Cloning of Phytase Gene in Probiotic Bacterium

Bacillus coagulans

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Abstract

Phytase enzymes can increase the nutritional value of food and feed by liberating inorganic phosphate from phytate, the major storage form of phosphorus in plants. Bacillus coagulans is a lactic acid producing and sporeforming probiotic bacteria. In present study, we aimed to clone and express phytase gene in B. coagulans to create better probiotics for poultry. The phytase gene (phyC) from Bacillus subtilis VTT E-68013 was cloned and inserted into Escherichia coli-Bacillus sp. shuttle vector pMK3 and then transferred into E. coli by electrotransformation. pMK3P plasmid isolated from E. coli was used to introduced into B. coagulans DSM1 by electrotransformation. Insert and PCR analysis of pMKP from recombinant B. coagulans confirmed the phytase gene (phyC) fragment on agarose gel electrophoresis. Although phytase gene was cloned in B. coagulans, the phytase enzyme activity wasn’t detected on growth medium supplemented with wheat bran extract medium plates. Recombinant B. coagulans tested for phytase activity on test plates at various pH (5, 6, 7, 8, 9, 10) to obtain any changing in pH optimum of the enzyme in the new host but no phytase activity was observed on the plates.

Keywords: Cloning, phytase, Bacillus coagulans, probiotic
Introduction

Due to concerns about residues in animal products and the development of bacterial resistance to antibiotics, the potential exists for the implementation of a complete ban of the use of antibiotics in animal feed. As a consequence, the development of alternatives to antibiotics is receiving considerable attention (Turner et al., 2001). Probiotics are one potential alternative. Probiotics have been defined as “live microbial feed supplements that can benefit the host by improving its intestinal balance” (Fuller, 1989). The most common microorganisms found in the probiotic products currently available are Lactic Acid Bacteria (LAB), which are generally recognized as safe (GRAS), tolerant to acid and bile, able to adhere to the intestinal epithelium of the hosts and able to demonstrate antagonistic activity against pathogenic bacteria (Rial, 2000; Lonkar et al., 2005; Lin et al., 2006).

*Bacillus* species, a type of exogenous spore-forming bacteria, are not normally found in the gastrointestinal tract but have also been shown to be effective in keeping a favorable balance of microflora in the gastrointestinal tract and in improving animal performance (Zani et al., 1998; Adami and Cavazzoni, 1999; Kyriakis et al., 1999; Alexopoulos et al., 2004; Kritas and Morrison, 2005). Generally, when a *Bacillus* is used as a probiotic, it is used in the spore form and thus can be resistant to unfavorable conditions arising during transit through the gastrointestinal tract of animals (Guo et al., 2006).

*Bacillus coagulans* is a spore-forming, facultatively anaerobic, lactic acid producing, Gram-positive bacillus that has been administered as a biotherapeutic agent to chickens (Cavazzoni et al., 1998) and pigs (Adami and Cavazzoni, 1999). When they are administered to animals through feed or water can transform into active bacterial cells in existing specific environmental conditions such as body temperature, acidity, bile and other secretions of the upper digestive tract of poultry (Anonymous, 2007).

Cereals, legumes, and oilseed crops are grown in over 90% of the world’s harvested area. These crops serve as a major source of nutrients for humans and animals. An important constituent in these crops is phytic acid (*myo*-inositol hexaphosphate). The salt form, phytate, is the major storage form of phosphorus and accounts for more than 80% of the total phosphorus in cereals and legumes (Reddy et al., 1989). Phytases are enzymes capable of hydrolyzing phytic acid to less-phosphorylated myoinositol derivates. Monogastric animals, such as pig, poultry and fish, are not able to metabolize phytic acid, and therefore inorganic phosphate is added to their diets to satisfy the phosphorus requirement. This consequently contributes to phosphorus pollution problems in areas of intensive livestock production (Nayini and Markakis, 1983; Common, 1989; Nasi, 1990). Phytic acid also acts as an antinutritional agent in monogastric animals by chelating various metal ions needed by the animal, such as calcium, copper, and zinc (Lee et al., 1988; Graf, 1993; Lei et al., 1993). Therefore, the enzymatic hydrolysis of phytic acid into less-phosphorylated myo-inositol derivatives in the intestine of monogastric animals is desirable. Many attempts to enzymatically
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hydrolase phytic acid have been made to improve the nutritional value of feed and to decrease the amount of phosphorus excreted by animals (Simons et al., 1990; Lambrechts et al., 1992; Pen et al., 1993).

The present study is aimed to express phytase gene in B. coagulans to create recombinant probiotic for poultry, which combines beneficial effects of both probiotic and phytase enzyme. Therefore, the present study was carried out to evaluate the potential of B. coagulans to express Bacillus subtilis phytase.

Methods

Plasmids, Strains and Growth Media

Escherichia coli-Bacillus sp. shuttle vector pMK3 and E. coli-Saccharomyces cerevisiae shuttle vector pRS416 were obtained from BGSC (Bacillus Genetic Stock Centre, USA) and Stratagene, respectively. Bacillus subtilis VTT E-68013 and Bacillus coagulans DSM1 were purchased from VTT Biotechnology (VTT Technical Research Centre, Finland) and DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany), respectively. The host bacterium E. coli DH5α was purchased from Stratagene (USA). E. coli and B. subtilis cells were cultured in LB (Luria Bertani) at 37°C. B. coagulans was grown in MediumI (Peptone (meat) 7.8 g/l, Peptone (caseine) 7.8 g/l, Yeast Extract 2.8 g/l, NaCl 5.6 g/l, Glucose 1.0 g/l, pH:7.5) at 40°C. Growth media were supplemented with 40 µg/mL X-Gal plus 100 µg/mL Ampicillin (Amp) and 10 µg/mL Kanamycine (Km) to select recombinant E. coli and B. coagulans cells, respectively. Strains were tested for phytase production on LB and MediumI supplemented with wheat bran extract medium (wheat bran extract supplemented with 2% D-glucose, 0.4% sodium phytate, 0.2% CaCl₂, 0.5% NH₄NO₃, 0.05% KCl, 0.05% MgSO₄.7H₂O, 0.001% FeSO₄.7H₂O, 0.001% MnSO₄.H₂O, pH was adjusted to pH 7.5) (Powar and Jagannathan, 1982).

Recombinant DNA Techniques

All recombinant techniques were performed according to Sambrook et al. (1989) if otherwise is stated.

PCR Amplification of Phytase Gene (phyC)

To amplify the phyC coding sequence, PCR (Polymerase Chain Reaction) reactions were carried out by using chromosomal DNA of B. subtilis VTT E-68013 and the recombinant plasmid pMK3P from B. coagulans cells as the template in a 50 µl mixture containing 0.5 U of Taq DNA Polymerase, 1X standard PCR buffer, deoxynucleotide triphosphate (dNTP) at a concentration of 1 mM, and 100 ng of each primer with the following cycling conditions: 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min for 35 cycles in thermal cycler. The following oligonucleotide primers were used: forward primer 5’ CGGATCCACATTTTGAACAATTTTTCACA AAAAAAC 3’ and reverse primer 5’ GCTAGTCTAGATTTTCCGCTTTCTGT CGGTCAG 3’.
Construction of pMK3P

To construct pMK3P plasmid, the phytase gene (phyC) from *B. subtilis* VTT E-68013 was firstly amplified by PCR technique and inserted in pRS416 shuttle vector of *E. coli*-S. cerevisiae to construct pRS416P. The phytase gene from pRS416P was then inserted in HindIII and BamHI restriction site of the pMK3 plasmid. The plasmid pMK3P constructed was introduced into *E. coli* DH5α (Dower et al. 1988) and *B. coagulans* by electroporation in this study. Plasmid DNA was isolated from *E. coli* and *B. coagulans* according to Birnboim and Doly (1979).

Results

To construct pMK3P plasmid vector, 1300 bp HindIII/BamHI cut DNA fragment of phyC gene from pRS416P was transferred into pMK3 *E. coli*-Bacillus sp. shuttle vector. (Figure1).

![Figure 1. Structure of pMK3P plasmid (pMK3 (7200 bp) plus phytase gene (~1300 bp))](image)

Ligation mixture was used to introduce plasmids into *E. coli* cells and resulting *E. coli*/pMK3P transformants grew with non-recombinant pMK3 on LB/Agar medium including 40 µg/mL X-Gal plus 100 µg/mL Amp. To select recombinant *E. coli* cells bearing pMK3P against *E. coli*/pMK3 cells, white colonies grown on solid growth media were directly collected and the plasmid was isolated. pMKP plasmid vector (Figure1) carrying phyC gene was transferred into *B. coagulans* cells by electrotransformation. Recombinant *B. coagulans*/pMK3P colonies were observed on MediumI agar medium supplemented with Km. Phytase activity of transformed bacteria was detected on MediumI supplemented with wheat bran extract medium plates and clear zones were expected around of the recombinant colonies.

While original *B. coagulans* bacterium with no Km resistance gene didn’t grow on solid growth medium contain Km antibiotic, recombinant *B. coagulans*/pMK3P bearing Km resistance gene on the vector grew well on the medium supplemented with Km (Figure 2A). When these bacteria were
inoculated to detect phytase activity of *B. coagulans*/pMK3P, original and recombinant *B. coagulans* bacteria didn’t show phytase activity on the MediumI supplemented with wheat bran extract medium plates (Figure 2B).

**Figure 2.** Original *B. coagulans* and recombinant *B. coagulans*/pMK3P bacteria
A: Growing on the MediumI/Km/Agar medium, B: Phytase activity on the MediumI supplemented with wheat bran extract medium

The original pMK3 and recombinant pMK3P were isolated from *E. coli* and *B. coagulans* cells, respectively. They were subjected to insert analysis on 0.8% w/v agarose gel electrophoresis with PCR fragment of *B. subtilis* VTT E-68013 and *B. coagulans*/pMK3P phytase gene. Phytase gene fragment (~1300 bp) from PCR amplification and restriction analysis was determined on the range with line (Figure 3).

**Figure 3.** Insert and PCR analysis of *B.coagulans*/pMK3P plasmid on agarose gel
M: 1kb DNA Markers, 1: pMK3/HindIII+BamHI, 2: PCR fragment of *B. subtilis* VTT E-68013 phytase gene, 3: *B. coagulans*/pMK3P/HindIII+BamHI, 4: PCR fragment of *B. coagulans*/pMK3P phytase gene

*B. subtilis* VTT E-68013, *E. coli*/pMK3P and *B. coagulans*/pMK3P bacteria were inoculated on MediumI supplemented with wheat bran extract medium plates to determine phytase activity of recombinant *E. coli* and *B. coagulans* at various pH value (5, 6, 7, 8, 9, 10). For phytase activity of the
bacteria, clear zones were viewed around the colonies. To view zones better, plates were stained with iodine (Figure 4). Whereas *B. subtilis* VTT E-68013 strain showed phytase activity except pH 5 and 10, recombinant *E. coli* and *B. coagulans* bearing phytase gene hadn’t phytase activity all the pH ranges on plates.

![Figure 4](image)

**Figure 4.** Testing of various bacteria (*B. subtilis* VTT E-68013, *E. coli/pMK3P*, *B. coagulans/pMK3P*) for phytase activity on MediumI supplemented with wheat bran extract medium plates at various pH (5, 6, 7, 8, 9, 10) (Plates were stained with iodine to view zones better)

**Discussion**

The cloning and expression in *B. coagulans* of *phyC* phytase gene was studied. As a feed additive, *B. coagulans* has remarkable effects on growth promotion of animals (poultry, pigs, etc.) through the increase of digestibility and prevention of disease caused by infectious microbial flora, *E. coli* and *Staphylococci* in intestine. Without any pathological and pharmaceutical side effects *B. coagulans* has growth-promoting effects on poultry, pigs and other animals (Anonymous, 2007).

The PhyC phytase has several features favoring its use in feed applications. Firstly, the enzyme is derived from a GRAS classified microorganism, allowing its use as a feed additive. Secondly, the enzyme has a neutral pH optimum. This is particularly important since the enzyme should function in the small intestine (pH near neutral), where phosphate absorption takes place. Most fungal phytases are practically inactive in neutral pH. Thirdly, the PhyC is a thermostable enzyme. This is perhaps the most important feature of a feed enzyme, since during the pelleting process feed (and supplemented enzyme) is subjected to high temperatures. Fourthly, the enzyme is not prone to product - inhibition caused by inorganic phosphate that could decrease the enzyme activity.
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in the small intestine. Fifthly, the enzyme is not susceptible to proteases (Kerovuo, 2000).

To express phytase gene in the bacterium, a vector called pMK3P-bearing \textit{phyC} was constructed and then electrotransferred into \textit{B. coagulans}. HindIII and BamHI digestion of the recombinant plasmids from \textit{B. coagulans} yielded 1300 bp fragment of DNA carrying the gene encoding \textit{phyC} and 7200 bp fragment of pMK3 vector on agarose gel electrophoresis. Phytase gene of recombinant vector pMK3P was also amplified by PCR and visualized by agarose gel electrophoresis to support the results. Although phytase gene was cloned in \textit{B. coagulans}, phytase enzyme activity wasn’t detected on Wheat Bran Extract/Sodium-phytate/LB/Agar plates. Recombinant \textit{B. coagulans} tested for phytase activity on MediumI supplemented with wheat bran extract medium plates at various pH (5, 6, 7, 8, 9, 10) to obtain any changing in pH optimum of the enzyme but no phytase activity was observed on the test plates.

Kerovuo and Tynkkynen (2000) was expressed the phytase (\textit{phyC}) from \textit{B. subtilis} VTT E-68013 in \textit{Lactobacillus plantarum} strain 755. The recombinant phytase was secreted at a slower rate in comparison to the native proteins of \textit{L. plantarum} 755. They mentioned that, the expression and secretion level of phytase in \textit{L. plantarum} 755 was low in relation to the levels needed for industrial production.

The value of expression systems based on strong and tightly regulated promoters is well recognized in modern biotechnology. In the study \textit{lacZ} promoter or own promoter of the phytase gene was used for expression of phytase gene in \textit{B. coagulans}. The promoters may be not strong enough for expressing the gene. Therefore, Kerovuo et al (2000) were developed a new expression system for \textit{B. subtilis} to express phytase gene (\textit{phyC}). The expression system developed showed promising results when applied to the production of recombinant \textit{Bacillus} phytase.

\textit{B. subtilis} and related \textit{Bacillus} species have been used for decades for the bulk production of industrial enzymes, in particular for the production of native secretory enzymes such as amylases and proteases (Sarvas, 1995; Quax, 1997). Despite the high secretion capacity of \textit{Bacillus} species for native enzymes, the use of these bacteria in the production of heterologous proteins (as in the case of all other production organisms) has frequently resulted in low expression levels (Bron et al., 1998). Molecular chaperons and targeting factors are potential limiting factors for high-level secretion of heterologous proteins in \textit{Bacillus} (Kontinen and Sarvas, 1993; Bron et al., 1998). Besides, numerous proteases secreted by these expression hosts are often responsible for the degradation of most heterologous and even homologous secreted gene products (Doi, 1991; Kunst et al., 1997). Thus, PhyC secreted from recombinant \textit{B. coagulans} may be not enough amounts to degrade of phytate or inactivated by proteases of the host.

In conclusion, the phytase (\textit{phyC}) from \textit{B. subtilis} VTT E-68013 was not expressed in \textit{B. coagulans}. However, this was the first report to our knowledge that \textit{phyC} was attempted to clone in \textit{B. coagulans}. 
References


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