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# Phytase gene expression in *Lactobacillus* and analysis of its biochemical characteristics

**Ruiyu Zuo, Juan Chang, Qingqiang Yin*, Liying Chen, Qixin Chen, Xia Yang, Qiuhong Zheng, Guangzhi Ren, Hua Feng**

*College of Animal Science and Veterinary Medicine, Henan Agricultural University, Zhengzhou 450002, PR China*

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## Summary

A 1.4-kb DNA containing the coding region of phytase gene from *Aspergillus ficuum* (*A. ficuum*) was connected with the plasmid of plAβ8 to construct shuffling vector, which was inserted into *Lactobacillus casei* (*L. casei*) by electroporation. The *Lactobacillus* with phytase gene was selected and incubated in anaerobic liquid medium. The results indicated that the highest phytase activities in the supernatant and cells were 22.12 and 4.49 U $ml^{-1}$ ($P<0.05$) at the fourth day of incubation; the optimum pH and temperature of phytase were 5.0 and 40–80 °C, respectively ($P<0.05$); the lowest pH value in the anaerobic medium was 3.35 ($P<0.05$) at the third day incubation; and the molecular weight of the expressed phytase was 39.2 kDa.

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## Introduction

Phytic acid exists in the diets of non-ruminant animals and serves as a phosphorus (P) reservoir. It is a powerful chelating agent making many nutrients low soluble and digestible through the formation of phytate complexes (Selle 1997). Phytase can catalyze the hydrolysis of phytate by releasing organic P and phytate-bound nutrients (Wodzinski and Ullah 1996; Murry et al. 1997). Because there is little phytase activity in the digestive tracts of the non-ruminant animals (Bitar and Reinhold 1972), these animals cannot make use of most minerals and nutrients effectively, especially the phytate-bound P (Sweeten 1992). P is an important mineral in animal nutrition, and calcium phosphate dibasic is generally added in animal diets to provide P. The addition of phytase in animal diet can reduce the supplementation of inorganic P through the release of phytate-P. This will reduce P excretion and pollution (Selle 1997).

At present, phytase is one of the important enzymes for non-ruminant animal production. How to reduce cost and improve phytase production and

*Corresponding author. Tel.: +86 371 6355 4237; fax:+86 371 6355 8998
*E-mail address:* QQZ22@yahoo.com.cn (Q. Yin).

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availability becomes more and more important. It was reported that microorganisms might be a more feasible source of the enzymes (Reddy et al. 1982). The first microbial phytase was isolated and purified from *Aspergillus niger* (Nelson et al. 1968). Because the original fungi and bacteria produce low level of phytase, phytase gene expression in *A. niger* was first used to improve its production for commercial use (Van et al., 1993). By now, phytase genes isolated from *A. niger*, *Escherichia coli* and *Bacillus* sp. have been successfully over-expressed in *A. niger* (Van et al., 1993), yeast (Dassa et al. 1990) and *Bacillus subtilis* (Kim et al. 1998) with high phytase activity. The study on phytase gene expression will continue to be a hot research field due to its high commercial value.

Probiotics such as lactobacilli can help animal's growth as well as improve animal body's resistance to the infectious agents by equilibrating gut microflora and stimulating the immune system (Kyriakis et al. 2003; Salminen et al. 1998). Furthermore, probiotics are natural, harmless bacteria and have no drug residues in edible animal products after being fed to the animals, and thus they can replace antibiotics for safe food production.

If phytase gene is inserted and expressed in *Lactobacillus*, the transformed *Lactobacillus* will have both functions of phytase and probiotics. Once the transformed *Lactobacillus* is given to animals, it will survive in animal gut to play the roles of secreting phytase and probiotics. The aim of this research was to construct the transformed *Lactobacillus* with phytase gene to make a new strain of bacterium for degrading phytate, decreasing digestive diseases, and improving nutrient availability. Animal production will be increased by using this transformed *Lactobacillus* in the diets of animals.

## Materials and methods

### Extract genomic DNA from *Aspergillus ficuum*

*A. ficuum* was selected, incubated and shaken with 150 RPM at 30 °C for 3 days in a cone bottle with PDA medium. The PDA medium was made as follows: in an Erlenmeyer flask, 6 g soluble starch, 2 g yeast extract, 5 g peptone, 20 g glucose, 2 g $KH_2PO_4$, and 0.3 g $MgSO_4$ were added and dissolved into 200 ml distilled water. The solution was then transferred to a 1 L volumetric flask and diluted to 1 L with distilled water. The extracted protocol was conducted according to the previous report (Raeder and Broda 1985). The genomic DNA was examined by agarose gel electrophoresis.

### Phytase gene isolation and analysis

Phytase gene with molecular weight of 1.4 kb was amplified from genomic DNA of *A. ficuum* by PCR with the following primers. An *Xba*Isite at 5′ end and a *Kpn*Isite at 3′ end were added to the primers.

Upstream: 5′-ATGTCTAGACTGGCAGTCCCCGCCT-CGAGA-3′;
Downstream: 5′-CTAGGTACCCTAAGCAAAACACT-CCGCCCAATC-3′.

The PCR reaction mixtures contained 250 ng of genomic DNA as template, 100 pmol of each primer, 5 U of AmpliTaq DNA polymerase, 12.5 mM $MgCl_2$, 200 μmol of each dNTP, and the volume was adjusted to 50 μl with deionized water. The reaction was performed using the GeneAmp PCR system 2400 (Perkin Elmer, Norwalk, CT, USA). The thermal program consisted of 1 cycle at 94 °C for 3 min, 30 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min, 1 cycle of 72 °C for 10 min and stored at 4 °C. PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. They were introduced into *E. coli* by using the pGEM-T vector (Promega, Madison, Wis. USA). The plasmids containing a unique insert of the appropriate size were purified by the QIAprep spin miniprep kit and were subjected to DNA sequence analysis, and the result was deposited in GenBank (Ref. GenBank nucleotide sequence database, EF206311).

### Construction of shuffle vector cassettes

The recombinant pGEM-T vectors was partially digested with *Xba* I and *Kpn* I to separate phytase gene and then purified with the QIAprep spin miniprep kit. The plasmid of pIAβ8 (Biomedal Company of Spain, Figure 1) was also partially digested with *Xba* I and *Kpn* I, and purified with the kit. Ligase was used to construct the recombinant of pIAβ8 vector. The promoter and signal peptide for galactosidase expression were used to manipulate phytase expression in pIAβ8. The recombinant shuffle vector was inserted into *E. coli* by chemical method (Sambrook and Russell 2001), and purified with the kits. The antibiotics for selecting the positive colonies were ampicillin.

### Electroporation

The competent cell of *Lactobacillus casei* was prepared as follow: One fresh colony was selected and incubated overnight in a 15 ml anaerobic roll

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