

Isolation and Characterization of Phytase from Chicken Manure Bacteria

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ABSTRACT

Cereals in animal feed contain anti-nutrients of phytic acid that has capability of chelating proteins and cations. Phytase can be employed to reduce phytic acid through hydrolyzing phytic acid into free phosphate group and lower derivate of inositol phosphate. The aim of the study was to isolate and characterize phytase obtained from chicken manure bacteria. The study included the screening of phytase-producing bacteria from chicken manure, the homology analysis of bacterium, and the determination of phytase activity. Phytase activity was measured from concentration of free phosphate. The results showed that one phytase-producing isolate obtained from chicken manure grew in the medium containing 5% rice bran extract at 37°C for 5 d. Based on a phylogeny is tree analysis of the genes related to 16S rRNA, the isolate was identified as *Acinetobacter* sp. TZ1. The extracellular phytase expressed by *Acinetobacter* sp. TZ1 exhibited optimum reactions at pH 5 and 50°C. The enzyme showed activity of 64,6 nmol mL⁻¹min⁻¹ and specific activity of 236 nmol min⁻¹mg⁻¹. Relative molecular mass of phytase TZ1 was ~35 kDa. Phytase obtained is potential to improve animal feed quality by hydrolyzing phytic acid.

Keywords: chicken manure bacteria, phytase, rice bran

ABSTRAK

Biji-bijian yang terdapat dalam pakan ternak mengandung zat antinutrisi berupa senyawa asam fitat (inositol fosfat) yang mampu membentuk kompleks dengan protein dan kation. Fitase dapat dipakai untuk mengurangi asam fitat dalam pakan ternak dengan cara menghidrolisis asam fitat menjadi fosfat dan derivat inositol fosfat yang lebih rendah. Tujuan dari penelitian ini adalah mengisolasi dan mengkarakterisasi fitase dari bakteri feses ayam. Metode penelitian yang dilakukan adalah isolasi dan penapisan bakteri dari feses ayam, penentuan hubungan kekerabatan bakteri, serta penentuan aktivitas enzim. Aktivitas fitase diperoleh dengan cara menghitung fosfat bebas yang terbentuk setelah reaksi enzimatik. Hasil penapisan bakteri dari feses ayam menghasilkan isolat bakteri yang mampu memproduksi fitase pada medium tumbuh ekstrak bekatul 5% pada 37°C selama 5 hari. Berdasarkan analisis filogeni urutan gen 16S rRNA, isolat bakteri tersebut diidentifikasi sebagai *Acinetobacter* sp. TZ1. Fitase yang dihasilkan *Acinetobacter* sp. TZ1 merupakan enzim ekstrasel dan memiliki aktivitas optimum pada pH 5 dan 50°C dengan unit aktivitas sebesar 64,6 nmol mL⁻¹ min⁻¹ dan aktivitas spesifik 236 nmol min⁻¹ mg⁻¹ protein. Massa molekul relatif fitase TZ1 yang diprediksi dari SDS-PAGE adalah ~35 kDa. Fitase yang dihasilkan berpotensi untuk meningkatkan kualitas nutrisi pakan melalui hidrolisis asam fitat.

Kata Kunci: bakteri feses ayam, bekatul, fitase

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INTRODUCTION

Animal feed is made from grains which contain phytic acid (myo-inositol hexakisphosphate) (Kerovuo *et al.* 1998). Phytic acid act as anti-nutritional agent in animal body due to its ability to form chelates with amilase and cations such as Ca^{2+} , Fe^{2+} , Mg^{2+} and Zn^{2+} (Bohn *et al.* 2008; Raghavendra & Halami 2009). Adding inorganic phosphate to fulfill animal nutrition is needed to increase feed stock quality. However, inorganic phosphate addition into animal feed may raise water body pollution near to live stock (eutrophication), algal bloom, and water animal death (Bohn *et al.* 2008). Therefore, an enzyme is required to hydrolyze and decrease anti-nutritional effect of phytic acid so that raising the bioavailability of phosphate and cations (Raghavendra & Halami 2009; Kerovuo *et al.* 2000).

The feed and food can influence the type of microbes that live in animal and human digestion track (Tremaroli & Bäckhed 2012). Chicken feed consists of grains which contain phytic acid, so that it is needed to screen chicken manure bacteria with the assumption that there is phytic acid-hydrolyzing bacteria in chicken manure. The aims of this study were to screen phytase-producing bacteria in chicken manure for hydrolyzing phytic acid, to identify bacteria type through 16S rRNA gene analysis, and to characterize phytase activity of chicken manure bacteria.

MATERIALS AND METHODS

Screening of Phytase-Producing Bacteria from Chicken Manure. Chicken manure (1 g) was diluted gradually into 100 mL NaCl 0,85% solution. 100 mL solution was pipetted and spread on the 5% rice brand extract solid medium. The medium was incubated at 37°C for 2 d. Bacteria which grown on the medium were streaked on fresh medium and incubated at 37°C for 2 d. Single colony of bacteria that was grown on the surface of 5% rice bran extract medium were grown on fresh solid medium at 37°C for 5 d. Phytase activity was qualitatively identified by coloring the solid medium surface of bacterial growth (Bae *et al.* 1999). Phytase-producing bacteria was identified by clearing zone appearance after coloring assay.

Sequencing of 16s rRNA Gene and Bacterial Genetic Relationship. Bacterial DNA chromosome was isolated by Wizard® Genomic DNA Purification Kit and amplified by

PCR (primer BactF1 and UniB1) for 30 cycles and the DNA ribbons were visualized by gel electrophoresis. Sequencing of 16S rRNA gene from phytase-producing bacteria was done by MacroGen Korea. Bacterial identification was implemented by comparing 16S rRNA nucleotide from isolated bacteria with GeneBank data using BLAST on www.ncbi.nlm.nih.gov. Phylogeny tree was created using ClustalX which based on alligning several bacterial 16S rRNA nucleotide and genetic relationship was visualized by TreeView.

Bacterial Growth and Cultivation. Single colony was grown on 5% rice brand extract liquid medium in shaker incubator at 37°C 150 rpm and measured by spectronic at 600 nm every day to determine bacterial growth. Bacteria was cultivated by centrifuge (9820×g for 30 min) after incubating single colony on 5% rice brand extract liquid medium at 37°C for 5d. Supernatant was presipitated by ammonium sulphate (0–80%). The consentrared protein was collected by centrifuge (16.260×g for 15 min) and dialyzed with 5 mM pH 7 Tris-Cl buffer.

Phytase Assay. Phytase activity was determined by measuring free phosphate after enzymatic reaction using modified method of Fiske & Subbarow 1925. 200 µL crude enzyme and 200 µL substrate (sodium phytate 5 mM in 50 mM pH 7 Tris-Cl buffer) were reacted for 10 min at 37°C. Reaction was stopped by heating in boiling water for 30 min. Solution was mixed by vortex and centrifuged at 16.260×g for 10 min. Supernatant (100 µL) was dilluted 10 times by adding 900 µL aquadest and reacted with 200 µL dye solution for 30 min. Dye reagent contained 5% ammonium heptamolybdate solution and 10% ascorbic acid in 1M H_2SO_4 with 1:5 ratio. Absorbance of solution was determined by spectrophotometer at 650 nm. Unit activity was defined as the amount of enzyme to liberate 1 nmol free phosphate in 1 min and free phosphate standard was K_2HPO_4 (10–150 µM).

Characterization of Phytase. Characterization of phytase was determined by measuring phytase activity at 50 mM Na-acetate buffer pH 3, 4, 5, 6, and 50 mM Tris-Cl buffer pH 7, 8, 9. And also, temperature characteristic was determined by measuring phytase activity at 30, 37, 50, 60, 70, 80, dan 90°C. Phytase mass molecular size profile was visualized by *Commassie Brilliant Blue* coloring SDS-PAGE

and phytase screening coloring Zymogram (Bae *et al.* 1999) using 5% stacking gel and 12% separating gel.

RESULTS AND METHODS

Phytase-Producing Bacteria from Chicken Manure.

Chicken manure was used to obtain phytase-producing bacteria. Screening of bacteria using 5% rice bran extract solid medium generated 4 kinds of bacteria colony (Figure 1a). There was no bacterial growth in the first day of incubation. The bacteria were observable since second day of incubation and it became obvious after several days. Therefore, bacteria were incubated for 5 d to gain better visualization.

Determining phytase-producing bacteria was done by coloring the surface of bacterial growth medium using cobalt chloride, ammonium heptamolybdate, and sodium metavanadate (Bae *et al.* 1999). Clearing zone on the surface of bacterial growth medium indicated that the isolate was phytase-producing bacteria. Gram identification showed that isolate 1 was Gram negative bacteria which had coccus shape and did not have flagella.

Isolate 1 DNA chromosome was isolated by *Wizard® Genomic DNA Purification Kit* and amplified by PCR using universal primer BactF1 and UniB1 to produce 16S rRNA gene. DNA fragment that showed on gel electrophoresis was about 1500 bp (Figure 2). DNA sequencing resulted

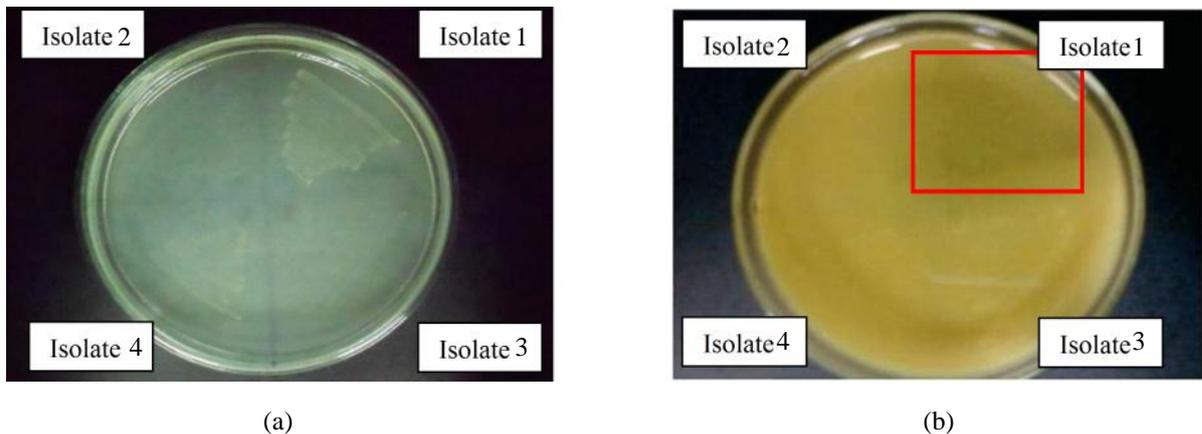


Figure 1 Screening of phytase-producing bacteria from chicken manure (a) profile of bacterial from chicken manure which incubated for 5 d, and (b) profile of coloring screening of phytase-producing bacteria

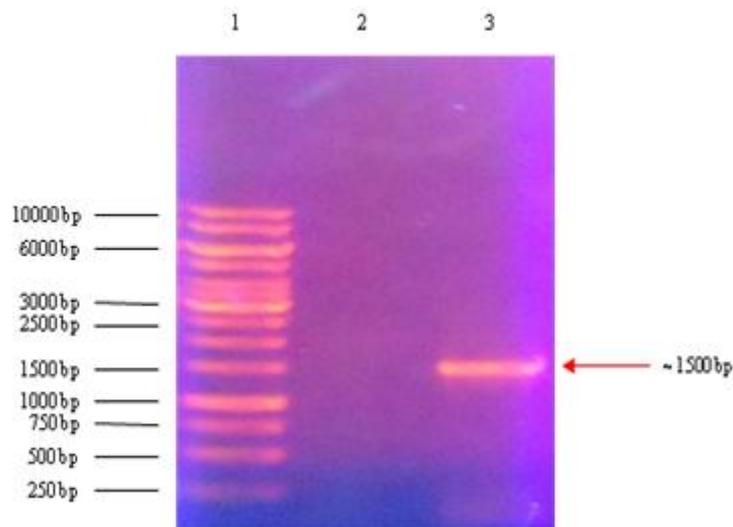


Figure 2 Electrophoresis profile of 16S rRNA gene. (1) 1kb DNA Ladder (Fermentas). (2) Negative control. (3) Isolated and amplified DNA from isolate 1.

1692 pb by BactF1 primer and 1606 bp by UniB1 primer. Both of sequences was combined by *Seqman* and developed 1315 bp of 16S rRNA gene.

Genetic relationship of isolate 1 with bacterial data on GeneBank was determined by BLAST on www.ncbi.nlm.nih.gov based on 16S rRNA gene sequence. It showed that isolate 1 had 99% maximum similarity with 100 kinds of *Acinetobacter* sp., such as *Acinetobacter* sp. DR.Y12 (GeneBank no. DQ226213.1), *Acinetobacter oleivorans* (GeneBank no. JX020951.1) dan *Acinetobacter calcoaceticus* (GeneBank no. JX164201.1). Phylogeny tree analysis based on 16S rRNA gene sequence of *Acinetobacter* genus was done by ClustalX and visualized by TreeView. Result showed that isolate 1 had genetic relationship with *Acinetobacter calcoaceticus* (Figure 3). Thus, isolate 1 which was obtained from screening of chicken manure bacteria was called *Acinetobacter* sp. TZ1.

Growth Characteristic of *Acinetobacter* sp. TZ1.

Growth characteristic of *Acinetobacter* sp. TZ1 in 5% rice bran extract liquid medium was shown in Figure 4. Bacteria adapted with the medium in the first day and went through doubling cell cleavage from second until fourth day. Stationary phase of bacterial growth was reached at fifth day and death phase was started from sixth day of

incubation. Bacterial growth was very slow because the medium for incubating the bacteria had minimum nutrition and the bacteria needed 5 d to reach maximum cell growth. *Acinetobacter* sp. TZ1 produced extracelullular phytase, and crude enzyme was obtained by collecting supernatant from cultivation of *Acinetobacter* sp. TZ1 on stationary phase of growth or at the bacteria reached maximum optical density (Figure 4).

Characteristic of Phytase TZ1. Phytase TZ1 activity was determined at several conditions of pH and temperature to obtain phytase characteristic. Phytase TZ1 activity reached optimum value wich achieved 69 $\text{nmol min}^{-1} \text{mL}^{-1}$ at pH 5 (Figure 5a). Phytase TZ1 activity was still obsevable about 70% at the pH range 3–7, but the activity went down at $\text{pH} > 7$. This result indicated that phytase TZ1 was grouped in acid phosphatase.

On the other hand. Phytase TZ1 activity was observed at temperature range between 30–90°C, and optimum activity was achived at 50°C which indicated that the phytase TZ1 was mesophylic enzyme (Fig 5b). Unit activity and spesific activity of phytase TZ1 at optimum temperature were 64,6 $\text{nmol min}^{-1} \text{mL}^{-1}$ and 236 $\text{nmol min}^{-1} \text{mg}^{-1}$, respectively. Around 80% of the enzyme activity in hidrolyzing phytic acid was observable at 30°C and

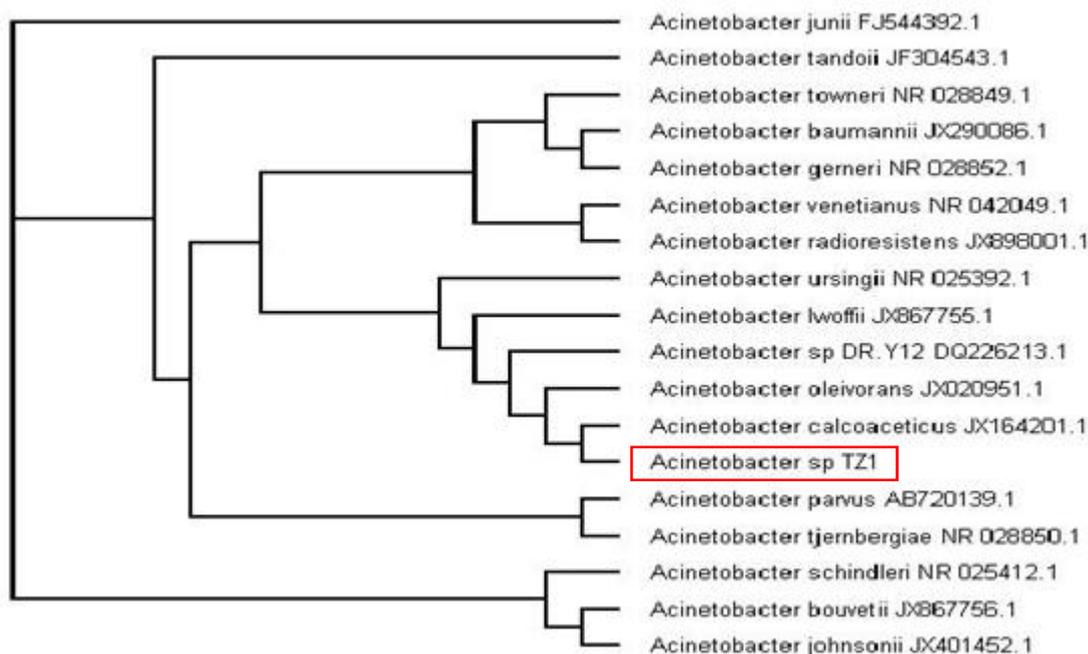


Figure 3 Phylogeny tree of *Acinetobacter* sp. TZ1

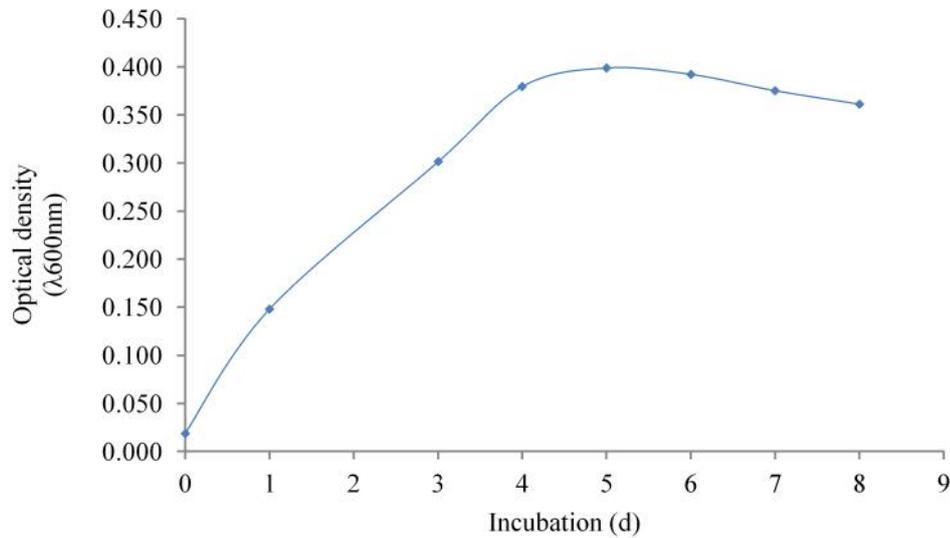


Figure 4 Growth profile of *Acinetobacter* sp. TZ1 in 5% rice bran extract liquid medium

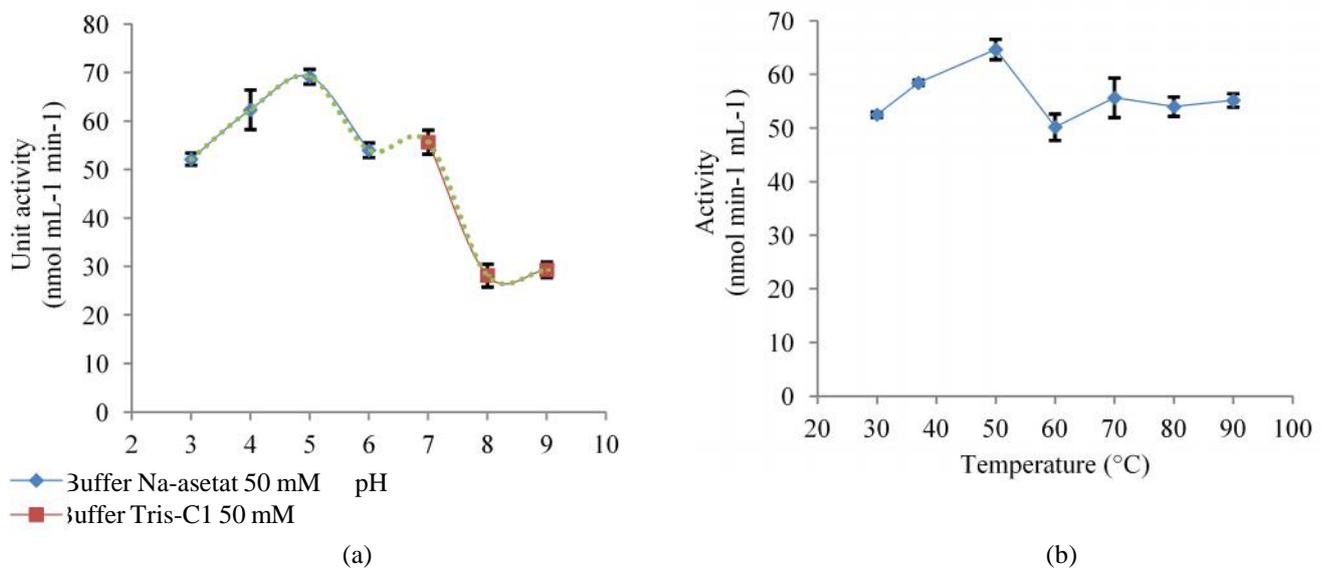


Figure 5 Phytase TZ1 activity. (a) Unit activity depend on several pH condition. (b) Unit activity depend on several temperature condition

temperature range between 60–90°C. The phytase TZ1 characteristic was interesting for industrial application.

Mass molecular of phytase TZ1 was determined by SDS-PAGE (Figure 6). Protein band that was shown on the gel was about 35 kDa, but the real size of phytase TZ1 couldnot be determined yet. So that, protein band which appeared on gel couldnot be stated as the real mass molecular size of phytase TZ1.

Acinetobacter sp. TZ1 was coccus shape without flagela and Gram negative bacteria. It was grouped in γ -Proteobacteria sub-class and Moraxellaceae family, non-motile, non-fermentative, strictly aerob, and its shape was about 1,5 μ m. Genus *Acinetobacter* had coccus or coccobacilli shape and was able to grow in low nutrition medium at temperature 37°C or below. The *Acinetobacter* sp. was difficult to differ because 16S rRNA gene similarity among these species was high which was about >97% (Visca *et al.* 2011). It had about 1500 bp of 16S rRNA gene

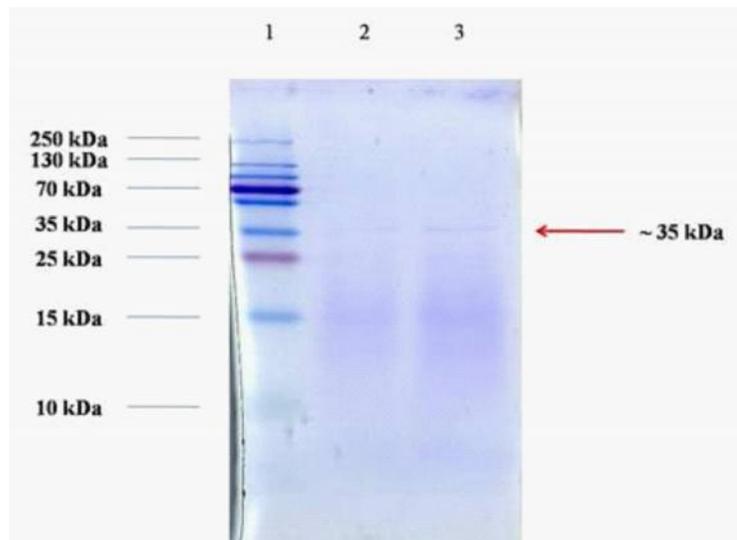


Figure 6 Relatif mass molecular profile of phytase TZ1 on SDS–PAGE and Zymogram. (1) Protein ladder. (2) 4.52 µg concentrated protein. (3) 9.04 µg concentrated protein

(Chang *et al.* 2005). The characteristics of *Acinetobacter* sp. were similar with the result of *Acinetobacter* sp. TZ1 identification in this study.

Acinetobacter sp. was able to grow in minimal salt medium containing triacontane (n-alkane with 30 carbon) at 28°C and it achieved optical density about 0.6 after 5 d (Sakai *et al.* 1994). Moreover, it was capable to grow until 4 d in minimal salt medium containing 0.1% n-parafin (Koma *et al.* 2001).

Palacios *et al.* (2008) reported about bacteria that lived in chicken digestion track which was able to hydrolyze phytic acid. The result of his study said that screening of phytase-producing bacteria from chicken digestion track resulted *Bifidobacterium* dan *Lactobacillus*. These bacteria had specific activity at pH 5 dan 50°C between 4,8–266,5 nmol min⁻¹ mg⁻¹. The phytase characteristic of Palacios was similar with the characteristic of phytase that produced by *Acinetobacter* sp. TZ1.

Previous study which investigated phytase from chicken digestion tract couldnot determine the mass molecular size of phytase appropriately (Raghavendra & Halami 2008; Palacios *et al.* 2008), but the mass molecular size of phytase produced by bacteria was about 10–55 kDa (Lei & Porres 2003; Liu *et al.* 1998).

CONCLUSIONS

One bacterial colony which was screened from chicken manure was identified as phytase-producing bacteria. According to 16S rRNA gene analysis, the isolate was named as *Acinetobacter* sp. TZ1. Phytase from *Acinetobacter* sp. TZ1 was assumed to have relative mass molecular about 35 kDa, and it had optimum activity at pH 5 and 50°C.

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