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Studying Culture Conditions for High Level Phytase Production from *Aspergillus fumigatus* ET3

Nguyen Van Tinh[*], *Nguyen Thi Ngoc Diep and Duong Thi Huong Giang*

Biotechnology Research and Development Institute, Can Tho University
**Correspondent author: vantinhnguyen1106@gmail.com*

Abstract

This research aimed to isolate and study culture conditions for high level phytase biosynthesis of potential phytase producing *Aspergillus fumigatus* from rice soil samples in some provinces of Mekong Delta, Viet Nam. Among selected fungal isolates, the isolate ET3 exposed highest phytase activity on phytase screening medium. Based on morphological characteristics and sequences of 18S rRNA gene, ET3 appeared to belong to *Aspergillus fumigatus* species. The results also showed that the best conditions for high level phytase production from *A. fumigatus* ET3 on solid-state culture medium which contained wheat flour as a phytate substrate, was inoculated with 10^8 spores/ml of one-day-old spores, supplemented with 0.5% malt extract, 1% KH_2PO_4, 0.5% glucose and incubated for 2 days at pH 4, 35 ^oC.

Keywords: *Aspergillus fumigatus*, extracellular phytase, phytate, 18S rRNA gene.

1. Introduction

Phosphorus (P) is a vital element in living organisms. Naturally, the main storage form of phosphorus in plants is phytate (1). However, there are some drawbacks of phytate. First, human and monogastric animals cannot use the phosphorus in this form since they lack phytase enzyme (2). Consequently, the undigested phytate phosphorus in the manure accelerates eutrophication (3). Second, phytate is considered as an anti-nutritive factor because it is a strong chelator of cations ($Ca^{2+,}$ Zn^{2+} and Fe^{2+}…), proteins and vitamins in feeds or foods originated from cereals or legums (4, 5).

In fact, phytase (EC 3.1.3.8) can catalyze the hydrolysis of phytate into an inositol sugar and salt of ortho-phosphoric acid. As a result, phytase supplemented in animal feeds, supporting to digest the phytate, will efficiently improve nutritive values of phytate and decrease environmental pollution. The phytase has been found in some bacteria, yeast and fungi. Nonetheless, the fungi belonging to *Aspergillus* genus (*A.carbonarius* (6), *A. niger* (7), *A. oryzae* (8), *A. fumigatus* (9)) are considered as the most active sources of extracellular phytase. Currently, most of the commercial phytases are produced from *A. ficuum* NRRL3135 (10). The commercial phytases include, Allyzeme Phytase (Alltech Ireland Ltd, Co Meath), PhyzymeTM (Dversao/Danisco A/S).

The enzyme applied for commercial feed industry must have special characteristics as thermal stability and activity over broad pH range. A heat resistant phytase reported in *A. fumigatus*, which can remain 90% activity after being heated at 100 ^{o}C for 20 min (11). Therefore, *A. fumigatus* phytase with high ability of refolding in heat denaturation process (12) is suitable for implementing in feed production. Phytase production can be carried out in submerged and solid-state fermentation. However, solid-state fermentation can bring several economical and practical benefits as higher product level and simple cultivation equipment in comparison with submerged fermentation. For these reasons, the research was conducted to isolate *A. fumigatus* with high phytase activity and enhance phytase production by investigating culture conditions for solid-state fermentation such as phytate substrate, incubation time, spore inoculum size, pH, and temperature, age of spore inoculums, supplementary KH_2PO_4, nitrogen source and carbon source.

2. Materials and Methods

2.1 Materials

Rice soil samples were collected in different places of Can Tho City, Soc Trang Province and Vinh Long Province, Mekong Delta, Vietnam. Media included isolation medium (13), selective medium M2 (10), PGA – Potato Glucose Agar, solid state medium.

2.2 Methods

2.2.1 Isolation and identification of phytase-producing fungal strains

Each soil sample (10 g) was added to Erlenmeyer flask containing 30 ml of distilled water and mixed well. The mixture was then deposited. The supernatant solution was spread on Petri dishes containing isolation medium. The disks were incubated at 30^{o}C for 3 days. Single colonies were picked up and transferred to fresh medium until pure colonies obtained. The pure colonies with hydrolytic zones on selective medium (14) were subjected to check thermal stability. One selected isolate with highest phytase activity and thermal stability then was determined species by morphological and biomolecular methods.

2.2.2 Solid-state fermentation

Solid-state fermentation was carried out in a plastic bags in temperature chambers. The medium in each plastic bag contained 10 g of rice husk, 30 g of phytate substrate and 25 ml of mineral salt solution pH 5.5 (0.1 g/l of $MgSO_4.7H_2O$, 0.5 g/l of KCl, 0.01 g/l of $FeSO_4$, 0.01 g/l of $MnSO_4$, 0.1 g/l of NaCl, 5 g/l of $CaCl_2$). Then, the bag was autoclaved at 121^{o}C for 20 minutes before inoculating 1 ml of conidial spore solution. Finally, the bag was put into the chamber for fermentation.

2.2.3 Studying culture conditions for high phytase production from the isolate

The experiments were carried out to elucidate the effects of important factors for high phytase production such as:

- Interactive effect of phytate substrate sources (wheat, corn and soybean) and incubation time (1, 2, 3, 4, 5, 6 day(s)),
- Effect of spore density: 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} spore/ml,
- Interactive effect of pH (2, 3, 4, 5) and temperature (30 ^{o}C, 35 ^{o}C, 40 ^{o}C).
- Effect of age of spore inoculums: 1, 2, 3, 4, 5, 6, 7 day(s) old.
- Effect of supplementary phosphorus (KH_2PO_4): 0%, 0.5%, 1%, 1.5%, 2%, 2.5%
- Effect of nitrogen sources (malt extract, yeast extract and ammonium sulfate) with different concentrations (0%, 0.25%, 0.5%, and 0.75%).
- Effect of carbon sources (glucose, fructose, maltose, sucrose) with different concentrations (0%, 0.5%, 1%, and 1.5%).

2.2.4 Crude phytase extract

Crude enzyme was extracted by mixing fungal biomass with 90ml of Na-acetate buffer pH 5.5. Then, the mixture was filtered by filter cloth and centrifuged at 13000 rpm to remove fungal biomass. The supernatant solution was used to determine phytase activity.

2.2.5 Protein determination method and phytase activity assay

Protein determination was based on Bradford method (15).

Phytase activity was determined by modified method of (16). Reaction mixture containing 5µl of 5%Ca^{2+} ($CaCl_2$) and 30µl of phytase solution was incubated at 37°C for 20 min. Next, the reaction solution was added 20 µl Na-phytate and incubated at 55°C for 60 min. Then, the reaction was stopped by adding 45 µl of 15% TCA. After adding 0.8 ml of a coloring reagent (70 ml/l of H_2SO_4 98%, 6 g/l of $(NH_4)_6Mo_7O_{24}4H_2O$, 0.11454 g/l of $KSbOC_4H_4O_6$, 930 ml of distilled water) and 0.6 ml of distilled water, the released phosphate was determined at 880 nm. One unit of phytase activity (U) was defined as 1 µmol of inorganic phosphate liberated per minute.

2.4. Data Analysis

Microsoft Excel and Statgarphic 15 software were used to analyze the experimental data.

3. Results and Discussion

3.1 Isolation of phytase producing fungal strains

Eight fungal strains were screened on isolation medium. Only three of them: ET3, ET7 and ET8 gave hydrolytic zones on M2 (Figure 1). It meant that they showed the highest phytase activity.

In fact, *A. fumigatus* is a thermophilic species (19); it can grow at high temperatures (55 °C) and keep stable at temperature up to 70 °C while other fungi in *Aspergillus* genus are hardly to develop at 40 °C. This is a significant characteristic to distinguish between *A. fumigatus* and other species in *Aspergillus* genus such as *A. flavus*, *A. niger*, vl *A. terreus* (20). The above three selected strains were further tested for their temperature stability on M2 at 45 °C. The information on Figure 2 showed that the ET3 isolate was the earliest one to give a halo just on the first day of incubation, while ET7 and ET8 only developed later on the second day of incubation, perhaps, they needed more time to adapt to the high temperature condition. It could be concluded that ET3 was able to be thermo stability, therefore, it was chosen for the next experiment.

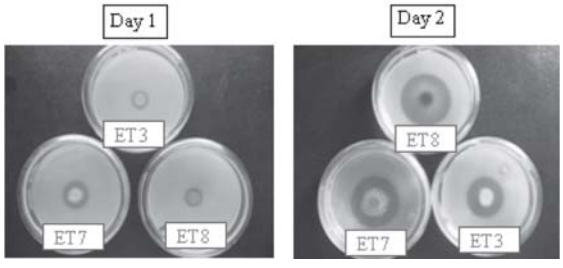

Figure 1. Halo zones of three strains ET3, ET7 and ET8 on M2 medium at 30 °C

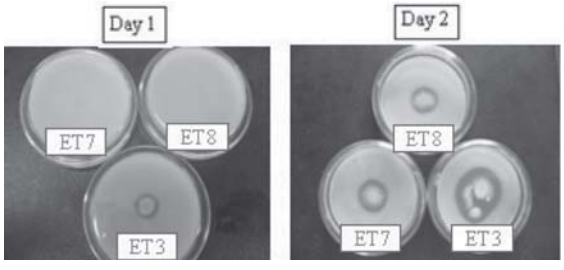

Figure 2. Halo zones of three strains ET3, ET7 and ET8 at 45°C

3.2 Identification of phytase producing fungi

Identification of ET3 was firstly based on micro-morphology of conidia and conidiophore. The fungal mycelium under light microscopes with objective E40 was shown in Figure 3. Its vegetative mycelia had septum and branched off. Conidiophores were short, no branching-off, and no pigment. Vesicle was pigment like conidiosphore, beared philiade or sterigmata with a lot of

conidia. These characteristics were similar to micro-morphological characteristics of *A. fumigatus* reported by (21, 22).

It was used by the primers of (23) (ITS1: 5'-TCCGTAGGTGAACCTGCGG-3';ITS4:5'-TCCTCCG CTTAT TGATATGC-3'). The ITS region of ET3 contained 533 nucleotides. The result was compared with Genbank database on NCBI website by BLAST SEARCH. This sequence had 98% homology with *A. fumigatus* sequence registered on Genbank and with E-Value equal 0. In sum, micro and macro-morphology and biomolecular determination has proved that ET3 belonged to *A. fumigatus* species and designated strain ET3.

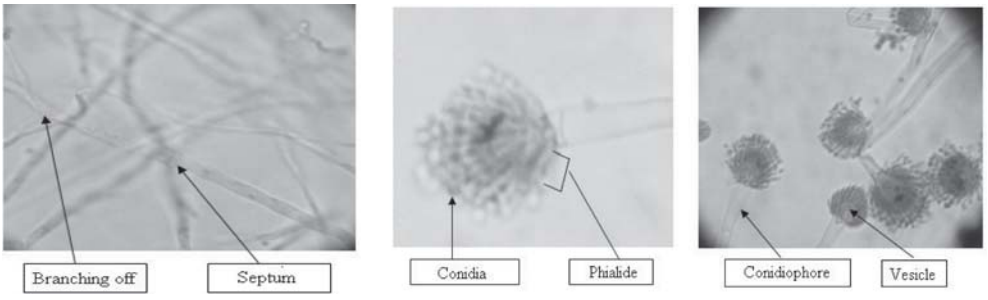

Figure 3. Vegetative and reproductive mycelia of ET3

3.3 Factors effecting on phytase production of *A. fumigatus* ET3

3.3.1. The effect of phytate substrate and incubation time

When culturing *A. fumigatus* ET3 on different phytate substrates and incubation time, levels of phytase production were various (Figure 4). In case of wheat substrate, the phytase activity of *A. fumigatus* ET3 reached highest value (0.584U/g fresh biomass) after fermenting for 2 days.

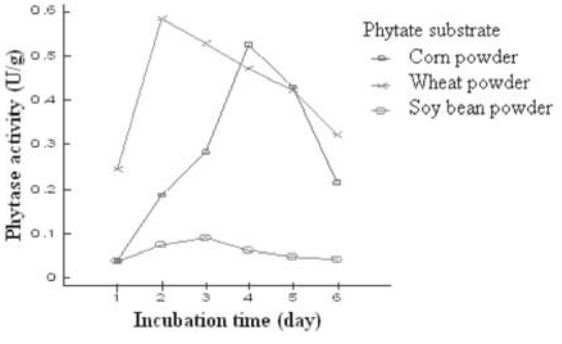

Figure 4. The interaction effect of phytate substrate and incubation time on phytase production

In contrast, growing on corn and soybean substrates, the higest phytase production was at day 3 and 4, respectively. Then, the phytase activity rapidly decreased in day 5 and day 6 on all substrates. In the second day, the biosynthesis of enzyme occurred rapidly since the fungus adapted to culture conditions to produce high level of phytase to develop. After 5 days, since there was a significant decrease of nutrients and moisture in the medium, the phytase production fell dramatically.

Phytate subtrate is an important factor affecting microbial phytase production (24). Results showed that wheat powder was appropriate phytate substrate for the isolate to produce high level of phytase. The phytase production of the isolate was lowest in soybean substrate. In fact, soybean is rich in protein, *A. fumigatus* species can produce protease to use the protein in soybean (25); unfortunately the protease breaks down the phytase produced. Following study researches of (26, 27) on *A. niger* NRRL 3135; suitable phytate substrate was corn powder. It is different with the best substrate for phytase production of the isolate.

3.3.2 The effect of spore inoculum size

Results on Figure 5 presented that phytase activity (0.688U/g) peaked at 10^8 spores/ml. (28), the low spore density (10^6 spores/ml) negatively affected on the efficiency of phytase production due to insufficient number of microbial cells. However, the spore density was too high e"10^{10}, so there were not enough nutrients in the culture medium for the fungus in order to grow. As a result, the phytase activity was low (29).

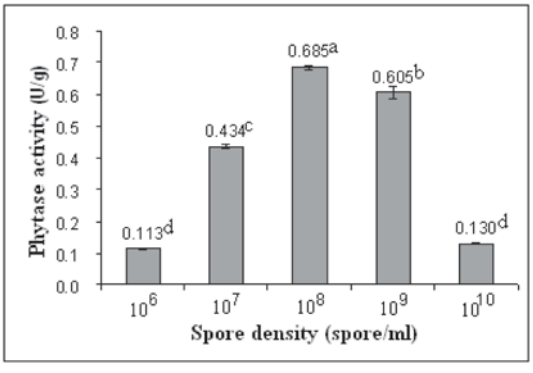

Figure 5. The effect of spore density on phytase production

3.3.3 The interactive effects of initial pH and incubation temperature

The contour plot (Figure 6) is graphical represented based on the regression equation. The coefficient R-squared = 92.2626 % and R-squared (adjusted for d.f.) = 90.9735 % of the significant factors were very high, indicating a high significance of the model (30, 31).

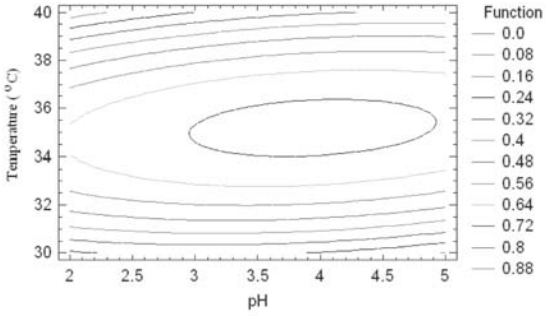

Figure 6. Contour plot of phytase production from *A. fumigatus* ET3 showing interaction between incubation temperature and initial pH.

The statistical values and contour plot presented that pH region from 3-5 was optimum for high phytase production at 35 °C. Within this range pH 4 was the best point because this pH was the natural pH of mineral salt solution. It can be concluded that this fungal isolate could develop well at acidic pH. Interestingly, the optimal pH range for the *A. fumigatus* isolate was lower than that of other bacteria and fungi (pH 5-7) (32). Similarly, (33), also demonstrated that the optimal temperature range for biosynthesis of phytase in microoganisms was about 25-37 °C. It was different with the optimal temperature for phytase synthesis of *A. niger* NCIM 563 (30 °C) on liquid medium reported by (34).

3.3.4 The effect of the age of spore inoculum on phytase production by *A. fumigatus* ET3

Based on the results in Figure 7, the productivity of the enzyme reached maximum with one-day-old spore inoculum (1.347 U/g). The phytase activity decreased significantly (1.5 folds) in the second day and started to fall slightly in the next days. It meant that this isolate was potential to develop during short time. In the first day, the fungus adapted to culture conditions to produce high level phytase for growth. After two days, the phytase production dropped because there was a faster exhausting of the nutrition substances in the media in case of higher amount of biomass imported with the inoculums. In addition, in the longer culture time, the spores entered into the dormant

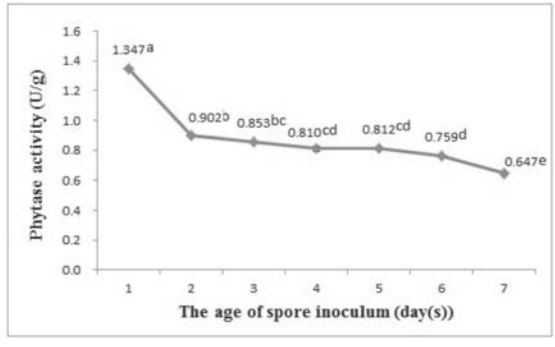

Figure 7. Effect of the age of spore inoculums on phytase production by *A. fumigatus* ET3

phase, so they took a longer time to germinate. This led to low enzyme activity (35).

3.3.5 The effect of supplementary phosphorus concentrations (KH_2PO_4) on phytase production by A. fumigatus ET3

Phytase production of *A. fumigatus* ET3 was significantly affected by supplementary phosphorus (Figure 8). The enzyme activity (1.861 U/g) was highest at the KH_2PO_4 concentration of 1%.

The similar result was studied by (31), who reported that the suitable amount of KH_2PO_4 for optimal phytase biosynthesis in liquid medium was 1 g/l (1% w/w). This observation was also in accordance with the results published by (36, 37). They reported that phytase biosynthesis was inhibited at high phosphorus content since the microorganism would preferably use the free phosphorous source in the medium instead of that from phytate degradation.

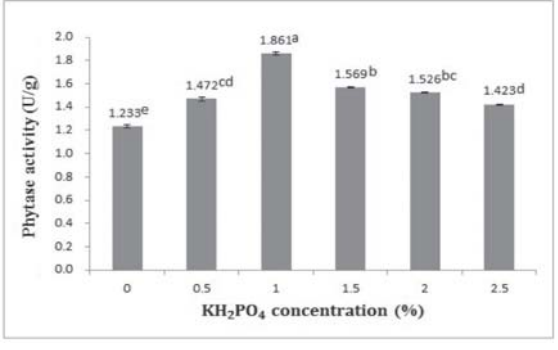

Figure 8. Effect of supplementary KH_2PO_4 concentrations on phytase production by *A. fumigatus* ET3

3.3.6 The effect of supplementary nitrogen sources and their levels on phytase production by *A. fumigatus* ET3

As indicated in Figure 9, the nitrogen sources and their levels also influenced phytase production. Obviously, the maximum activity of phytase was obtained in the medium containing malt extract at 0.5%.

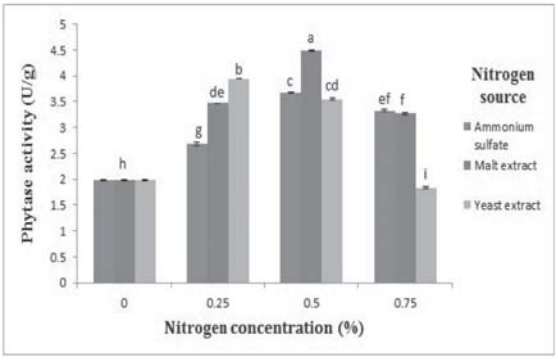

Figure 9. Effect of supplementary nitrogen sources and their levels on phytase production by *A. fumigatus* ET3

Among various nitrogen sources tested, malt extract supported high phytase production by *A. fumigatus* ET3 as compared with other nitrogen sources, followed by yeast extract. These results were corresponding to those of (38, 39) reporting that organic nitrogen sources were generally better for protein production than inorganic nitrogen sources. High nitrogen supplementation (more than 0.5%) interestingly did not yield better phytase production, the phytase activity with these sources even dropped remarkably.

3.3.7 The effect of supplementary carbon sources and their levels on phytase production by *A. fumigatus* ET3

Like the nitrogen sources, carbon sources also significantly affected the phytase production by the fungus (Figure 10).

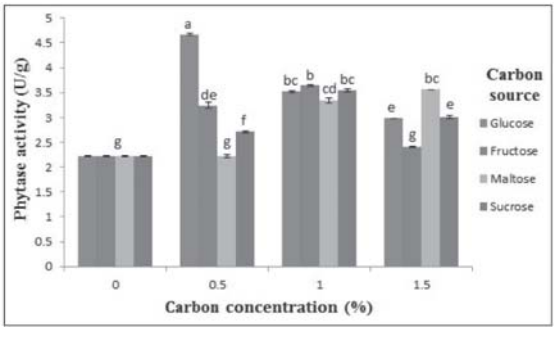

Figure 10. Effect of supplementary carbon sources and their levels on phytase production by *A. fumigatus* ET3

Results indicated that adding glucose at level of 5% resulted in the highest enzyme production of 4.673 U/g. The high glucose concentrations inhibited the enzyme production, therefore, lower amounts of phytase were produced. The similar finding was reported by (40), who presented that the production of phytase enzyme by *A. ficuum* was reduced by high glucose concentrations. This was the same with the research on *L. amylovorus* (41) and *P. anomala* (42) which glucose was a suitable carbon source for phytase production.

4. Conclusions

A. fumigatus ET3 with high phytase activity was isolated. The optimal culture conditions being able to enhance phytase synthesis of *A. fumigatus* ET3 were determined. This fungus exhibited maximum enzyme yield on the medium containing wheat flour as a phytate substrate, 0.5% of malt extract, 1% of KH_2PO_4, 0.5% of glucose, and one-day-old spore inoculum of 10^8 spores/ml when fermenting at 35 ^oC and pH 4 for 2 days.

5. Acknowledgement

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