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# Isolation, Characterization and Production of Phytase from Endophytic Fungus its Application for Feed

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**Abstract:** Thirty four isolates of endophytic fungus produce phytases were isolated from leaf, stem and root fragments of soybean. Two isolates were the best of phytases enzyme producer and identified as *Rhizoctonia* sp. and *Fusarium verticillioides*. The phytase production was induced by phytate in medium used. The crude preparations were used in subsequent characterization studies, pH and temperature optimum and compared to other phytases tested and is thus a promising candidate for animal feed applications. The results showed that optimal production of phytase from *Rhizoctonia* sp. were pH 4.0 and temperature 50°C and pH 5.0, temperature 50°C for *Fusarium verticillioides*.

**Key words:** Endophytic fungus, phytase, soybean, *Rhizoctonia* sp., *Fusarium verticillioides*

## INTRODUCTION

Phytate (*myo*-inositol-hexaphosphate) is the major form of phosphorus stored in cereals, pollens, legumes and oil seeds. Phytate is known as an anti-nutrient factor, since it chelates various metal ions such as $Mg^{+2}$, $Ca^{+2}$, $Zn^{+2}$, $Fe^{+2}$, $Fe^{+3}$ and forms complex with proteins (Pallauf and Rimbach, 1996; Martin *et al*., 2005; Cao *et al*., 2007; Liu *et al*., 2007). Morever, phytate is not metabolized by monogastric animals, which have low levels phytate-degrading enzymes in their digestive tracts, Thereby, inorganic phosphate has to be added to feeds to ensure a sufficient phosphate supply for these animals. Consequently, the phytate in animal feeds is discharged in feces of these animals into waterways, which contributed to eutrophication for surface waters, particularly in areas of livestock production (Takizawa, 1998).

One way to enhance phosphate utilization from phytate is the use of phytase. To obtain a good source of phytase, a variety of microorganisms, animals tissue and plant have been screened for enzyme. Several plant phytases in wheat, barley, bean, corn, soybean, rice and cotton have been studied extensively (Greiner and Konietzny, 2006). Microbial sources are *Bacillus* sp. (Poward and Jagannathan, 1982), *Eschericia coli* (Greiner *et al*., 1993a,b), *Enterobacter* (Yoon *et al*., 1996) *Raoutella* sp. (Greiner *et al*., 1997; Shah and Parekh, 1990), *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus terreus* (Howson and Davis, 1983) and ruminal bacteria (Yanke *et al*., 1998). Several types of fungal phytase are available on the market from several companies such as Gist Brocades Co, Novo Nordisk Co and Kyowa Hakko Kogyo Co (Takizawa, 1998). At the end of 20th century, annual sales of phytase as feed additive were estimated at US$ 500 million and are continuing to rise (Vats and Banerjee, 2004). In Indonesia, the phytase has been new research. The possibility of using these phytases in industry has not investigated. However, more work needs to be done to obtain superior enzyme for industrial applications. It includes screening for strain that produce high phytase with better physicochemical properties, including high thermostability and suitable pH, along with gen cloning. The objectives of this study were to isolate, characterization production of phytase enzyme from endophytic fungus and its applications for feed.

## MATERIALS AND METHODS

Calcium phytate was made in the laboratory by adding phytic acid into a saturated calcium hydroxide solution. Sodium phytate and sodium dodecyl sulfate were sourced from Sigma. All other reagents were domestic products of analytical grade.

**Isolation of endophytic fungus:** Isolation of phytase producers was performed by the agar plate of method Quan *et al*. (2001). Leaf, stem and root fragments sample of soybean [*Glycine max* (L.) Merril] were obtained from a farmer garden in Padang, Indonesia. All leaf stem and root samples were washed twice in distilled water then surface sterilized by immersion for 1 minute in 70% (v/v) ethanol, 4 minutes in sodium hypochlorite [3% (v/v) available chlorine] and 30 sec in 70% (v/v) ethanol and then washed three times in sterilized distilled water for 1 min each time. After surface sterilization, the samples were cut into 5-7 mm pieces and aseptically transferred to plates containing 0.1% Ca-phytate; 1.5% glucose; 0.2% $NH_4NO_3$; 0.05% KCl; 0.05% $MgSO_4 \cdot 7H_2O$; 0.03% $MnSO_4 \cdot 4H_2O$; 0.03% $FeSO_4 \cdot 7H_2O$ and 1,5% agar. The final pH was adjusted

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to 5.5. Cultivation carried out at 28°C for 2-5 days. Fungal colonies, capable of hydrolyzing Ca-phytate which can be recognized by their surrounding clear halo, were selected and repeatedly streaked onto solid Potato Dextrose Agar (PDA) plates. Colonies which developed on the plates were inspected for their morphology. Pure colonies were obtained by replating single colonies. Identification of fungal phytase was determined with using of methods Samson and Van Reenen-Hoekstra (1988); Barnett and Hunter (1972).

**Screening of endophytic for phytase produser:** Each of isolated strains was grown in 50 ml of liquid medium (0.1% Ca-phytate; 1.5% glucose; 0.2% $NH_4NO_3$; 0.05% KCl; 0.05% $MgSO_4 \cdot 7H_2O$; 0.03% $MnSO_4 \cdot 4H_2O$; 0.03% $FeSO_4 \cdot 7H_2O$, pH 5.5) in 500-ml Sakaguchi flask and incubated at 28°C for 48 h on reciprocal shaker (200 rpm). Cells collected from 1 ml of culture by centrifugation at 5000 x g for 10 min in cood room (4°C). Then, the collected cells were resuspended in acetate buffer (0.2 M, pH 5.5) and used for the phytase activity assay.

**Measurement of enzymatic activity:** The phytase activity assay was determined by measuring the amount of liberated inorganic phosphate according to a method of Quan *et al*. (2001). Reaction mixture consisted of 0.8 ml acetate buffer (0.2 M, pH 5.5) containing I mMNa-phytate and 0.2 ml of cell suspension. After incubation for 30 min at 37°C, the reaction was stoped by adding 1 ml of trichloroacetic acid. A 1 ml aliquot was analyzed for inorganic phosphate liberated by method Kim and Lei (2005). One unit of enzyme activity was defined as the amount of enzyme liberating I nmol of inorganic phosphate per minute.

**Enzymatic characterization studies:** The effect of the pH on the activity of phytase was examined from pH 2.0-8.0 in 100 mM buffer. The buffers used were as follows: pH 1.0-3.5: Gly-HCl; pH 3.5-6.0: NaAc-NaOH; pH 6.0-7.0: Tris-HAc; pH 7.0-8.0: Tris-HCl. Temperature versus enzyme activity was measured over a range of 28-80°C.

## RESULTS

**Identification of isolates:** A total 34 endophytic fungal strains were screened for their ability to produce extracellular phytase. Only two strains, forming clear peripheral zones on turbid agar plate, were isolated from root samples and their activities were determined using liquid culture. According to the results of morphological observation were classified as fungi. They are *Rhizoctonia* sp. and *Fusarium verticillioides* (Fig. 1). The changes of phytase activity in fermentation were shown in Fig. 2. The phytase activity of *Rhizoctonia* sp. was 0.77-2.72 U/ml and *F. verticillioides* was 0.79-6.11 U/ml.

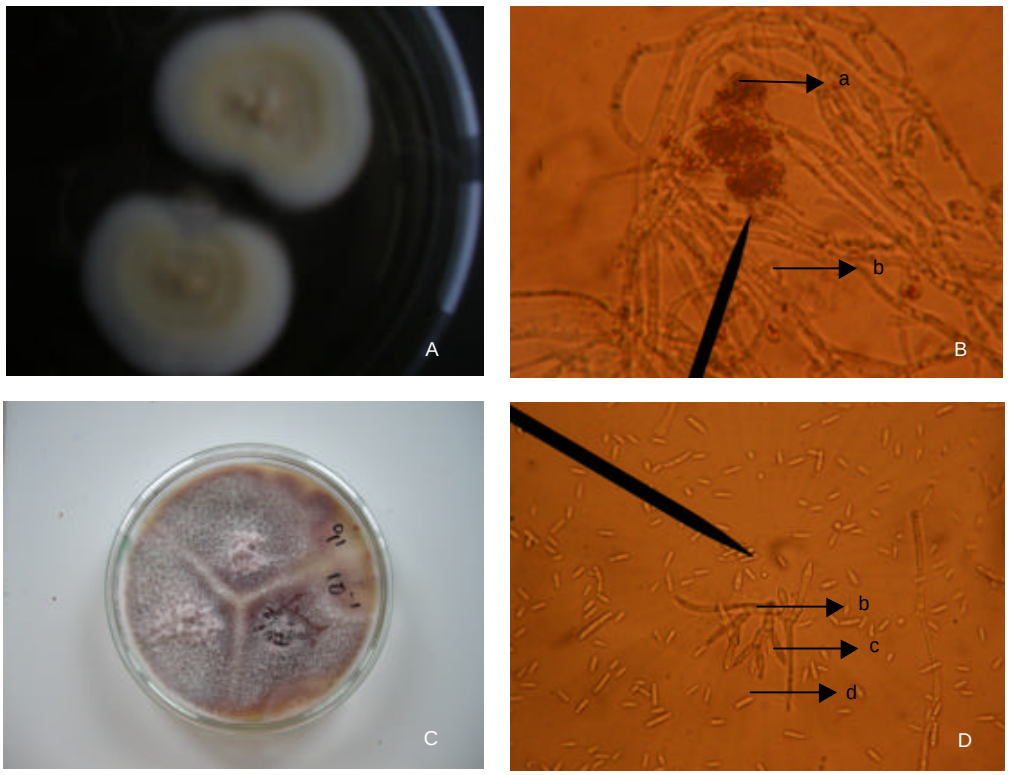


Fig. 1: Morphology of endophytic fungal phytases. A-B = *Rhizoctonia* sp.; C-D = F. *verticillioides*; (100x); a = sclerotia; b = hifa; c = phialid; d = macroconidia

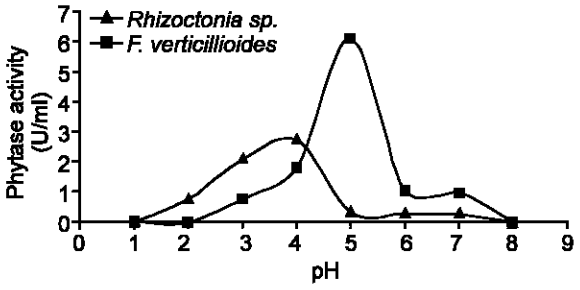


Fig. 2: pH versus activity profiles of phytase from *Rhizoctonia* sp. and F. *verticillioides*

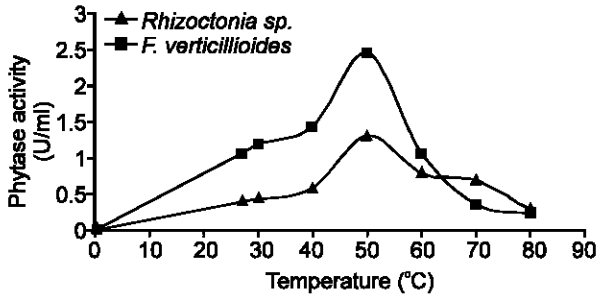


Fig. 3: Temperature versus activity profiles of phytase from *Rhizoctonia* sp. and F. *verticillioides*

**Enzymatic characteristics:** Phytase production in culture of *Rhizoctonia* sp. and *F. verticillioides* by submerged fermentation reached its stationary growth phase after cultivation for 24 h. During exponential growth phase, its produced small amounts of phytase. Phytase production occurred in late stage of the exponential growth phase and the activity phytase increased gradually with increasing incubation time. Both of enzyme activity increased abruptly and reached the maximal value of 0.46 U/ml and 0.77 U/ml respectively.

**pH:** The phytase activity of *Rhizoctonia* sp. reached the highest point at pH 4.0 for 15 min and phytase activity of *F. verticillioides* at pH 5.0 (Fig. 2).

**Temperature:** Both fungal phytase displayed maximum activity were at temperature of 50°C (Fig. 3). At pH 8.0 and at temperature 80°C almost all enzymatic activity of both fungal were lost.

## DISCUSSION

Endophytic fungal phytase was isolated from leaf, stem and root fragments sample of soybean and identified as *Rhizoctonia* sp. and *F. verticillioides* was identified for further study. This is the first report of *Rhizoctonia* sp. and *F. verticillioides* are extracellular phytase and exhibiting high phytase activity. The phytase synthesis in *Rhizoctonia* sp. and *F. verticillioides* by phytate in the culture medium. It may be concluded that only phytate induced these enzymes. Many other phytase producing strains as *Eschericia coli*, *Pseodomonas* sp and *Raoutella* sp. also were induced by phytate (Shah and Parekh, 1990; Konietzny and Greiner, 2004). Enzyme induction is due to physiological change in a whole microbial population and it involves an accelerated rate of renewed formation of enzyme in response to a relatively specific nutritional stimulus (Rhodes and Fletcher, 1966). The phytase was induced early phase of cultivation. It seems that the phosphates are released from phytate.

Phytase often has a low-pH optimum range (pH 4.5-6.0) with a rapid drop in activity at pH value above 6.0. Yeast phytases also have an optimal range 4.0-5.0 (Cao *et al*., 2007; Quan *et al*., 2001; Nakamura *et al*., 2000). The phytase in both *Rhizoctonia* sp. and *F. verticillioides* have pH 4.0 and 5.0 respectively and most stable at pH range 2.0-7.0. were very compatible with the internal environment of monogastric animals' stomach such as in pigs and poultry. Compared to many other phytase producing strains which exhibit low enzyme activity at pH values associated with the upper digestive tract, the *Rhizoctonia* sp. and *F. verticillioides* phytase activity is significantly higher, reaching levels of commercial acceptability. The optimum temperature of the these phytase did not reveal differences between phytase from *Aspergillus niger* N-3 and Natuphos phytase, the latter exhibiting maximum activity at 50°C (Martin *et al*., 2005). At present, a major drawback to the widespread use of phytases in animal feed is the constraint of thermal stability required for these enzymes to withstand inactivation during the feed-pelleting or expansion processes (Cao *et al*., 2007). Both of phytases exhibited maximum activity as high as Natuphos and pGP209 phytase, were at 50°C (Martin *et al*., 2005). This phytase reached levels of commercial acceptability. This phytase is worthy of further research as retains activity over a wide range of pH values characteristic of digestive tract and could conceivably be more suited to increasingly higher feed processing temperatures currently employed in the animal feed industry.

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