

Identification Characterization and Production of Phytase from Endophytic Fungi

Yetti Marlida , Rina Delfita , Neni Gusmanizar, and Gita Ciptaan

Abstract—Phytases are acid phosphatase enzymes, which efficiently cleave phosphate moieties from phytic acid, thereby generating myo-inositol and inorganic phosphate. Thirty four isolates of endophytic fungi to produce of phytases were isolated from leaf, stem and root fragments of soybean. Screening of 34 isolates of endophytic fungi identified the phytases produced by *Rhizoctonia* sp. and *Fusarium verticillioides*. The phytase production were the best induced by phytic acid and rice bran compared the others inducer in submerged fermentation medium used. The phytase produced by both *Rhizoctonia* sp. and *F. verticillioides* have pH optimum at 4.0 and 5.0 respectively. The characterization of phytase from *Fusarium verticillioides* showed that temperature optimum was 50°C and stability until 60°C, the pH optimum 5.0 and pH stability was 2.5 – 6.0, and substrate specificity were rice bran>soybean meal>corn> coconut cake, respectively.

Keywords—endophytic fungus, phytase, soybean, *Rhizoctonia* sp., *Fusarium verticillioides*,

I. 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 [1]-[4]. Moreover, 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 [5].

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 [6]. Microbial sources such as *Bacillus* sp. [7], *Eschericia coli* [8], *Enterobacter* [9], *Raoutella* sp. [10], *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus terreus* [11]. The possibility of using these phytases in industry has not investigated. The objectives of this study were to isolate, characterization production of phytase enzyme from endophytic fungi.

II. 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 [12]. Leaf, stem and root fragments sample of soybean (*Glycine max* (L.) Merrill) 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 seconds in 70% (v/v) ethanol and then washed three times in sterilized distilled water for 1 minute 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 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 medium 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 [13], [14]

Screening of endophytic for phytase producer

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 hours on reciprocal shaker (200 rpm). Cells collected from 1 ml of culture by centrifugation at 5000 x g for 10 minutes in cool room (4°C). Then, the

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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 [12]. Reaction mixture consisted of 0.8 ml acetate buffer (0.2 M, pH 5.5) containing 1 mM Na-phytate and 0.2 ml of cell suspension. After incubation for 30 minutes at 37 °C, the reaction was stopped by adding 1 ml of trichloroacetic acid. A 1ml aliquot was analyzed for inorganic phosphate liberated by method [15]. One unit of enzyme activity was defined as the amount of enzyme liberating 1 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 to 8.0 in 100 mM buffer. The buffers used were as follows: pH 1.0 to 3.5: Gly-HCl; pH 3.5 to 6.0: NaAc-NaOH; pH 6.0 to 7.0: Tris-HAc; pH 7.0 to 8.0 :Tris-HCl. Temperature versus enzyme activity was measured over a range of 28-80 °C. The substrate specificity of the enzyme were studied using rice bran, soybean meal, corn, and coconut cake as substrate amount of inorganic phosphate liberated was determined according to a method of [12].

III. RESULTS AND DISCUSSION

Isolation Screening and Identification

Thirty four of endophytic fungi were isolated from leaf, stem and root fragments sample of soybean and screened for their ability to produce extracellular phytase on medium containing calcium phytic acid as inducer. Measurement of their ability for degrading phytic acid was determined by the size of clear zone formed after growing them for 5 days (Fig 1a and 1b). Only two strains, forming clear peripheral zones on turbid agar plate, were isolated from root samples and their activities were determined using liquid culture. The strains were identified as strains Phy2 and Phy4, whereas other 32 isolates did not clear zone around the colony (Fig 1c and 1d). It means the phytase that produce by those isolate close linked with root physiology of soybean. The root have function as absorb of micro and macro mineral and by natural has mechanism specific in absorption of P that binding with phytic acid. [16] reported that capability of endophytic fungi in produce secondary metabolic such as enzyme suitable with host of physiology. In addition by [17] the synthesis of phytase from the isolates was response that changing in physiology. The results also showed that the difference of the phytase produce from the isolates, the Phy4 showed that the highest ability to degrade phytic acid (> 5 mm) compared to Phy2 (3-4 mm). These results show that different strains of endophytic fungi have different ability to degrade phytic acid. The same results also reported by [18] for different ability of newly isolated strains of endophytic fungi for production of raw starch degrading enzyme.

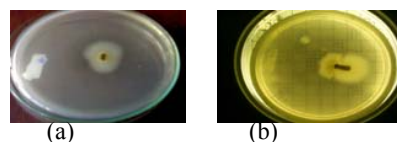


Fig. 1. Clear zone formed by endophytic fungi, a = positive isolate; b = negative isolate producing phytase

Several criteria were used to identify the endophytic fungi isolates by taxonomy and the results obtained are described in Figure 2. Figure 2a-2b shows the morphological characteristics of the endophytic fungi grown on solid media. The two isolates were identified as *Rhizoctonia* sp. (Phy2) and *Fusarium verticillioides* (Phy4). The morphological characteristics of these organisms were similar to those described by [13] for *Rhizoctonia* sp; [14] for *Fusarium verticillioides*.

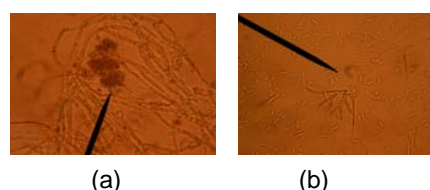


Fig. 2. Photomicrograph of conidiospore (100x) of *Rhizoctonia* sp (a) and *F Verticillioides* (b) incubation at 27°C for 5 days.

Characterization of Phytase

The *Rhizoctonia* sp and *F Verticillioides* endophytic fungi were grown on liquid medium containing phytic acid as inducer at 27°C for 96 h and each 24 h the sample were taken to detected of the phytase activity. Fig 3, showed that *F Verticillioides* the highest yield of phytase (0.78 unit/ml) compared to phytase from *Rhizoctonia* sp (0.46 unit/ml) at 48 h of incubation. The increase of incubation time the phytase production were decreased at 70 h of incubation the activity lost until 50% and the end of incubation (96 h) the activity lost 70%. Based on the activity for continuing the research we used the *F Verticillioides* for characterization, purification and application for feed.

Temperature optimum and stability

The phytase produced from *F Verticillioides* displayed optimum activity at 50°C (Fig 4) and was fully stable at this temperature for 30 min incubation. Rapid inactivation occurred above that temperature whereas 30% of activity was lost at 60°C and only 10% activity at 80 °C. Similar finding has been reported by [6], [2], [19] The temperature stability of the phytase was 30 - 60 °C.

pH optimum and stability

The pH optimum of the enzyme can be shown at Fig 5, whereas the enzyme has pH optimum 5.0 and showed broad pH stability 2.5 – 6.0, with 50 -100% of maximum activity observed in the pH range, although dropping to 18% residual activity at pH 7.0. Similar results also has been reported by [2] for Allzyme dan Natuphos phytase from *A.fumigatus* (commercial phytase) pH stability was 2.5 – 6.0.

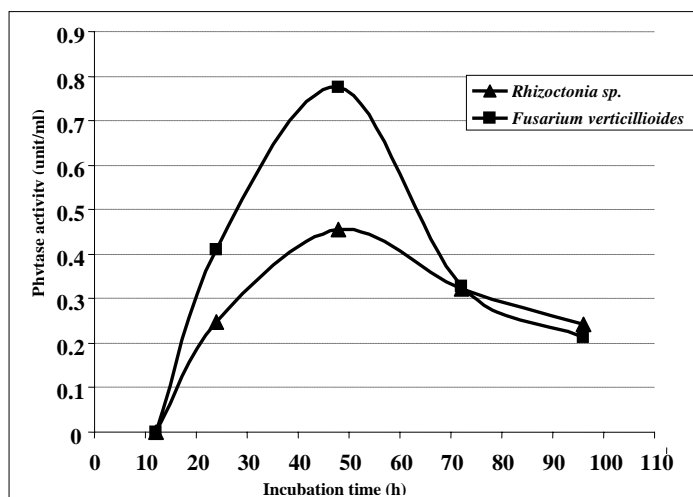


Fig. 3. Production of phytase on basal medium by *Rhizoctonia sp.* and *F. Verticillioides* incubation at 27°C for 2 days.

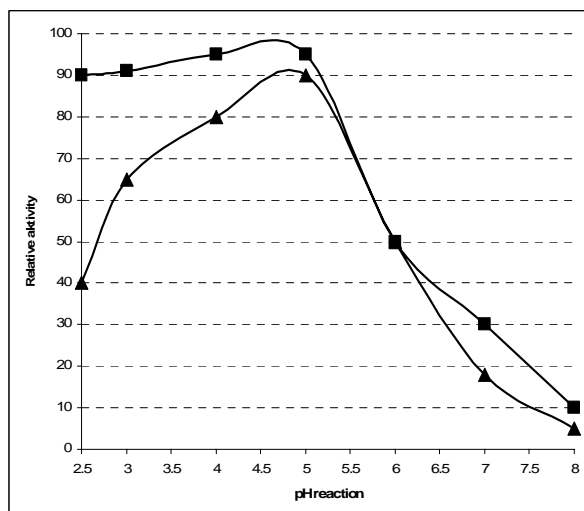


Fig. 5. pH optimum and stability of phytase produced by *F. Verticillioides*

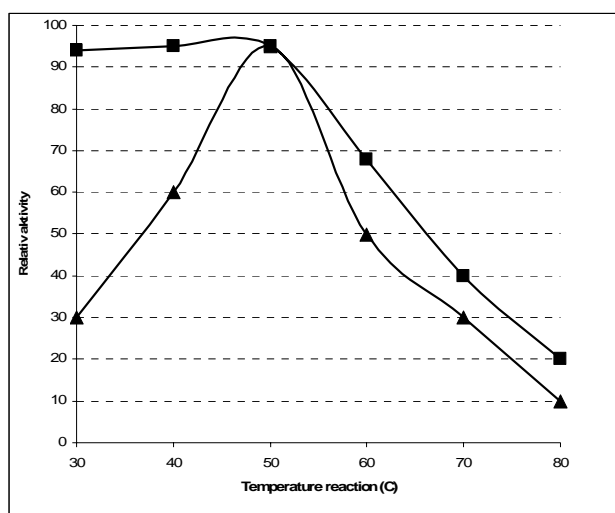


Fig. 4. Temperature optimum and stability of phytase produced by *F. Verticillioides*

Substrate specificity

The substrate specificity of the enzyme was measurement using various feed as substrate such as: rice bran, soybean mill, corn, and coconut cake, and Na-phytate as control. The relative hydrolysis rates of various substrate by the partial purified of phytase are presented in TABLE I. The enzyme is not only capable of hydrolyzing pure Na-phytate but also phytic acid that is inside of feed. The substrate preference of the enzyme for hydrolysis can be arranged in the the following order: rice bran>soybean meal>corn> coconut cake.

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TABLE I SUBSTRATE SPECIFICITY OF PHYTASE FROM *F. VERTICILLIOIDES*

No	Substrates	Relative activity (%)
1	Na-phytate	100
2	Rice bran	97
3	Soybean mill	86
4	Corn	65
5	Coconut cake	45

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