Note

High-level Expression of a Recombinant Thermostable Phytase in *Bacillus subtilis*

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An efficient expression system was developed in *Bacillus subtilis* for the large scale production of phytase. The phytase gene with a native promoter derived from *Bacillus amyloliquefaciens* was cloned in the *Bacillus* expression vector pJH27 under a strong BJ27 promoter and its expression was optimized. The expression of the phytase gene occurred during late exponential growth and the extracellular phytase production was 2.0 units/ml, which constituted over 90% of the total protein. The yield was 100-fold higher than wild type *Bacillus amyloliquefaciens D511.*

Key words: *Bacillus subtilis*; phytase; phytate; overexpression

Phytate (*myo*-inositol hexakisphosphatase) is the predominant form of phosphate in cereal grains, oil seeds, and legumes. It is an enzyme capable of hydrolyzing phytate to inorganic phosphate, *myo*-inositol, and inositol mono- to pentaphosphates. Since the digestive tracts of monogastric animals including pigs and poultry lack phytase, phytate-phosphate in the feed can’t be used by these animals and thus inorganic phosphorus has to be added to secure sufficient phosphate supply. As a result, phytate and phosphate are excreted in manure, causing environmental problems in areas of intensive livestock production. In addition, phytate forms complexes with proteins and multivalent metal ions such as iron, zinc, and calcium, thereby showing anti-nutritional effects. So, the enzymatic hydrolysis of phytate into less-phosphorylated inositol derivatives in the intestine of monogastric animals is desirable. Many attempts to hydrolyze phytate with phytase have been made to decrease the amount of phosphorus excretion and improve the nutritional value of feed.

Previously, we purified and characterized a thermostable phytase from *Bacillus amyloliquefaciens D511* and the gene was cloned and expressed in *E. coli.* This phytase is very valuable for its thermostability, which allows it to withstand inactivation during the feed pelleting and expansion processes, and thus decreases costly formulations to limit activity loss. For animal feed applications, it is necessary to develop an efficient expression system in *B. subtilis* that is biologically safe and cost-effective in large-scale production. *B. subtilis* is also regarded as an attractive host for the secretion of endogenous as well as heterologous proteins because of its nonpathogenic nature, high secretion capacity, and existence of a great deal of fermentation technology. Therefore, in this study we constructed a recombinant plasmid for this phytase expression in *B. subtilis.* The 2.2-kb *HindIII* DNA fragment encoding the phytase of pKP1 was subcloned into the *HindIII* site of pJH27 containing a strong *Bacillus* promoter (BJ27), producing pJKP. Therefore, in pJKP, the phytase gene and its own promoter were downstream from BJ27. pJKP was used to transform *B. subtilis* DB104. The expression of the phytase gene was first studied with *B. subtilis* cells harboring pJKP growing in LB medium with 25 μg/ml kanamycin at 37°C. The phytase activity of the culture supernatant reached 0.3 U/ml when the cell growth reached \( A_{420} \) = 11.0, which was 17 times higher than that of the wild type strain, *B. amyloliquefaciens* D511. But the enzyme activity decreased rapidly after 12 h of culture time and it showed little activity at the late stationary phase (Fig. 1). The culture supernatant was then analyzed by SDS-PAGE (Fig. 2). The phytase band with a molecular mass of 44 kDa was clearly shown in the culture supernatant at 11 h of fermentation, but it was undetected after 24 h. This result suggests that it may be as

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Fig. 1. Growth and Phytase Expression in *B. subtilis* DB104 Harboring pJKP in LB Medium.

The strain was grown in LB medium at 37°C with periodic observations of growth by spectrophotometer at 420 nm and for enzyme activity in the culture supernatant by measuring the increasing rate of inorganic phosphate. Growth (■) and enzyme production (●) by *B. subtilis* DB104 harboring pJKP; growth (○) and enzyme production (○) of *B. subtilis* DB104 as a control.

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a result of proteolytic degradation of the translated gene product.

Initially, LB medium was used as the growth medium for phytase expression. This medium can support only modest growth in shake flasks, since it is not well buffered. To improve the growth characteristics of the cells and further expression of phytase gene in *B. subtilis*, we tested a richer and better-buffered modified LB medium which contained in addition to LB medium 10 g K2HPO4/l, 0.45 g MgSO4/l, 40 g glucose/l, and 2 ml/l of trace elements solution. The expression of phytase and cell growth was observed throughout the cultivation time (Fig. 3). With the modified LB broth as the growth medium, both cell and enzyme yields were improved greatly, reaching a level of about 2 U/ml with \( A_{420	ext{ nm}} = 53 \). The transformant produced phytase about 100 times higher than that of *B. amyloliquefaciens* DS11 expressed under the control of its native promoter. Also, phytase production was very stable even after 24 h incubation and remained constant up to 48 h. The high production of phytase encoded by a foreign gene in *B. subtilis* did not impede the growth of the host cell significantly. The protease activity was checked throughout the cultivation time. The secretion of protease was observed to be slower and lower in modified LB medium, which may be the reason of suppressed degradation of phytase in this medium. In addition, a study was done to identify which components added to the LB medium were most effective on the stable production of the enzyme using different media compositions, but right now we are unable to draw any conclusions. SDS-PAGE analysis of the culture supernatant of *B. subtilis* harboring pJPK showed a major distinct protein band at 24 h of cultivation (Fig. 4). The protein band corresponding to phytase was identified by activity staining (data not shown). The specific activity in this case was 14 U/mg protein, which accounts to about 90% of the total protein in the culture supernatant. On the other hand, there was no detectable phytase activity in the cell extract, indicating that the phytase is effectively secreted into the culture medium. Phytase produced by *B. subtilis* had similar thermostable characteristics to that of the native one. That means, the optimum temperature for phytase activity was 70°C and about 50% of its original activity was retained after incubation at 90°C for 10 min in the presence of 5 mM CaCl2. The instability of recombinant plasmids in the hosts is a problem often encountered in the application of recombinant DNA technology. Several reports have described segregational and structural instability of recombinant plasmids in *B. sub-
However, *B. subtilis* harboring pPK has not shown any problems of instability such as deletion or rearrangement of the plasmid. These results indicate that this particular plasmid-host combination is suitable for large-scale production of phytase. Therefore, the *Bacillus* strain transformed by this plasmid seems to be a promising candidate for industrial applications. Work is now in progress studying large-scale fermentor conditions controlling aeration, pH, and respiratory quotients for high yields.

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**References**


