Bitar, K. and J.G. Reinhold, 1972. Phytase and alkaline phosphatase activities in intestinal mucosae of rat, chicken and man. Biochim. Biophys. Acta., 268: 442-452.
5. Sweeten, J.M., 1992. Livestock and Poultry Waste Management: A National Overview. In Blake, J. D. and Magette, W. (Ed.), National livestock, poultry and aquaculture waste management. Am. Soc. Agric. Eng., St. Joseph, Minnesota, pp: 4-15.
6. Reddy, N.L., S.K. Sathe and D.K. Salunkhe, 1982. Phytates in legumes and cereals. Adv. Food. Res., 28: 1-92 .
7. Nelson, T.S., T.R. Shieh, R.J. Wodzinski and J.H. Ware, 1968. The availability of phytate P in soybean meal before and after treatment with a mold phytase. Poult. Sci., 47: 1842-1848.
8. van Hartingsveldt, M., C.M.J. van Zeijl, G.M. Harteveld, R.J. Gouka, M.E.G. Suukerbuyk, R.G.M. Luiten and P.A. van Paridon, 1993. Cloning, characterization and overexpression of the phytase-encoding gene (phyA) of *A. niger*. Gene, 127: 87-94.
9. Dassa, J., C. Marck and P.L. Boquet, 1990. The complete nucleotide sequence of the *E. coli* gene appA reseals significant homology between pH 2.5 acid phophatase and glucose-1-phosphatase. J. Bacteriol., 172: 5497-5500.
10. Kim, Y.O., J.K. Lee, H.K. Kim, J.H. Yu and T.K. Oh, 1998. Cloning of the thermostable phytase gene (phy) from *Bacillus* sp. DS11 and its overexpression in *A. niger*. FEMS Microbiol. Lett., 162: 185-191.
11. Caldwell, D. R. and M. P. Bryant, 1966. Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. Applied Microbiol., 14: 794.
12. Yin, Q.Q. and Q.H. Zheng, 2005. Isolation and identification of the dominant Lactobacillus in gut and faeces of pigs with carbohydrate fermentation and 16S rDNA technique. J. Biosci. and Bioeng., 99: 68-71.
13. Rodriguez, E., Y.M. Han and X.G. Lei, 1999. Cloning, sequencing and expression of an *A. niger* acid phosphatase/phytase gene ($appA_2$) isolated from pig colon. Biochem. Biophys. Res. Comm., 257: 117-123.
14. Peng, R.H., A.S. Xiong, X. Li, H.Q. Fan, Q.H. Yao,M.J. Guo and S.L. Zhang, 2002. High expression of a heat-stable phytase in *Pichia pastoris*. ACTA Biochimica et Biophysica Sinica., 34: 725-730.
15. Brown, T.A., 1990. Gene Cloning, 2nd Edn., Chapman and Hall New York, pp: 14-15.
16. Ullah, A.H.J. and D.M. Gibson,1987. Extracellular phytase (E.C.3.1.3.8) from *A. ficuum* NRRL 3135: Purification and characterization. Prep. Biochem., 17: 63-91.
17. Li, J., C.E. Hegeman, R.W. Hanlon, G.H. Hanlon, G.H. Lacy, M.D. Denbow and E.A. Grabau, 1997. Secretion of active recombinant phytase from Soybean cell-suspension culture. Plant Physiol. (Rockville), 114: 1103-1111 .
18. Greiner, R., U. Kontietzny and K.D. Jany, 1993. Purification and characterization of two phytases from *A. niger*. Arch. Biochem. Biophys., 303: 107-113.
19. Jurgens, M.H., 1997. Animal Feeding and Nutrition, 8th Edn., Kendall/Hunt Publishing Company, Iowa, pp: 39-42.