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# ***Aspergillus niger* Str 3 and *Neurospora sitophila* for phytase production on coconut oil cake supplemented with rice brand in solid-state fermentation**

**A Kanti<sup>1</sup>, I Idris<sup>1</sup> and I M Sudiana<sup>1</sup>**

<sup>1</sup>Microbiology Division, Research Center for Biology-LIPI, Jl. Raya Jakarta-Bogor Km 46, Cibinong Science Center, Bogor, 16911, Indonesia  
Email: atityeast@gmail.com

**Abstract.** The use of phytase as a feed supplement is getting popular. However, the production system of this enzyme should be optimized to reduce production cost. The objective of this study was to evaluate the ability of 10 fungi isolates i.e., *Aspergillus niger* (5 isolates), and *Neurospora crassa* InaCC F226 to produce phytase, and select best phytase producer for phytase production on coconut oil cake supplemented with rice brand in solid-state fermentation. *A. niger* Str3 and *N. sitophila* produced phytase of 4.6 and 3.4 unit respectively were selected for phytase production owing to its ability to produce phytase in submerging fermentation with glucose as the primary carbon sources. These potential isolates were then used for phytase production on coconut oil cake supplemented with rice brand in solid-state fermentation. The effect of inoculants type, initial moisture content, and additional carbon sources were evaluated to obtain the optimum condition for phytase production. Media contained coconut oil cake supplemented with rice brand at a ratio of 20 to 50% could be used for phytase production. Initial moisture content and incubation time affect phytase production. Optimum initial moisture content was about 60-70%. This work concludes *A. niger* and *N. sithophyla* were good inoculant for phytase production using formulated media contained coconut oil cake and rice brand in solid-state fermentation.

## **1. Introduction**

Intensive studies have been focused on how to increase nutrient absorption by a monogastric animal such as poultry and swine [1]. Majority of poultries feed are plant origin and contain an appreciable amount of phytate phosphorus [2]. Due to the inability of monogastric animals to produce phytase, then this organically bound form of phosphorus is poorly absorbed in their digestive systems [3]. Phytasehydrolyzesphytate phosphorus into *Myo*-inositol and releases phosphorus in the form of available phosphate to the animal [4]. Phytase supplement into animal feed has double merits: increasing available phosphate, Ca, Zn, and Fe for animal [5], less undigested phytate entering water body hence reducing the risk for eutrophication.

The use of filamentous fungi for the production of phytase through solid-state fermentation (SSF) is gaining popularity [6]. SSF is more preferential than submerged fermentation (SmF) for the industrial scale of phytase production due to less wastewater generated and lower energy input, simpler fermentation media, easier to control bacterial contamination, and higher organic loading [7]. Phytase also produced by some bacteria and yeast, but the use of filamentous fungi as an inoculant is preferable in SSF systems. This system could be due to higher production yields and more acid tolerance for feed production [8]. Several SSF parameters were optimized, including particle size, pH, incubation temperature, initial moisture content, aeration, and inoculums size to obtain the highest phytase production [9]. Fungal phytase, including strain selections (*Mucor*, *Aspergillus*, and *Rhizopus* sp.) has been intensively explored [10]. However, the use of agricultural waste to reduce enzyme production cost has not been intensively explored. Coconut oil cake is selected due to commonly



available in the traditional market, and commonly rice brand was selected as media since the substrate is low-cost substrate containing phytate, and has not been fully utilized yet.

Here, we present the possibility of using mixed media contained rice brand and coconut oil cake to produce phytase. We also explored the effect of adding additional carbon sources to increase phytase production. The objective of the present study was to determine the effect of media formulation on the production of phytase by the selected phytase producing fungi as well as to optimize the various process parameters that influence the enzyme synthesis.

## 2. Materials and Methods

### 2.1. Culture maintenance

All strains tested were first grown in PDA medium for 120 h, at 30°C. These strains were then evaluated for their ability to produce phytase under submerged culture.

### 2.2. Screening for phytase production

Ten isolates representing *Aspergillus niger* (5 isolates), *Rhizopus oligosporus* (2 isolates), *Mucor rouxii* (1 isolate), *Neurospora crassa* (1 isolate), and *Neurospora sitophila* (1 isolate) were grown at described in session 2.1 to obtain phytase producing fungi. Enrichment culture media containing 0,5% calcium phytate as the sole phosphorus and glucose were used for the primary screening of phytase producers. The method was based on the estimation of phosphate solubilization from calcium phytate in aqueous media. The strains were grown under shaking condition at 150 rpm, at 30°C for 96 hours. After 96 hours of incubation time, the fungal biomass was discarded by centrifugation at 8000 rpm for 20 minutes, and the supernatant was then estimated for phytase production using a method described by Idriss *et al.*[11]

### 2.3. Inoculum preparation for SSF

The fungal cultures were grown and maintained on potato dextrose agar (PDA) slants. The slants were stored at 4°C and sub-cultured fortnightly. Five-day-old fully sporulated slant was used for inoculant preparation. In brief, the inoculant preparation was started by added 10 ml sterile distilled water containing 0.1% Tween-80 into the slant, and spores were scraped with a sterile needle. The inoculant obtained contained  $4.7 \times 10^7$  spores per ml.

### 2.4. Substrates preparation for SSF

Rice bran (RB), coconut oil cake (COC), were used as substrates for the phytase production. RB and COC were obtained from a local market. Ten grams of the dried substrate taken in cotton plugged 250 ml Erlenmeyer flask was supplemented with 6.0 ml of a salt solution containing (%)  $\text{NH}_4\text{NO}_3$  0.5,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.1 and NaCl. Media for phytase production were contained percentage a mixture of COC/RB as the following: 100/0; 90/10; 80/20; 70/30; 60/40; 50/50; and 60/40.

### 2.5. Moisture optimization

The moisture was adjusted to the required level by adding distilled water to estimate the effect of initial moisture on phytase production. Substrates were sterilized at 121°C and 15 psi for 15 min, cooled and inoculated with 1.0 ml spore suspension ( $4.7 \times 10^7$  spores per ml) of fungal strain. The flasks were incubated at 30°C for 96 h unless otherwise mentioned. All experiments were carried out in two replicates.

### 2.6. The effect of additional carbon sources

Five of additional carbon sources were selected to study the effect of additional carbon on phytase production. Additional carbon sources evaluated were starch, glucose, sucrose, lactose, and mannitol at the concentration of 0.5%.

### 2.7. Effect of starch concentration on phytase production

During the experiment, we found that starch was effective additional carbon sources to increase phytase production. Then varying starch concentration (0.5 to 2,5%) were augmented to the best formula (RC and COC). Phytase productions were determined after 96 h incubation.

### 2.8. Enzyme extraction

Enzyme extraction was carried out using distilled water with 0.1% Tween-80. Known quantities of fermented substrates were mixed thoroughly with the required volume of distilled water (so that the final extraction volume was 100 ml) by keeping the flasks on a rotary shaker at 180 rpm for one hour. The suspension was centrifuged at 8000xg for 20 min and the clear supernatant obtained was assayed for phytase activity.

### 2.9. Phytase assay

Phytase activity was assayed by measuring the amount of inorganic phosphorus released from sodium phytate solution using the method of Harland and Harland [12]. One unit of enzyme activity was defined as the amount of phytase required to release one  $\mu\text{mol}$  of inorganic phosphorus per minute under the assay conditions.

### 2.10. Protein estimation

The soluble protein content of the crude samples was determined spectrophotometrically according to the method described by Lowry *et al.* [13] using bovine serum albumin as standard.

### 2.11. Biomass estimation

Fungal biomass estimation was carried out by determining the N-acetyl glucosamine released by the acid hydrolysis of chitin present in the cell wall of the fungi [14]. For this, 0.5 g (dry wt) of the fermented matter was mixed with concentrated sulfuric acid (2 ml), and the reaction mixture was kept for 24 h at room temperature (30°C). This mixture was diluted with distilled water to make a 1 N solution, autoclaved for one h, neutralized with 1 N NaOH and the final volume was made up to 100 ml with distilled water. The solution (1 ml) was mixed with 1 ml acetyl acetone reagent and incubated in a boiling water bath for 20 min. After cooling, ethanol (6 ml) was added, followed by the addition of 1 ml Ehrlich reagent, and the resulting mixture was incubated at 65°C for 10 min. Once cooled the optical density of the reaction mixture was read at 530 nm against a reagent blank. Glucosamine (Sigma) was used as the standard. The results obtained are expressed as mg glucosamine per gram dry substrate (gds).

## 3. Results and Discussion

### 3.1. Production of phytase by fungi

The initial screening for phytase activity revealed some differences between the strains found (table 1). Three groups can be distinguished after 96 h incubation: no activities, moderate and extensive Ca-Phytate degradation. Highest phytase production was observed in *Aspergillus niger* Str3. All strains *A.niger* tested produced phytase. The second group with moderate activities was *Neurospora crassa*

and *Neurospora sitophila*. The third group with low phytase production was *Rhizopus oligosporus* and *Mucor rouxii*.

*A. niger* has been intensively studied for their ability to produce phytase [15], and *A. ficuum* was proposed by Shieh and Ware [16] to produce phytase. We found other isolates such as *N. crassa* and *N. sitophila* also produced phytase. We observed that phytase was induced Ca-phytate. When Ca-phytase was unavailable, Phytase was not produced (data is not shown). This finding verifies that phytase is an inducible enzyme. *N. crassa* and *N. sitophila* were selected due to these fungi commonly encountered in traditional fermented food in Indonesia. Other important isolates are *Rhizopus oligosporus* as inoculants for tempeh production [17], and *Mucor rouxii* known as lipid accumulating fungi [18]. The best idea could be phytase production system is utilizing generally well known as safe microorganism.

**Table 1.** Phytase activity of fungi grown on glucose with 0.5% Ca-phytate contained (g per liter) glucose 30,0; MgSO<sub>4</sub>, 0.5 g; KCl, 0.4 g; FeSO<sub>4</sub>, 0.08 g; and NaNO<sub>3</sub> 8.5 g, grown under shaking condition at 30°C.

No.	Species name	Acid phytase production (Unit)
1	<i>Aspergillus niger</i> Str1	2.6 ± 0.1
2	<i>Aspergillus niger</i> Str2	3.2± 009
3	<i>Aspergillus niger</i> Str 3	4.6± 009
4	<i>Aspergillus niger</i> Str4	1.2± 009
5	<i>Aspergillus niger</i> Str 5	3.9± 009
6	<i>Mucor rouxii</i>	1.9± 009
7	<i>Neurospora crassa</i>	3.2± 009
8	<i>Neurospora sitophila</i>	3.4± 009
9	<i>Rhizopus oligosporus</i>	1.4± 009
10	<i>Rhizopus oligosporus</i>	1.2± 009

### 3.2. Activity of Phytase in formulated media

Media formulation was chosen as a strategy to reduce phytase production cost. Gradient composition of coconut oil cake and rice brand were explored to achieve cost-effective phytase production. Coconut oil cake was selected due to the low price of this media in the traditional market, and rice brand is commonly used for phytase production. As shown by table 2, media composition and inoculants type affect phytase production. *A. niger* (Str2 and Str 3), and *Neurospora crassa* and *N. sitophila* were good inoculants, and COC:RB at a ratio from 30 to 60 could be used to produce phytase. Optimization of a medium component to achieve higher phytase production was introduced by Sunithaet *al.* [19]. Coconut oil cake inoculated with *R. oligosporus* in solid-state fermentation was conducted by Sabuet *al.* [20]. They observed maximal enzyme production was 14.29 U/g of dry substrate, occurred at pH 5.3, 30 degrees C, and 54.5% moisture content after 96 h of incubation.

We observed that using formulated media contained rice brand and coconut oil cake was able to increase phytase production by more than 40% (Table 2). Not only coconut oil cake, but sesame oil cake was also used by Singh and Satyanarayana [21] for phytase production using *Sporotrichum thermophile* in solid-state fermentation. They found *Sporotrichum thermophile* TLR50 increased phytase production to 180 U/g of dry moldy residue in sesame oil cake at 120 h and 45° C. The initial substrate-to-moisture ratio of 1:2.5 and aw of 0.95. Addition of carbon and nutrient sources i.e., glucose and ammonium sulfate enhanced phytase titer (282 U/g of dry moldy residue). An overall

76% enhancement in phytase production was achieved owing to optimization. Not only phytase, but this fungi also excreted amylase, xylanase, and lipase. While Roopesh *et al.* [22] compared the phytase production of wheat bran and oil cake using *Mucor racemosus* as an inoculant. To the best of our knowledge, only our study introduced formulated media containing coconut oil cake and rice brand for phytase production. Those optimization of media and microorganism in solid-state fermentation are a common strategy to reduce phytase production cost. There is significant different phytase production character in solid-state and submerged fermentation. *A. niger* NCIM 563 produce more phytase type in the solid-state than under submerge fermentation [23]. Various oil cakes (sesame, groundnut) and mixed substrate contained those oil cake were studied by Ramachandran *et al.* [24].

**Table 2.** Phytase activity of fungi in formulated media (U gds<sup>-1</sup>)

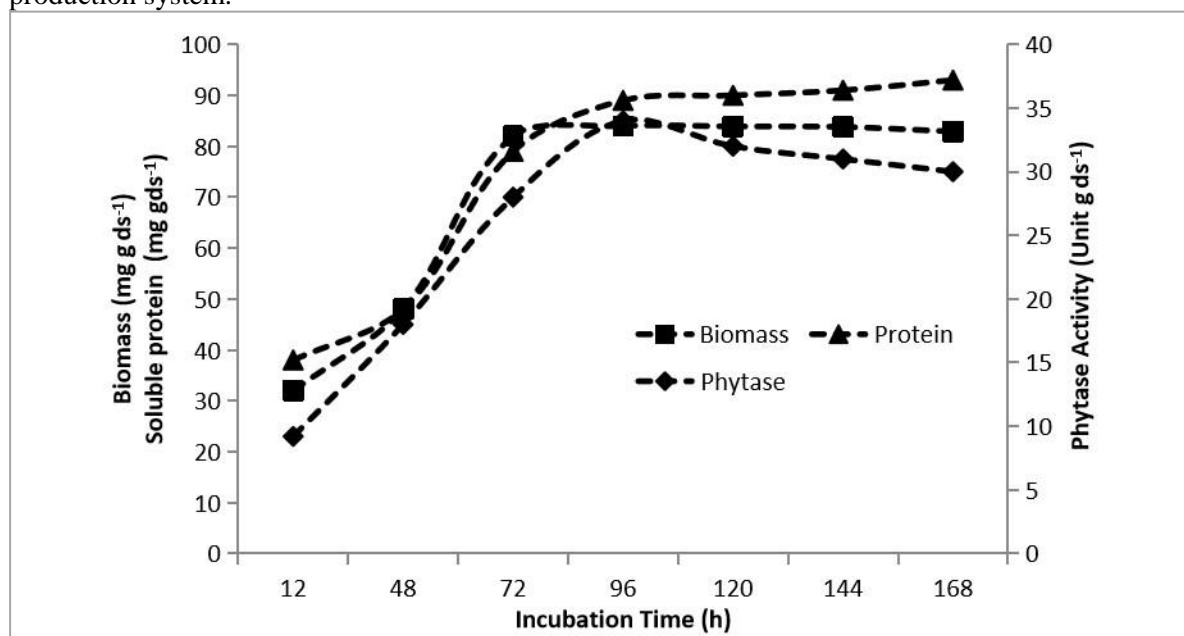
Species name	Media composition (COC: RB)						
	0:100	10:90	20:80	30:70	40:60	50:50	60:40
<i>Aspergillus niger</i> Str2	31.3 ±0.4	31.5±0.3	27.5±0.2	34.2±0.2	31.1±0.3	27.4±0.6	27.1±0.7
<i>Aspergillus niger</i> Str 3	27.3±0.6	28.6±0.3	29.8±1.5	34.2±1.4	33.2±1.3	34.5±0.1	33.9±0.2
<i>Neurospora crassa</i>	26.3±0.3	28.5±0.6	34.5±3.4	34.2±1.4	31.1±0.3	26.8±0.6	27.1±0.5
<i>Neurospora sitophila</i>	19.6±0.5	26.5±0.5	35.5±2.2	34.2±2.3	31.1±0.5	25.4±0.5	30.8±0.5
<i>Rhizopus oligosporus</i>	8.1±0.5	12.5±0.5	15.5±0.6	16.2±0.4	22.1±0.5	27.4±0.4	27.1±0.3

Comparisons were made for phytase production using wheat bran and oilcakes as substrates in solid-state fermentation by *Mucor racemosus* NRRL 1994 [22]. Sesame oil cake served as the best carbon source for phytase synthesis by the fungal strain as it gave the highest enzyme titers (30.6 U gds<sup>-1</sup>). Ground nut oil cake also produced a reasonably good quantity of enzyme (24.3 U gds<sup>-1</sup>). They found, the wheat brand which commonly known as the best media for phytase production, produce much less phytase than sesame oil cake. Increased phytase production was obtained when the wheat brand was supplemented with sesame oil cake (1:1). The highest phytase production (44.5 U gds<sup>-1</sup>) was attained through optimizing various process parameters such as incubation time, initial moisture content, and inoculum concentration, implying that substrate formulation and environmental manipulation are crucial for phytase production.

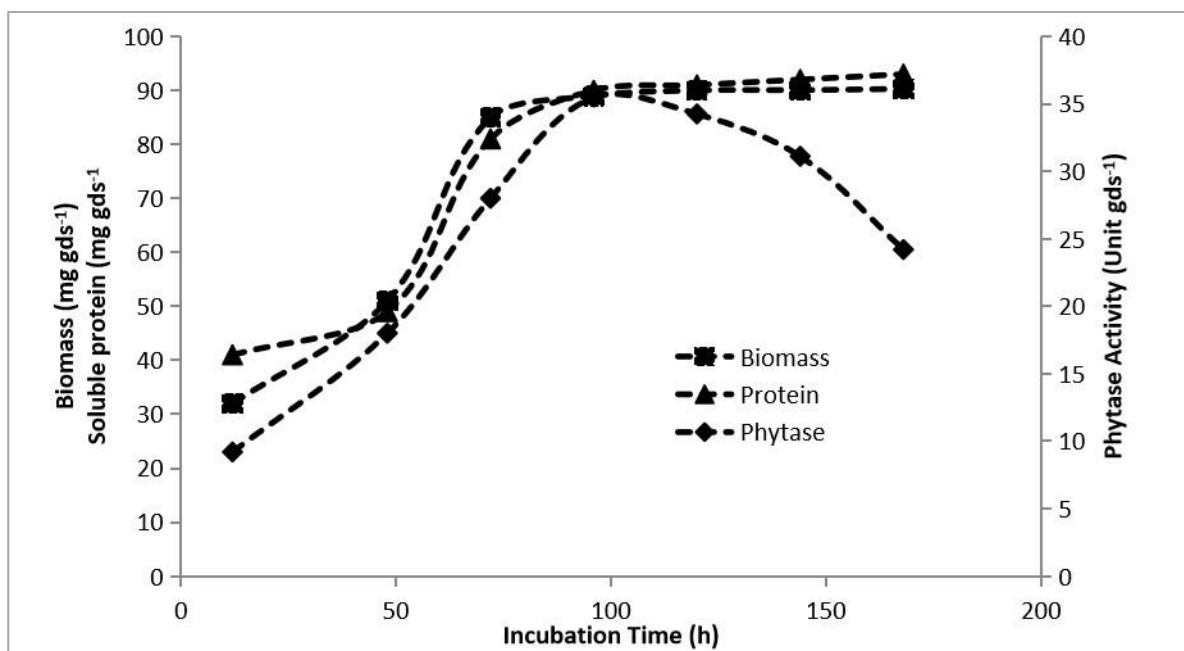
### 3.3. Biomass growth, protein production, and enzyme activities

Enzymes production is concomitant with biomass growth and protein synthesis (figure1 and 2) for *A.niger* and *N. sitophila*, respectively. It might suggest that biomass formation requires more phosphate from their surrounding and thus stimulating phytase production (figure 3 and 4). Enzyme production was maximum at 96 h. This result is reaffirmation the previous study of Roopesh *et al.* [22], which noted that optimum production of phytase in oil cake based media was attained after 96 hours. Protein synthesis is slightly increased, which may indicate that these fungi produced other enzymes as reported by Singh&Satyanarayana [25] who evaluate enzyme hydrolyzes by *S. thermophile*. Beside phytase, this thermophilic fungi also produced amylase, xylanase, and lipase when grown in sesame cake oil. Production of other hydrolytic enzymes could be beneficial to increase nutrient availability when mixed with animal feed. Both *A.niger* Str 3 and *N. sitophila* showed a similar pattern, but phytase production *N. sitophila* significantly decreased after 100 h fermentation period (figure2).

Therefore it is essential to monitored enzyme production profile to obtain the most efficient phytase production system.



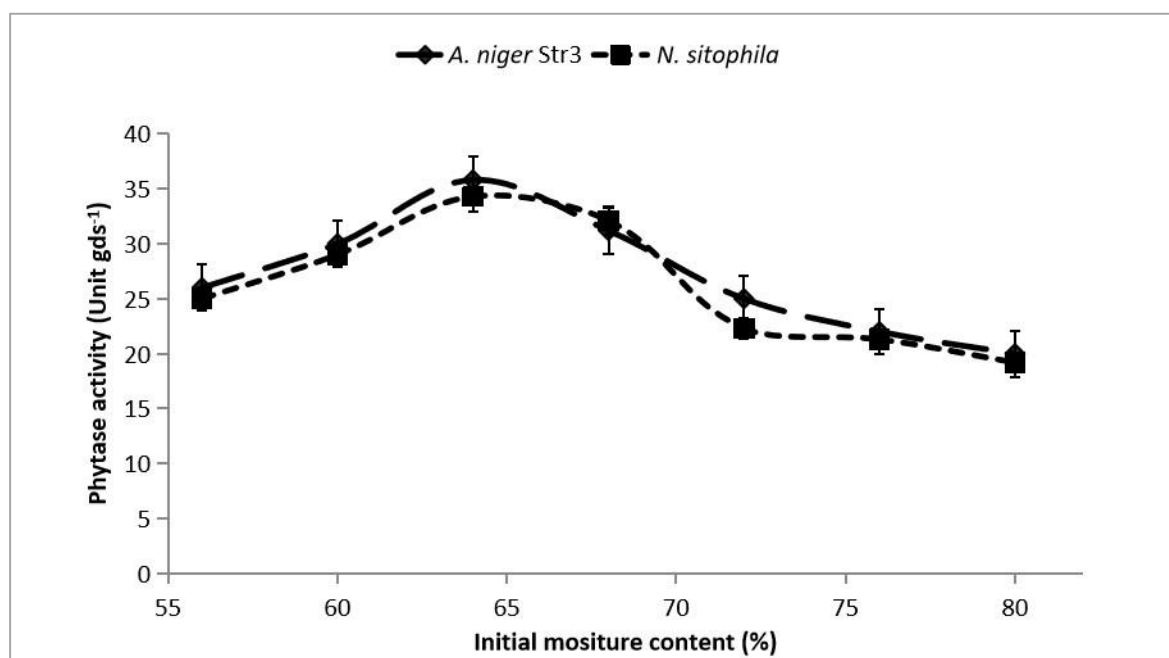
**Figure 1.** Phytase activity, growth, and soluble protein content at different time intervals of *A. niger* Str3 in formulated media COC:RB of 30:70 respectively.



**Figure 2.** Phytase activity, growth, and soluble protein content at different time intervals of *N. sitophila* in formulated media COC:RB of 30:70 respectively.

### 3.4. Effect of initial moisture

The profile of phytase production was affected by the initial moisture content of the media. Both *A. niger* Str 3 and *N. sitophila* showed a similar pattern. When the initial moisture content higher than 70%, the phytase production slowing down (figure3). Substrate moisture is a critical parameter for solid-state fermentation. We found that substrate moisture also affects the growth of mycelia in the substrate. When initial moisture was too high (>85%), mycelia grow very slow, and media easily contaminated by other microorganisms (bacteria). The effect of moisture content would be thorough, limiting oxygen diffusion for mycelia growth [26] and due to much water media became sticky. We have tried some sterilization technique to get the most suitable initial moisture content for stimulating mycelia growth and hence stimulating enzyme production. The best initial moisture of phytase production is 64% for both fungal strains with phytase activity 35.8 U gds<sup>-1</sup> (*A. niger* Str 3) and 34.3 U gds<sup>-1</sup> (*N. sitophila*).



**Figure 3.** Effect of initial moisture on phytase activity on a substrate containing COC:RB of 30:70 respectively, inoculated with *A. niger* Str 3 and *N. sitophila*.

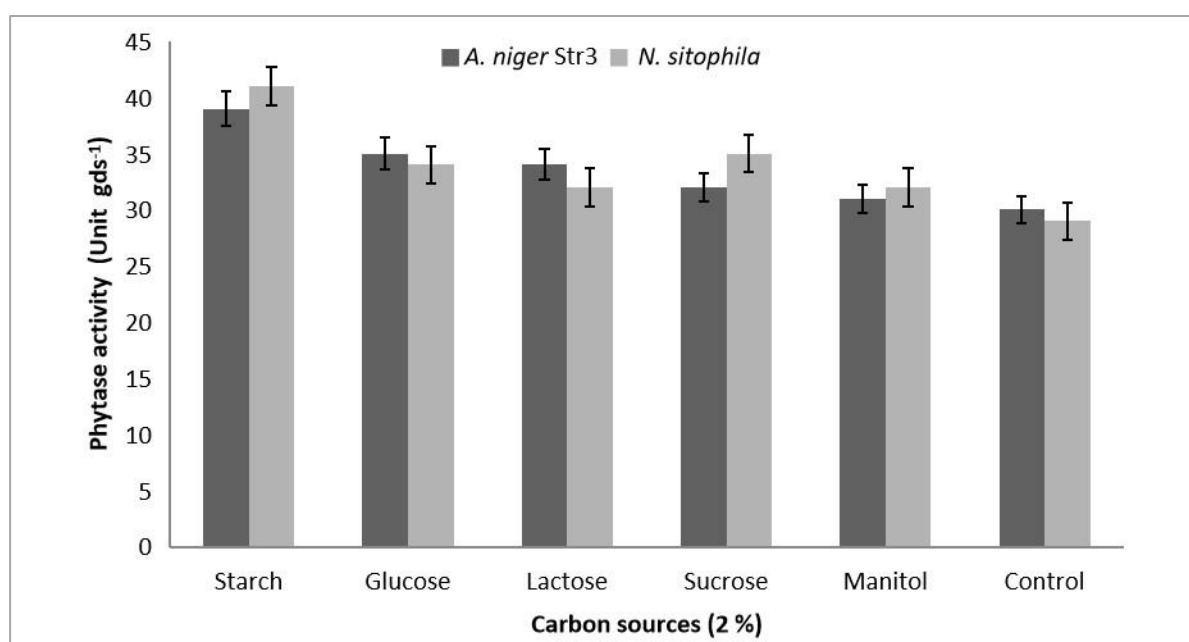
### 3.5. Effect of additional carbon sources

All of the carbon source types used in this study gave a positive effect to phytase production by *A. niger* Str 3 and *N. sitophila*. Figure 4 showed the effect of carbon source on phytase activity of *A. niger* Str 3 and *N. sitophila*. Starch is the most effective carbon source for phytase production compare to glucose, lactose, sucrose, and mannitol. The order of the effectiveness for *A. niger* Str 3 was starch > glucose > lactose > sucrose > mannitol and for *N. sitophila* was starch > sucrose > glucose > lactose > mannitol. Carbon source related to the availability of energy source that will be utilized by fungal to grow. Starch has been reported before as the best carbon source for phytase production [27], [22]. Several simple sugars used in this study did not give a significant increase to phytase production. It was suggested that phytase production by fungal strains involved accessory enzymes. The accessory enzymes have better utilization of starch, which enhanced the phytase production [22].

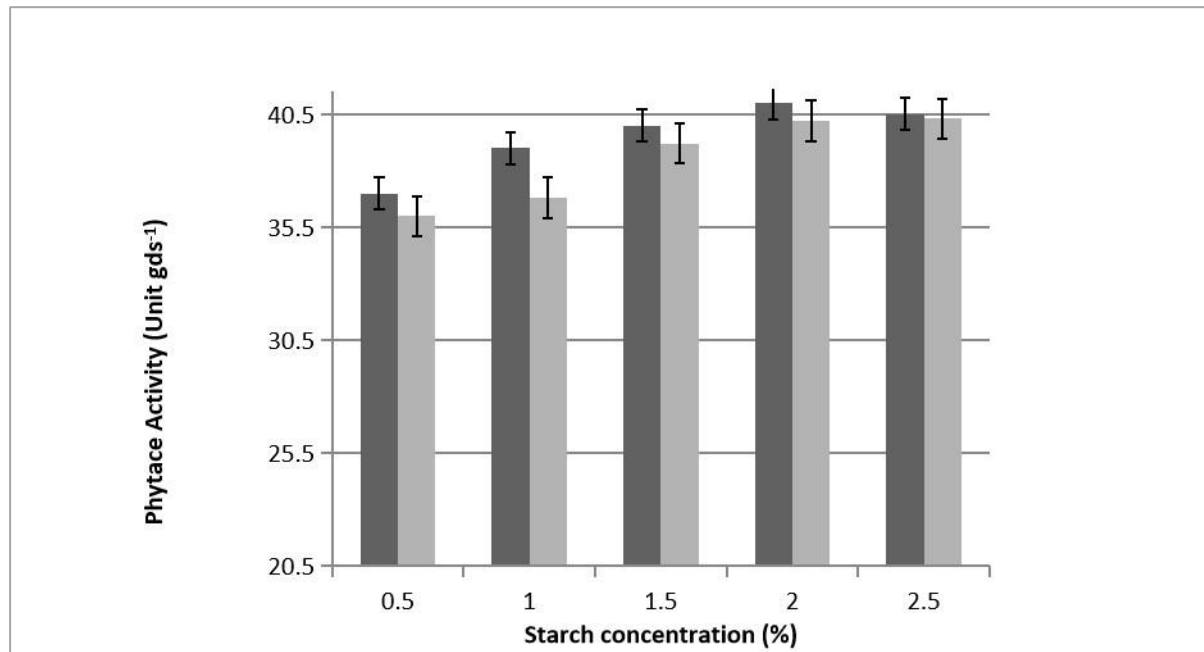


### 3.6. Effect of starch concentration

Variation of starch concentration was conducted to observe the effective concentration of starch, which enhanced the phytase production. The phytase production was performed within five levels of starch concentration. Our study was found that 2% of starch addition was the optimum concentration to enhanced phytase production, and both fungal strains gave the same result (figure 5). This finding supported the previous finding, phytase production by *Mucor racemosus* is effectively enhanced in 2% starch addition [22]. Phytase activity of *A. niger* Str3 increased from 37.0 Unit gds<sup>-1</sup> to 41.0 Unit gds<sup>-1</sup> and further increased of starch concentration decreased the phytase production. Phytase activity of *N. sitophila* increased from 36.0 Unit gds<sup>-1</sup> to 40.2 Unit gds<sup>-1</sup>, and increasing in starch concentration did not change the phytase production.



**Figure 4.** The effect of additional carbon sources on phytase production, fermentation period (96 h, initial humidity (60%) with COC: RB of 30:70 respectively inoculated with *A. niger* Str3 and *N. sitophila*.



**Figure 5.** Effect of starch concentration on phytase production, at 96 h fermentation time, 60% initial moisture content on media composition COC:RB of 30:70 respectively, inoculated with *A.niger* Str 3 and *N.sitophila*.

#### 4. Conclusion

*A. niger* Str3 and *N. sitophila* were found as the best strain which produced the highest phytase among tested strains. The optimum condition for phytase production by both fungal strains was within 96 h of incubation, 60% of initial moisture, media composition COC:RB of 30:70, with 2% of starch addition. Starch addition enhanced the phytase production by 2%, and further increase tends to inhibit phytase production.

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