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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 500C and stability until 600C, 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 P HYTATE (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]. 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 [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 Yetti Marlida Author is with the Department of Animal Nutrition, Faculty of Animal Science, Andalas University,Padang, West Sumatera, Indonesia, (corresponding author to provide phone: 62-81363445528; fax: 62-0751 71464 e-mail: yettimarlida@yahoo.com,) Rina Delfita is with the Department of Biology, Faculty Mathematic and Natural Sciences, Andalas University, West Sumatera Padang, Indonesia Neni Gusmanizar Author is with the Department of Animal Nutrition, Faculty of Animal Science, Andalas University,Padang, West Sumatera, Indonesia Gita Ciptaan Author is with the Department of Animal Nutrition, Faculty of Animal Science, Andalas University,Padang, West Sumatera, Indonesia 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.) 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 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% NH4NO3; 0.05% KCl; 0.05% MgSO4·7H2O; 0.03% MnSO4·4H2O; 0.03% FeSO4·7H2O and 1,5% agar. The final pH was adjusted to 5.5. Cultivation carried out at 28 0C 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% NH4NO3; 0.05% KCl; 0.05% MgSO4·7H2O; 0.03% MnSO4·4H2O; 0.03% FeSO4·7H2O, pH 5.5) in 500-ml Sakaguchi flask and incubated at 28 0C 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 (40C).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 [12]. Reaction mixture consisted of 0.8 ml acetate buffer (0.2 M, pH 5.5) containing I mM Na -phytate and

0.2 ml of cell suspension. After incubation for 30 minutes at 37 0C, the reaction was stoped 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 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 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 0C. 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 DISSCUSSION 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 ofthe 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. (a) (b) 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. (a) (b) Fig. 2. Photomicrograph of conidiaspore (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 acis as induser 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. 0.8 Phytase activity (unit/ml) 0.7 0.6 0.5 0.4 0.3 Relativeaktivity 0.2 0.1 Fig. 3. Production of phytase on basal medium by Rhizoctonia sp Fig. 5. pH optimum and stability of phytase produced by F and F Verticillioides incubation at 27◦C for 2 days. Verticillioides TABLE I SUBSTRATE SPECIFICITY OF PHYTASE FROM F. VERTICILLIOIDES No Substrates Relative activity (%) 1 Na-phytate 100 Relativaktivity 2 3 4 Rice bran Soybean mill Corn 97 86 65 5 Coconut cake 45 REFERENCES [1] J. Pallauf, and G. Rimbach. Nutritional Significance of Phytic Acid and Phytase. Arch. Anim. Nutr. . 1996; 50: 301-319. [2] J. A., Martin, R. A. Murphy and R. F. F. Power. Purrification and Fig. 4.Temperature optimum and stability of phytase produced by F Physico- Chemical Characterisation of Genetically Modifed Phytases Verticillioides Expressed in Aspergillus awamori. Bioresource Technology 2005. pp 97. [3] L., W. Cao, Wang., C. Yang., Y. Yang., J. Diana., A. Yakupitiyage., Z. Substrate specificity Luo And D. Li.. Application of Microbial Phytase in Fish Feed. J. Enzyme and Microbial Technology. 2007 40: 497-507. The substrate specificity of the enzyme was measurement [4] Z., H. Liu, Wang., Xiu-E Wang., H. Xu., D. Gao., G. Zhang,. P. Chen using various feed as substrate such as: rice bran, soybean And D. Liu. Effect of Wheat Perling on Flour Phytase Activity, Phytic mill, corn, and coconut cake, and Na-phytate as control. The Acid, Iron and Zinc Content. Food Sci and Technology.2007. [5] N. Takizawa.. Utilization of Recombinan Phytase on the Avoidance of relative hydrolysis rates of various substrate by the partial Water Pollution by Phosporus of Excretory Organ of Liverstock. purified of phytase are presented in TABLE I. The enzyme is Seitbutsu-kakagu. , 1998, pp 76. not only capable of hydrolyzing pure Na-phytate but also [6] R Greiner,., Haller, E., Konietzny, U. and K. D. Jany.. Purification and phytic acid that is inside of feed. The substrate preference of Characterization of A Phytase From Klebsiella Terrigena. Arch. Biochem. Biophys. 1997, 341: 201-206. the enzyme for hydrolysis can be arranged in in the the [7] V. K. Powar, and V. Jagannathan.. Purfication and Properties of Phytate- following order: rice bran>soybean meal>corn> coconut cake. Specific Phosphatase from Bacillus subtilis. J. Bacteriol. 1982, 151: 1102-1108. ACKNOWLEDGMENT [8] R,Greiner, Konietzny, U. and Jany, K. D.. Purification and This work was supported by Grant from DIKTI (Hibah Characterization of Two Phytases From Escherichia coli. Arch. Biochem. Bersaing). The authors are grateful to the laboratories staff at Biophys. 1993, 303: 107-113. [9] S. J Yoon,., Y. J. Choi., H. K. Min., K. K Cho., J. W. Kim., S. C. Lee and Microbiology Lab. Universitas Andalas for their support Y. H.. Jung. Isolation and Identification of Phytase-Producing during the experiment. Bacterium, Enterobacter sp. 4, and Enzymatic Properties of Phytase Enzyme. Enzyme Microbiol. Technol. 1996., 18: 449-454. [10] V, Shah, and Parekh, L. J.. Phytase From Klebsiella sp. No. Pg-2: Purification and Properties. Indian J. Biochem. Biophys. 1990, 27: 98-102. [11] S. J, Howson,. and R. J. Davis.. Production of Phytate-Hydrolysing Enzyme by Fungi. J. Enzyme Microbiol. Technol. 1983, 5: 377-382. [12] C., L. Quan, Zhang., Y. Wang and Y. Ohta.. Prpduction of Phytase in Low Phosphate Medium by a Novel Yeast Candida krusei. Journal of Bioscience and Bioenginering. 2001, 9 (2): 154-160. [13] R. A Samson, and E. S. van Reenen-Hoekstra.. Intoduction to Food- Borne Fungi. 1988, Centraalbureau voor Schimmelcultures. Wageningen. [14] H. L ,Barnett, and B. B, Hunter.. Ilustrated Genera of Imperfect Fungi. Third Edition. 1972, Burgess Publishing Company. USA. [15] T. W Kim, and X. G. Lei. 2005. An Improved Method for a Rapid Determination of Phytase activity in animal feed. J. Anim. Sci. 83: 1062- 1067. [16] McKee, Trudy,.. Biochemistry: An Intoduction. Secend Edition. 1999, McGraw-Hill.

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