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Isolation of Phytase-Producing *Pseudomonas* sp. and Optimization of its Phytase Production

KIM, YOUNG-HOON, MOON-NAM GWON, SI-YONG YANG, TAE-KYU PARK, CHAN-GIL KIM, CHANG-WON KIM[1], AND MIN-DONG SONG*

Department of Biotechnology, Konkuk University, Chungju 380-701, Korea
[1]Department of Dairy Science, Konkuk University, Seoul 143-701, Korea

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Abstract Phytase (*myo*-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8) catalyzes the hydrolysis of phytate (*myo*-inositol hexakisphosphate) to release inorganic phosphate. A bacterial strain producing phytase was isolated from soil around a cattle shed. To identify the strain, cellular fatty acids profiles, the GC contents, a quinine-type analysis, and physiological test using an API 20NE kit were carried out. The strain was identified to be a genus of *Pseudomonas* sp. and named as *Pseudomonas* sp. YH40. The optimum culture condition for the maximum productivity of phytase by *Pseudomonas* sp. YH40 were attained in a culture medium composed of 1.0% (w/v) glycerol, 2.0% (w/v) peptone, and 0.2% (w/v) $FeSO_4 \cdot 7H_2O$. Within the optimal medium condition, the production of phytase became highest after 10 h of incubation, and the maximal phytase production by *Pseudomonas* sp. YH40 was observed at 37°C and pH 6.0.

Key words: *Pseudomonas* sp., phytase, cellular fatty acids, GC contents, quinone, optimum medium

Phytases (*myo*-inositol hexakisphosphate phosphohydrolase: EC 3.1.3.8) are phosphatase enzymes that hydrolyze phytate (*myo*-inositol hexakisphosphate) to *myo*-inositol and inorganic phosphate. Such phytases have already been found in molds, yeast, bacteria, and plants [1, 2, 3, 9, 13, 15, 16, 20, 23, 24]. Phytate is the major storage form of phosphorus and accounts for more than 70–80% of the total phosphorus in cereals and legumes [15, 27, 30]. However, since monogastric animals including pigs, poultry, and fish lack phytase, phytate in the feed cannot be utilized and, therefore, inorganic phosphate is added to their diets for the phosphorus requirement [4, 11, 17, 21].

*Corresponding author
Phone: 82-43-840-3612; Fax: 82-43-851-4169;
E-mail: minds@kku.ac.kr

The phytate is seen as a way to reduce the level of phosphorus pollution that results from the excretion of phytic acid and phosphate supplements, which then contributes to water pollution problems in areas of intensive livestock production [6, 21]. In addition, phytate acts as an antinutritional agent in monogastric animals by chelating minerals (such as, calcium, copper, magnesium, and zinc), protein, and vitamins. Therefore, the reduction of the phytate content in seed meal via its enzymatic hydrolysis is desirable [7, 14, 19, 20, 22, 27].

During the last 30 years, extensive research has been conducted to improve the availability of phosphorus. However, the high cost of the enzyme production, compared to the cost of inorganic phosphate, has prevented its use. Recently, there has been a renewed interest in phytase due to its low production cost by recombinant DNA technology.

A number of phytase-producing microorganisms have already been reported, including *Bacillus subtilis* [11, 21, 23], *Bacillus* sp. [3, 12], *Enterobacter* sp. [13, 29], *Pseudomonas* sp. [23], yeast [15], and molds [9, 20, 28, 30].

Accordingly, the object of the current study was to isolate and identify a phytase-producing *Pseudomonas* sp., and optimize its culture conditions for phytase production.

Materials and Methods

Isolation of Phytase-Producing Bacteria

To isolate a phytase-producing bacterial strain, various soil samples were collected from a cattle shed. Nine milliliters of a sterile 0.9% (w/v) NaCl solution was add to 1 g of soil. After 30 min of standing, serial dilutions of the samples were prepared and plated on BHI agar medium, and then incubated at 37°C for 48 h. The colony-forming bacteria on the BHI agar medium were selected. To screen phytase-producing bacteria, the bacteria were streaked onto a

phytase screening medium (PSM, 1.5% glucose, 0.5% NH_4NO_3, 0.5% calcium phytate, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 4H_2O$, and 2.0% agar), and incubated at 37°C for 24 h. The bacteria with a clear zone around the colony were selected, then the isolated strains were cultivated in a PSM broth at 37°C for 24 h with vigorous shaking. After centrifugation, each culture supernantant was then tested for phytase activity.

Identification of Phytase-Producing Bacteria

The bacterial strain with the highest phytase activity was then identified by Gram staining, as well as morphological, biochemical, and physiological tests. Thereafter, the strain was identified according to *Bergey's Manual of Determinative Bacteriology* [8] based on its morphological, biochemical, and physiological characteristics. The morphology of the isolated bacteria was observed using a scanning electron microscope (SEM). To study the biochemical and physiological properties of the isolated strain, an API 20NE kit (Bio-Merieux, France) was used to test and measure the quinone composition and DNA GC mol%. Analysis of the quinone composition was carried out as described by Shin *et al.* [25], by thin layer plate chromatography (TLC) and HPLC. The band with the same R_f value as the quinone standards on a Kiesel-Gel TLC plate, which was developed by a solvent of hexane:diethyl ether (85:15, v/v), was scraped off and eluted with acetone. Quinones were determined by HPLC under the following conditions: column: Spherisorb ODS2 column (4.6×250 mm); mobile phase: methanol: isopropyl ether (3:1, v/v for ubiquinone; 4:1, v/v for menaquinone); flow rate: 1 ml min^{-1}; detector: UV detector at 275 nm for ubiquinones and at 270 nm for menaquinones. The mol% GC was analyzed by HPLC under the following conditions: C18 Symmetry column (4.6×250 mm); 0.5 M triethylamine phosphate (pH 5.1):water:methanol (1:18:5, v/v/v); mobile phase flow rate of 1 ml min^{-1}; and UV detector at 254 nm. In addition, the major cellular fatty acid composition was analyzed by gas liquid chromatography [10], and the results analyzed using the MIDI aerobe method saved on chemstation version 4.02.

Phytase Assay

The phytase activity assay was carried out based on the rate of increase in inorganic orthophosphate by the ascorbic acid method [5]. The reaction mixture for phytase determination consisted of 400 µl of a 0.1 M Tris-HCl buffer, pH 7.0, containing 2 mM sodium phytate and 100 µl of the enzyme preparation, and the mixture was incubated at 37°C for 30 min. The reaction was stopped by the addition of 500 µl of 5% (w/v) trichloroacetic acid. The released inorganic phosphate was measured by incubating 4 ml of the assay mixture [6N H_2SO_4:2.5% (w/v) ammonium molybdate:10% (w/v) ascorbic acid:H_2O, 1:1:1:2] at 37°C. After 30 min, the absorbance at 820 nm was measured. One unit of phytase activity was defined as the amount of enzyme required to liberate 1 µmol of P_i per min.

Optimization of Culture Condition for Phytase Production

To optimize the medium composition for phytase production by *Pseudomonas* sp.YH40, the effects of carbon, nitrogen, and mineral sources on phytase production were determined after 10 h of culture. A basal medium containing 1% (w/v) glucose, 1% (w/v) peptone, and 0.1% (w/v) $MgSO_4 \cdot 7H_2O$ was adjusted to pH 7.0 before sterilization, unless otherwise stated. The effect of various nitrogen sources on phytase production was investigated by replacing the peptone in the basal medium with tryptone, beef extract, malt extract, yeast extract, casein hydrolysate, NH_4NO_3, and $(NH_4)_2NO_4$. The other major studied ingredients were carbon sources (glucose, glycerol, maltose, mannose, fructose, sucrose, lactose, soluble starch, and wheat bran), inorganic salts (KCl, $FeSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, NaCl, and $CaCl_2$), and phosphate salts (Na-phytate, Ca-phytate, $NaHPO_4$, and K_2HPO_4). Generally, 50 ml of the resulted medium in a 250-ml Erlenmeyer flask was aerobically cultured at 37°C for 10 h on a rotary shaker (180 rpm). The culture broth was then centrifuged (5,000 ×g, 4°C, 10 min), and the supernatant was used for the enzyme assay. After establishing the optimal medium composition, the effects of the initial pH and culture temperature on the production of phytase were also investigated. All experiments were carried out at least twice until the optimal culture conditions were found, as such, the data given in this paper are representative.

RESULTS AND DISCUSSION

Isolation and Identification of Phytase-Producing *Pseudomonas* sp.

Phytase-producing bacterial strains were isolated from soil using PSM agar media. Sixteen strains showed phytase activity on PSM agar. Finally, the highest phytase-producing strain, YH40, was selected (Fig. 1). The morphological characteristics of the strain YH40 are summarized in Table 1. As such, strain YH40 was found to be a Gram-negative, motile, non-spore forming bacteria, and it formed yellow colonies. Figure 2 shows the shape of strain YH40 under an SEM (scanning electron microscope); it was rod-shaped (0.4–1.0 × 1.0–3.0 µm). The results obtained for the biochemical and physiological characteristics of strain YH40 are summarized in Table 2. The strain YH40 exhibited positive results with catalase test, reduced nitrates to nitrites, arginine dihydrolase, esculin hydrolysis, and gelatine hydrolysis, while showing negative results with indole production, glucose acidification, urease, and β-galactosidase. In addition, strain YH40 was able to

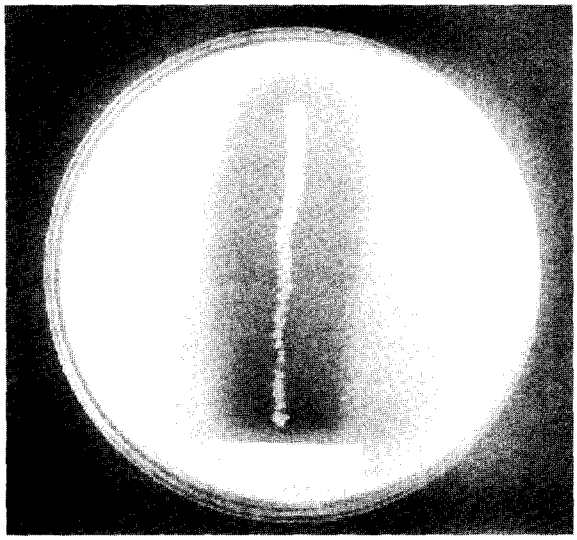

Fig. 1. Phytase activity on PSM agar medium (1.5% glucose, 0.5% NH_4NO_3, 0.5% calcium phytate, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 4H_2O$, and 2.0% agar) with strain YH40.

utilize glucose, mannitol, *N*-acetyl-glucosamine, gluconate, caprate, adipate, malate, and citrate as carbon sources. The GC mol% of strain YH40 was 63%. The major components of quinone type of strain YH40 was ubiquinone-8 and ubiquinone-9. The cellular fatty acid analysis of strain YH40 is shown in Table 3 and Fig. 3. The cellular fatty acid analysis revealed the presence of C10:0 3OH, C12:0, C12:0 2OH, C12:0 3OH, C16:0, C18:1 w7c, C19:0 cyclo w8c. On the basis of its morphological, physiological, and biochemical characteristics, strain YH40 was identified as a species of *Pseudomonas* and named as *Pseudomonas* sp.YH40.

Bacterial Growth and Phytase Activity

To determine the relation of cell growth and phytase activity, *Pseudomonas* sp. YH40 was cultured in a BHI broth at 37°C for 28 h with shaking (180 rpm). Figure 4 shows the bacterial growth curve and phytase activity of *Pseudomonas* sp. YH40 at various times. The enzyme activity of crude phytase was detected after 4 h and maximum activity was achieved after 10 h.

Table 1. Morphological characteristics of strainYH40.

Characteristics	Strain YH40
Gram reaction	-
Cell shape	Rod
Motility	+
Size (μm)	0.4–1.0 × 1.0–3.0
Spore	-
Color of colony	Weak yellow

+, positive; –, negative.

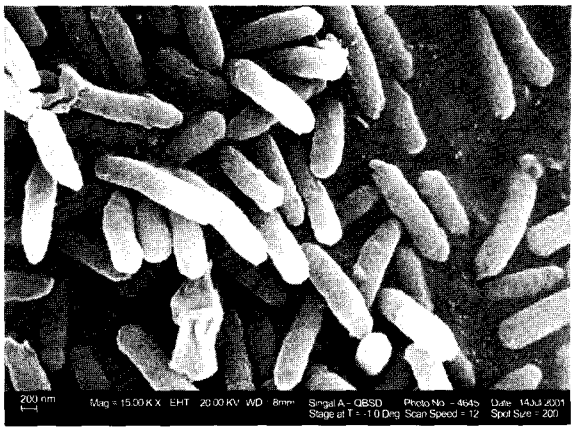

Fig. 2. Scanning electron microscopic observation of strain YH40.

Optimization of Culture Conditions for Phytase Production

To determine its optimal culture conditions, *Pseudomonas* sp.YH40 was cultured in various carbon sources, nitrogen sources, inorganic salts, and phosphate salts at 37°C for 10 h with vigorous shaking.

Effect of Nitrogen Sources on the Phytase Production

The effect of nitrogen sources on phytase production by *Pseudomonas* sp. YH40 was investigated in a medium

Table 2. Physiological and biochemical characteristics of strain YH40.

Characteristics	Strain YH40
Cytochrome oxidase	+
Reduction of nitrates to nitrites	+
Indole production	-
Glucose acidification	-
Arginine dihydrolase	+
Esculin hydrolysis (β-glucosidase)	+
Gelatine hydrolysis (protease)	+
Urease	-
β-galactosidase	-
Assimilation of	
Glucose	+
Arabinose	-
Mannose	-
N-acetyl-glucosamine	+
Maltose	+
Gluconate	+
Caprate	+
Adipate	+
Malate	+
Citrate	+
Phenyl-acetate	-
GC mol%	63%
Quinone type	Ubiquinone-8, ubiquinone-9

+, positive; –, negative.

Table 3. Major cellular fatty acids in strain YH40.

Retention time	Fatty acids	%
4.061	C 10:0 3OH	4.01
4.581	C 12:0	4.19
5.948	C 12:0 2OH	5.02
6.322	C 12:0 3OH	5.18
10.231	C 16:0	32.98
13.388	C 18:1 w7c	29.98
15.266	C 16:0 cyclo w8c	3.90

containing 1.0% glucose, 0.1% $MgSO_4 \cdot 7H_2O$, and 0.5–3.0% of each different nitrogen source, respectively. After the bacteria was grown at 37°C for 10 h, the phytase activity was assayed as described in Materials and Methods. As shown in Table 4, high levels of phytase activity were detected in the medium containing 2.0% peptone, 3.0% peptone, or 1.0% tryptone as the nitrogen source. However, no phytase activity was detected in the medium containing malt extract, yeast extract, casein hydrolysate, NH_4NO_3, or $(NH_4)_2SO_4$.

Effect of Carbon Sources on Phytase Production

The effect of carbon sources on phytase production by *Pseudomonas* sp. YH40 was investigated in medium containing 2.0% peptone, 0.1% $MgSO_4 \cdot 7H_2O$, and 0.5–4.0% each of different carbon sources, respectively, and the results are shown in Table 5. High levels of phytase activity were detected in the medium containing 1.0% glycerol, 2.0% glycerol, or 2.0% glucose as the carbon source. However, a very low level of phytase activity was detected in the media containing other carbon sources, such as lactose, mannose, and sucrose, while no phytase activity was detected in the medium containing soluble starch of wheat bran. These results indicated completely different patterns of phytase production with different microorganisms such as *Bacillus* sp. and *Aspergillus terreus*. Maltose has been reported to be the most effective carbon source for *Bacillus* sp. KHU-10 [3], while wheat bran and rice bran are the most effective carbon sources for *Bacillus* sp. DS11 [12] and *A. terreus* [29].

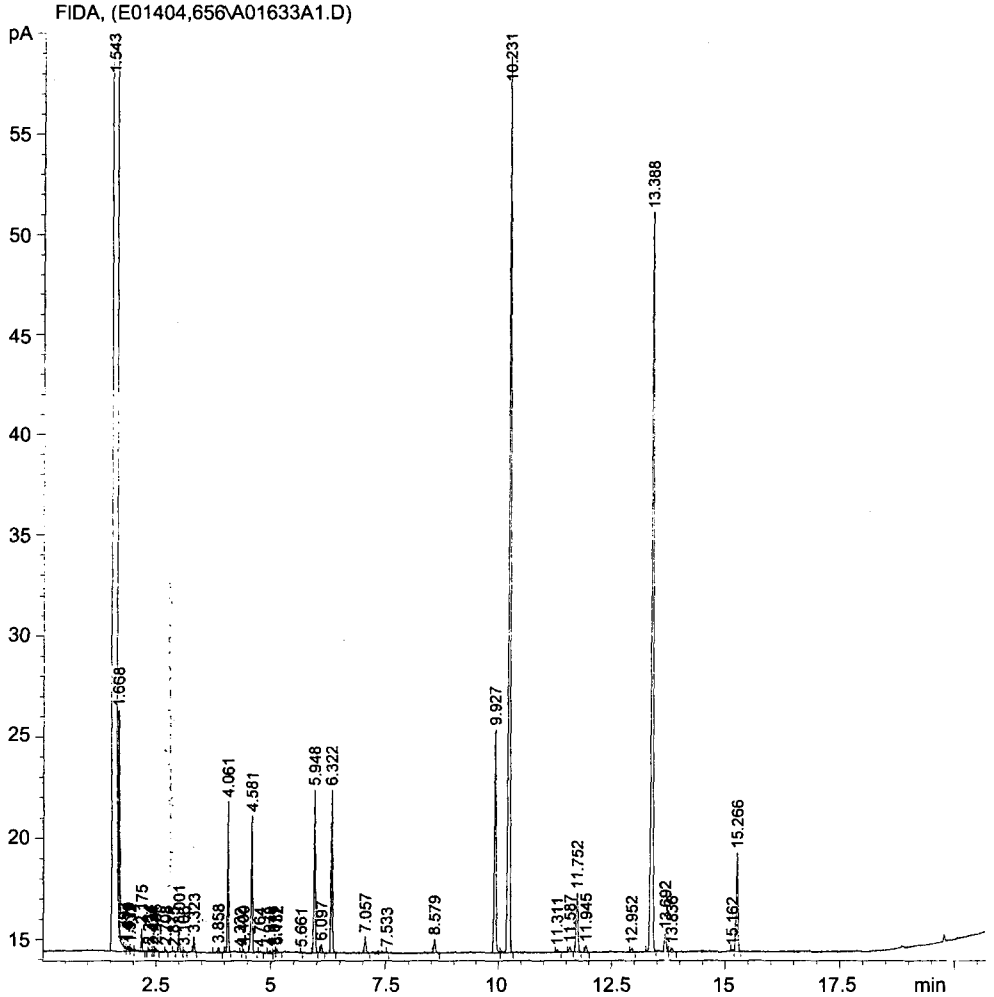

Fig. 3. Gas chromatogram of cellular fatty acids from strain YH40.

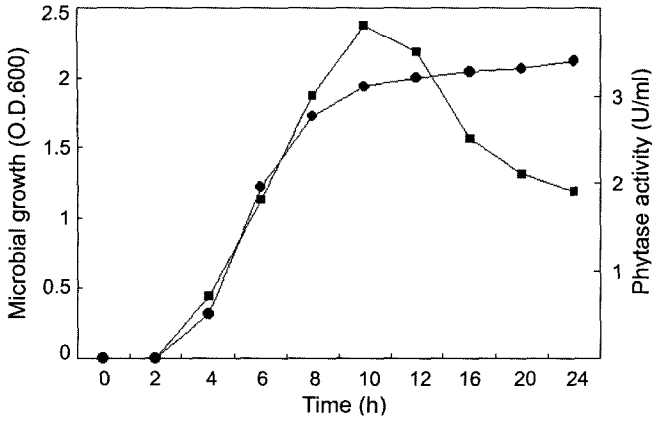

Fig. 4. Time course of microbial growth and enzyme activity of *Pseudomonas* sp. YH40.
■, Phytase activity, U/ml; ●, Microbial growth, O.D. at 600 nm.

Effect of Inorganic and Phosphate Salts on Phytase Production

Table 6 shows the effect of inorganic salts on the phytase production by *Pseudomonas* sp. YH40, which was investigated in medium containing 1.0% glycerol, 2.0% peptone, and 0.01–0.5% of each various inorganic salts, respectively. It has been previously reported that *Aspergillus ficuum* and other *Aspergillus* sp. [9, 22, 29, 31] exhibit repressed phytase production with the addition of most inorganic salts, while *Bacillus* sp. DS11 [12] and *B. subtilis* (natto) [24] exhibit enhanced production with the addition of Ca^{2+} ions to the culture medium. However, in the present study, $FeSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, and KCl all induced a high level of phytase activity by *Pseudomonas* sp. YH40, and the addition of 0.2% $FeSO_4 \cdot 7H_2O$ to the medium enhanced the phytase activity by about 34%, when compared to the control medium. The effect of phosphate salts, such as Ca-phytate, Na-phytate, Na_2HPO_4, and K_2HPO_4, on the phytase production is shown in Table 7. None of the examined phosphate salts had any effect on enhancing the phytase production by *Pseudomonas* sp. YH40. In fact, Na_2HPO_4 and K_2HPO_4 both decreased the phytase production.

Table 4. Effect of nitrogen sources on phytase production by *Pseudomonas* sp. YH40.

Nitrogen sources	Activity (U/ml)	Relative activity (%)
0.5% peptone	0	0
1.0% peptone	1.3	46
2.0% peptone	2.8	100
3.0% peptone	2.4	84
0.5% tryptone	0.5	23
1.0% tryptone	2.3	81
2.0% tryptone	1.7	62
0.5% beef extract	1.2	42
1.0% beef extract	1.2	44
2.0% beef extract	1.0	37
0.5% malt extract	0	0
1.0% malt extract	0	0
2.0% malt extract	0	0
0.5% yeast extract	0	0
1.0% yeast extract	0	0
2.0% yeast extract	0	0
0.5% casein hydrolysate	0	0
1.0% casein hydrolysate	0	0
2.0% casein hydrolysate	0	0
1.0% NH_4NO_3	0	0
1.0% $(NH_4)_2SO_4$	0	0

Culture conditions; 37°C, 180 rpm for 10 h.
Basal medium; 1.0% glucose, 0.1% $MgSO_4 \cdot 7H_2O$.

Table 5. Effect of carbon sources on phytase production by *Pseudomonas* sp. YH40.

Carbon sources	Activity (U/ml)	Relative activity (%)
0.5% glucose	1.3	42
1.0% glucose	1.6	51
2.0% glucose	2.8	89
3.0% glucose	2.4	74
0.5% glycerol	1.7	53
1.0% glycerol	3.2	100
2.0% glycerol	3.0	92
3.0% glycerol	2.5	78
1.0% lactose	0.7	21
1.0% maltose	0.4	14
1.0% mannose	0.3	10
1.0% sucrose	0	0
1.0% soluble starch	0	0
1.0% wheat bran	0	0

Culture conditions; 37°C, 180 rpm for 10 h.
Basal medium; 2.0% peptone, 0.1% $MgSO_4 \cdot 7H_2O$.

Table 6. Effect of inorganic salts on phytase production by *Pseudomonas* sp. YH40.

Inorganic salts	Activity (U/ml)	Relative activity (%)
None	3.0	100
0.01% KCl	2.9	98
0.1% KCl	3.7	124
0.2% KCl	3.6	121
0.01% $FeSO_4 \cdot 7H_2O$	2.8	94
0.1% $FeSO_4 \cdot 7H_2O$	3.7	125
0.2% $FeSO_4 \cdot 7H_2O$	4.1	134
0.01% $MgSO_4 \cdot 7H_2O$	3.1	102
0.1% $MgSO_4 \cdot 7H_2O$	3.9	129
0.2% $MgSO_4 \cdot 7H_2O$	3.8	127
0.01% $CaCl_2$	2.8	96
0.1% $CaCl_2$	2.7	91
0.1% $MnSO_4 \cdot H_2O$	1.8	59
0.1% NaCl	0.8	27

Culture conditions; 37°C, 180 rpm, 10 h.
Basal medium; 1.0% glycerol, 2.0% peptone.

Table 7. Effect of phosphate salts on phytase production by *Pseudomonas* sp. YH40.

Phosphate salts	Activity (U/ml)	Relative activity (%)
None	4.0	100
0.1% Na-phytate	3.9	98
0.5% Na-phytate	4.1	102
0.1% Ca-phytate	3.8	95
0.5% Ca-phytate	3.8	95
0.1% Na_2HPO_4	3.2	80
0.5% Na_2HPO_4	2.6	65
0.1% K_2HPO_4	3.4	85
0.5% K_2HPO_4	3.3	83

Culture conditions; 37°C, 180 rpm, 10 h.
Basal medium; 1.0% glycerol, 2.0% peptone, 0.2% $FeSO_4 \cdot 7H_2O$.

Effects of Initial pH and Temperature on Phytase Production

The effects of the initial pH and temperature on the phytase production by *Pseudomonas* sp. YH40 were examined after the strain was cultured for 10 h in an optimal medium containing 1.0% (w/v) glycerol, 2.0% (w/v) peptone, and 0.2% $FeSO_4 \cdot 7H_2O$. The effect of temperature on phytase production is shown in Fig. 5b. The maximal phytase production by *Pseudomonas* sp. YH40 was observed at 37°C and the optimum initial pH for phytase production was at pH 6.0 (Fig. 5a).

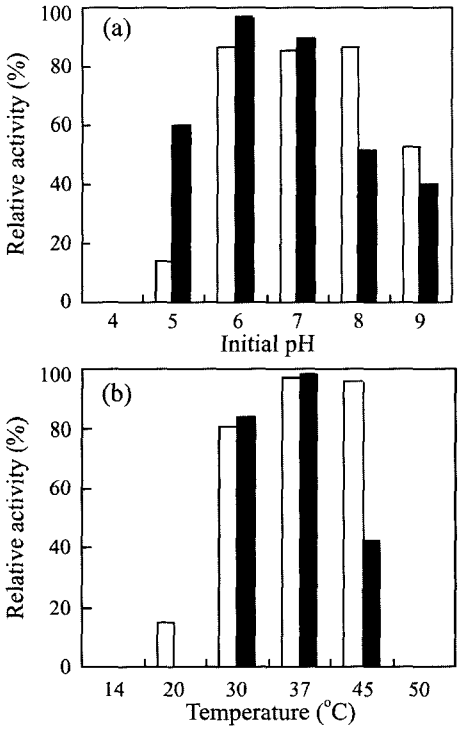

Fig. 5. Effects of initial pH of media (a) and temperature (b) on microbial growth and phytase production by *Pseudomonas* sp.YH40.
□, Microbial growth; ■, Phytase activity. The media contained 1% (w/v) glycerol, 2.0% (w/v) peptone, and 0.2% (w/v) $FeSO_4 \cdot 7H_2O$.

Effects of pH and Temperature on Crude Phytase Activity and Stability

The phytase activity was measured at various pHs ranging from pHs 3.0 to 11.0 using 50 mM glycine-HCl (pHs 3–4), 50 mM Na-acetate-acetic acid (pHs 4.5–6.0), 50 mM Tris-HCl (pHs 7.0–8.0), and 50 mM boric acid-NaOH (pHs 9.0–11.0) buffers. The optimum pH was found to be 7.0, as shown in Fig. 6a. pH stability was also determined 30 min after the incubation with buffer. The phytase was stable between pHs 5.0–7.0. When enzyme activity was measured between 20–70°C, the optimum temperature was found to be at 40°C and the activity was reduced at above 50°C (Fig. 6b).

CONCLUSION

A new phytase-producing bacterium was isolated from soil. The isolated strain was identified as a *Pseudomonas* sp. and named *Pseudomonas* sp. YH40. Phytase activity by *Pseudomonas* sp. YH40 was detected after 4 h and reached a maximum activity after 10 h of culture. The optimum culture conditions for phytase production by *Pseudomonas* sp. YH40

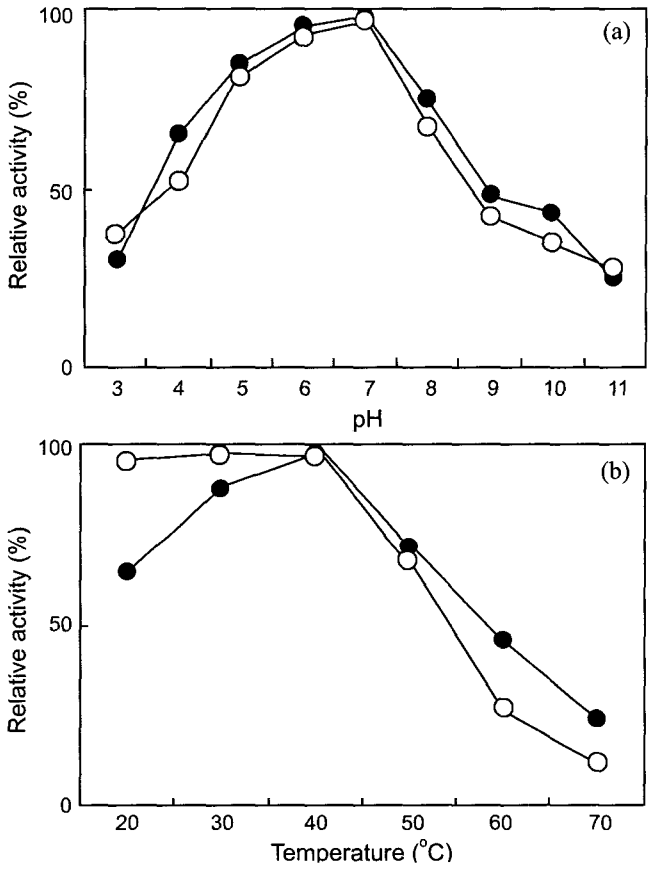

Fig. 6. Effects of pH (a) and temperature (b) on crude phytase activity and stability.
●, phytase activity; ○, stability.

was observed in a culture medium composed of 1.0% (w/v) glycerol, 2.0% (w/v) peptone, and 0.2% (w/v) $FeSO_4 \cdot 7H_2O$, at 37°C and pH 6.0. This medium composition is completely different from that for other phytase-producing bacteria. Further study is currently in progress to purify and characterize the phytase from *Pseudomonas* sp. YH40. In addition, the phytase-coding gene will be cloned to produce a highly effective and low-cost recombinant phytase. Crude phytase had an optimum pH of 7.0 and was stable at pH values ranging from 5.0–7.0, and its optimum temperature was at 40°C.

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