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Culture Conditions and Characterizations of a New Phytase-Producing Fungal Isolate, *Aspergillus* sp. L117

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A novel fungal strain *Aspergillus* sp. L117 that produced acid-stable and thermostable phytase was isolated on basis of the clearing zone on PSM plate and the ability of Na-phytate hydrolysis. The phytase of isolate showed a 3-fold higher activity than that of *A. ficuun* NRRL3135. The *Aspergillus* sp. L117 produced maximal level of phytase at initial pH of 5.0 and 30°C. The optimal pH and temperature for phytase activity were 5.5 and 50°C, respectively. The phytase showed totally stable activity after 20 min of exposure between 30 and 90°C, and even at 100°C. The highest level of residual phytase activity was obtained at pH 5.5, and still retained the stability at the broadest pH ranges (2.0 to 7.0) of all the aforementioned phytases. Storage stability of phytase was preserved over 96% of initial activities for 60 days at 4, –20, and –70°C and to retain even 70% of the initial activity at room temperature.

KEYWORDS: Acid-stable and thermostable phytase, *Aspergillus* sp., Phosphatase

Phytase (EC 3.1.3.8) has many potential applications in such fields as animal diets, the food industry, and health and environmentally related areas (Cromwell and Coffey, 1991; Lihono *et al*., 1997; Q'Quinn *et al*., 1997; Zhou and Erdman, 1995). Phytase is capable of hydrolyzing phytate, which is a major storage form of phosphate in plant seeds during maturation (Mitchell *et al*., 1997). However, despite its role in phosphate storage, phytase acts as an anti-nutrient factor in animal feed by forming insoluble complexes with protein and divalent cations (DeBoland *et al*., 1975; Reddy *et al*., 1982; Sharma *et al*., 1978). As a result, the phytate in animal feedstuffs is not digested by monogastric animals such as pigs, fish, and poultry, because they lack the microorganisms that produce phytases in their digestive tracts (Cromwell *et al*., 1995). The undigested phytate is then excreted in the manure, thereby causing serious phosphate pollutions, especially in the area of intensive livestock production (Cromwell and Coffey, 1991). A way to solve this problem is the supplement of phytase in feedstuff to improve the availability of both phosphates, and protein and divalent cations (Murry *et al*., 1997; Nelson *et al*., 1971).

Phytases are found in plants (Brearley and Hanke, 1996; Lolas and Markakis, 1977; Ullah and Gibson, 1988) and in a variety of microorganisms such as bacteria (Greiner *et al*., 1997; Greiner *et al*., 1993; Jareonkitmongkol *et al*., 1997; Kerovuo *et al*., 1998; Shimizu 1992; Yanke *et al*., 1999; Kim *et al*., 1998), yeasts (Howson and Davis, 1983; Lambrechts *et al*., 1992; Yanke *et al*., 1998; Quan *et al*., 2001) and filamentous fungi (Mitchell *et al*., 1997; Pasamontes *et al*., 1997; Shieh and Ware, 1968; Dvorakova *et al*., 1997; Shimizu, 1993; Gargova *et al*., 1997; Kim *et al*., 1999; Fujita *et al*., 2003). Currently, most commercial phytases are produced by *Aspergillus ficuum* NRRL3135 (Ullah and Gibson, 1987). Natuphos, a phytase commercially produced from a recombinant *Aspergillus* sp., has been approved as a feed additive in most European countries, and parts of Asia (Wodzinski and Ullah, 1996). In the United States, phytase has been commercially available as a food additive since the FDA approved a GRAS (generally recognized as safe) petition for the use of phytase in food (Wodzinski and Ullah, 1996). However, in view of its commercial importance, the high cost of phytase production makes it difficult to add phytase to animal feed. The enzyme also requires the stabilities of pH and temperature at the reaction in digested trait and at the pellet process of animal feed. Therefore, for the reduction of production costs, it is desirable to isolate the microorganism that produce the new and high extracellular phytase from as many sources as possible and that have the properties of enzyme such as pH and temperature.

Accordingly, *Aspergillus* sp. L117 has been isolated from soil as a result of screening tests, producing high extracellular phytase activity. Here we report the isolation of a new phytase-producing fungal isolate, *Aspergillus* sp. L117, the establishment of culture conditions for production of extracellular phytase, and the characterization of the enzyme.

Materials and Methods

Screening for phytase-producing microorganisms. Soil samples were collected in the regions of Gyeong-

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sangnam-do, Korea. The impurities were filtered through a sieve (diameter 1~2 mm). Soil (1 g) was suspended in 10 *ml* of 0.85% NaCl solution. The suspension was filtered through a glass filter and used for the isolation of thermostable and acid stable phytase producing microorganisms. The filtrate was plated out onto a modified phytase-screening medium (PSM) (Howson and Davis, 1983) containing 5 g sodium phytate, 10 g sucrose, 2 g $(NH_4)_2SO_4$, 3 g tryptone, 2 g yeast extract, 0.5 g KCl, 0.5 g $MgSO_4$, 0.01 g $MnSO_4 \cdot 5H_2O$, 0.01 g $FeSO_4$, 1 g Triton X-100, and 15 g agar l^{-1}; pH was adjusted to 7.0 with 1.0 M NaOH. After incubating the plates at 30°C for 3 days, each colony producing a clearing zone was selected and inoculated into PSM medium without sodium phytate and agar. The cells were grown at 30°C for 3 days and after centrifugation, the supernatant was used for analysis of phytase. *A. ficuum* NRRL 3135 was purchased from the Korea Research Institute of Bioscience and Biotechnology for a comparison of phytase activity. For a counterstaining method to detect phytase activity, the fungal strain was eliminated on the PSM plate (Bae *et al*., 1999). The PSM plate was flooded with a 2% (w/v) aqueous cobalt chloride solution. After 5 min incubation at room temperature, the cobalt chloride solution was replaced with a freshly prepared solution containing equal volumes of a 6.25% (w/v) aqueous ammonium molybdate solution and 0.42% (w/v) ammonium vanadate solution. Following 5 min incubation, the ammonium molybdate/ammonium vanadate solution was removed and the plate examined for clearing zone. The fungal strain was observed for Morphological characteristics by a light microscope (Nikkon K, Japan) using differential interference contrast optics and agar plates.

Culture conditions. The strain for phytase production was incubated on PDA (Difco, Ditroit, USA) at 30°C for 5 days. The strain was maintained by periodic transfer on PDA slants and stored at 4°C. For the preparation of the inoculum, *Aspergillus* sp. L117 was incubated at 30°C for 7 days on solid slant medium. After 7 days of incubation, 5 *ml* of 0.001% Tween 80 was added and vigorously agitated for 5 min. The mixture was aseptically filtered through a glass filter. The filtrate was centrifuged for 10 min at 3,000 ×g and the precipitate was diluted with sterilized water, producing an approximate spore number of 2×10^7/*ml*. Culture was studied in the starch medium containing 5% starch, 3% glucose, 0.8% $NaNO_3$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, and $KH_2PO_4 \cdot 7H_2O$ (pH 5.5). A sterile starch medium (50 *ml*) in a 500 *ml* Erlenmeyer flask was inoculated with 1 *ml* of spore suspension, which had been prepared by transferring 2×10^7 spores per ml from a stock slant. The culture was incubated at 30°C at 200 rpm in a rotary shaker for 5 days. After culture under different conditions, the culture broth was filtered through a filter paper (Toyo No. 2) to remove any fungal mycelia. The filtrate was centrifuged at 3,000 ×g and the cell-free supernatant fluid was then used to estimate activities of phytase and phosphatase, and concentration of extracellular protein. At least three cultures were carried out in repeated test.

Biomass. A sample (10 *ml*) from the *Aspergillus* sp. L117 culture was harvested by filtration and washed twice with distilled water. Biomass was determined after drying at 80°C for 48 h. The biomass was defined as the mycelial weight per 100 *ml* of culture broth.

Enzyme assays. The phytase activity was determined by the incubation of 1 *ml* of the culture sample at 37°C for 30 min in a 0.2 M sodium acetate buffer (pH 5.5) containing 0.5% sodium phytate (dodecasodium salt of phytic acid $[C_6H_6O_{24}P_6Na_{12}]$; Sigma, St. Louis, Mo. USA). The reaction was terminated by addition of 1 *ml* of trichloroacetic acid (15% [w/w]). After the addition of 2 *ml* of a coloring reagent (3.66 g of $FeSO_4 \cdot 7H_2O$, 0.5 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 1.6 *ml* of concentrated H_2SO_4 in 50 *ml* of distilled water), the chilled sample in ice water was followed by incubation for 10 min at 30°C. The released phosphate was determined at 750 nm using a UV-VIS spectrophotometer (JASCO International Co. Ltd., Tokyo, Japan). A standard curve for sodium phosphate was made for each independent experiment. One unit was defined as 1m mole of phosphate produced per min.

Effect of different pHs and temperatures on phytase activity was studied by diluting the enzyme in one of the following buffers: 0.2 M glycine-HCl between pH 2.0 and 3.0, 0.2 M sodium acetate between pH 3.5 and 5.5, 0.2 M imidazole-HCl between pH 6.0 and 6.5, and 0.2 M Tris-HCl of pH 7.0, and was studied by incubating the enzyme in one of the temperatures between 30 and 90°C. Free inorganic phosphate was measured at 750 nm as outlined above.

For thermostability assays, phytase was diluted in 0.2 M sodium acetate, pH 5.5, and incubated for 20 min at each of the following temperatures: 30, 40, 50, 60, 70, 80, 90, and 100°C. The samples were placed on ice for 30 min, and the enzymatic activity with phytate was measured at pH 5.5 as outlined above. For stability assays of pH with the *Aspergillus* sp. L117 phytase, the enzyme was incubated at pH 2, 2.5, 3.0, 3.5, 4.0, 5.0, 5.5, 6.0, and 7.0 for 20 min.

Phosphatase activity was determined by a modified method of Pasamontes *et al.* (1997). The enzyme activity was measured in the reaction mixture, which consisted of 2.5 *ml* of 0.5 M sodium citrate buffer, pH 5.5, containing 0.8 mM *p*-nitrophenyphosphate and 10 μl of enzyme. One unit was expressed as the amount of enzyme that will lib-

erate 1 μmol phosphate per min.

Storage stability of phytase was studied by comparing the residual activity at room temperature, 4, −20, and −70°C during two months.

Results

Screening of phytase-producing fungal strains. Phytase-producing fungal strains were extensively isolated from various soil samples on PSM-agar plate and their production pattern was compared in liquid medium. Five colonies, fungal strain L002, L102, L116, L117 and L121 displaying the largest clearing zones were selected and then inoculated into Erlenmeyer flasks on PSM medium without sodium phytate and agar. To compare phytase activity of the isolated fungi in this study with *A. ficuum* NRRL 3135, each fungal strain was inoculated into 100 *ml* of dextrose medium (Shieh and Ware, 1968) and incubated for 5 days at 30°C. Among five colonies, fungal strain L117 showed the best activity of phytase (6.96 U/*ml*), specific activity (2.32 U/g), and yield (1,354 U/g), which were 2.9 and 3.1 and 1.9 times respectively, higher than those of *A. ficuum* NRRL 3135 (Table 1). The fungal strain L117 as the best candidate was used in the following test. In additional screening, counterstaining method to eliminate a mistaken positive zone caused by acid production of the fungal strain was used (Bae *et al.*, 1999). The presence of phytase activity was identified by evident zone of clearing on counterstaining agar medium containing phytate (Fig. 1A).

Morphological characteristics. A selected fungal strain L117 was studied for morphological properties by a light microscope. The colonies of fungal strain L117 thus appeared to be dark-brown with hyphae showing multinucleate condition (Fig. 1A and 1B). The strain commonly occurred in conidiophore with one raw of sterigmata. The hyphae were separated by septum, resulting in a food cell. According to "The Genus *Aspergillus*" (Raper and Fennell 1965), the isolated strain L117 was identified as a strain of *Aspergillus* with an unnamed species. Thus, the isolated fungal strain was named as *Aspergillus* sp. L117.

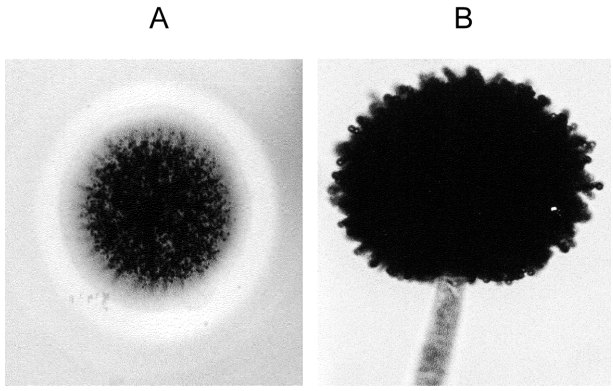

Fig. 1. The effect of counterstaining assay on the clearing zone formed by the fungal strain L117 phytase (A) and morphological appearance of *Aspergillus* sp. L117 (B).

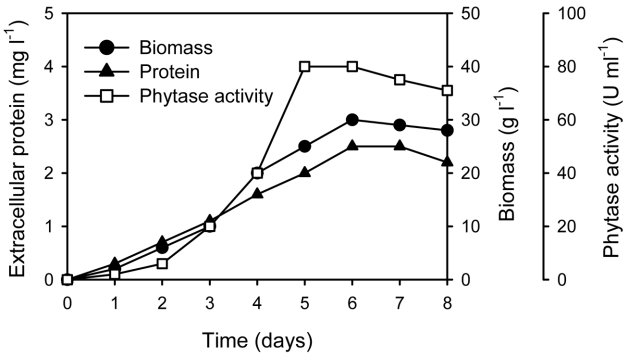

Fig. 2. Time course of phytase peoduction by *Aspergillus* sp. L117 in starch medium at initial pH 6.8 at 30°C, 180 rpm.

Effect of culture time on enzyme production. When the course of phytase production was studied in starch medium (pH 6.5) at 30°C, the phytase activity appeared to be maximal (80 U/*ml*) after 5 days of culture with a parallel increase in biomass and extracellular protein, and there was no further activity increase after 5 days (Fig. 2). Culture time in the following experiments was, however, fixed to 5 days.

Effect of pH and temperature on enzymes production. The effect of environmental factors on productions of

Table 1. Phytase activities from fungi isolated from soil compared with *Aspergillus ficuum* NRRL3135

Strain	Activity (U/*ml*)	Specific activity (U/g protein)	Yield (U/g DMW)
Aspergillus ficuum NRRL 3135	2.44 (±0.21)	0.74 (±0.23)	701 (±26)
Fungal strain L002	3.41 (±0.28)	1.29 (±0.17)	512 (±27)
Fungal strain L102	3.27 (±0.26)	1.34 (±0.21)	549 (±29)
Fungal strain L116	3.58 (±0.28)	1.13 (±0.22)	651 (±28)
Fungal strain L117	6.96 (±0.51)	2.32 (±0.25)	1354 (±59)
Fungal strain L121	3.08 (±0.15)	1.11 (±0.23)	666 (±31)

*Parentheses show S.E. of estimated parameters.

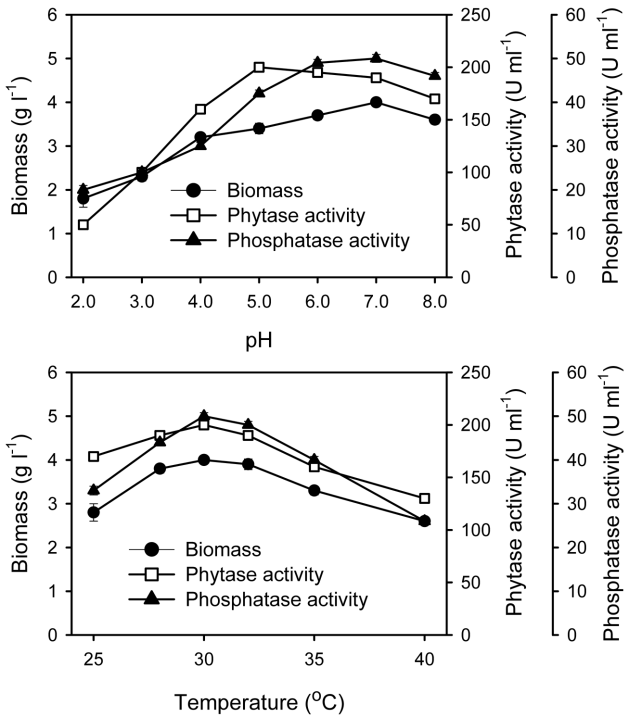

Fig. 3. Effect of initial pHs or culture temperatures on the production of phytase, phosphatase, and biomass of *Aspergillus* sp. L117. The strain was cultured in 100 *ml* of nutrient medium at different pHs (between 2.0 and 7.0) and temperatures (between 30 and 90°C) for 5 days. Error bars show S.E. of estimated parameters.

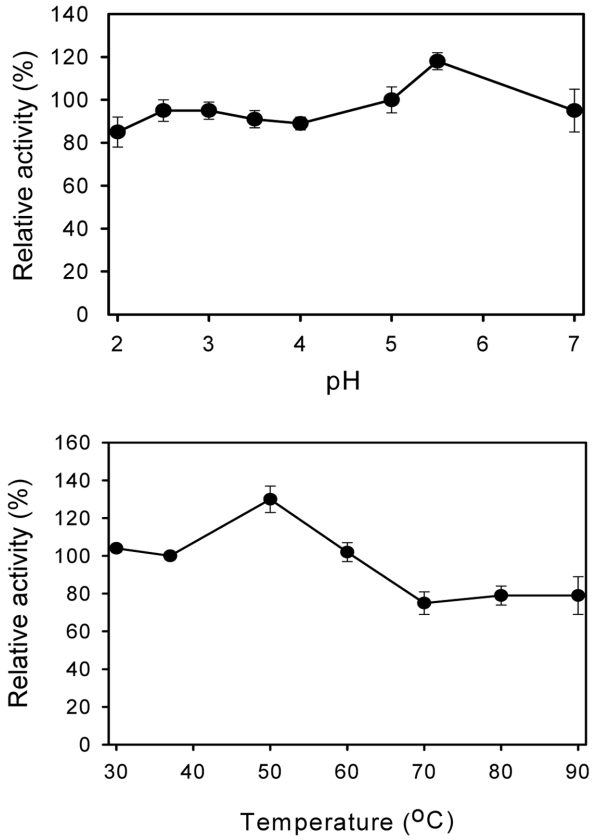

Fig. 4. Effect of pHs or temperatures on the activity of *Aspergillus* sp. L117 phytase. Error bars show S.E. of estimated parameters.

phytase and biomass by *Aspergillus* sp. L117 was studied in shake flasks. Production of phytase, phosphatase, and biomass was determined at initial pHs in the range from 2.0 to 8.0 after 5 days of culture (Fig. 3). Maximal phytase activity was observed when the initial pH of the medium was adjusted to 5.0, and relatively high activity of phytase showed at initial pHs from 5.0 to 7.0 (Fig. 3). Phosphatase activity and biomass increased as the initial pH of the medium moved from acidic towards neutral range, but above pH 7.0, a decline was observed. The initial pHs of each medium began to decrease as the culture started, and there was a sharp decline in pH after 3 days of culture, stabilizing the final pH in the range 2.1 to 1.8 (data not shown). When growth was carried out at different temperatures (25, 28, 30, 32, 35, and 40°C), the *Aspergillus* sp. L117 grew best at 30°C with a sharp decline at 35°C and above (Fig. 3). Maximum phytase production was obtained when the strain was incubated at 30°C. Moreover, all of activities of phytase and phosphatase as well as biomass showed similar patterns according the change of culture temperature.

Properties of phytase and storage stability. The optimal pH for activity of *Aspergillus* sp. L117 phytase was 5.5 (Fig. 4). The phytase hydrolyzed phytic acid at broader pH range (2.0 to 7.0), and over 80% of the activity occurred at all pH ranges. These results confirm that we have isolated an interesting phytase. The optimal temperature of phytase activity was studied at various temperatures, and was found to be 50°C (Fig. 4). The phytase produced by *Aspergillus* sp. L117 showed broad pH and temperature optima for enzymatic activity. As shown in Fig. 5, *Aspergillus* sp. L117 phytase showed totally stable activity after 20 min of exposure at various temperatures (30 to 90°C) and pHs (2.0 to 7.0). Thermostability of the phytase revealed that even after 20 min of exposure at 100°C, the enzyme still retained 80% of its initial activity (Fig. 5). The highest level of residual phytase activity was measured at pH 5.5, and roughly 85% of the activity retained between pH 2.0 and 7.0 (Fig. 5). Thus, *Aspergillus* sp. L117 phytase showed high thermostability as well as still retained the stability at the broadest pH ranges of all the aforementioned phytases. Storage stability of phytase was studied at room temperature, 4, −20, and −70°C (Fig. 6). Phytase retained over 96% of activities for 7 days at the tested all temperatures. Also phytase continued to preserve the same activities for 60 days and to retain even 70% of the initial activity at room temperature.

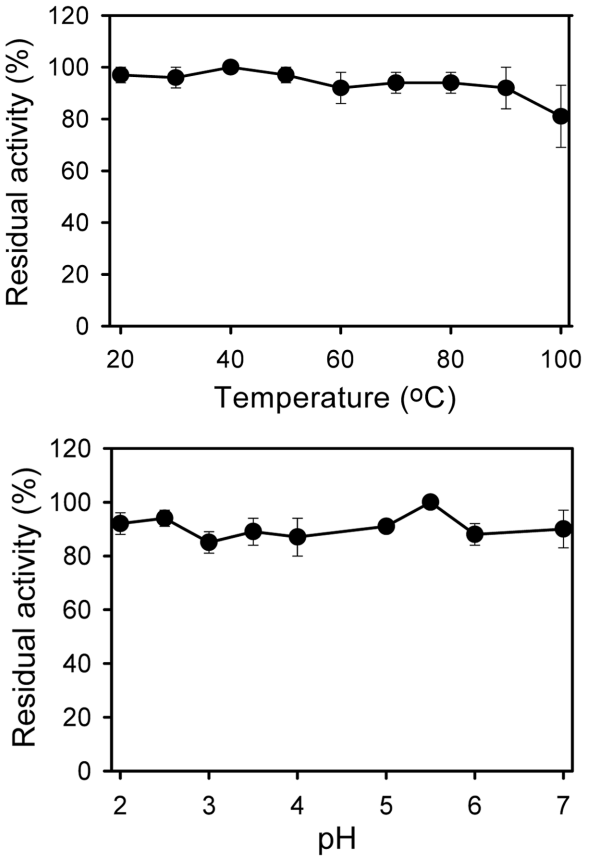

Fig. 5. pH stability and thermostability of *Aspergillus* sp. L117 phytase after exposure for 20 min to the indicated temperature or pH. Error bars show S.E. of estimated parameters.

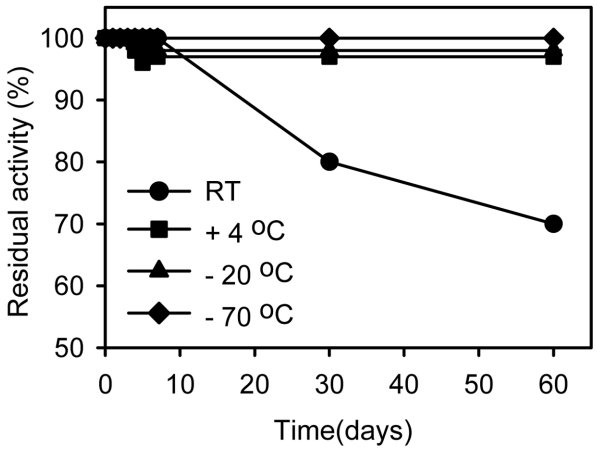

Fig. 6. Storage stability of phytase solution.

Discussion

As a phytase-producing fungal strain, *Aspergillus* sp. L117 showed the best activity of phytase (6.96 U/*ml*), specific activity (2.32 U/g), and yield (1,354 U/g), which were 2.9 and 3.1 and 1.9 times respectively, higher than those of *A. ficuum* NRRL 3135. This strain was interesting for phytase production. In addition, the method comparing size of clearing zone formed on plate was often used in screening of phytase-producing microorganisms. However, this method was hindered by various acids produced during the metabolic pathway in the microorganisms. Because of this, counterstaining method was used (Bae *et al*., 1999; Chelius and Wodzinski, 1994). In our result, *Aspergillus* sp. L117 phytase showed clearing zone around colonies on the solid medium and this result could be due to acids produced by *Aspergillus* sp. strain. As our counterstaining result on PSM plate to eliminate a mistaken positive zone caused by acids, the presence of phytase activity was identified by evident zone of clearing on counterstaining plate. We also found the clearing zone formed by acids and it was eliminated by the counterstaining method. Thus, *Aspergillus* sp. L117 produced phytase hydrolyzing Na-phytate.

When the course of phytase production was studied in starch medium (pH 6.5) at 30°C, the maximal value of phytase activity was 80 U/*ml* after 5 days of culture and the phytase activity was no further increase (Fig. 2). This result was different from the phytase activity produced by *Aspergillus ficuum* NRRL 3135, which consistently increased with culture time until 10 days (Utt 1987). The effect of environmental factors on productions of phytase and biomass by *Aspergillus* sp. L117 was studied in shake flasks. Maximal value of phytase activity was observed when the initial pH of the medium was adjusted to 5.0, and relatively high activity of phytase showed at initial pHs from 5.0 to 7.0 (Fig. 3). Phosphatase activity and biomass increased as the initial pH of the medium moved from acidic towards neutral range, but above pH 7.0, a decline was observed. This result differs from acid phosphatase from *Aspergillus* sp. 307, which produced maximally below pH 4.0 and phytase from *Aspergillus* sp. 307, which optimally produced at initial pHs of 3.0 to 5.0 (Gargova *et al*., 1997).

Aspergillus sp. phytase showed optimal activity at pH 5.5 (Fig. 4). The phytase hydrolyzed phytic acid at broader pH range (2.0 to 7.0), and roughly over 80% of the activity occurred at all pH ranges. The pH dependence of *Aspergillus* sp. L117 phytase resembles that described for the phytases of *A. fumigatus* (Pasamontes *et al*., 1997) and *Aspergillus* sp. 5990 (Kim *et al*., 1999), but clearly differ from those of *A. niger* T213 (Wyss *et al*., 1998), *A. terreus* 9A1, *M. thermophila* (Mitchell *et al*., 1997; Pasamontes *et al*., 1997), and *A. ficuum* (Kim *et al*., 1999). These results confirm that we have isolated an interesting phytase. The optimal temperature of phytase activity was found to be 50°C (Fig. 4). The phytase produced by *Aspergillus* sp. L117 showed a broad pH optimum for enzymatic activity and had a lower optimum temperature for catalysis than that of *A. ficuum* NRRL

3135, which suggests higher thermo stability.

The *Aspergillus* sp. phytase showed totally stable activity at pH and temperature. Thermostability of the phytase revealed that even after 20 min of exposure at 100°C, the enzyme still retained 80% of its initial activity (Fig. 5). Roughly 85% of the phytase activity retained between pH 2.0 and 7.0 (Fig. 5). Similarly, incubation of the *A. fumigatus* phytase for 20 mm at 90°C had no discernible effect on the activity, while phytases of *A. terreus* 9A1 and *M. thermophila* lost 50% of their initial activity within 20 min of exposure to 50 and 55°C, respectively (Pasamontes *et al*., 1997). The phytase from *Aspergillus* sp. 5990 was found to be remarkably thermostable with temperature optima at 65°C (Kim *et al*., 1999). Similarly, *Aspergillus ficuum* NRRL 3135 phytase was found to be thermostable with an optimum temperature of 58°C (Ullah and Gibson, 1987). Thus, *Aspergillus* sp. L117 phytase showed thermostability such as *A. fumigatus* phytase as well as still retained the stability at the broadest pH ranges of all the aforementioned phytases. At storage stability, phytase retained over 96% of activities for 7 days at the tested all temperatures (Fig. 6). Also phytase continued to preserve the same activities for 60 days and to retain even 70% of the initial activity at room temperature.

In conclusion, we isolated a novel fungal strain that produced acid-stable and thermostable phytase. Phytase that produced by the *Aspergillus* sp. L117 had the ability of enzyme to withstand high temperatures, which normally reached during the feed-pelleting process without a significant loss of activity. Moreover, the broad pH range over which the enzyme reacts makes this phytase an excellent candidate for commercial applications. Storage stability of phytase was preserved over 96% of initial activities for 60 days at 4, −20, and −70°C and to retain even 70% of the initial activity at room temperature.

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