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Comparison of *Neurospora crassa* and *Neurospora sitophila* for phytase production at various fermentation temperatures

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Abstract. *Kanti A, Sudiana IM. 2016. Comparison of* Neurospora crassa *and* Neurospora sitophila *for phytase production at various fermentation temperatures. Biodiversitas 17: 769-775.* There is general consensus that the presence of phytate in poultry negatively influences protein in and energy utilization by poultry, and these influences would be mitigated by augmentation of hydrolytic enzymes. The objective of this study was to evaluate phytase production by *Neurospora crassa* and *Neurospora sitophila* on solid state fermentation. The isolates were isolated from *oncom* Bogor. Phytase production ability was first determined on submerge fermentation (SmF) using glucose as the main C-sources, and on solid state fermentation (SSF) on media containing maize 30%, soybean 30%, and rice brand 40% w/w. Maximum enzyme activities were observed at 96 h incubation. SSF produce higher phytase than SmF. Optimum temperature for phytase production was 35°C. Highest phytase production by *Neurospora crassa* was 45.25 Unit per g substrate, while 40.78 Unit per g substrate was produced by *Neurospora sitophila.* Peptone and yeast extract were good N-sources for both isolates. Starch supplement increased phytase activity. Increased amylase activity was also observed when starch supplement was added on SSF. This study proves that *Neurospora crassa* and *Neurospora sitophila* can be used to produce phytase for better poultry nutrition.

Keywords: *Neurospora crassa*, *Neurospora sitophila*, phytase, solid state fermentation

INTRODUCTION

Morphological observations, physiological studies, ecology and genetics of the occurrence of *Neurospora* on natural and artificial substrates were reported (Steele and Trinci 1975). The organism is ubiquitous in moist tropical or subtropical climates (Tian et al. 2009). Because dormant ascospores are activated by heat, blooms occur on burnt vegetation (Galagan et al. 2003). The most important finding is that *Neurospora* is generally recognized as safe. Never, in more than a century of observation and experimentation has the genus been implicated in human disease or observed to cause disease in animals or plants (Perkins and Davis 2000; Znameroski et al. 2012). *Neurospora* is a common fungi, it has been used in many experiments, and vast genetic information has been obtained through DNA sequencing (Galagan et al. 2003). Several isolates have been found in traditional fermented food in Indonesia (Perkins and Davis 2000; Liu 2003). More than 1000 loci have been sequenced and mapped on the chromosome (Powell et al. 2007). *Neurospora* have been studied since 1843, and the species *N. crassa* has been a focus of intensive research on traditional fermented food fermentation, and enzymes production (Springer and Yanofsky 1989). There are five important species of conidiating *Neurospora* which include *N. crassa, N. sitophila, N. intermedia, N. tetrasperma,* and *N. discreta.* They can be determined by their distinctive orange color, rapid growth, and profuse production of powdery conidia (Jacobson 1992). *Neurospora* are also important fungi for bioprocess based industry include enzyme for feed (Zhou et al. 2006).

Reduction of feed cost for poultry is the main interest of many scientists. Inclusion of phytase in poultry diet has increased remarkably during the past decade. This is due to a high concentration of phytate in cereal (barley, maize, sorghum and wheat) ranging from 1.86-2.89 ($g.kg^{-1}$). Higher phytate are found in oilseed meals (4.0-9.11 $g.kg^{-1}$), and the highest 8.79-24.20 Phytate-P ($g.kg^{-1}$) are found in rice brand and wheat brand (Maga 1982; Heaney et al. 1991; Haraldsson et al. 2005). Phytase hydrolyzed phytate which eliminate the intense of phytate bound on mineral, carbohydrate and protein (Liebert and Portz 2005). Thus augmentation of phytase will reduce feed costs and increase the efficiency of utilization of phosphate and other nutrients in cereal based feed ingredients (Leytem et al. 2008). It is expected that inclusion of phytase will result in economic and environmental benefits. Not only phytase, but other hydrolytic enzymes (amylase and cellulase) are important components of feed ingredients (Kim et al. 2007). Up to now, inclusion of hydrolytic enzymes in feed ingredients is mostly focused on phytase production, and *Aspergillus niger* is the most popular phytase producer. Several other fungi such as *N. crassa*, *N. sitophila, Rhizopus oryzae,* and *Rhizopus oligosporus* could be important microbes for production of hydrolytic enzymes (Zhou et al. 2006). Those fungi are well known to play a major role in traditional fermented food in Indonesia such as *oncom* and *tempeh*. Solid state fermentation offers higher enzyme production, and less expensive and easier process control. Solid state fermentation has been effectively used to produce phytase. Temperature and

carbon nitrogen sources could be important factors influencing phytase production. The objective of this study was to assess the effect of temperature, and the augmentation of nitrogen and starch on phytase production by *Neurospora crassa* and *N. sitophila* isolated from *oncom*.

MATERIALS AND METHODS

Isolation and identification of *Neurospora*

Isolation of fungi from *oncom* was performed following methods described by Choi et al. 1999. The *oncom* was obtained from the local market in Bogor, West Java, Indonesia. To isolate the fungi from the sample, 1.0 g of the sample was diluted in 9 mL sterilized water and vortex-mixed. One-tenth of a milliliter of successive decimal dilutions was spread on acidified Dichloran Rose Bengal agar chloramphenicol agar (OXOID, Cat.1076012). This selective medium was used because bacteria growth is prevented, and spreading of molds is suppressed.

Plates were incubated for 5 days at room temperature. Strain purification was done at least twice by selecting one of each type of fungi colony and purified twice for single colonies. The plates were incubated at 27°C for 3 days. Representative colonies were picked, purified and maintained on Potato Dextrose Agar (PDA)(OXOID, Cat.CM 0139). Morphological observation of fungi was conducted following Jacobson (1992).

rDNA sequence determination

Fungi DNA template was prepared from freshly-grown cells on the Potato Dextrose Broth and used for extracting the DNA (Butinar et al. 2005). PCR amplification of the partial Internal Transcribed Spacer (ITS) ribosomal subunit with primers ITS 4: 5`– TCC TCC GCT TAT TGA TAT GC – 3`and Primer ITS 5: 5`– GGA AGT AAA AGT CGT AAC AAG G –3` (White et al. 1990) using GoTaq master mix (Promega, M7122). PCR products were visualized on 2% agarose and sequenced with both primers using Big Dye terminator v3.1. Cycle Sequencing Ready Reaction Kit (Applied Biosystems) following the manufacturer's instructions. The partial 26S sequences determined in this study were compared to those in the EMBL/GenBank/ DDBJ databases using the nucleotide Basic Local Alignment Search Tool (BLASTn) (Altschul et al. 1997).

Culture maintenance

The isolated strains of *Neurospora* were first grown in PDA (Potato Dextrose Agar) medium for 120 h, at 30°C. These strains were then evaluated for their ability to produce phytase under submerge culture and solid state fermentation at various temperatures and media composition.

Screening for phytase production

The isolated strains of *Neurospora*-were grown in PDA medium. Enrichment culture media containing 0.5% sodium phytate as the sole phosphorus and glucose were used for the primary screening of phytase producers. The method was based on estimation of phosphate solubilization from sodium phytate in aqueous media. The strains were grown under shaking conditions at 150 rpm, at 30°C for 96 hours. The culture was grown as submerge fermentation (SmF). After 96 hours of incubation time the fungal biomassa was discarded by centrifugation at 8000 rpm for 20 minutes and the supernatant was then used for estimation of phytase production using the method described by Liu et al. (1999).

Inoculum preparation for SSF

The culture was grown and maintained on potato dextrose agar (PDA) slants. The slants were stored at 4°C and sub-cultured for 4 days. Five-day-old fully sporulated slant was used for inoculant preparation. For this, 10 mL sterile distilled water containing 0.1% Tween-80 was added to the slant and spores were scraped with a sterile needle. The inoculant obtained contained 4.7 x 10^7 spores per mL.

Substrates preparation for SSF

The media composition for phytase production were maize 30%, soybean 30%, rice bran 40%, which were obtained from a local market. Ten grams of the dried mixed substrate taken in a cotton plugged 250 mL Erlenmeyer flask were supplemented with 6.0 mL of salt solution containing (%) 0.5, $MgSO_4$.7H2O 0.1 and NaCl.

The effect N-sources

To study the effect of nitrogen sources, the media of SSF was added with either sodium nitrate 0.1%, urea 0.5%, yeast extract 0.5%, and peptone 0.5%. The incubation temperature was maintained at 30°C, 35°C and 40°C. The activity of phytase was determined at 3, 4 and 5-days. The activity was expressed in units defined as µmol phosphate release by 1.0 mL enzymes per minute at 37°C.

The effect of additional carbon source

Starch as additional carbon source was selected to study the effect of additional carbon on phytase production. Additional carbon sources were evaluated at concentration of 0-4%.

Enzyme extraction

Enzyme extraction was carried out using distilled water with 0.1% of 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 8000 · g for 20 min at 4ºC and the clear supernatant obtained was assayed for phytase activity.

Phytase assay

Phytase activity was assayed by measuring the amount of inorganic phosphorus released from sodium phytate solution using the method of Singh et al. 2013. One unit of enzyme activity was defined as the amount of phytase required to release one micromole of inorganic phosphorus per minute under the assay conditions.

Amylase assay

Alpha amylase was assayed by adding 0.5 mL of enzyme to 0.5 mL soluble starch (1%, w: v) in 0.1 M phosphate buffer, pH 6.0, for 30 min at 40 °C. The reaction was stopped and reducing sugar was determined with dinitrosalicylic acid according to the method of Bernfeld (Saqib and Whitney 2011). An enzyme unit is defined as the amount of enzyme releasing 1 μMol of glucose equivalents from the substrate per hour at 40°C.

Protein estimation

Protein was determined using a UV spectrophotometer (UV mini 1240 UV/VIS Shimadzu) taking readings at 280 nm. Water or buffer was used as a blank. A standard curve for the conversion of OD readings to mg protein was obtained using a series of dilutions of bovine serum albumin.

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 (Jost et al. 2011). For this, 0.5 g (dry weight) of fermented matter was mixed with concentrated sulphuric 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 1 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 540 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).

RESULTS AND DISCUSSION

Isolation and identification of *Neurospora*

Morphological observation on hyphal morphology of *Neurospora* colonies grown on PDA plate showed that the hyphal can be divided into two regions: the periphery and interior of the colony. The morphology of *Neurospora* are shown in Figure 1. In the colony periphery, the leading hyphae grew relatively straight and had a subapical branching pattern with primary hyphae exhibiting apical dominance over its branches. This is a wide, fast-growing hypha located at the colony periphery. It consists of an apical, tip-growing hyphal compartment interconnected with subapical compartments separated by perforated septa that provide continuity by allowing the passage of nuclei, other organelles, and cytoplasm. The leader hyphae undergo subapical branching and their growth contributes to the increase in colony diameter. Trunk hypha is located in the colony interior. It is wide and composed of hyphal compartments that typically become highly vacuolate (Riquelme et al. 2011). These hyphal compartments are typically shorter than those of leader hyphae because of the greater frequency of septa. The septal pores of trunk hyphae are frequently occluded.

Fusion hypha is typically a narrow, dichotomously branching hypha that arises from a trunk hypha. It exhibits positive tropisms, homing towards other fusion or trunk hyphae and anastomosing with them. In contrast to the hyphae described above, aerial hyphae grow in or on the surface of solid or liquid media, and grow away from the medium surface into the air. This is believed to be a result of forming hydrophobic hydrophobin proteins on their surfaces (Steele and Trinci 1975; Kasuga and Glass 2008; Wallrath and Elgin 2012).

Macroconidiophore is the specialized hypha that gives rise to macroconidia. Macroconidium (blastoconidium) is large, multinucleate (typically 3-6 nuclei) asexual spore that grows and develops by repeated budding of the apical cell of the conidiophore. The primary hyphae and their branches usually grew in such a way that they actively avoided neighboring hyphae (negative autotropism). Nevertheless, these hyphae occasionally made contact with each other but this did not result in hyphal fusion. In these cases, two alternative behaviors were observed: (i) the growth vector of the intersecting hypha would change, usually beginning just prior to contact, and resulting in subsequent parallel growth of this hypha along the side of a resident hypha or (ii) the growth vector of the intersecting hypha did not change, resulting in the contact of the hyphal tip with a resident hypha.

Molecular analyses of the ITS region confirmed that the two isolates belonged to *Neurospora crassa* and *N. sitophila*. The phylogenetic affiliation of *Neurospora crassa* and *N. sitophila* is shown in Figure 2.

Production of phytase by fungi

N. crassa and N. sitophila originated from *oncom* produced phytase on submerged culture from 2.1 to 7.4 unit (Figure 2). Fermentation time affected enzyme production profile. Maximum phytase production was achieved after 4 days fermentation. The activity of phytase produced by *Neurospora crassa and N. sitophila* was much higher than that produced by *Pichia anomala* (Olstorpe et al. 2009).

Activity of phytase in formulated media

Nitrogen sources affect phytase activity (Figure 3). In the case of *N. sitophila*, yeast extract and peptone supplement increased phytase production by 25%, but urea and sodium nitrate was less effective. However *N. crassa* appeared to use various nitrogen sources (sodium nitrate, yeast extract and peptone). N-supplement increased phytase production by 30%. Various influences of N-supplement were observed by several scientists. Yeast extract were good N-sources for phytase producing *Bacillus subtilis* (Singh et al. 2013), whose introduced nitrogen sources at the 0.5% yeast extract, sodium nitrate, ammonium sulphate, urea and ammonium acetate. This study reveals that *N. crassa* and *N. sitophila* used more variable nitrogen sources than *B. subtilis*.

Effect of incubation temperature

Temperature affected phytase production by *N. crassa* and *N. sitophila* (Figure 4,5 and 6). The highest temperature for phytase production was at 35°C. The optimum temperature for *N. crassa* and *N. sitophila* is lower than *B. subtilis* (Singh et al. 2013).

Effect of starch supplement

Starch supplement increased phytase production, optimum starch supplement was 3% (Figure 7). Starch will be used by fungi after hydrolysis by amylase (Juanpere et al. 2005; Lim et al. 2008). This indicates that *N. crassa* and *N. sitophila* produced amylase (Murthy et al. 2009). Both phytase and amylase are used for feed supplement of monogastric animal (Cowieson and Adeola 2005).

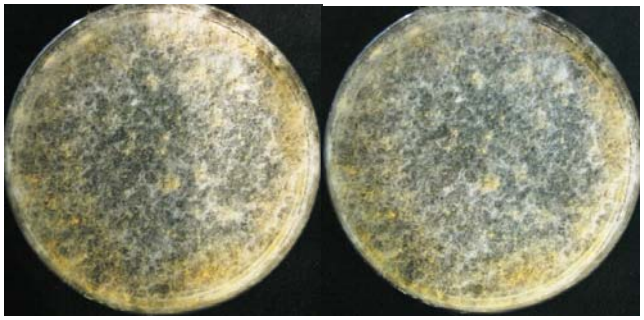
Alpha amylase activity

We observed an increase of phytase activity due to the addition of starch, and therefore we determine the activity of alpha amylase (Figure 8). The activity of alpha amylase increased in both cultures, and maximum enzyme activity was observed at 72 hour fermentation. This implies that the two isolates were able to use starch as carbon sources. The ability of fungi to produce amylase is variable. Higher alpha amylase production was observed on *Aspergillus niger* using sorghum than starch as a media (Abu et al. 2005). Starch supplementation increased carbon sources and finally trigger higher phytase production by the two isolates.

Biomass and protein

Biomass increased and then stabilize after 72 hour incubation (Figure 9 and 10). The biomass production of *Neurospora sitophila* is slightly higher than *N. crassa*. Protein also increased during fermentation (Figure 9 and 10). Increasing protein content in SSF was also observed on raw starch inoculated with *Bacillus* sp. (Hamilton et al. 1999), on sorghum and starch by *Aspergillus niger* (Viniegra et al. 2003; Pothiraj et al. 2006). Increased protein will be good for feed nutrition (Cao et al. 2007). *Neurospora crassa* and *N. sitophila* are important fungi for traditional fermented food. The increase of protein is also good for increasing the nutritional quality of traditional fermented food like *tempeh* and *oncom*.

Animal feed contain bound phosphorus from 18- 88% of total phosphorus content, as phytate. This phytate phosphorus cannot be directly utilized by monogastric animals like poultry and pigs due to a lack of intrinsic phytase in their gastrointestinal tracts. Phytate behave as an antinutrient through chelating various cations such as Ca^{2+}, Fe^{2+}, Zn^{2+}, and Mg^{2+} thereby reducing their bioavailability. Supplementation of phytase into animal feed will break down phytate into inositol and phosphate. Our finding revealed that *N. crassa* and *N. sitophila* produce phytase under submerge fermentation as well as under solid state fermentation. Production of phytase under solid state fermentation was higher than SmF which implies that SSF is more favorable for phytase production. Development of SSF technology to optimize phytase is then necessary. In addition to temperature, substrate composition influences phytase production. Those parameters will be optimized for future study.



A **B**

Figure 1. Colony morphology of *Neurospora crassa* (A) and *Neurospora sitophila* (B) grown in PDA after 3 days incubation at 30ºC

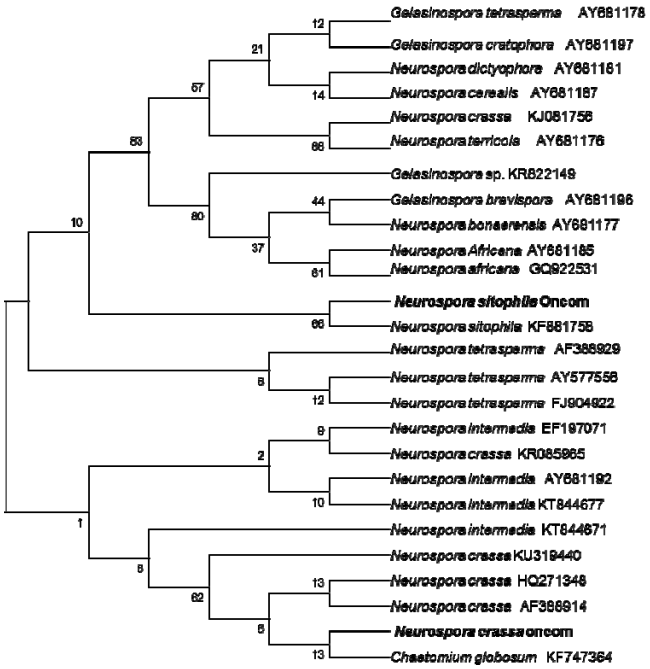


Figure 2. A rDNA-ITS-based phylogenetic tree of *Neurospora* showing the position of isolate. The numbers after the species name represent the accession numbers of isolates in GenBank. The numbers in each branch points denote the percentages supported by bootstrap. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Neim 1993). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein 1985) is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches . Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 26 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 510 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

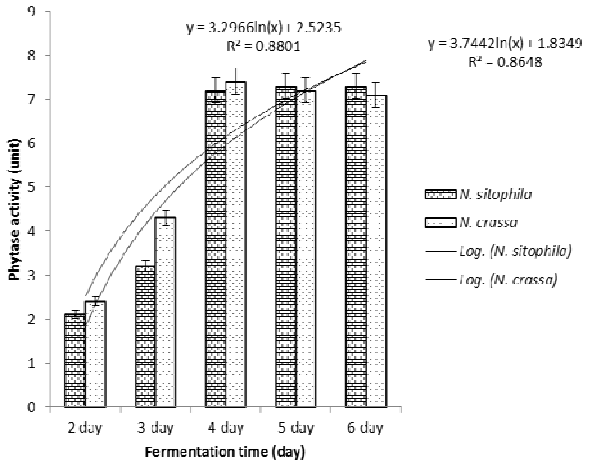


Figure 3. Phytase activity of *N. crassa* and *N. sitophila* in submerge fermentation at 30°C

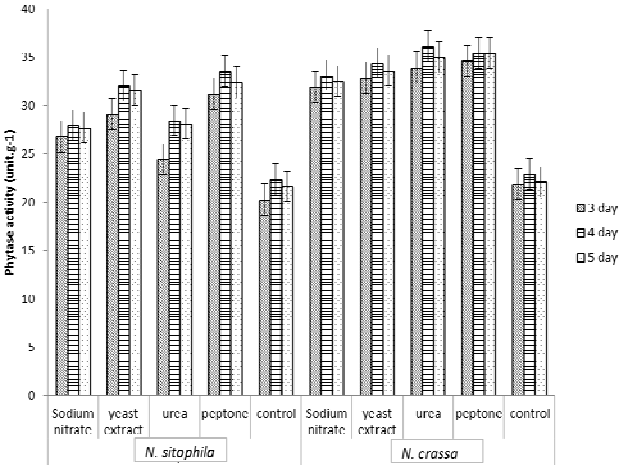


Figure 6. The effect of N-sources on activity of phytase at 40°C on various fermentation times

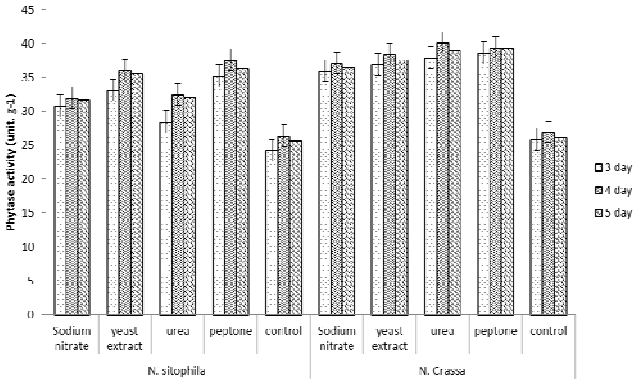


Figure 4. The effect of N-sources on the activity of phytase at 30°C on various fermentation times

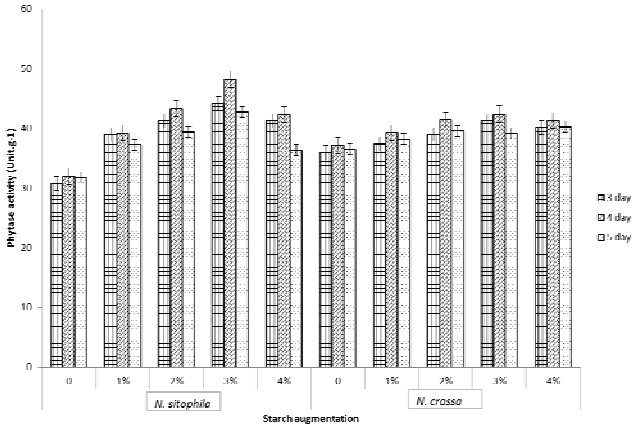


Figure 7. The effect of starch supplement on phytase activity at 35°C

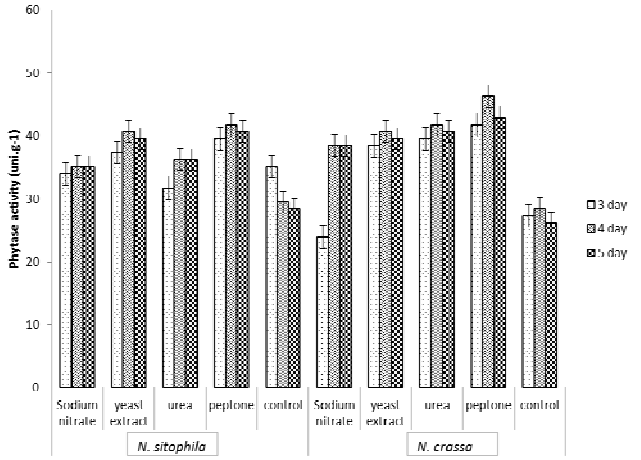


Figure 5. The effect of N-sources on the activity of phytase at 35°C on various fermentation times

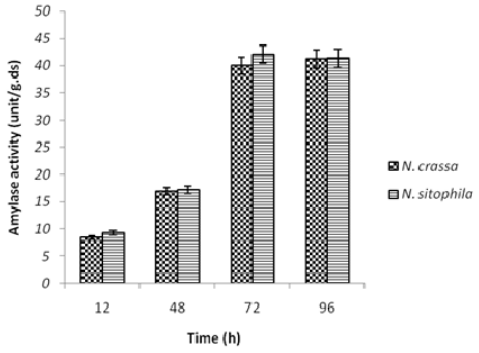


Figure 8. Profile of amylase during solid state fermentation inoculated with either *Neurospora crassa* or *Neurospora sitophila*

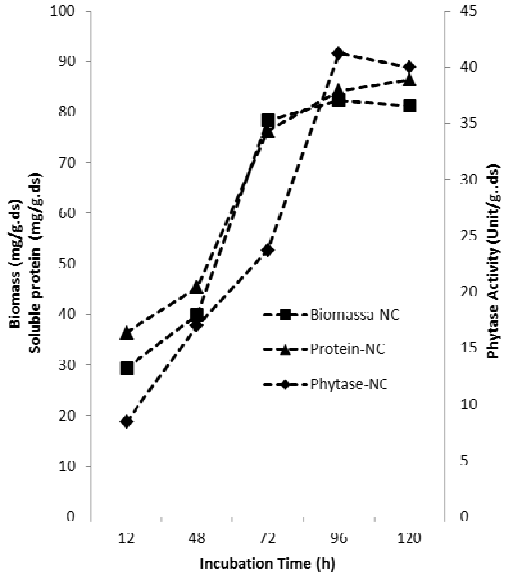


Figure 9. Profile of phytase, biomass and protein during fermentation by *Neurospora crassa*

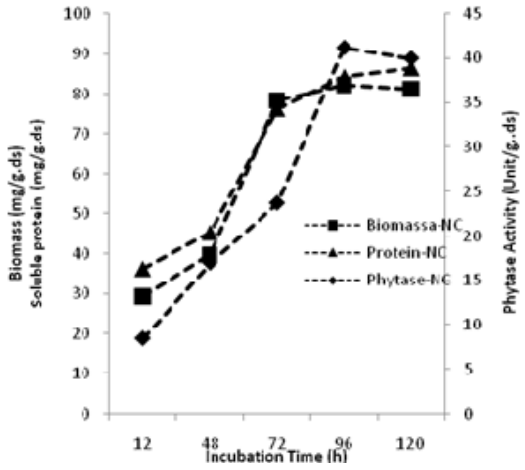


Figure 10. Profile of phytase, biomass and protein during fermentation by *Neurospora sitophila*

Neurospora crassa and *N. sitophila* produced phytase in high amounts (45.25 unit). The phytase production was influenced by media formula especially carbon and nutrient sources. Optimal temperature for phytase production is 35°C on solid state fermentation after 4-days. The ability of *N. crassa* and *N. sitophila* to use starch as carbon sources implies that these isolates also produce other hydrolytic enzymes. *N. crassa* and *N. sitophila* are potential isolates for phytase production which finally can be used as good supplement for monosgastric animals.

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