PRODUCTION AND CHARACTERIZATION OF CRUDE INTRACELLULER PHYTASE FROM RECOMBINANT BACTERIA *pEAS1AMP*

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ABSTRACT

This research was aimed at producing a crude intracellular phytase characterized from recombinant bacteria. The recombinant bacteria (*pEAS1AMP*) was produced by way of transforming *pET-22b(+) +pEAS1* into competent *E. coli BL21* and *E. coli BL21(DE3)* cells. Crude intracellular phytase production was induced using 1,5 mM *Isopropyl-β-D-thiogalactopyranosid* (IPTG). Recombinant bacteria product and enzyme activity test followed the Sajidan method. *E. coli BL21(+)pEAS1* and *E. coli BL21 (DE3)(+)pEAS1* recombinant bacteria showed growth after 20 hours and 10 hours of transformation. Phytase activity of *E. coli BL21 (DE3)(+)+pEAS1* showed higher than those of *E. coli BL21(+)+pEAS1*. Crude intracellular phytase of *pEAS1AMP* recombinant bacteria has an optimum activity at pH 5, 40°C, incubation period of 60 minutes, substrate concentration of 2%, molecular weight (MW) of 47.3 kDa, Km = 15.91 υM and Vm = 2.41 υM/second. Mg^{2+} acts as a cofactor but Fe^{3+} (10^{-4} M) acts as an inhibitor.

Keywords: bacteria recombinant pEAS1AMP, competent cells, crude intracellular phytase

INTRODUCTION

Commercial poultry feed uses P from inorganic P. However, it is now known that the usage of P inorganic produces non digestible P and thus is excreted into the feces. The rapid growth of poultry production has resulted in the increase of pollutant which in turn increases eutrofication. Organic P sources needs to be developed as an alternative source of P for poultry feeds. Organic P is much easier to be digested by poultry animals and reduce the occurance of eutrofication. Phytase can reduce pollution caused by inorganic P, thus eutrofication on water surfaces can be prevented (Pen *et al*., 1993, Volfova *et al*., 1994, Shin *et al*., 2001).

Poultry feed produced from plants (grain) is mostly comprised of agriculture waste products. About two-thirds of plant derived phosphor (P) is in the form of phytate. Phytate is a phosphate complex, in plants it is stored in the seeds (Reddy *et al*., 1989). Phytase in categorized in the phosphatase enzyme group and has the ability to hydrolyze phytate compounds (Greiner *et al*., 1997).

According to Phillippy dan Mullaney (1997), wild type microbes produces lower amounts of phytase when compared to recombinant microbes and recombinant microbes has produced phytase more effectively on large scale production. Phytase that was produced from recombinant bacteria showed specific activity about 1000 times higher than those produced from wild-type bacteria, futhermore, recombinant phytase has the ability to hydrolyze phytate compound up to Inositol (2) monophosphate and was also able to degrade other organic and inorganic phosphates.

Gen phytase from *Klebsiella pneumonia* has been expressed into *pET-22b(+)* (Sajidan, 2002). BL21 competen cells can be used to express a stable gene utilizing a recombinant DNA on vector plasmid *pET,* thus, it was able to effectively code target proteins for the continuity of cell growth.

Environmentally friendly organic P sources derived from agricultural waste products must be developed. Agricultural wastes containing phytic acid can be hydrolyzed by phytase resulting inorganic phytase. Before phytase can be utilized to hydrolyze agricultural wastes, it needs to be produced and characterized. The capability of production and characterization of recombinant bacteria phytase utilizing gene transformation of vector plasmid *pET-22b(+)+pEAS1* into *E. coli BL21/DE3* was a fascinating subject investigate.

MATERIALS AND METHODS

The study used *pET22b(+)-Plasmid-Vector* (Novagen 69744-3) with phytase gene *EAS1* (AS23[front:1-27]:5'atgcaagacatcaggggctgttacgcc3' and AS22[behind:1.257-1.233]:5'cggcaggaccatggctaccgccgg3'), set of competent cells BL21 (Novagen 70232-3) LB media, SOC (*Super Optimum Reppresive Catabolic*) media, IPTG/*Isopropyl-β-D-thiogalactopyranoside* (Promega V395D), Na-phytat (EMerck), Na-acetate (EMerck) dan STOP solution.

Phytase production via over expression of phytase recombinant gene

Phytase gene fragment produced from PCR has been successfully cloned into *pET22b(+)-Plasmid-Vector* (Novagen 69744-3) using phytase gene (*EAS1*) with His-Tag sequence on C-terminal. *Plasmid vector-insert* will be transformed into competent *E. coli BL21/(DE3)* (Novagen 70232-3) to be used for recombinant phytase production

Transformation was conducted by mixing 200 µl competent *E. Coli BL21* and *BL21 (DE3)* with 20 µl *pET22b(+)+pEAS1* in an ice-bath. The mixture was then incubated for 90 second at 42ºC and quickly resubmerged in the ice-bath. 2 ml of SOC liquid medium was then added and followed with 2 hours of incubation in a shaker at 37ºC and 100 rpm. Transformation culture production (recombinant colony) was then planted on LB (*Luria Bertany)* media containing 50 µg/ml amphycillin and incubated at 37ºC for 10 hours for *E. coli BL21 (DE3)* and 20 hours for *E. coli BL21* (Sajidan, 2002; Nuhriawangsa *et al*., 2008a).

Enzyme production was produced by Sajidan method (2002) with modification. Enzyme production starts with collecting one recombinant colony and planting in 5 ml LB media containing amphycillin (25 µg/ml) and incubated on a shaker at 37ºC and 100 rpm at 8 hours. 200 µl liquid media contaning recombinant bacteria was taken and inserted into 9.8 ml LB media containing amphycillin 25 µg/ml, after 90 minutes 10 υl 1 mM IPTG was added to induce enzyme production. Culture was cultivated after one night and centrifuged at 4500 G for 10 minutes at 4ºC. Intracellular enzyme was extracted by extracted the pellet extracted from the crude enzyme. 100mM Na-acetate pH 5 was added to the pellet with a ration of pellet : buffer = 1 : 1 and stored in a -80 ºC freezer for 10 minutes then thawed for 20 minutes, this process was replicated twice. Then the mixture was centrifuged at 10,000 G at 4ºC for 30 minutes. Supernatant was collected as a source of crude intracellular phytase.

Measuring recombinant phytase activity

Activity of the resulting recombinant phytase was measured. Measurement was conducted following the Sajidan method (2002): 50 µl enzyme, 150 µl substrate (0.4 % Na-phytate in 100 mM Na-acetat pH 5) incubated at 37°C for 60 minutes. The reaction was stopped using 400 µl STOP solution. The yellow color emitted by phosphomolibdat was measured using a spectrophotometer at λ 415 nm.

Characterization of phytase activity

Phytase activity was characterized against pH, temperature, incubation period, substrate concentration, and effects of metallic ions. Phytase characterization examined its activity against N-phytate at various pH, temperature, incubation period, substrate concentration [S], and metallic ions (concentration 10^{-3} M and 10^{-4}M) (Greiner *et al*., 1997; Sajidan, 2002).

Km and Vm values were revealed using Robyt and White method (1997) by comparing the product before and after hydrolysis (observation period of 30 and 60 minutes) using the Lineweaver-Burk graph approach. Concentration determination used a standard graphic from an organic P source (KHPO4).

Phytase was separated according to its molecular weight using SDS-PAGE electrophoresis method (BioRad, 2000). As the standard protein, a protein marker was used (Bio-Rad catalog 161-0318) with a protein sequence of *Carbonic anhydrase* (36.3 kDa) and Ovalbumin

(52.2 kDa). Molecular characterization was carried out using SDS-PAGE with 12% polyacrylamid gel and 20 ul sample buffer.

Statistical Analysis

Result of the research was statistically analyzed using descriptive quantitative approach by incorporating observation and count results (Steel and Torrie, 1993).

RESULTS AND DISCUSSIONS

Transformation results of *pET22b(+) +pEAS1* into competent cells *E. coli BL21* and *E. coli BL21 (DE3)* after incubation showed the development of recombinant bacteria colony after 20 hours and 10 hours. Plasmid *pET22b(+)* contains resistant gene against amphicillin up to a dosage of 100 υg/ml. Gene *EAS1* of *Klebsiella pneumonia* was inserted into *pET22b(+)*, resulting in *vector plasmid pET22B(+)+pEAS1* formation which will be referred to as *pEAS1* (Sajidan, 2002). Transformation utilizing competent cell *E. coli BL21/(DE3)* was given freezer shock to insert plasmid *pET22b(+) +pEAS1*, resulting in the production of recombinant bacteria resistant to amphicillin and was able to produce phytase. This research showed that recombinant bacteria *E. coli BL21+pEAS1* and *E. coli BL21 (DE3)(+)+pEAS1* can grow on LB/Amp 50 υg/ml media. It indicates that plasmid *pEAS1* can be transformed into competent *E. coli BL21/(DE3)*cells.

Transformation of vector plasmid into competent cells can be utilized as an expression mean to produce phytase. This is inline with the opinion of Lassen *et al.* (2001) who also expressed phytase genes from *Peniophora lycii*, *Agrocybe pediades*, *Ceriporia sp.* and *Trametes pubescens* into *Aspergillus oryzae*, resulting in the production of pure characterized enzymes. Koul *et al.* (2000) was also able to express DNA genes from *Mycobacterium tuberculosis* $H_{37}Rv$ into *E. coli* to produce *tyrosine phosphatase*. Golovan *et al.* (2000) has achieved overproduction by taking advantage of gene *appA* expressed into *E. coli*. Han *et al.* (1999) was able to express *phyA* from *Aspergillus niger* gene into *Saccharomyces cerevisiae* resulting in a positive activity.

Results of measuring the crude extracellular enzyme activity from recombinant bacteria *E. coli BL21(+)+pEAS1* and *BL21(DE3)(+)+pEAS1* is shown on Figure 1. Results showed that recombinant bacteria *E. coli BL21(DE3)(+) +pEAS1* possess a relatively better activity than *E. coli BL21(+)+pEAS1*. *E. coli BL21 (DE3)* has a regulator which binds to vector plasmid.

Phytase is a monomer of protein, so its molecular weight can be determined (Pandey *et al.*, 2001). This research used *EAS1* gene from *K. pneumoniae*. This *EAS1* gene has a MW of 46 kDa (Sajidan *et al.*, 2004). Electrophoresis results are shown on Figure 2, depicting the existance of protein (phytase) positioned between MW 36.3 and 52.2 kDa (47.3 kDa), this showed a difference compared to other research findings, but its MW range is correct. The MW range of phytase is between 40 to 100 kDA (Pandey *et al.*, 2001). Gene *mptpA* and *mptpB* gene from

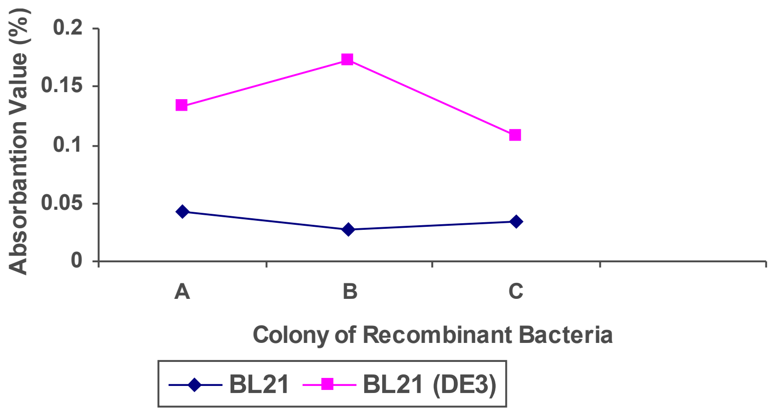

Figure 1. Absorbance value of crude enzyme activity from recombinant bacteria*E. coli BL21(+)+pEAS1*and*BL21(DE3)(+)+pEAS1*observed underλ415 nm

Mycobacterium tuberculosis has MW of 56 and 59 kDa (Koul *et al*., 2000). Gene appA and agp gene from *E. coli* has MW of 46 dan 45 kDa (Golovan *et al*., 2000). Gene p*hyC* from *B. substilis* gene has MW of 43 kDa (Kerovuo *et al*., 1998).

Results (Figure 3) showed that phytase has an optimum at pH 5. The optimum pH of phytase is within the range of 3,5, to 6 (Greiner and Konietzny, 2006). Native phytase on *E. coli* has an optimum pH of 4 and *Klebsiella pneumonia* is very active at pH 5 (Sajidan *et al*., 2005). Phytase from *E. coli BL21 (DE3)(+)pET-appA* has an optimum pH of 4.5 (Golovan *et al*., 2000). Crude phytase (Nuhriawangsa *et al*., 2008a) and pure (Nuhriawangsa *et al*., 2008b) extracellular from recombinant bacteria *pEAS1AMP* has an optimum activity at pH 5.

Results (Figure 4) showed that phytase has an optimum temperature at 40ºC. Hydrolisis activity of phytase is at the range of 35 to 80ºC (Greiner and Konietzny, 2006). Temperature optimization of native phytase *E. coli* is at 50-55 °C dan *K. pneumonia* phytase at 45-50°C (Sajidan *et al*., 2005). *E. coli BL21 (DE3) (+) pET-appA* has an optimum temperature range of 55 to 60ºC (Golovan *et al*., 2000). Pure and crude extracellular phytase from recombinant bacteria *pEAS1AMP* has an optimum temperature of 45ºC (Nuhriawangsa *et al*., 2008b) and 40ºC

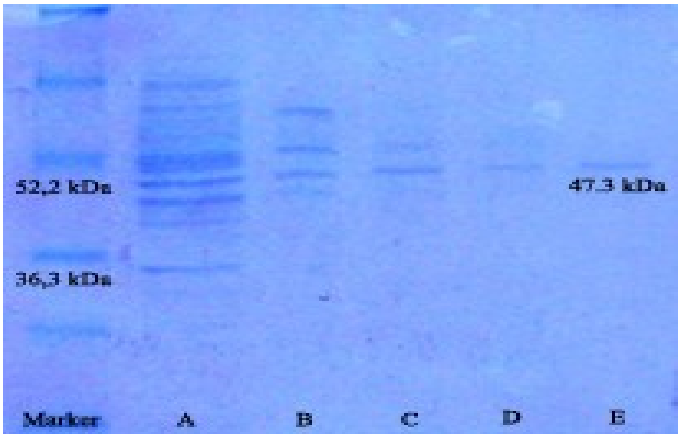

Figure 2. SDS-PAGE 12% MW crude phytase and pure intracellular from recombinant bacteria. M: Marker, A: crude intracellular phytase, B: imidazole 10 mM, C: imidazole 50 mM, D: imidazole 100 mM, E: pure intracellular phytase (imidazole 200 mM)

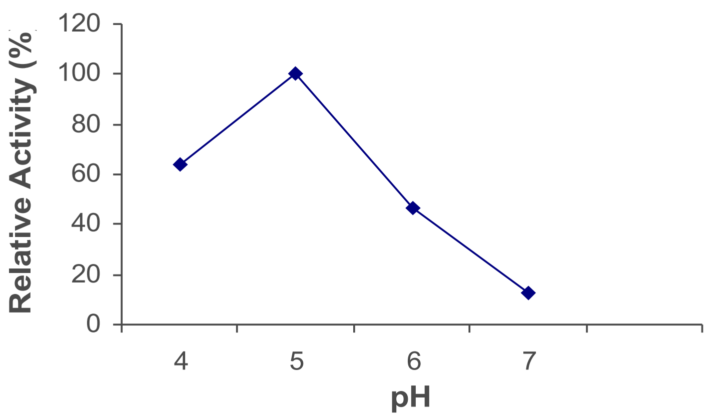

Figure 3. Relative activity value optimized pH of phytase

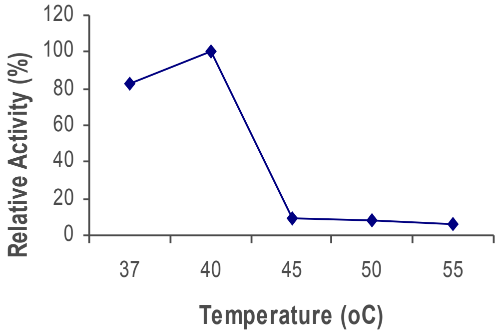

Figure 4. Relative activity value optimized temperature (^{O}C) of phytase

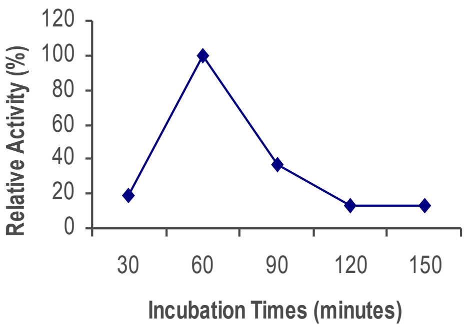

Figure 5. Relative activity value from incubation time of phytase (minutes)

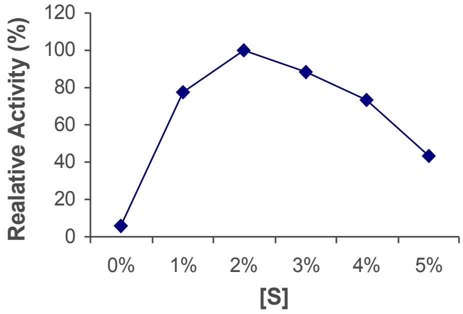

Figure 6. Relative activity from substrat concentration of phytic acid (%)

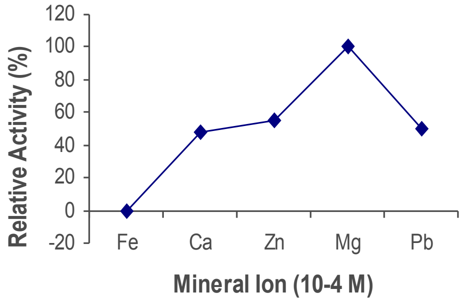

Figure 7. Relative activity value about mineral ions with concentration 10^{-4}M

(Nuhriawangsa *et al*., 2008a).

Results (Figure 5) showed that incubation time of phytase has an optimum at 60 minutes. Enzyme activity increased with the increasing of incubation time. Thus, enzyme activity had maximum when the speed of active bound maximum to product P from S (Nelson dan Cox, 2000). Crude phytase (Nuhriawangsa *et al*., 2008a) and pure phytase (Nuhriawangsa *et al*., 2008b) from recombinant bacteria *pEAS1AMP* has an optimum incubation time at 60 minutes.

[S] had relative activity that was maximum on 2% (Figure 6). Speed of enzyme activity increased when active site of enzyme to bind substrat was increased. Its constant while active site of enzyme was saturated substrat. That condition was not to increased product (Nelson and Cox, 2000). Crude and pure extracellular phytase from recombinant bacteria *pEAS1AMP* has an optimum substrat concentration of 1% (Nuhriawangsa *et al*., 2008a) and 3% (Nuhriawangsa *et al*., 2008b).

Results of metal ion test (Fe^{3+}, Ca^{2+}, Zn^{2+}, Mg^{2+}, Pb^{3+}) on phytase activity was shown at Figure 7. Enzyme had specific character catalytic activity. Catalytic enzyme was arranged by ion or molecule (Rahayu, 1991). Characteristics of ion were influenced by the activity of enzyme, It inhibitor or activator (Nelson dan Cox, 2000).

Result showed that phytase activity was inhibited by Fe^{2+} (10^{-4}M). It was good activity on Mg^{2+} (10^{-4}M) ion. Its means that Fe^{2+} (10^{-4}M) decreased enzyme activity but Mg^{2+} (10^{-4}M) was on the contrary. Enzyme activity was decreased by Fe^{2+} and Fe^{3+} ion (5 mmol l^{-1}) (Yanke *et al*., 1999). Enzyme activity was increased by Ca^{2+} and Mg^{2+} ion (Maenz, 2005). Crude and pure extracellular phytase from recombinant bacteria *pEAS1AMP* has activator Mg^{2+} (10^{-3} and 10^{-4} M) but, It has inhibitor Fe^{3+} (10^{-4} M) (Nuhriawangsa

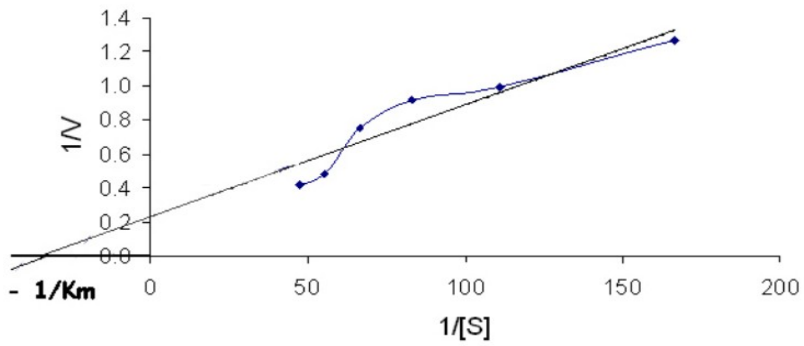

Figure 8. Lineweaver-Burk Curve for determination of Km and Vm of phytase (Y = 0.2304 + 0.0066, R2= 0.9437)

et al., 2008a).

Definition of Km is specific [S] on ½ Vm (Nelson and Cox, 2000). Result of Km and Vm were calculated by Lineweaver-Burk Curve (Figure 8) that were 15.91 υM and 2.41 υM/ minutes. Value of Km was different from another research, but It was on average of phytase Km value. Fungal phytase has average Km value at 10.6 to 23,2 υM Wyss *et al*. (1999). *Klebsiella sp*. had Km value at 280 υM (Sajidan *et al*., 2004), and recombinant bacteria *E. coli BL21 (DE3)pLysSpET-29a(+)-phyA* at 96 υM (Philippy dan Mullaney, 1997). Crude extracellular phytase from recombinant bacteria *pEAS1AMP* has Km and Vm value at 12.33 υM and 1.37 υM/second (Nuhriawangsa *et al*., 2008a). Pure extracellular phytase from recombinant bacteria *pEAS1AMP* has Km and Vm value at 54.82 υM and 30.3 υM/ minutes (Nuhriawangsa *et al*., 2008b).

CONCLUSION

Recombinant phytase was produced by transformation *pET-22b(+)+pEAS1* plasmid into competent cells *E. coli BL21* and *E. coli BL21 (DE3)*. Relative activity phytase of recombinant bacteria *E. coli BL21+pEAS1* was lower than thse of *E. coli BL21 (DE3)+pEAS1*. Crude intracellular phytase from recombinant bacteria *pEAS1AMP* had optimum activity at pH 5, temperature 40^{o}C, incubation time 60 minutes, substrat concentration 2%, molecule weight 47.3 kDa, Km 15.91 υM and Vm = 2.41 υM/second. Mg^{2+} was cofactor but Fe^{3+} (10^{-4} M) was inhibitor.

IMPLICATIONS

Phytase from *pEAS1AMP* recombinant bacteria is used to poultry feeding. It is mixed on feed to produce P organic, so feces which is produced will be safe for the environment.

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