ISOLATION AND CHARACTERIZATION OF THE PHYTASE GENE PROMOTER FROM *Bacillus licheniformis* DSM13

Nguyen Thanh Trung[1*], Nguyen Minh Hung[1], Nguyen Huy Thuan[1], Trinh Thanh Trung[2], Nguyen Quoc Trung[3], Trinh Thi Thu Thuy[3], Nguyen Hoang Anh[4]

[1]Center for Molecular Biology, Institute of Research and Development, Duy Tan University, Danang
[2]Institute of Microbiology and Biotechnology, Vietnam National University, Hanoi
[3]Faculty of Biotechnology, Vietnam National University of Agriculture, Hanoi
[4]Faculty of Food Science and Technology, Vietnam National University of Agriculture, Hanoi

Email[*]: trungnt@duytan.edu.vn

Received date: 09.20.2017 **Accepted date: 25.03.2017**

ABSTRACT

Phytases are a group of enzymes that hydrolyze phytate to release inorganic phosphate and *myo*-inositol phosphate intermediates. The phytase encoding gene (*phyL*) of *Bacillus licheniformis* DSM13 is strongly induced during phosphate starvation conditions. In this study, we analyzed the activity of the phytase promoter by constructing a recombinant strain containing a plasmid bearing the *phyL* promoter fragment and the reporter gene *xynA* (encoding the xylanase) fusion. The recombinant strain was grown in conditions without and with the addition of different concentrations of sodium phytate. The results showed that the phytase promoter was strongly induced when less than 5 mM of phytate was added to the growth medium. Furthermore, growth analysis experiments revealed that phytate was an important alternative phosphate source for *B. licheniformis* cells to overcome phosphate starvation conditions.

Keywords: *Bacillus licheniformis*, gene expression, phosphate starvation, phytate, phytase.

Phân lập và khảo sát sự biểu hiện của promoter của gen phytase từ *bacillus licheniformis* DSM13

TÓM TẮT

Phytase là một họ enzyme thủy phân cơ chất phytate để giải phóng ra phosphate vô cơ cùng các sản phẩm trung gian *myo*-inositol phosphate. Gene mã hóa enzyme phytase (*phyL*) ở *Bacillus licheniformis* DSM13 cảm ứng biểu hiện mạnh trong các điều kiện thiếu hụt phosphate. Trong nghiên cứu này, chúng tôi thiết kế vector biểu hiện sử dụng promoter của gene *phyL* để điều khiển sự biểu hiện của gen *xynA* (mã hóa enzyme xylanase). Hoạt tính promoter phyL được xác định thông qua mức độ biểu hiện của protein XynA. Chủng tái tổ hợp được nuôi cấy trong điều kiện môi trường không có hoặc có bổ sung phytate ở các nồng độ khác nhau. Kết quả cho thấy hoạt động của promoter *phyL* được cảm ứng mạnh khi nồng độ cơ chất phytate bổ sung vào ở giá trị ≤ 5 mM. Ngoài ra, thí nghiệm phân tích sinh trưởng của vi khuẩn trong môi trường có bổ sung phytate chỉ ra rằng phytate là một nguồn phosphate thay thế quan trọng giúp cho vi khuẩn *B. licheniformis* vượt qua các điều kiện đói phosphate.

Từ khóa: *Bacillus licheniformis*, biểu hiện gene, phytate, phytase, thiếu hụt phosphate.

1. INTRODUCTION

Phytate, the salt of phytic acid, is the main storage form of phosphate in most mature plant seeds such as cereal and oilseeds (Reddy *et al.*, 1982; Rao *et al.*, 2009). It accounts for up to 80% of the total seed phosphorus and contributes as much as 1.5% to the seed dry weight (Bohn *et al.*, 2008). Phytate forms insoluble complexes with nutritionally important minerals, such as

Fe^{2+}, Zn^{2+}, Mg^{2+}, and Ca^{2+} (Harland and Oberleas, 1999), and it is poorly utilized by non-ruminant animals including swine, poultry, and fish (Roy *et al.*, 2009).

Phytases are a diverse group of enzymes that hydrolyze phytate (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate) to release inorganic phosphate (Pi) and a series of *myo*-inositol phosphate intermediates (Kerovuo *et al.*, 2000; Singh *et al.*, 2011). Based on the catalytic mechanism, phytases can be divided into histidine acid phosphatase, cysteine phosphatase, β-propeller phytases, and purple acid phosphatase (Mullaney and Ullah, 2007). Phytases have been found widely in microorganisms, plants, and some animals (Konietzny and Greiner, 2002; Rao *et al.,* 2009). Among them, phytase-producing rhizobacteria play an essential role in the mineralization of soil organic phosphorus. They include *Pseudomonas* sp. (Richardson and Hadobas, 1997), *Bacillus* sp. (Kerovuo *et al.*, 1998; Choi *et al.*, 2001), *Raoultella* sp. (Sajidan *et al.*, 2004), *Escherichia coli* (Greiner *et al.*, 1993), and *Enterobacter* (Yoon *et al.*, 1996).

The expression of the phytase gene in microorganisms depends on the growth medium components, pH, and phytate as the phosphate source. Phytate has been identified as an inducer substrate for the expression of the phytase encoding gene in *Klebsiella terrigena* (Greiner *et al.*, 1997) and *Mitsuokella jalaludinii* (Lan *et al.*, 2002). The expression of phytase genes in rhizosphere *Bacillus* species is also induced when phytate is added to the soil (Jorquera *et al.*, 2013). However, phytate has no influence on the expression of the phytase gene in *E. coli* (Greiner *et al.*, 1993) or *Bacillus subtilis* (Antelmann *et al.*, 2007). It has been reported that *B. licheniformis* cells can produce and secrete high levels of phytase protein into the extracellular medium under phosphate starvation conditions (Hoi *et al.*, 2006). However, little information is known about the effect of phytate on the expression of the phytase gene in *B. lichenifomis* under these growth conditions.

In the present study, we constructed a phytase gene promoter (P_{phyL})-reporter fusion to study the activity of the phytase promoter under phosphate starvation conditions with and without the addition of phytate. Furthermore, the effect of different concentrations of phytate on the expression of the phytase promoter was also investigated.

2. MATERIALS AND METHODS

2.1. Strains and cultivation

All bacterial strains and plasmids used in this study are listed in Table 1. Belitzky Minimal Medium (BMM) was used in all growth experiments (Stülke and Hillen, 2000). The cells were cultivated at 37°C, 180 rpm in BMM containing 15 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4{\cdot}7H_2O$, 27 mM KCl, 7 mM sodium citrate dihydrate, and 50 mM Tris-HCl (pH 7.5) supplemented with 0.6 mM KH_2PO_4, 2 mM $CaCl_2{\cdot}2H_2O$, 1 µM $FeSO_4{\cdot}7H_2O$, 10 µM $MnSO_4{\cdot}4H_2O$, and 11 mM glucose. For the phosphate starvation experiments, the concentration of phosphate was reduced to 0.15 mM. The activity of the P_{phyL} was also examined by adding sodium phytate to the medium. In these experiments, sodium phytate was added at the $OD_{500\ nm}$ of 1.0 (after 8 h of cultivation), if not indicated otherwise.

Table 1. Bacterial strains and plasmids used in this study

Strain	Relevant genotype	Reference
B. subtilis 168	TrpC2	DSMZ, Germany
B. licheniformis DSM13	Wild-type	DSMZ, Germany
B. licheniformis MW3	Δ*hsdR1,* Δ*hsdR2*	Waschkau *et al*. (2008)
B. licheniformis TH4	Δ*hsdR1,* Δ*hsdR2,* pKUC4	This study
pKUC	pUC18 fused with pKTH290	Truong (2006)

pKUC4	pKUC bearing the *phyL'-'xynA* fusion fragment	This study

Table 2. Sequences of primers used in this study

Primer number	Sequence 5´-3´
1	GAGTATCTAGAATCCATCCTGCTCGGGATC
2	GTTTTTTTTAAATTTAAACATATATTAACCTCCTTTTG
3	CAAAAGGAGGTTAATATATGTTTAAATTTAAAAAAAAC
4	GTCGAGGTACCATAGAAAAAGAGCATTTTTTG

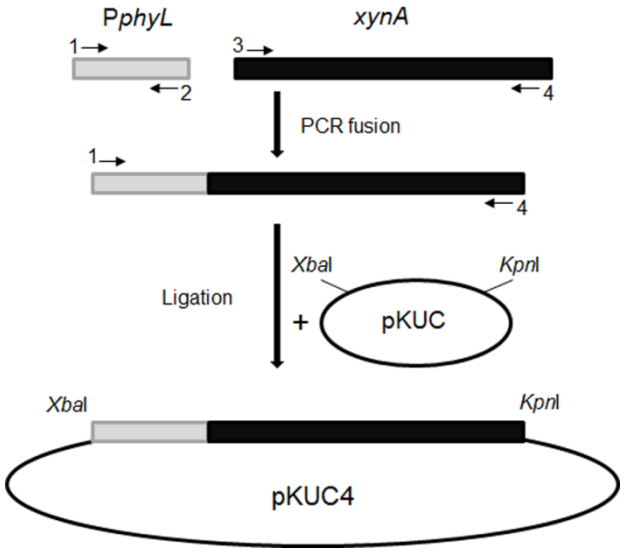

Figure 1. Schematic presentation of the cloning procedure for the construction of vector pKUC4

2.2. Construction of recombinant bacterial strain

In this study, the activity of the P_{phyL} in *B. licheniformis* was analyzed by means of the promoter-reporter gene fusion method. First, the P_{phyL} region (300 bp in length from the start codon of the *phyL* gene) was amplified using the primer pair 1/2 (Table 2). The reporter gene *xynA* (coding for xylanase) from *B. subtilis* 168 was then amplified with the primer pair 3/4 (Table 2). The *phyL'-'xynA* fusion fragment was generated by fusion PCR technique (Yon and Fried, 1989) using the primer pair 1/4 (Table 2). The PCR product was then inserted into the *Xba*I and *Kpn*I sites of the multiple-copy plasmid pKUC (Truong, 2006) resulting in vector pKUC4 (Fig. 1). This vector was then transformed into the *B. licheniformis* MW3 resulting in strain TH4.

2.3. Analysis of enzyme activity

In this experiment, samples of the supernatant of *B. licheniformis* strain TH4 were taken at the exponential growth phase, the transient phase, and 2, 4, 6, 8, 10, and 12h after the onset of the stationary phase. The xylanase activity in the supernatant samples was measured using the modified dinitrosalicylic acid (DNSA) method (Bailey *et al.*, 1992). Xylan from beech wood (Sigma-Aldrich Co, USA) was used as substrate at a concentration of 1% (w/v) in 0.05 M $NaPO_4$-Buffer (pH 6.0). The calibration curve was prepared from a concentration of xylose ranging from 0.5 mM to 50 mM. A 60 µL sample was added to 540 µL

1% Xylan (pre-warmed at 50°C) and incubated for exactly 10 min at 50°C. The reaction was terminated by adding 600 µL DNSA-solution and incubated for 10 min at 100°C. The reaction mix was cooled down on ice for 5 min and the absorbance was measured at 540 nm against the reaction blank. One unit of xylanase activity was defined as the amount of enzyme that liberates one micromole of reducing sugars equivalent to xylose per minute under the assay conditions described.

2.4. Analysis of the extracellular proteins

The proteins in the supernatant samples were separated by one-dimensional (1D-) SDS-PAGE. 20 µL samples were mixed with 5 µL of SDS sample buffer (50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromphenol blue) and denatured at 50°C for 10 minutes. The separation according to protein mass was conducted using a Protean II Cell system (BIO-RAD). Electrophoresis was run at 150 V for one hour (separating gel: 10% "Acrylamide-Solution (30%)-Mix 37.5:1" (AppliChem), 0.4 M Tris (pH 8.8), 0.1% SDS, 0.1% APS, 0.04% TEMED; stacking gel: 4% Acrylamide-Solution, 0.125 M Tris (pH 6.8), 0.1% SDS, 0.05% APS, 0.1% TEMED). Protein bands were visualized by Coomassie Brilliant Blue (CBB) staining.

3. RESULTS AND DISCUSSION

3.1. Construction of the expression vector

In this experiment, the promoter *phyL* (P_{phyL}) from *B. licheniformis* was fused with the reporter gene *xynA* from *B. subtilis* to generate an expression cassette by the PCR fusion method. We used the entire *xynA* gene, including its signal peptide sequence, to fuse with the P_{phyL} and thus the produced XynA protein would be secreted into the growth medium. The PCR fragment P_{phyL}-*xynA* was then inserted into the *Xba*I and *Kpn*I sites of the multiple-copy plasmid pKUC resulting in vector pKUC4 as shown in Figure 1. The expression vector pKUC4 was used to transform the *B. licheniformis* MW3 cells by the electroporation transformation method. The transformants were then selected and used for plasmid isolation. By restriction enzyme digestion with *Xba*I and *Kpn*I, the insertion of the expression cassette within the vector pKUC4 was confirmed.

3.2. Growth analysis

Phytate is an organic phosphate source that many bacteria can use to overcome phosphate starvation conditions. Therefore, in this experiment we added different concentrations of phytate to the growth medium of *B. licheniformis* strain MW3 to verify if this bacterial strain could use phytate as an additional phosphate source and how the concentrations of phytate affected the growth rate of this bacterium. Sodium phytate was added to the growth medium when cells came into the stationary phase (after 8h of cultivation). The results showed that, under the growth conditions without the addition of sodium phytate, *B. lichenfiormis* cells reached a maximal $OD_{500\ nm}$ of 1.0 during the stationary growth phase. However, when 1 mM phytate was added to the growth medium, the growth rate started to increase 2h after the addition of phytate and reached a maximal $OD_{500\ nm}$ of 2.0. When a higher concentration of phytate (5 mM) was added, it took a longer time (after 6h) for cells to increase the growth rate. However, the final $OD_{500\ nm}$ reached 2.7 after 20h of cultivation (Figure 2). These results indicated that *B. licheniformis* cells were able to use phytate as an additional phosphate source when cells were subjected to phosphate starvation conditions. Furthermore, the constant OD value during the stationary phase in the growth condition without phytate additionally revealed that phytate was not the only phosphate source for *B. licheniformis* cells to overcome the phosphate starvation conditions. This result was in agreement with

the study of Hoi *et al.* (2006) who found that *B. licheniformis* cells induce many genes encoding for nucleases which mobilize phosphate groups from intracellular and extracellular ribonucleic and deoxyribonucleic acids.

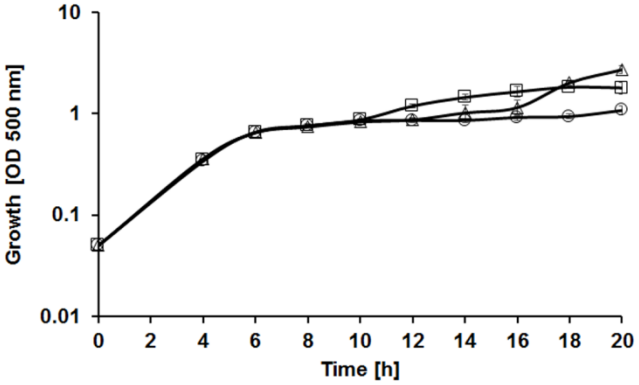

Figure 2. Growth of *B. licheniformis* MW3 in phosphate-starved BMM medium without and with the addition of phytate

Note: Sodium phytate was added at an $OD_{500\,nm}$ *of 1.0 (after 8 hours cultivation). Circles: without the addition of sodium phytate; squares: with the addition of 1 mM sodium phytate; triangles: with the addition of 5 mM sodium phytate (n=3 independent cultivations)*

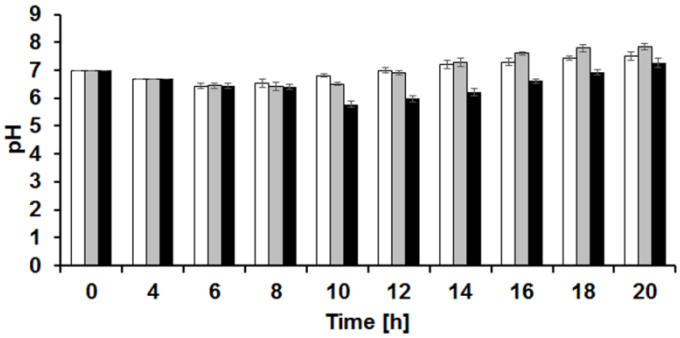

Figure 3. pH value of the growth medium in the conditions with and without the addition of phytate

Note: White bars: without the addition of sodium phytate; grey bars: with the addition of 1 mM sodium phytate; black bars: with the addition of 5 mM sodium phytate

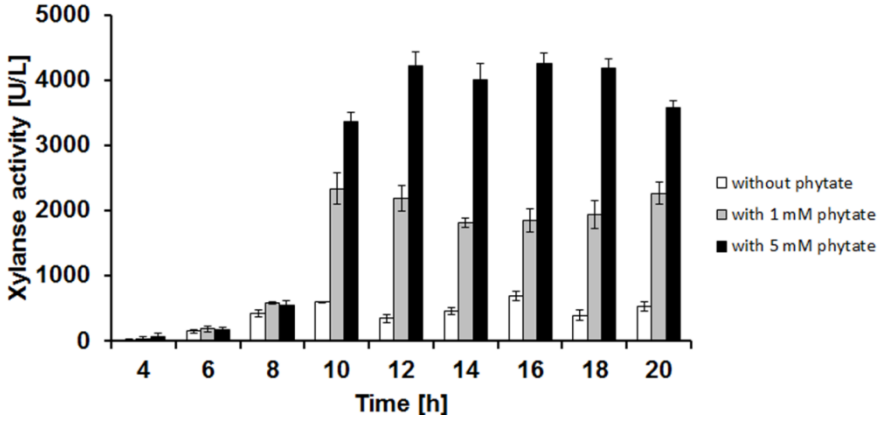

Figure 4. Xylanase activity in strain TH4 grown in the conditions with and without the addition of phytate

Note: White bars: without the addition of sodium phytate; grey bars: with the addition of 1 mM sodium phytate; black bars: with the addition of 5 mM sodium phytate

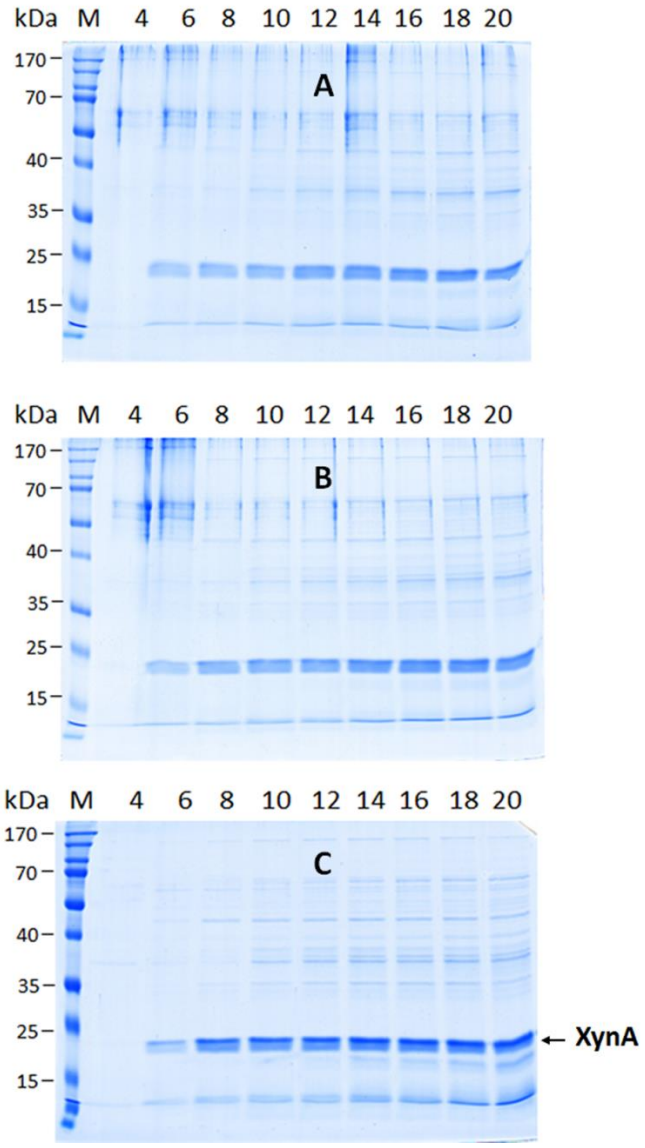

Figure 5. One-dimensional polyacrylamide gel electrophoresis separation of extracellular proteins

Note: 20 µL of culture medium of the TH4 strain after removal of cells by centrifugation was used. Sodium phytate was added at an $OD_{500\ nm}$ of 1.0 (after 8 hours cultivation). XynA protein bands are indicated by arrows. A: without the addition of sodium phytate; B: with the addition of 1 mM sodium phytate; C: with the addition of 5 mM sodium phytate

In this experiment, the addition of a high concentration of sodium phytate (5 mM) resulted in lowering the pH value to 5.8 at the time of addition (Figure 3). Therefore, bacterial cells required a longer time to increase their growth rate than in the growth conditions with a lower phytate addition as shown in Fig. 2. This result was in agreement with the study of Ibrahim *et al.* (2013) in that pH values lower

than 6.0 resulted in a lower growth rate for *B. licheniformis* BT5.9.

3.3. Analysis of the phytase promoter activity

In this study, the activity of P_{phyL} was analyzed in the growth conditions with and without the addition of different concentrations of sodium phytate. Under the growth conditions without the addition of sodium phytate, the *phyL'-'xynA* fusion strain TH4 showed a maximal xylanase activity of 750 U/L. However, when 1 mM sodium phytate was added to the growth medium, the xylanase activity reached a higher maximal value of 2500 U/L. Interestingly, the addition of 5 mM sodium phytate resulted in significant increase of xylanase activity up to 4200 U/L (Figure 4). The expression pattern of the xylanase gene under the control of P_{phyL} was also checked by SDS-PAGE (Figure 5). These results indicated that the activity of the *phyL* promoter depended on the sodium phytate concentrations. Moreover, sodium phytate (at concentrations less than or equal to 5 mM) played as an inducer for the expression of the phytase promoter.As shown by Hoi (2006), the addition of a three-fold higher concentration of phytate (15 mM) resulted in the repression of phytase promoter activity. Furthermore, Hoi *et al.* (2006) reported that the expression of the phytase gene in *B. licheniformis* is only induced under phosphate starvation conditions. Therefore, the addition of a high concentration of phytate could increase the inorganic phosphate level by the phytate hydrolysis activity of the phytase enzyme, and thus repress phytase gene expression.

4. CONCLUSIONS

The activity of the phytase promoter in *B. licheniformis* was strongly induced under phosphate starvation conditions. Furthermore, sodium phytate was an important alternative phosphate source for *B. licheniformis* cells to overcome the phosphate starvation conditions. In addition, sodium phytate (at a concentration less than or equal to 5 mM) was able to induce the expression of the *phyL* promoter of *B. licheniformis* under phosphate starvation conditions.

Acknowledgements

This work was supported by the National Foundation for Science and Technology Development of Vietnam (NAFOSTED) [106.16-2012.23]. We also thank Claudia Borgmeier (Universität Müster, Germany) for providing the *B. licheniformis* MW3 strain.

REFERENCES

Antelmann H., Töwe S., Albrecht D. and Hecker M. (2007). The phosphorus source phytate changes the composition of the cell wall proteome in *Bacillus subtilis*. J. Proteome. Res., 6:897 - 903.

Bailey J.M., Biely P. and Poutanen K. (1992). Interlaboratory testing of methods for assay of xylanase activity. J. Biotechnol., 23: 257 - 270.

Bohn L., Meyer A.S. and Rasmussen S.K. (2008). Phytate: impact on environment and human nutrition. A challenge for molecular breeding. *J*. Zhejiang Univ. Sci. *B*., 9:165 - 191.

Choi Y.M., Suh H.J. and Kim J.M. (2001). Purification and properties of extracellular phytase from *Bacillus* sp. KHU-10. J. Prot. Chem., 20:287 - 292.

Greiner R., Konietzny U. and Jany K.D. (1993). Purification and characterization of two phytases from *Escherichia coli*. *Arch. Biochem. Biophys*., 303: 107 - 113.

Greiner R., Haller E., Konietzny U. and Jany K.D. (1997). Purification and characterization of a phytase from Klebsiella terrigena. Arch. Biochem. Biophys., 341: 201 - 206.

Harland B.F. and Oberleas D. (1999). Phytic acid complex in feed ingredients. In: Coelho MB, Kornegay ET (eds) Phytase in animal nutrition and waste management. BASF Corp, Mount Olive, pp. 69 - 76.

Hoi L.T. (2006). Genome-wide analysis of nutrient starvation responses of *Bacillus licheniformis*. Dissertation, University of Greifswald.

Hoi L.T., Voigt B., Jurgen B., Ehrenreich A., Gottschalk G., Evers S., Feesche J., Maurer K.H., Hecker M. and Schweder T. (2006). The phosphate-starvation response of *Bacillus licheniformis*. Proteomics, 6: 3582 - 3601.

Ibrahim D., Zhu H.L., Yusof N., Isnaeni and Hong L.S. (2013). *Bacillus licheniformis* BT5.9 isolated from Changar hot spring, Malang, Indonesia, as a potential producer of thermostable α-amylase. Trop. Life. Sci. Res., 24: 71 - 84.

Jorquera M.A., Saavedra N., Maruyama F., Richardson A.E., Crowley D.E., del C Catrilaf R., Henriquez E.J. and de la Luz Mora M. (2013). Phytate addition to soil induces changes in the abundance and expression of *Bacillus* ß-propeller phytase genes in the rhizosphere. FEMS. Microbiol. Eco., 83: 352 - 360.

Kerovuo J., Lauraeus M., Nurminen P., Kalkinnen N. and Apajalahti J. (1998). Isolation, characterization, molecular gene cloning and sequencing of a novel phytase from *Bacillus subtilis*. Appl. Environ. Microbiol., 64: 2079 - 2085.

Konietzny U. and Greiner R. (2002). Molecular and catalytic properties of phytate-degrading enzymes (phytases). Inter. J. Food Sci. Technol., 37: 791 - 812.

Moling S.D., Douglas M.W., Hohnson M.L., Wang X., Parsons C.M., Koelkebeck K.W. and Zimmerman R.A. (2000). The effects of dietary available phosphorus levels and phytase on performance of young and older laying hens. Poult. Sci., 79: 224 - 230.

Mullaney E.J. and Ullah A.H.J. (2007). Phytase: attributes, catalytic mechanisms and applications. Inositol phosphates: linking agriculture and the environment (Turner BL, Richardson AE and Mullaney EJ, eds), CABI Publishing, Oxfordshire, UK, pp. 97 - 110.

Lan G.Q., Abdullah N., Jalaludin S. and Ho Y.W. (2002). Culture conditions influencing phytase production of *Mitsuokella jalaludinii*, a new bacterial species from the rumen of cattle. J. Appl. Microbiol., 93: 668 - 674.

Rao D.E., Rao K.V., Reddy T.P. and Reddy V.D. (2009). Molecular characterization, physicochemical properties, known and potential applications of phytases: An overview. Crit. Rev. Biotechnol., 29: 182- 198.

Reddy N.R., Sathe S.K. and Salunkhe D.K. (1982). Phytates in legumes and cereals. Adv. Food. Res., 28: 1- 92.

Richardson A.E. and Hadobas P.A. (1997). Soil isolates of *Pseudomonas* spp. that utilize inositol phosphates. Can. J. Microbiol., 43: 509 - 516.

Roy T., Mondal S. and Ray A.M. (2009). Phytase-producing bacteria in the digestive tracts of some freshwater fish. Aquac. Res., 40: 344 - 353.

Sajidan A., Farouk A., Greiner R., Jungblut P., Müller E.C. and Borriss R. (2004). Molecular and physiological characterization of a 3-Phytase from the rhizobacterium *Klebsiella pneumoniae* ASR1. Appl. Microbiol. Biotechnol., 65: 110 - 118.

Singh B., Kunze G. and Satyanarayana T. (2011). Developments in biochemical aspects and biotechnological applications of microbial phytase. Biotechnol. Mol. Biol. Rev., 6:69 - 87.

Stülke J. and Hillen W. (2000). Regulation of carbon catabolism in *Bacillus* species. Annu. Rev. Microbiol., 54: 849 - 880.

Truong L.V. (2006). Characterization of the pectinolytic enzymes of the marine psychrophilic bacterium *Pseudoalteromonas haloplanktis* strain ANT/505. Dissertation, University of Greifswald.

Yoon S.J., Choi Y.J., Min H.K., Cho K.K., Kim J.W., Lee S.C. and Jung Y.H. (1996). Isolation and identification of phytase-producing bacterium, *Enterobacter* sp. 4, and enzymatic properties of phytase enzyme. Enzyme Microbial. Technol., 18: 449 - 454.

Yon J. and Fried M. (1989). Precise gene fusion by PCR. Nucleic Acids Res., 17: 4895.