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Isolation of a Phytase-Producing *Bacillus* sp. KHU-10 and Its Phytase Production

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Abstract A bacterial strain producing high level of an extracellular phytase was isolated from cooked rice and identified as a strain of *Bacillus* sp. and designated as *Bacillus* sp. KHU-10. Optimum culture conditions were investigated for the maximum productivity of phytase by *Bacillus* sp. KHU-10. 1.0% Maltose and 1.0% peptone with 0.5% beef extract were the best carbon source and nitrogen source, respectively. The addition of $CaCl_2$ stimulated the enzyme productivity with concentration between 0.01% and 0.2%, in the medium. Although sodium phosphate increased the cell mass, the enzyme activity decreased. Calcium phytate and wheat bran containing phytate did not enhance the enzyme production. Under the optimum medium, the production of the phytase reached the highest level of 0.2 unit/ml after 4 days of incubation.

Key words: Phytase, phytic acid, *Bacillus* sp. KHU-10

Phytic acid (myo-inositol(1,2,3,4,5,6)hexakisphosphate) is the major storage form of phosphorous in the seeds of plants [17]. In particular, animal feeds made from the byproducts of oil industry contain high amounts of phytate from 1% up to 5% [2, 3, 14, 15, 20]. Phytic acid in animal feed is an antinutritional factor due to its ability to form complexes not only with divalent metal ions such as Ca^{2+}, Mg^{2+}, Zn^{2+}, and Fe^{2+}, but with proteins. These phytate complexes are not easily utilized by monogastric animals such as pig and chicken [4, 13, 19], and result in the phosphorus pollution problems in the areas of intensive livestock production [9]. Phytase (EC 3.1.3.8) hydrolyzes phytic acid to myo-inositol and phosphoric acid. These phytases are present in fungi [8, 9], bacteria [10, 21], yeast [12], and plants [1, 5]. Because monogastric animals, including man, lack phytase, phytate is not degraded in the small intestine during digestion and the phosphate is not available. Therefore, the addition of phytase to the food and animal feed can not only enhance the bioavailability of phytate, but reduce the environmental pollution due to undigested phytate [14]. Phytase for animal feed is on the market by several companies including Gist Brocades Co. Most phytases on the market are produced from fungi. Enzymes originated from fungi need more time to produce than bacterial ones and are more active under acidic pH compared with bacterial ones. The objective of this research was to isolate a bacterial strain capable of producing phytase with high productivity and to examine the culture conditions for high production using the isolated bacterium.

For the isolation of a phytase-producing bacterial strain, about 100 strains from soil, food, and stock were investigated using the medium containing 1.0% peptone, 0.5% beef extract, 1.0% glucose, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% $CaCl_2$, and 0.1% calcium phytate. The microorganisms were aerobically incubated at 37°C, and after the centrifugation, each culture supernatant was tested for phytase activity.

The phytase activity was measured by incubating 0.1 ml of enzyme solution with 0.9 ml of 2 mM sodium phytate in 0.1 M Tris-HCl buffer (pH 7.0). The enzyme reaction was carried out at 37°C for 10 min and then the reaction was stopped by adding 0.75 ml of 5% trichloroacetic acid. The liberated phosphate was measured at 700 nm after adding 1.5 ml color reagent, which was prepared just before the experiment by mixing four volumes of 2.5% ammonium molybdate solution in 5.5% sulfuric acid and one volume of 2.5% ferrous sulfate solution. One unit of phytase activity was defined as 1 μmole of phosphate liberated per minute under the assay condition. Among 100 bacterial strains, KHU-10, which was isolated from cooked rice, was shown to have the highest phytase activity. The KHU-10

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strain was a motile, gram-positive, rod-shaped, and spore-forming bacterium. On the basis of the results of the morphological and biochemical tests (Table 1), the strain KHU-10 could be assigned to a strain of *Bacillus* sp. or its variant strain.

The effect of carbon sources on the production of phytase by *Bacillus* sp. KHU-10 was examined using the medium containing 1.0% peptone, 0.01% $MgSO_4 \cdot 7H_2O$ and with 1.0% each of different carbon sources. As shown in Table 2, *Bacillus* sp. KHU-10 synthesized the phytase using all carbon sources such as monosaccharide (glucose, fructose, and mannose), disaccharide (maltose and lactose), sugar alcohol (sorbitol), and starch. Although the best carbon source varied according to culture time, maltose was the

Table 1. Morphological and physiological characteristics of the isolated strain.

Morphological characteristics.			
Cell shape (rod), gram (+), Spore formation			
Biochemical and physiological characteristics			
Casein hydrolysis	+	Catalase	+
Starch hydrolysis	+	Oxidase	+
Gelatine hydrolysis	+	Urease	-
Acid formation from			
Sucrose	+	Inulin	+
Tagatose	-	Ribose	-
Glucose	+	Trehalose	-
Arabinose	+	Palatinose	+
Xylose	-	Sorbitol	+
Mannitol	+	*N*-Acetyl-D-glucosamine	-
Raffinose	-	Amylopectin	-
Salicin	-	Arabitol	-
Amygadalin	-		
Glucose fermentation in the presence of			
KCN	+	Sodium acetate	+
7% NaCl	+	Polyamido-hydrogrostrepin	+
Mandelic acid	+	Nalidixic acid	-
Oleandomycin	-	Esculin	+
Growth at 55°C		+	
Reduction of tetrazolium red		+	

Table 2. Effect of carbon sources on the phytase production.

Carbon source (1%)	Cell growth at 660 nm	Phytase activity (unit/ml)
Glucose	7.8	0.038
Fructose	7.5	0.040
Mannose	8.0	0.046
Maltose	8.2	0.064
Sucrose	7.7	0.052
Lactose	6.5	0.034
Galactose	5.8	0.039
Sorbitol	6.2	0.050
Soluble starch	7.6	0.053

Cultivation was carried out aerobically at 37°C for 3 days in the medium (pH 7.0) consisting of 1.0% peptone and 0.01% $MgSO_4 \cdot 7H_2O$. 2.0 ml of the seed culture was inoculated into 80 ml medium in the 300-ml flask.

Table 3. Effect of nitrogen sources on the phytase production.

Nitrogen source	Cell growth at 660 nm	Phytase activity (unit/ml)
Peptone	7.8	0.064
Polypepton	7.6	0.050
Casein	6.8	0.048
Casamino acid	6.2	0.038
Casitone	7.2	0.049
Skim milk	6.5	0.030
Bacto-soytone	6.5	0.058
Malto extract	5.4	0.028
Beef extract	4.5	0
Meat extract	5.4	0.028
Yeast extract	5.4	0.030
1% Peptone + 0.5% Meat extract	9.2	0.085
1% Peptone + 0.5% Yeast extract	8.9	0.075
1% Peptone + 0.5% Beef extract	8.8	0.096

Cultivation was carried out aerobically at 37°C for 3 days in the medium (pH 7.0) consisting of 1.0% maltose and 0.01% $MgSO_4 \cdot 7H_2O$. 2.0 ml of the seed culture was inoculated into 80 ml medium in the 300-ml flask.

Table 4. Effect of inorganic salts on the phytase production.

Inorganic salt	Cell growth at 660 nm	Phytase activity (unit/ml)
Control	7.8	0.096
0.1% $CuSO_4$	8.0	0.065
0.1% $Fe_2Cl_3 \cdot 6H_2O$	8.2	0.082
0.1% $FeSO_4$	7.7	0.084
0.1% KCl	6.5	0.060
0.1% LiCl	5.8	0.068
0.1% $MnCl_2$	6.2	0.073
0.1% NaCl	7.6	0.035
0.1% $SnCl_2$	7.9	0.056
0.1% $ZnCl_2$	8.5	0.115
0.001% $CaCl_2$	8.8	0.125
0.01% $CaCl_2$	8.8	0.135
0.1% $CaCl_2$	8.9	0.138
0.2% $CaCl_2$	9.5	0.112
0.3% $CaCl_2$	9.8	0.076
0.4% $CaCl_2$	9.8	0.075

Cultivation was carried out aerobically at 37°C for 3 days in the medium (pH 7.0) consisting of 1.0% maltose, 1.0% peptone, 0.5% beef extract, and 0.01% $MgSO_4 \cdot 7H_2O$. 2.0 ml of the seed culture was inoculated into 80 ml medium in the 300-ml flask.

best one with 0.064 U/ml after 3 days of incubation. Kim [11] reported that, as for *B. amyloliquefaciens*, phytase was not induced by glucose, fructose, maltose, and sucrose but by wheat bran containing phytate. This result suggested that *B. amyloliquefaciens* produced the phytase by the induction of phytate. On the other hand, *Bacillus* sp. KHU-10 synthesized the phytase in all carbon source media tested showing that this stain does not need phytate for the phytase production.

Table 3 shows the effect of various nitrogen sources on the phytase production in the basal medium containing 1.0% maltose, 0.1% $MgSO_4 \cdot 7H_2O$. Although pepton was found to be effective for the phytase production as an individual nitrogen source, the highest activity (0.096 U/ml) was obtained when *Bacillus* sp. KHU-10 was cultivated in the basal medium supplemented with 1.0% peptone and 0.5% beef extract as a nitrogen source.

Table 4 shows the effect of the addition of inorganic salts on the phytase production using the basal medium containing 1.0% maltose, 1.0% peptone, 0.5% beef extract, and 0.01% $MgSO_4 \cdot 7H_2O$. The addition of 0.01 and 0.1% $CaCl_2$ to the medium resulted in the enhancement of the phytase productivity by approximately 30% compared with no addition. Even though the relationship between Ca^{2+} ions and the phytase synthesis is not known, Ca^{2+} ions is supposed to contribute to stabilize the produced phytase in the cell broth. Kim [10] and Shimizu [18] reported that Ca^{2+} ions stabilize each phytase produced by *Bacillus* sp. DS11 and *B. subtilis* (natto), respectively.

To investigate the effect of phosphate salts and phytate on the production of phytase, sodium phosphate, calcium phytates and wheat bran containing phytate were added to the medium mentioned above supplemented with 0.1% $CaCl_2$ (Table 5). Although phosphate salts increased cell mass, phytase activity decreased in the cell broth. This result is supposed to be due to the feedback inhibition of phosphate for the phytase. Calcium phytate and wheat bran containing phytate were reported to enhance the phytase production in cell broth for *Aspergillus ficuum* [7] and *B. subtilis* [16], but there was no effect on phytase production for *Bacillus* sp. KHU-10.

Table 5. Effect of phosphate salts and phytate on the phytase production.

Chemical compound	Cell growth at 660 nm	Phytase activity (unit/ml)
Control	9.5	0.138
0.01% Wheat bran	9.2	0.120
0.1% Wheat bran	9.4	0.125
1.0% Wheat bran	9.5	0.118
0.01% Ca-phytate	9.2	0.130
0.1% Ca-phytate	9.8	0.128
1.0% Ca-phytate	10.8	0.110
0.001% Na_2HPO_4	10.0	0.133
0.01% Na_2HPO_4	11.8	0.115
0.1% Na_2HPO_4	12.8	0.085

Cultivation was carried out aerobically at 37°C for 3 days in the medium (pH 7.0) consisting of 1.0% maltose, 1.0% peptone, 0.5% beef extract, 0.01% $MgSO_4 \cdot 7H_2O$, and 0.1% $CaCl_2$. 2.0 ml of the seed culture was inoculated into 80 ml medium in the 300-ml flask.

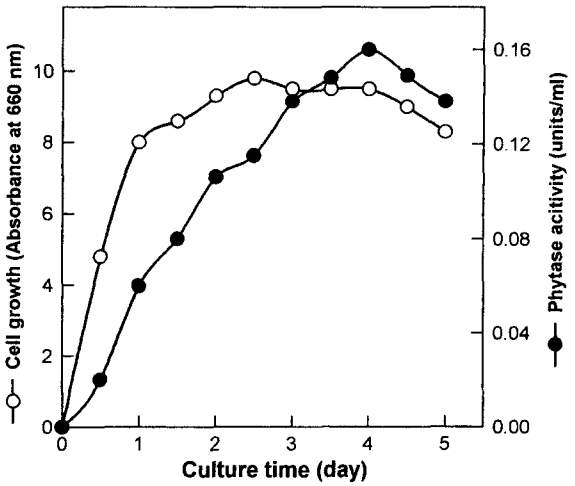

Fig. 1. Time course of growth and enzyme production in a culture of *Bacillus* sp. KHU-10.

The time course of the phytase production was examined using the optimum medium investigated for the enzyme production. As shown in Fig. 1, the kinetics of the phytase production during incubation revealed that the cell growth reached the stationary phase after 3 days of incubation and the maximum level of the enzyme activity which had produced 0.2 U/ml was observed after 4 days of incubation. The maximum production of the phytase by *B. subtilis* [21] and *B. subtilis* (natto) [18] were reported to be reached after 5 days incubation. *E. coli* [6] was reported to produce the maximum phytase during the late stationary phase. From these results, bacteria were supposed to produce the maximum level of phytase after the stationary phase, but *Bacillus* sp. DS11 [10] was reported to reach the maximum phytase productivity after 1 day of cultivation.

For use of the phytase from *Bacillus* sp. KHU-10 as an additive of the animal feed to be made possible, further research on the enzyme characteristics and enhancement of the enzyme stability remains a challenging task.

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