Phytase production by fungi based on palm oil mill effluent

A Sugiharto[1]

[1] Research Center for Biology, Indonesian Institute of Sciences
Jl. Raya Bogor Km 46, Cibinong Science Center, Cibinong 16911, Indonesia.

E-mail: one.myconano@yahoo.co.id

Abstract. The utilization of phytase enzyme containing feed to monogastric and digastric livestock could increase the efficiency of nutrients uptake and livestock resistance to disease attacks. Palm oil mill effluent (POME) is one type of waste that has not been widely used in enzyme production. Some fungi that grow on POME indicate capable of producing phytase. The aims of this study is to utilize POME as a media production of phytase through fermentation by fungi with the eddition of C and N as sources. The experiment was arranged by complete randomized design with 5 replications. The result showed that isolated fungi from palm oil mill effluent (POME) was able to grow with pH value of 4 to 8. Furthermore, the two isolated fungi could optimally grow in pH value of 5 to 7. Through spores microscopic of the cross section of two fungi, the fungi were identified as *Aspergillus niger* and *Neurospora crassa.* Further study showed different results regarding on phytase enzyme activity of these two fungi in a modified media consists of 1% and 5% of POME with the addition of sucrose as the C source and 1% of peptone as the N source. The highest phytase production by *Aspergillus niger* was 0.393 U/mL substrate in media contain with 5% of POME and addition of 1% of peptone, for 96 hours and at 30^0C. While for *Neurospora crassa,* the highest phytase production was 0.115 U/mL substrate, in media contain 1% of POME, 1% of sucrose, for 48 hours and at 30^0C.

1. Introduction

The palm oil sludge and palm oil mill effluent (POME) has received considerable attention in recent years [1]. Most of this material is just disposed away and polluted the environment [1-8]. POME also is most expensive and difficult waste to manage [6], becausethe factory requires a relative large amount of money to dispose the waste. POME have not been efficiently utilized by the factory except for fertilizer. Beside that, POME as the one of wastes still contains large concentrations of carbohydrate, proteins, nitrogen compouds, lipid and minerals [4,6]. So POME is an excellent raw material for bioconversion by biotechnological technique [9]. Utilization of POME as a raw material to produce phytase enzyme has never been report. Through preliminary research, it is known that some of fungi are able to grow in POME and have ability to produce phytase enzyme.

Phytase enzyme plays an important role to help breaking the phytate bond in animal feed derived from nuts. Phytase compounds are classified as anti-nutritional substances. The existence of this bond in animal feed can result in the feed not being easily digested. This bond will bind the essential elements for animal

in the feed, such as protein and minerals [10]. The absence of the phytase enzyme in the gastrointestinal tract in monogastric animals results in low feed digestibility value [8,11-14].

The use of phytase as a mixture of monogastric and digastric animal feed has been done in several countries, but it has not been done in Indonesia. It might be caused by the expensive production costs. Nowadays, some innovation to utilize cheap materials for phytase production must be continuously pursued. Phytase production is available on fungal sources [15], but there is no report on phytase production by fungi based on POME as a media.

The aims of this study is to utilize POME as a media production of phytase through fermentation by fungi with the eddition of C and N as sources. The experiment was arranged by complete randomized design with 5 replications. The results of this study are expected to be able to provide better alternative on POME utilization and provide a new method to produce phytase enzyme using a cheap material.

2. Materials and Methods

2.1. Materials

POME, *Aspergillus niger, Neurospora crassa.,* potato dextrose agar (PDA)(Oxoid), buffer solution, mixed reagent, Glucose, Sucrose, Peptone, (Merck) Yeast (Difco), Ca-phytate (Sigma), distillated water, $MnSO_4$, $CaCl_2$, $MgSO_4$, $FeSO_4$, KCl, and NaCl (Merck) were used as materials in this study. *Aspergillus niger* and *Neurospora crassa* used in this study were obtained from our own culture collection, through isolation from POME derived from Palm Oil Processing, PT Perkebunan 4, Medan, North Sumatera.

2.2. Methods

2.2.1. Fungi inoculants

The *Aspergillus niger* and *Neurospora crassa* were cultivated in PDA and incubated for 96 hours, at pH 7, and 30^0C. After incubation, 20 ml of distillated water was added. This suspension was used as inoculants. Biomass inoculants were measured using a method from Garraway and Robert [16].

2.2.2. Phytase production in POME media

The mixture of 1% and 5% POME, 1% of sucrose and peptone solutions, and 200 ml of solution consists of 6 g of $C_6H_{12}0_6$; 0.1 g of $MgSO_4.7H_2O$; 0.1 g of KCl; 0.02 g of $FeSO_4$; and 0.02 g of NaOH were added to erlenmeyer flask. The solution was then homogenized and sterilized for 20 minutes in temperature of 121^0C and pressure of 1 atm. This liquid medium was conditioned for 24 hours in temperature of 30^0C. The prepared inoculant was then added to the solution and fermented in room temperature, and under stirring of 150 rpm.

2.2.3. Measurement of phytase

The test of phytase production capability by *A. niger* and *N. crassa* was done in 1% and 5% of POME medium and with addition of 1% of sucrose as carbon source and 1% of peptone as nitrogen source. The phytase analysis was performed at 30^0C for 48 hours, 96 hours and 144 hours after fermentation.

Phytase activity was measured using Engelen, *et al*.[17]. The centrifugation supernatant was taken for 50 µL and transferred into test tube. After that 50 µL of calcium phytate 1% was added to the extract enzyme and incubated for 30 minutes. Then 160 µL of mixed reagent prepared by mixing 10 mL of H_2SO_4 5N, 6 mL of ammonium molybdate 0.0032 M, 3 mL of ascorbic acid 0.1 M, and 1 mL of potassium antimonyl 0.0086 M was added into the solution. After that the sample was incubated for 3 hours, and then the phytase activity was measured using UV-spectrophotometer at wavelength of 880 nm.

One unit of phytase activity (U) is expressed as the amount of enzyme released for 1 µM phosphorus/min. The phytase activity unit is calculated using formula below:

$$\text{Enzyme Activity (U/g)} = \frac{\frac{[\text{phosphate}]\text{mg}}{1} x 1000\mu \frac{\text{g}}{\text{mg}} x \frac{1\text{L}}{1000\text{mL}} x 5\ \text{mL} \frac{\text{enzyme}}{1} g}{\text{BM } x \text{ v } x \text{ t}} x\ \text{fp}$$

Note:

v : Phytase volume

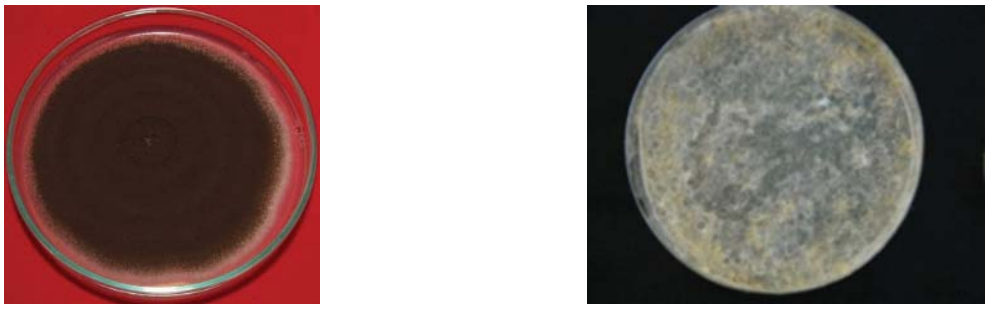
t : Incubation time

BM : Molecular weight

Fp : Dilution factor

3. Results and Discussion

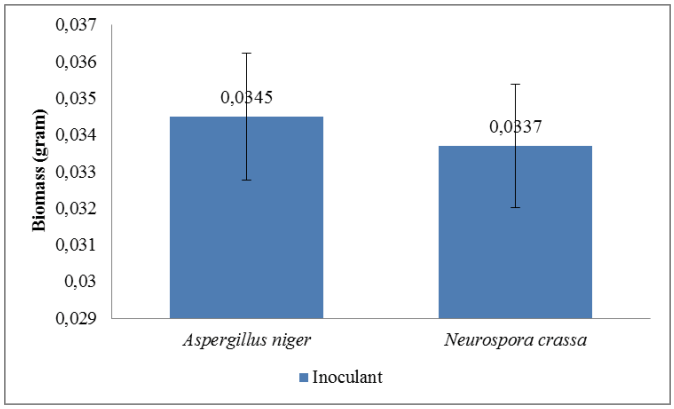
Aspergillus niger and *Neurospora crassa* were grown on PDA for 4 days at 30^0C and pH 7 (**Figure 1**). The biomass used from both isolates *Aspergillus niger* and *Neurospora crassa* was about 0,0345 g and 0.0337 g (**Figure 2A**). This biomass was obtained by growing both isolates on PDA slank at 30^0C, pH 7 for 5 days using in reaction tube with 2.5 cm diameter.



A B

Figure 1. Morphology of *A. niger* (A) and *N. crassa* (B) grown in PDA after 4 days incubation at pH 7 and 30^0C.

The results of pH and POME concentration treatment on the phytase activities showed that both isolates, *Aspergillus niger* and *Neurospora crassa,* had quite different results (**Figure 2B**). In general, *N. crassa* had higher phytase activities than that of *A. niger* on both treatments of 1% or 5% of POME and different pH levels. However, the addition of POME may not always increase the phytase activities. The highest phytase activity for *A. niger* was 0.0389 U/mL substrate, at pH 5 and 5% of POME. While for *N. crassa,* the highest phytase activity was 0.0653 U/mL substrate at pH 6 and 5% of POME.



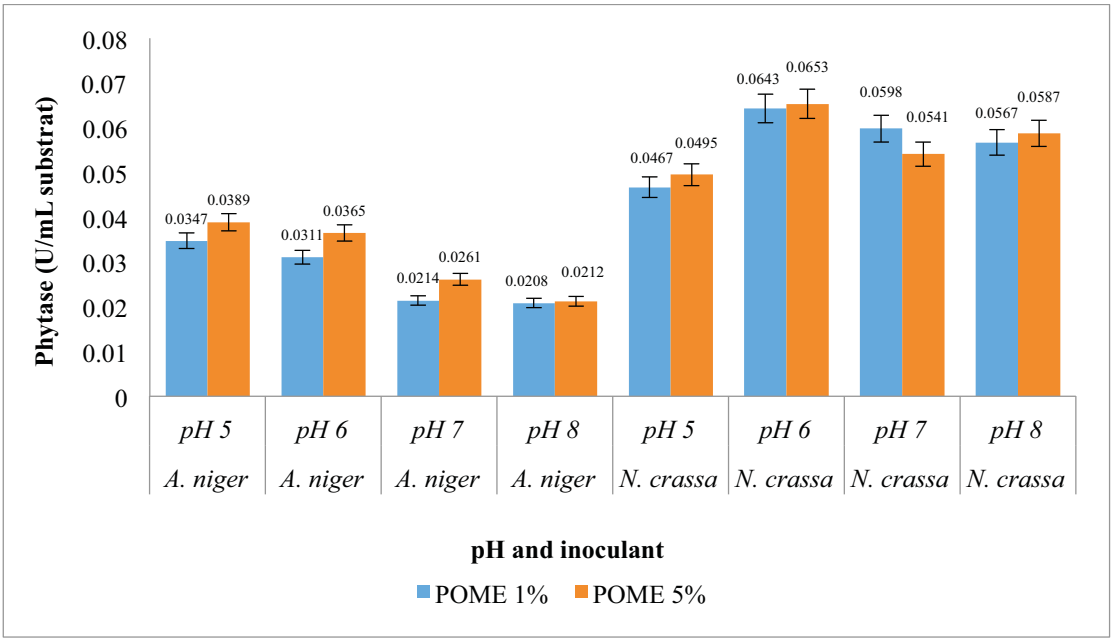
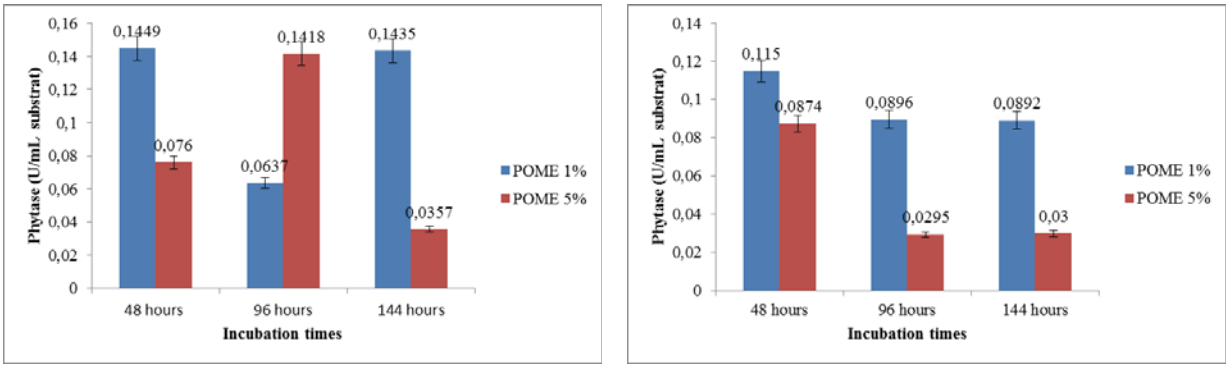


Figure 2. Biomass for *A. niger* and *N. crassa* grown in PDA slank after 96 hours (above), Phytase actvity for *A. niger* and *N. crassa* in different pH values with addition 1% and 5% POME (below)

The effect of adding 1% of sucrose to the production of phytase from *Aspergillus niger* and *Neurospora crassa* on media containing 1% and 5% of POME showed different results. In general, the addition of 1% of sucrose can increase the production of phytase compared to the control (**Figures 2B** and **3A**). In *A. niger* , the maximum results were obtained on 1% of POME for 48 and 144 hours incubation, 0,145 U/mL substrate and 0.144 U/mL substrate. While in 5% of POME the maximum result was 0.142 U/mL substrate, and wasobtained after 96 hours incubation (**Figure 3A**). This condition is different from the isolate of *N. crassa*, where the addition of 1% of sucrose, in 1% and 5% of POME will decrease the phytase production as increases in incubation time. The maximum result was 0.115 U/mL substrate, for 48 hours incubation and 1% of POME (**Figure 3B**). This condition indicates that the carbon source of sucrose is better used for *A. niger*. The carbon source used will greatly affect the phytase

production [18,19] and sucrose is the best carbon source for phytase production by *A. niger* [19]. This condition is possible because the addition of carbon source will increase cell production after 24 hours [20]

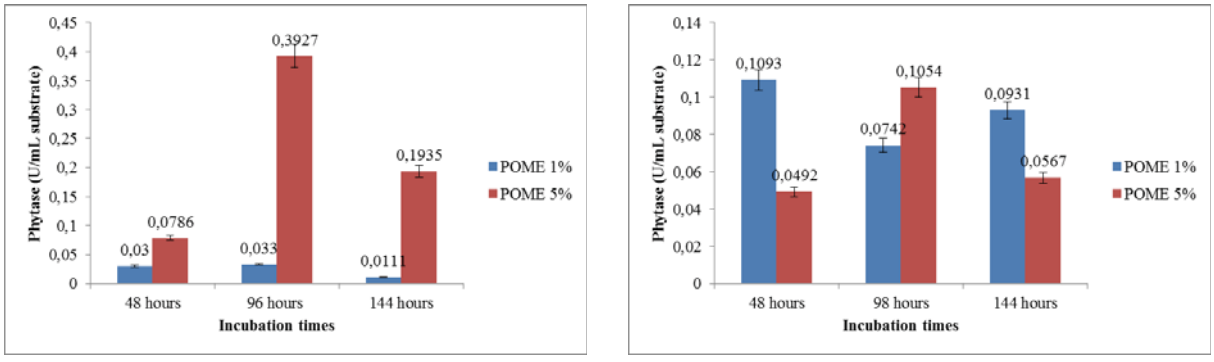


A B

Figure 3. Phytase activity of A. niger at pH 5, and at 30^{0}C with 1% and 5% of POME and 1% of sucrose (A) Phytase activity of N. crassa at pH 6, and at 30^{0}C with 1% and 5% of POME and 1% of sucrose (B)

Different results were obtained in addition of 1% of peptone in medium containing 1% and 5% of POME for the same isolate. In *Aspergillus niger*, the addition of 1% of peptone gave better phytase activity on media containing 5% of POME. The highest phytase activity for *A. niger* was 0,393 U/mL substrate obtained at incubation time of 96 hours (**Figure 4A**)

In *Neurospora crassa*, addition 1% of peptone on 1% and 5% of POME produced different results. The maximum of phytase of 0.109 U/mL substrate was obtained at 1% of POME medium after 48 hours incubation period, meanwhile 5% of POME had the best result at 98 hours incubation with 0.105 U/mL substrate (**Figure 4B**).



A B

Figure 4. Phytase activity of A. niger at pH 5, and at 30^{0}C with 1% and 5% of POME and 1% of peptone (A), Phytase activity of N. crassa at pH 5, and at 30^{0}C with 1% and 5% of POME and 1% of peptone (B)

Several bacteria, fungi, yeasts and actinomycetes were identified having capability to produce phytase in several different environmental and media conditions [13,18-19, 21-24]. Some fungi such as *Aspergillus niger* and *Neurospora crassa* have the ability to produce phytase [21,22]. Purwadaria *et al* [21] has reported that *Aspergillus oryzae* has abilities to produce phytase with POME and rice bran media.

The results indicated that the pH and type of inoculant used were important factors in controlling the production of phytase enzymes (**Figure 2B**). *Aspergillus niger* and *Neurospora crassa* have different abilities in the production of phytase in POME media. As shown in **Figure 3**, the phytase production by *A. niger* was optimum at pH 5 (0.0389 U/mL substrate) and *N. crassa* was at pH 6 (0,0653 U/mL substrate). The productivity of some fungi in producing phytases is very different and one of the factors to be considered is pH [8,18,19]. Sucrose became one of the best sources of carbon to produce phytase by *A. niger* [19]. *A. niger* has the ability to produce phytase on POME [21], and it is commonly used for production of phytase [25]. Addition of small amounts of nitrogen to the fermentation medium can trigger the production of phytase [18]. Phytase production are available on fungal sources [26] but there is no report on phytase production by fungi base on POME as a media. The content of the material, pH and the eddition of C or N in the media is known to affect the microbes activity in producing phytase enzymes [18-20].

4. Conclusion

Both *Aspergillus niger* and *Neurospora crassa* are types of fungi which are able to grow well in POME. The optimal phytase production of *A. niger* was 0.3927 U/mL substrate, and was obtained in the addition of 1% of peptone with 5% of POME, and incubated for 96 hours, at 30^0C. The optimal phytase production of *N. crassa* was 0.115 U/mL substrate, and was obtained in the treatment of 1% of POME, after 48 hours of incubation time with the addition of 1% of sucrose at 30^0C.

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