Characterization of phytase enzymes as feed additive for poultry and feed

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Abstract. One of the obstacles to utilizing rice bran as feed is the presence of antinutrition in the form of phytic acid which binds in minerals to form complex compounds with P, Mg, Mn, Fe, Zn, Ca. Phytic acid and its salts are the main forms of P, Mg, Mn, Fe, Zn, Ca deposits contained in cereals, legume and grains, about 60-90% of total minerals P, Mg, Mn, Fe, Zn, Ca in the form of phytic acid or phytate salts. Phytate is one of the enzymes belonging to the phosphatase group capable of hydrolyzing phytate compounds of myo-inositol (1,2,3,4,5,6) hexsa phosphat into myo-inositol and organic phosphat. The aim of this study was to obtain characterization of phytase enzymes from isolate Actinobacillus sp., Bacillus pumilus, Bacillus vallimortis and IBR-1. Determination of phytase activity and the absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 392 nm. The result of Actinobacillus sp., Bacillus pumilus, Bacillus vallimortis, IBR-1 each having optimum temperature were 50 °C, 40 °C, 45 °C, 45 °C, and optimum pH were 4, 4, 5.5. Bacteria especially Actinobacillus sp, Bacillus pumilus, Bacillus vallimortis, IBR-1 are proven capable of producing the high enough phytase enzymes required for mineral availability for livestock and fish.

1. Introduction
Phosphor has an important role in the broilers metabolic and growth processes in the chicken’s body. This is due to the fact that phosphorus is a nutrient that has the third highest economic value in broiler diet formulation after energy and amino acids, and its use should be optimized [1]. The use of phytic acid as a source of phosphorus in chicken’s diet through phytate-bound phosphate hydrolysis process is expected to improve the efficiency of phosphorus use.

The drawback of the use of rice bran as poultry feed in high phytic acid content is phytic acid will form insoluble salts when the phytic acid binds with phosphorus and other minerals. Thus these minerals cannot be absorbed by the digestive tract of poultry. Phytic acid has a negative charge at low, neutral and high pH. This enables phytic acid to bind with metal ions such as P, Ca, Mg and Zn as well as positive proteins as a terminal amino group at pH below the isoelectric point. The formation of insoluble phytate-mineral or
phytate-protein compounds can cause a decrease in the availability of minerals and the nutritional value of proteins [2]. Phytate is the primary form of phosphorus and is found in cereal grains, legumes and oilseed meal used in the diet of monogastric animals such as poultry which be used due to their lack of endogenous phytase enzymes. To meet poultry’s need for phosphor, inorganic phosphate is added to poultry’s feed. The phosphorus excreted in feces becomes the primary cause of the problems of environmental pollution. As an alternative to this, microbial phytase can be used. Microbial phytase has a beneficial effect on the growth performance, feed efficiency, protein digestibility, energy utilization, mineral retention and bone growth of broilers as a direct hydrolytic effect on phytate [3,4]. Phytase is one of the enzymes belonging to phophatase group that is capable of hydrolyzing phytic compounds such as myo-inositol 1,2,3,4,5,6-hexa into myo-inositol phosphatase and organic phosphate.

The abovementioned problem can be overcome by using phytase as a feed additive in animal feed. Degradation of phytic acid is the disconnection of bond between inositol group and myo-phosphoric acid group of phytase produced by rumen microbes [5]. The phosphate released will be used as a source of phosphorus minerals for livestock [6], and the beneficial effect of exogenous phytase in poultry diet is the direct hydrolytic effect on the increase in the availability of phytate and minerals, amino acids, and energy [7]. There are two types of phytase, namely 6-phytase, which is found in plants, and 3-phytase, which is produced by fungi [5]. Phytic acid can be classified as an anti-nutrient component in feed, thus a sitrate-producing enzyme capable of hydrolyzing phytic acid is needed. Rumen bacteria from ruminant animals (Actinobacillus sp. and Bacillus pumilus) are expected to produce phytase as a feed additive to produce good feed quality in terms of high availability of proteins and minerals P, Mg, Mn, Fe, Zn and Ca. Local feed ingredients that can potentially be used as poultry feed are rice bran, palm kernel cake, sludge palm oil, coconut cake and other agricultural and industrial waste. Rice bran has been widely used as an animal feed ingredient for poultry. The use of higher portion of rice bran in diet will lower the production costs as its price is relatively cheap. The aim of this study was to find out the characterization of phytase originating from isolates Actinobacillus sp., Bacillus pumilus, Bacillus vallimortis and IBR-1.

2. Methodology

2.1 Phytase producing bacteria

The bacteria, Actinobacillus sp., Bacillus pumilus and Bacillus vallimortis, are the pure isolates from proteomic, Laboratory Institute of Tropical Disease. The bacteria were from the rumen of cow.

2.2 Production of crude extracts of phytase

The single rumen selected was rumen isolate that had the highest phytase activity, grown in 20 mL of liquid LB medium at a temperature of 52°C, shuffed using a 200 rpm incubator shaker for 24 hours. Furthermore, 10 % of the liquid culture was inoculated in 100 mL of filter medium at a temperature of 50°C, shuffled using a 200 rpm incubator shaker for 16-18 hours. The suspension was centrifuged at 4,000 rpm at a temperature of 4°C for 20 minutes. The supernatant obtained was the crude extract of phytase.

2.3 Determination of phytase activity

The Liquid LB medium was composed of: 1 % (w/v) bacto-tryptone, 0.5 % (w/v) yeast extract and 1 % (w/v) NaCl. The composition of the solid LB medium was the same as that of the liquid LB medium, but in the solid LB medium 2 % (w/v) bacto-agar was added. The pharmaceutical screening medium was composed of: 2 % (w/v) glucose, 0.4 % (w/v) Na-phytate, 0.2 % (w/v) CaCl₂, 0.5 % (w/v) NH₄NO₃, 0.05 % (w/v) KCl, 0.05 %(w/v) MgSO₄.7H₂O, 1.5 % bacto-tryptone, 0.001 % (w/v) FeSO₄.7H₂O and 0.001 % (w/v) MnSO₄.7H₂O [8]. The method used in the determination of physiological activity was based on the method of Wang [9] with a slight modification. One mL of supernatant from each liquid culture on the
screening medium was incubated for 5 minutes at a temperature of 55°C. Also incubated was 2 mL of 0.1 M phosphate buffer solution at pH 6 containing 2 mM of Na-phytate and 1 mM of CaCl₂ at a temperature of 55°C for 5 minutes. Furthermore, this solution was added to 1 mL of supernatant from liquid culture, mixed and incubated again for the next 15 minutes. Afterwards, 3 mL of 5% TCA was added, and the mixture of the substrate was kept at a temperature of 4°C for 15 minutes and then centrifuged at 4,000 rpm for 20 minutes. In addition to the supernatant, vanadate-molybdate reagent was added at the same volume ratio, then they were mixed homogeneously. Finally, the absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 392 nm.

3. Results and Discussion

3.1 Characterization of phytase

*Actinobacillus* sp., *Bacillus pumilus*, *Bacillus vallimortis*, IBR-1 produce phytase enzymes. The production capabilities of phytase enzymes vary from one another (table 1 and table 2)

| Temperature °C | Phytase Enzymes Activities (Unit/mL) |
|----------------|-------------------------------------|
|                | Actinobacillus sp | *Bacillus pumilus* | *Bacillus vallimortis* | IBR-1 |
| 40             | 0.246              | 0.267              | 0.202                  | 0.214 |
| 45             | 0.257              | 0.237              | 0.254                  | 0.229 |
| 50             | 0.278              | 0.144              | 0.212                  | 0.203 |
| 55             | 0.175              | 0.126              | 0.180                  | 0.139 |
| 60             | 0.147              | 0.120              | 0.209                  | 0.154 |

| pH | Phytase Enzymes Activities (Unit/mL) |
|----|-------------------------------------|
|    | Actinobacillus sp | *Bacillus pumilus* | *Bacillus vallimortis* | IBR-1 |
| 4  | Acetate              | 0.237              | 0.257                  | 0.216 | 0.265 |
| 5  | Acetate              | 0.230              | 0.238                  | 0.228 | 0.272 |
| 6  | Tris-maleat         | 0.168              | 0.175                  | 0.199 | 0.196 |
| 7  | Tris-maleat         | 0.149              | 0.126                  | 0.188 | 0.185 |
| 8  | Tris-maleat         | 0.125              | 0.129                  | 0.171 | 0.178 |

Temperature affects the production of phytase. *Actinobacillus* sp. has an optimum activity at a temperature of 45-50°C, *Bacillus pumilus* 40-45°C, *Bacillus vallimortis* 45-50°C and IBR-1 40-45°C (table 1). At higher temperatures, the production of phytase by each bacterium will decrease. The fermentation temperature will affect the growth rate of biomass and enzyme production. The effective temperatures for growth depend on the type of microorganisms, be it bacterium or fungi. *Rhizopus oligosporus* grown on coconut husk medium at pH 5.3 resulted in the highest activity of phytase in 4 days of fermentation. *A. niger* produced the highest activity of phytase after 3 days of incubation on a medium containing 1 g/L of gum at a temperature of 30°C [10].

The production of phytase is also affected by pH. *Actinobacillus* sp., *Bacillus pumilus*, *Bacillus vallimortis* and IBR-1 had optimum activity at pH 4-5 (table 2). At higher pH the production of phytase by each bacterium will decrease. The pH factor affects the growth rate of biomass and enzyme production. The highest amount of phytase obtained from *Actinobacillus* sp. was 0.230 unit/L. The pH factor affects the growth of biomass and the production of phytase. *Aspergillus ficuum* produces phytase optimally at pH 6.8-7.0 [11,12]. Research on the production of phytase using tofu pulp is still not common. In general,
research on the production of phytase uses by-products of coconut oil, olive oil, palm oil and cottonseed [12]. The addition of phytase to soybean curd residue increases the nutritional value and promotes the growth of fish [13]. This research succeeded in using tofu pulp as a medium for producing phytase using A. niger, R. oryzae and N. sithophila.

Phytic acid is an anti-nutrient substance that can interfere with the nutritional function and components of food. Phytic acid is a very powerful chelating agent that can bind to minerals, proteins and some amino acids [14]. Livestock and fish feed generally contain phytic acid. The phytic acid in livestock and fish feed can decrease mineral availability for livestock and fish. Phytic acid in livestock and fish feed can be hydrolyzed by phytase by breaking phosphate bond from phytic acid into inositol and inorganic phosphate [15]. The presence of phytase enzymes in livestock and fish feeds can improve the nutrients for livestock, especially monogastric livestock and fish.

The presence of phytic acid that can bind to metal ions, proteins and amino acids can interfere with the absorption of these compounds by livestock and human gut. So it is absolutely necessary to have an enzyme that can break phytic acid bonds with proteins, amino acids and micro elements. The addition of the phytase is aimed to break the ester bonds that bind amino acids, fats and microelements to phytic acid. Bacteria, especially Actinobacillus sp., Bacillus pumilus, Bacillus vallimortis and IBR-1, are proven to be capable of producing high enough phytase required for the availability of minerals for livestock and fish.

4. Conclusion
The optimum temperatures of Actinobacillus sp., Bacillus pumilus, Bacillus vallimortis and IBR-1 were 50°C, 40°C, 45°C, 45°C, respectively, and the optimum pH were 4, 4, 5.5. Bacteria, especially Actinobacillus sp, Bacillus pumilus, Bacillus vallimortis and IBR-1 have been proven to be capable of producing high enough phytase required for the need of minerals of livestock and fish.

5. References
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