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Comparison of Two Nitrogen Sources for *Aspergillus* spp. Phytase Production

(Perbandingan Dua Sumber Nitrogen untuk Produksi Fitase dari *Aspergillus* spp.)

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ABSTRAK

Telah dilakukan penelitian tentang produksi enzim fitase dari tiga *Aspergillus* spp.: *Aspergillus ficuum* NRRL 3135 (AF1) dan NRRL 320 (AF2) serta *A. Niger* (AN). Penelitian ini bertujuan untuk mendapatkan sumber nitrogen yang murah untuk produksi fitase yang optimal. Analisis aktivitas enzim, protein dan gula dilakukan setelah inkubasi 2, 3, 4, 6, 8, dan 12 hari. Aktivitas fitase lebih rendah pada medium natrium nitrat untuk semua *Aspergillus* spp. Dibandingkan dengan pada medium amonium nitrat (2,39; 1,65; 1,65 *vs* 2,5; 1,65; 1,78 U/ml masing-masing untuk *AF1*, AF2, dan *AN*) pada suhu inkubasi 30°C. Aktivitas enzim ini dicapai selama waktu inkubasi 8 hari. Aktivitas fitase semua *Aspergillus* spp pada suhu inkubasi 37°C adalah 2,07, 1,22, 1,16 *vs* 2,97, 1,24, 1,36 U/ml untuk media natrium nitrat *vs* amonium nitrat dan dicapai pada masa inkubasi 4 hari untuk *A. ficuum* dan masa inkubasi 3 hari untuk *A. niger*. Terlihat jelas bahwa suhu inkubasi pada suhu 37°C mempengaruhi waktu fermentasi untuk mencapai aktivitas enzim yang optimal dan amonium nitrat menghasilkan aktivitas enzim yang lebih tinggi jika dibandingkan dengan media natrium nitrat

Kara kunci: *Aspergilus* spp., fitase, sumber nitrogen

ABSTRACT

The study on phytase enzyme production of the three *Aspergillus* spp.: *Aspergillus ficuum* NRRL 3135 (AF1), NRRL 320 (AF2), and *A. niger* (AN) had been conducted. The aimed of this study was to obtain the low-cost nitrogen source for optimum phytase production. All fungi were incubated in media M0 and M1 for 12 days for enzyme production. Analysis for enzyme activities, protein and sugar content and biomass production were performed at day 2,3,4,6,8, and 12 days of incubation periods. Phytase activity was lower in sodium nitrate medium for all *Aspergillus* spp than in ammonium nitrate medium (2.39, 1.65, 1.65 vs 2.5, 1.65, 1.78 U/ml for *AF1*, AF2 dan *AN* respectively) at 30°C of incubation temperature. These enzyme activities were reached for 8 days

incubation time. The phytase activities of all *Aspergillus* spp at 37°C incubation temperature were 2.07, 1.22, 1.16 vs 2.97, 1.24, 1.36 U/ml for sodium nitrate vs ammonium nitrate medium and reached at 4 days incubation period for both *A. ficuum* and 3 days incubation period for *A. niger*. It was clearly shown that incubation temperature at 37°C shorterned the fermentation period to reach optimal enzyme activity and ammonium nitrate produced higher enzyme activity when compared with sodium nitrate medium.

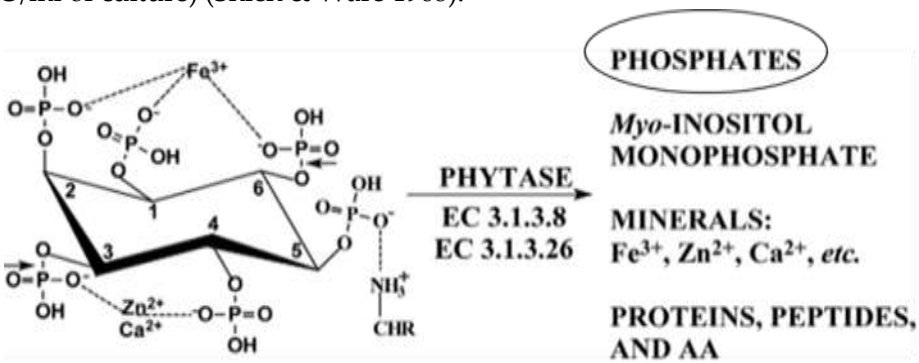
Key words: *Aspergillus* spp., phytase, nitrogen sources

INTRODUCTION

Phytic acid (myo-inositol 1,2,3,4,5,6-hexakis dihydrogenphosphate, empirical formula (Hill Notation) $C_6H_{18}O_{24}P_6$, molecular weight 660.04) and mixed cationic salts of phytic acid, designated as phytate or phytin, are a group of organic phosphorus compounds found widely in nature especially in legumes, cereals, and oilseed crops which serve as a major feed sources. Phytic acid salt or phytate, is organic-phosphorus storage form of more than 80% of the total phosphorus in cereals and legumes (Jongbloed & Kemme 1990; Savita et al. 2017). Phytates are also found at varying levels in different sorghum grain varieties (Wu et al. 2016) and can strongly chelate minerals, such as calcium, zinc, and iron, to form insoluble complexes, which could reduce the bioavailability of these minerals (Kruger et al. 2013). The ruminants digest phytate with the help of phytases produced by their anaerobic ruminal micro-flora. However, monogastric animals (pig, poultry) have only low levels of phytate-degrading enzymes in their digestive tracts, and since phytate itself is not absorbed in their gastro-intestine, inorganic phosphorus is supplemented to meet phosphorous requirement in non-ruminant feed. And for simple stomach animals, phytate in their feed become an antinutrition agent and can form complexes with proteins, amino acids (Pallauf et al. 1997) and a variety of mineral such as calcium, magnesium, iron and zinc.

Phytase (myo-inositol hexakis phosphate phosphohydrolase) is a phosphatase enzyme that catalyzes the hydrolysis of phytate and releases a usable form of inorganic phosphates (Fig. 1). Phytases had been found in ruminant and some animal tissue (Konietzny & Greiner 2002), plants (Grainer 2002), fungi and bacteria. Phytase from fungi and bacteria had been most detected and characterized (Greiner & Konietzny 2006). It has been reported that the fungus *Aspergillus niger* can produce phytase. Shieh & Ware (1968) first reported the isolation of phytase producing micro-organisms using selective phytase screening medium. It was an agar medium containing insoluble calcium phytate (Ca-phytate) and formed a turbid screening media, which turned transparent due to solubilization of Ca-phytate by diffused phytases from the isolates. After that, culture enrichment technique was used to isolate phytase-producing microorganisms. Shieh and Ware has traced over 2000 cultured of isolated microbes from soil and found that *Aspergillus ficuum* NRRL 3135

(AF1) the same group of *Aspergillus niger*, produced the highest activity of phytase (5.6 U/ml of culture) (Shieh & Ware 1968).



Phytases have been detected in different bacteria (Gram-positive, Gram-negative rods and cocci) viz. *Aerobacter aerogenes* (Yoon et al. 1996), *Escherichia coli*, *Klebsiella aerogenes* and *Pseudomonas* sp. (Greiner 2000; Sajidan et al. 2004; Kim, et al. 2003). Phytase derived from fungi other than Aspergillus have been widely explored, such as *Mucor* sp. and *Rhizopus* sp. Optimization of the production of phytase from *Aspergillus ficuum* NRRL 3135 and *Mucor racemosus* has been reported (Bogar et al. 2003). Production is carried out by solid substrate fermentation using wheat bran and coconut cake. Phytase is also produced by a type of heat-resistant bacteria isolated from soil that was identified as strain PH01 with activity to 10 U/ml at 37^{O}C and 48 hours-fermentation time have been reported (Popanich et al. 2003). Singh & Satyanarayana (2008) reported phytase production from thermophilic mold *Sporotrichum thermophile* Apinis using a solid substrate fermentation method. Enzyme activity reached 348.76 U/g dry matter. And methods for screening phytase from yeasts were deloped and reported (Olstorpe et al. 2009). However, the mold of the type of *Aspergillus* sp., *A. niger*, and *A. ficuum* were most widely explored for the purpose of commercial production (Pandey et al. 2001; Sabu et al. 2005). The first commercial phytase products were launched into market in 1991 (Hefner et al. 2005) and commercial phytase enzyme preparations are manufactured under the trade name Natuphos (BASF) .

Therefore, phytases are considered to be potential for use as an enzyme that gives great value in enhancing the nutritional quality of phytate-rich food and feed. In addition, phytase would be an eco-friendly product, reducing the amount of phosphorus entering the environment as supplementation of phytase in the diets for monogastric animals, reduces the fecal phosphate excretion up to 50%. In this paper, the effects of nitrogen sources ($NaNO_3$ and NH_4NO_3), and the effect of incubation temperature on enzyme production is reported. Sodium nitrate is more expensive than ammonium nitrate. Replacing sodium nitrate as originaly nitrogen source in phytase production with ammonium nitrate will lower the cost production. Observations were conducted on the measurement of enzyme activity, protein, sugars, biomass and the ability of the crude enzyme product to hydrolysis some

feedstuffs containing phytate (rice bran, pollard, rapeseed meal, coconut cake, palm kernel cake).

MATERIALS DAN METHODS

The materials used in the experiment were as follows: *Aspergillus* spp (*Aspergillus ficuum* NRRL 3135/AF1, NRRL 320 (AF2), and *Aspergillus niger* isolate 2 (AN) (collection of Indonesian Research Center for Veterinary Science, Bogor, Indonesia), SDA (Sucrose Dextrose Agar), Glucose, $FeSO_4.7\ H_2O$, KCl, K_2HPO_4, $MgSO_4.7H_2O$, $NaNO_3$ and NH_4NO_3, Tween 80, corn starch. Susbtrat : Phytic acid (50% solution, Tsuno Food Industrial Co., Ltd.TFI- Japan, CAS No.83-86-3), rice-bran, rapeseed meal, pollard, coconut cake and palm kernel cake. Phosphate Standard solution (1000ppm), acetate buffer pH 5.5 (1M), TCA (20%,), molibdovanadat reagent, 20% solution of sodium azide, Whatman filter paper No. 41; bovine serum albumin (BSA, protein standard), Na2CO3 (10%), NaOH (0.5 N), CuSO4.5H2O (1%), potassium-sodium tartrate (2%), NaOH (0,1N), Folin-Ciocalteu reagent. DNS reagent and glucose standard solution.

Inoculum and enzyme production

All the three fungi (AF1, AF2, and AN) were cultured in the SDA (Sucrose Dextrose Agar) medium (slant) until the age of 5 days. Spores were stored at a temperature of 4^OC until to be used. M0 media as described by Shieh and Ware (1968) was prepared which contains of (in every litre of basal medium) : corn starch 8%, glucose (30 g), $FeSO_4.7\ H_2O$ (0.18 g), KCl (0.5 g), K_2HPO_4 (0.2 g), $MgSO_4.7H_2O$ (0.5 g), and $NaNO_3$ (8.6 g), and then made up to pH 5. While the M1 media is the 0 where nitrogen sources ($NaNO_3$) is replaced by NH_4NO_3 (2.5 grams).

In time for production, to a five-days spores slant, sterile water containing 2% Tween 80 was added, gently scrapped and mixed with a sterile bend-glass pipette rod and inoculated into 50 ml of M0 and M1 media respectively and flasks were then incubated at 30 and 37^OC under shaking conditions (100 rpm). Harvesting was performed in day 2, 4, 6, 8, and 12 incubation time. For obtaining extracellular enzyme, the biomass was removed by centrifugation at 12000 x g for 10 minutes and the supernatant was assayed for phytase activity, protein content and sugar. Biomass yield was also determined.

Phytase assay

Phytase activity was determined using the method which was developed by Hahn and Galagher (1987) with a few modification. Two mixtures were prepared as follows:

Analytical mixture containing 2 ml of 1 M acetate buffer (pH 5.5), 2 ml phytic acid substrate (15 mM), and 1 ml of crude enzyme solution was prepared in a test tube.

Control mixture was prepared containing 3 ml of 1 mM acetate buffer (pH 5.5) and 2 ml of 15 mM phytic acid substrate. Both analytical and control mixtures were incubated at 37^{O} C for 30 min.

A total of 2 ml of analytical working solution consist of 0.2 cc of analytical mixture added to 0.8 cc of 20% TCA and added with 1 cc of water was reacted with the molybdo-vanadate reagent (3 ml, containing 2.5% Ammonium molybdate, $(NH_4)_6Mo_7O_{24}.4H_2O$; 0.125% Ammonium metavanadate, NH_4VO_3 in 5 M HCl), added 2 cc of water, and left for 10 minutes.

In the same time, a total of 2 ml of control mixture working solution cocnsist of, 0.8 cc of 20% TCA, 1 cc of water, 0,2 cc crude enzyme was reacted with molybdo-vanadate reagent (3 ml), and left for 10 minutes. Both analytical and control mixture was subjected for absorption measurement at 420 nm using a spectrophotometer. One enzyme unit was defined as the amount of enzyme liberating 1μmol of inorganic phosphate in 1 minute under the assay conditions (Hahn & Galagher, 1987)

Protein assay

Protein content in the culture was measured by Lowry method as modified by Hartree (1977). Bovine serum albumin (BSA) was used as a standard. A total of 1 ml liquid sample is added in to 0.9 ml solution containing 10% Na_2CO_3 in 0.5 N NaOH. The mixture was heated in a water bath at a temperature of 50^{O}C for 10 minutes. Stand to cool at room temperature and then 0.1 ml of reagent A (1% solution of $CuSO_45H_2O$ and potassium-sodium tartrate 2% in 0.1 N NaOH) was added, homogenized and 3 ml of diluted Folin-Ciocalteu reagent (basic solution diluted 15 X with distilled water) was added, the mixture was re-heated at 50°C for 10 minutes. Stand to cool and, absorbance was read at 610 nm. Standards are treated the same as sample.

Sugar analysis

Sugar content was determined using DNS reagent as described by Miller (1959). Crude enzyme solution was diluted several dilution and 1 ml of diluted crude enzyme fluid was added 3 ml DNS solution and 1 ml of water, then boiled for 15 minutes, cooled, and absorbance was measured at 575 nm. Glucose is used as a standard with a concentration of 100-600 μ g/ml.

Biomass production

Culture (50 ml) at a specific incubation period was taken and 0.5 ml of 20% sodium azide was added, stand at ambient temperature for 15 minutes and were blend using ultra- turrax for 1 minute. A total of 10 ml of a mixture of mold and residual medium was centrifuged at 12,000 rpm, at 4°C. Sediment (residue) was separated and washed with 5 ml of distilled water and re-centrifuged. Added back 5 ml of distilled water into the mold cell residue, stirred, and poured over Whatman filter paper No. 41 (9 mm diameter) that have weighed first. Filtering was conducted using vacuum filters (water jet), and then the filter paper and the residue dried at 105^{O}C until the weight remains.

Hydrolysis of organic-phosphorus from feed material

Culture filtrate having the highest enzyme activity used for elucidation capabilities of enzyme hydrolysis on various substrates are commonly used as feed material. Substrates used in this investigation are rice-bran, rapeseed meal, pollard, coconut, and palm kernel cake. A total of 2% substrate used for this purpose as follow: A total of 0.2 grams of substrate in 10 ml of acetate buffer pH 5.5, was added 1 ml of diluted enzyme was then incubated at 37°C for 30 minutes. To end the hydrolysis 3 ml of 20% TCA was added. To control the effect of dissolved phosphorus available in the materials, a set of the same samples was treated where the addition of the enzyme to control substrate was performed at the end of the incubation (after the addition of 20% TCA). Mixture of analytic and control were centrifuged at 3000 rpm for 10 minutes. A total of 1 ml of clear fluid was added to a tube containing 4 ml of molibdo-vanadate reagent. Allowed to stand for 10 minutes and absorbance was measured at 400 nm. Sodium phytate and phytic acid is used as a comparison.

RESULTS AND DISCUSSION

Biomass production of AF1, AF2 and AN at incubation temperature 30 and 37^{O}C in M0 and M1 media is shown in Fig. 2.

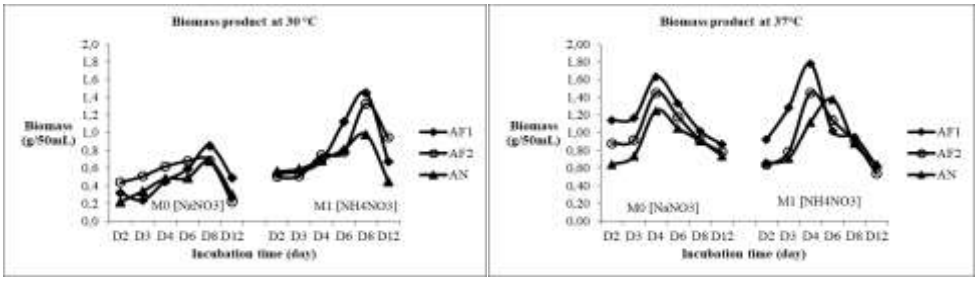


Fig. 2. Biomass yield for fermentation periods of 2-12 days at 30 and 37°C in M0 (sodium nitrate) and M1 (ammonium nitrate) media

The highest biomass product incubation at 30°Cwas shown in the 8th day incubation for AF1 and AF2 (1.45 and 1.33 g/50 ml) in M1 medium followed by AN (0.98 g/50ml). For the M0 medium, the highest biomass production shown also in the 8th day of incubation with lower values than M1. However, at 37°C incubation temperature, the highest values of biomass production was shortened to the 4th day incubation for all fungi in the M0 medium, and also for both AF1 and AF2 in M1 medium however, AN showed the highest yield at the 6th day. Biomass production was higher when ammonium nitrate as N source for both temperature incubations, however more amount of biomass formation at 37^{O}C was produced when compared with 30^{O}C incubation.

Nitrogen source is essential component for growth and enzyme production by the micro-organisms. It was reported that yeast extract, potassium nitrate, and sodium nitrate is a good sources for nitrogen in phytase production (Bogar et al. 2003), however ammonium nitrate was the worst nitrogen source for phytase production in this study. On the other hand, Ramachandran et al. (2005) stated that ammonium nitrate as inorganic nitrogen and peptone as organic nitrogen sources stimulated the phytase production in *Rhizopus* spp. and ammonium nitrate was more effective than peptone. To determine the optimum concentration of ammonium nitrate and peptone, their concentrations in the basal medium with 3.0% galactose as carbon source were varied from 0 to 5.0% (Man-jin 2008).

Sugar content in culture are shown in Fig. 3. The content of sugar in the culture medium using ammonium nitrate as a source of N was higher when compared with sodium nitrate for both incubation temperature. It was shown at 30^{O}C incubation, the sugar content was 750, 651, and 550 μg/ml for fermentation with fungi AF1, AF2, and AN respectively in M0 media at the 4th day and for M1 media were 1272, 1112, and 973 μg/ml for fermentation with fungi AF1, AF2, and AN. Incubation at 37°C showed a higher values of the sugar content for M0 media which were 1195, 925, and 1053 μg/ml on fermentation with AF1, AF2, and AN respectively and reached in the 3rd day of incubation. In the media M1, the values were sligtly lower for AF1 (1236 versus 1272 μg/mL for 30 and 37°C), and 1198 and 1488 μg/ml for AF2 and AN respectively. Fermentation temperature affected the sugar content in the filtrate as well as the nitrogen source, and shifted the sugar profile from 4th day to 3rd day incubation time. Media source contain glucose sugar which was added up to 3% (equivalent to 30,000 μg/ml) and corn starch and carbohydrates (corn, 8%). In this case the mold use sugar or carbohydrates as a source of energy to sustain life during the fermentation takes place. The use of sugar /carbohydrates was extremely high by mold on the medium with sodium nitrate as a source of N compared to ammonium nitrate and also in the lower incubation temperature sugar and carbohydrate was extremely required for mold growth. Ammonium nitrate is a weak salt compared to sodium nitrate and easily to be used by mold as a source of nitrogen for growth.

The extracellular proteins level in culture is shown in Fig. 4. Optimum formation of extracellular proteins in sodium nitrate medium for *AF1* (181 µg/ml) and AF2 (265 µg/ml) was reached on day 6th and AN (225 µg/ml) on day 4th at 30°C. Whereas at 37°C, the formation of extracellular proteins for AF1, AF2, and AN for M0 media were 429, 250, and 167 µg/ml achieved on day 4.

Optimum extracellular protein formation with ammonium nitrate as nitrogen source were 242, 295, and 361 µg/ml for AF1, AF2, and AN respectively at and were reached at the 6th day of incubation at 30°C. However at 37°C incubation the optimum cellular protein formation were achieved at day 4th. AF1 shown lower value of extracellular protein level in medium M1 when compared with medium M0. Extracellular protein formation was achieved when fungi using energy sources optimally. Energy sources that be found in the media are glucose and corn starch

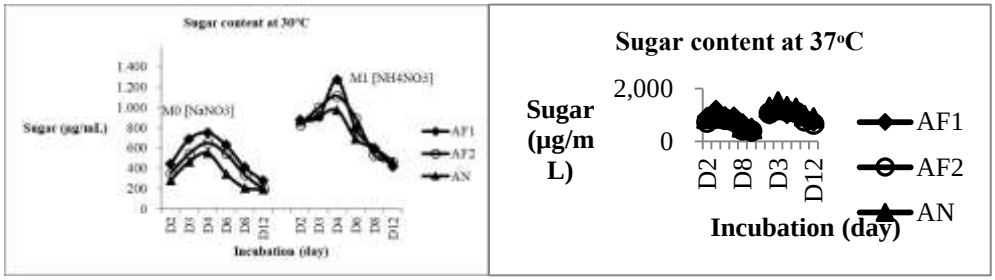


Fig. 3. Sugar content in the culture on fermentation periods of 2-12 days at 30 and 37°C in M0 and M1 media

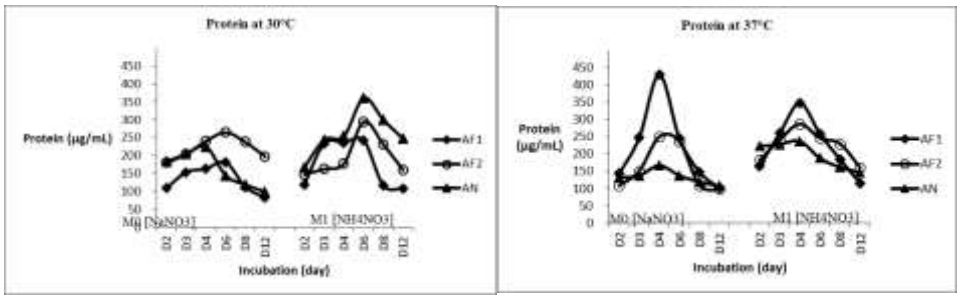


Fig. 4. Protein content in the culture on fermentation periods of 2-12 days at 30°C and 37°C in the M0 and M1 media

Phytase activity when incubation temperature was 30OC shown the same pattern between ammonium and sodium nitrate (Fig. 5). It was shown that there are two peaks activities for phytase of AF1: at day 4th (1.59 U/mL) and day 8th (2.39 U/mL) in M0 nedium and 3.01 U/mL at day 4th vs 4.19 at day 8th for M1 media. Other fungi (AF2 and AN) shown peak phytase activity at day 8th.in both media.

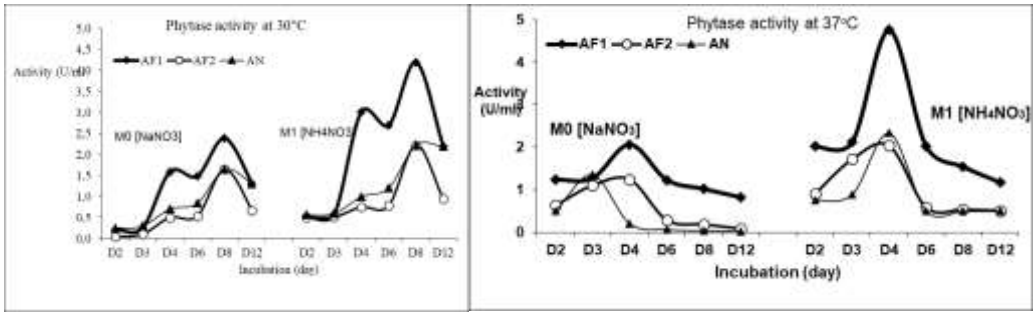


Fig. 5. Phytase activity in the culture on fermentation periods of 2-12 days at 30°C and 37°C in the M0 and M1 media

At incubation temperature of 37°C, M0 medium; optimum phytase activity for *AF1 and AF2 were* shifted the 4th day incubation period, the levels were 2.07 and 1,24 U/mL for AF1 and AF2 respectively. However, for *A. niger* was 1.36 U/ml in the 3rd day incubation period. And for M1 medium all fungi shown the same period of incunation (day 4th) to reach optimim protein level. It was clearly shown that incubation temperature shortened the fermentation period to reach enzyme activity, protein content and biomass production.

Previous results have been reported regarding incubation temperature for phytase production. Han & Galagher (1987) used 7-10 days incubation period on temperature of 30°C. Similarly, Nair et al. (1991), working at a temperature of 30°C on a solid substrate for 8 days incubation period. Others, Shieh & Ware (1968) when used incubation temperature of 28°C and reached the highest production in the 5th days of fermentation period, and Ahmad et al. (2000) at the same temperature incubation carried out for 10 days. In this study, the enzyme production was observed at incubation temperatures of 30 and 37°C with medium corn starch and sodium nitrate as nitrogen source such as Shieh & Ware (1968). Optimum activity for 30°C temperature reached at day 8 and at 37°C on day 4. However, for further production, incubation at 37°C is used because of the long incubation terms become more inefficient.

Table 1. Liberation of phosphorus in some feeding stuff by *AF1* enzyme

Feedstuff	Phosphor (◎mol /min, 37°C)
Rice bran	0,130
Rapeseed meal	0,022
Pollard	1,978
Coconut cake	0.050
Palm kernel cake	0,080
Sodium phytate, 3 mM	1,923
Phytic acid, 15 mM	2,896

The ability of Aspergillus phytase for de-phosphorylation of phytate in several feedstuffs were shown in Table 2. It was shown that organic-phosphor in the pollard was easily liberated when compared to other materials. Some reports had been published that *A. ficuum* phytase was used to hydrolyse phytate in soybean meal and kapok kernel cake (Han & Wilfred 19880), *A. usami* phytase was used to treat soybean meal (Ilyas et al. 1995), canola cake (Nair et al. 1991), and also in some fermented food products (Sudarmadji & Markakis 1977; Wang et al. 1980; Sutardi & Buckle 1988).

CONCLUSION

The production of enzyme clearly was affected by temperature of incubation. Temperature 37°C had higher biomass enzyme production, therefore higher tempertature shortened the incubation period to the 4th day when compared with 30^{O}C (8th day). Ammonium nitrate as nitrogen sources clearly produced higher phytase activity when compared to sodium nitrate for all fungi tested. However, *A. ficumm* NRRL 3135 show highest production of phytase. Shorter in incubation time and using ammonium nitrate as N source reduced cost of production.

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DAFTAR PUSTAKA

Ahmad T, Rasool S, Sarwar M, Haq A, Hasan Z. 2000. Effect of microbial phytase produced from a fungus Aspergillus niger on bioavailability of phosphorus and calcium in broiler chickens. Animal Feed Science Technology 83.103-114.

Bell FM, Marshall TR, Anderson EM. 1986. Microbiological and sensory tests of beef treated with acetic and formic acids. J Food Prot. 49:207-210.

Bogar B, Szakacs G, Linden JC, Pandey A, Tengerdy RP. 2003. Optimization of phytase production by solid substrate fermentation, J Ind Microbiol Biotechnol. 30:183-189.

Gargova S, Sariyska M. 2003. Effect of culture conditions on the biosynthesis of Aspergillus niger phytase and acid phosphatases. Enz Microb Technol. 32: 231-5.

Graf E. Phytic acid: Chemistry and applications. Minneapolis (USA): Pitlatus Press, 1986; 101-10.

Grainer R, Konietzny U. 2006. Phytase for Food Application. Food Technol. Biotechnol. 44:125-140

Greiner R. 2000. Properties of phytate degrading enzymes from germinated lupine seeds (*Lupinus albus* var. Amiga.). Proceedings of the 4th European Conference on grain legumes. Cracow, Poland: 398-9. Paris (France): AEP.

Greiner R. 2002. Purification and characterization of three phytases from germinated lupine seeds (Lupinus albus var. Amiga). J Agric Food Chem. 50:6858-64. PMID: 12405788.

Haefner S, Knietsch A, Scholten E, Braun J, Lohscheidt M, Zelder O. 2005. Biotechnological production and applications of phytases. Appl Microbiol Biotechnol.; 68: 588-97. PMID: 16041577.

Han YW, Galagher DJ. 1987. Phosphatases production by *Aspergillus ficuum*. J Ind Microb. 1:295-301.

Han YW, Wilfred AG. 1988. Phytate hydrolysis in soybean and cottonseed meals by *Aspergillus ficuum* phytase. J Agric Food Chem. 36:259-262.

Haros M, Bielicka M, Sanz Y. 2005. Phytase activity as a novel metabolic feature in Bifidiobacterium. Lett Microbiol. 247: 231-239.

Hartree EF. 1972. Determination of protein: a modification of the Lowry method that give a linier photometric response. Analytical Biochem. 48:422-427.

Ilyas A, Hirabayasi M, Matsui T, Yano H, Yano F, Kikishima T, Takebe M, Hayakawa K. 1995. A note on the removal of phytate in soybean meal using *Aspergillus usami.* AJAS. 8:135-138.

Jongbloed AW, Kemme PA. 1990. Apparent digestible phosphorus in the feeding of pigs in relation to availability, requirement and environment: 1. Digestible phosphorus in feedstuffs from plant and animal origin. Neth J Agric Sci. 38:567-575.

Kim HW, Kim YO, Lee JH, Kim KK, Kim YJ. 2003. Isolation and characterization of a phytase with improved properties from *Citrobacter braakii*. Biotechnol Lett. 25:1231-4. PMID:14514072

Konietzny U, Greiner R. 2002.Molecular and catalytic properties of phytate-degrading enzymes (phytases). Int J Food Sci Technol. 37:791-812.

Kruger J, Taylor JR, Oelofse A. 2012. Effects of reducing phytate content in sorghum through genetic modification and fermentation on *in vitro* iron availability in whole grain porridges Food Chemistry, 131, pp. 220-224.

Lambrechts C, Boze H, Moulin G, Galzy P. 1992. Utilization of phytate by some yeasts. Biotechnol Lett. 14: 61-6.

Liu BL, Rafiq A, Tzeng YM, Rob A. 1998. The induction and characterization of phytase and beyond. Enz Microb Technol. 22: 415-24.

Man-Jin In, Seo S, Oh N. 2008. Fermentative production and application of acid phytase by *Saccharomyces cerevisiae* CY strain. African JBiotechnology 7(17): 3115-3120.

Miller GL. 1959. Use of Dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chem. 31: 426 428.

Mitchell DB, Vogel K, Weimann BJ, Pasamontes L, van Loon APGM. 1997. The phytase subfamily of histidine acid phosphatases: isolation of genes for two novel phytases from the fungi *Aspergillus terrreus* and Myceliophthora. Microbiol. 143 pp. 245-252.

Mochizuki D, Takahashi H. 1999. Method for producing phytase, Patent: EP 0931837-A, 28 July.

Mullaney EJ, Ullah AH. 2003. The term phytase comprises several different classes of enzymes. Biochem Biophys Res Commun. 312:179-184. doi:10.1016/ j.bbrc.09.176. PMID 14630039.

Nair VC, Laflamme J, Duvnjak Z. 1991. Production of phytase by *Aspergillus ficuum* and reduction of phytic acid content in canola meal. J Sci Food Agric. 54, 355-365.

O'Dell BL, de Boland AR, Koirtyohann SR. 1972. Distribution of phytate and nutritionally important elements among the morphological components of cereal grains. J Agr Food Chem. 20:718.

Olstorpe M, Schnürer J, Passoth V. 2009. Screening of yeast strains for phytase activity. FEMS Yeast Research. 9: 478–488, https://doi.org/10.1111/j.1567-1364.2009.00493.x

Pallauf J, Rimbach G. Nutritional significance of phytic acid and phytase. 1997. Arch Tierernahr. 50: 301-19. PMID: 9345595.

Pandey A, Szakacs G, Soccol CR, Jose A, Rodriguez L, Soccol VT. 2001. Production, purification and properties of microbial phytase. Biores. Technol. 77:203-214.

Popanich S, Klomsiri C, Dharmsthiti S. 2003. Thermo-acido-tolerant phytase production from a soil bacterium in a medium containing rice bran and soybean meal extract. Bioresource Technology 87:295-298.

Ramachandran S, Roopesh K, Nampoothiri KM, Szakacs G, Pandey A. 2005. Mixed substrate fermentation for the production of phytase by *Rhizopus* spp. using oilcakes as substrates. Proc Biochem. 40:1749-1754.

Sabu AA, Pandey MJ, Daud, Szakacs G. 2005. Tamarind seed powder and Palm kernel cake: two novel agro residues for the production of tannase under solid-state fermentation by Aspergillus niger ATCC 16620. Biores Technol. 96:1223-1228.

Sajidan A, Farouk A, Greiner R, Jungblut P, Muller EC, Borriss R. 2004. Molecular and phycological characterization of a3-phytase from the bacterium Klebsiella pneumonia ASR1. Appl Microbiol Biotechnol. 65: 110-8. PMID: 14727093.

Sano K, Fukuhara H, Nakamura Y. 1999. Phytase of the yeast Arxula adeninivorans. Biotechnol Lett. 21:33-8.

Savita PD, Yallappa M, Nivetha N. 2017. Phytate solubilizing microorganisms and enzyme phytase to combat nutritional problems in cereal-based foods. J Bacteriol Mycol Open Access. 4(3):86-89. DOI: 10.15406/jbmoa.2017.04.00093.

Segueilha L, Lambrechts CM, Boze H, Moulin G, Galzy P. 1992. Purification and properties of the phytase from *Schwanniomyces castellii*. J Ferment Bioeng. 74:7-11.

Shieh TR, Ware JH. 1968. Survey of microorganisms for production of extracellular phytase. Appl Microb. 16:1348-1351.

Shieh TR, Wodzinski RJ, Ware JH. 1969. Regulation of the formation of acid phosphatases by inorganic phosphate in Aspergillus ficuum. J. Bacteriology,100(3) 1161-65.

Singh B, Satyanarayana T. 2008. Phytase production by a thermophilic mould Sporotrichum thermophile in solid state fermentation and its potential applications. Bioresource Technology. 99:2824-2830.

Sudarmadji S, Markakis P. 1977. The phytate and phytase of soybean tempeh. J Sci Food Agric. 28:381-383.

Sutardi, Buckle KA. 1988. Characterization of extra- and intra-cellular phytase from Rhizopus olygosphorus used in tempeh production. J Food Microb. 6:67-69.

Ulupi N, Sumantri C. 2015. Peranan kelompok gen triglyceride lipase, fatty acid synthase dan fatty acid binding protein pada metabolisme lemak ayam broiler. Wartazoa. 25:15-22.

Vohra A, Satyanarayana T. 2002. Purification and characterization of a thermostable and acid-stable phytase from Pichia anomala. World J Microbiol Biotechnol. 18:687-91.

Wang HL, Swain EW, Hesseltine CW. 1980. Phytase of molds used in oriental food fermentation. J Food Sci. 45:1262-1266.

Wu G, Johnson SK, Bornman JF, Bennett SJ, Singh V, Simic A, Fang Z. 2016. Effects of genotype and growth temperature on the contents of tannin, ghytate and in vitro iron availability of sorghum grains PloS One, 11 Article e0148712.

Yoon SJ, Choi YJ, Min HK. 1996. Isolation and identification of phytase-producing bacterium, *Enterobacter* sp. 4, and enzymatic properties of phytase enzyme. Enz Microb Technol. 18:449-54.

Yu S, Cowieson A, Gilbert C, Plumstead P, Dalsgaard S. 2012. Interactions of phytate and myo-inositol phosphate esters (IP1-5) including IP5 isomers with dietary protein and iron and inhibition of pepsin. J Anim Sci. 90:1824-1832. doi: 10.2527/jas.2011-3866