Production of Phytase, Amylase and Cellulase by *Aspergillus*, *Rhizopus* and *Neurospora* on Mixed Rice Straw Powder and Soybean Curd Residue

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Abstract. There is consensus that the presence of phytate in poultry diet negatively influence protein and energy utilization in poultry, and these influences could be enhanced by hydrolytic enzymes augmentation. The objective of study was to produce phytase, amylase and cellulase by *Aspergillus niger*, *Rhizopus oryzae* and *Neurospora sitophila* on solid state fermentation. Phytase production ability was first determined on submerge fermentation (SmF) using glucose as the main C-sources and on solid-state fermentation (SSF). The media composition for phytase production were a combination of rice straw powder and soybean curd residue at ratio of 0-100 % w/w. Maximum enzyme activities were observed at 96 h of incubation. SSF produced higher phytase than SmF. Maximum phytase production was achieved by *N. sitophila* (195.66 U/g) followed by *A. niger* and *R. oryzae*. Best media formula was 30.70 w/w of rice straw powder and soybean curd residue, respectively. At the same media formulation, the highest amylase was obtained by *A. niger* (141.85/g) followed by *R. oryzae* and *N. sitophila*. While for cellulase *A. niger* (92.0 U/g) followed by *N. sitophila* and *R. oryzae*. The ability of *A. niger*, *R. oryzae* and *N. sitophila* to produce phytase, amylase and cellulase on mixed substrate containing rice straw powder and soybean curd residue implies that those isolates are potential for hydrolytic production for poultry feed.

1. Introduction

Reduction of cost of feed in poultry is the main concern of many scientists. The inclusion of phytase in poultry diet has remarkably increased during the past decade. This is due to high concentration of phytate in cereal (barley, maize, sorghum and wheat) ranging from 1.86-2.89 (g.kg\(^{-1}\)). Higher phytate content is found in oilseed meals (4.0-9.11 g.kg\(^{-1}\)), and the highest 8.79-24.20 Phytate-P (g.kg\(^{-1}\)) is found in rice and wheat brands [1-3]. Phytase hydrolyses phytate which further eliminate the intense of phytate bound on mineral, carbohydrate and protein [4]. Thus, augmentation of phytase will reduce feed costs and increase the efficiency of phosphate utilization and other nutrients in cereal-based feed ingredients [5]. It is expected that the inclusion of phytase result in economic and environmental benefits. In addition, not only phytase but other hydrolytic enzymes such as amylase and cellulase are important component of feed ingredients [6].

Up to now, hydrolytic enzymes inclusion in feed ingredients is mostly focused on phytase production, and *Aspergillus niger* is the most popular phytase producer. Several other fungi especially *Neurospora crassa*, *N. sitophila*, *Rhizopus oryzae*, and *R. oligosporus* could be important microbes for...
hydrolytic enzymes production [7]. Those fungi are well known to play major role on traditional fermented foods in Indonesia such as oncom and tempeh. Solid-state fermentation offers higher enzyme production, less expensive and easier process control. Solid-state fermentation has been effectively carried out to produce phytase. Temperature and carbon nitrogen sources could be important factor influencing phytase production. A rice straw has been used as microbial sources for obtaining cellulytic microbes such as Trichoderma reesei and for ethanol production. T. reesei cellulase was produced by the solid-state culture on rice straw medium. The optimal pH and temperature for T. reesei cellulase production were 6 and 25°C, respectively.

Rice straw exhibited different susceptibilities towards cellulase to their conversion to reducing sugars. The rice straw bioconversion with cellulase was more current than the general trend by T. reesei. This enzyme effectively led enzymatic conversion of acid, alkali and ultrasonic pre-treated cellulose from rice straw into glucose, followed by fermentation into ethanol. The combined method of acid pre-treatment with ultrasound and subsequent enzyme treatment resulted the highest conversion of lignocellulose in rice straw to sugar and consequently, highest ethanol concentration after 7 days fermentation with Saccharomyces cerevisiae yeast. The ethanol yield in this study was about 10 and 11 g.L(-1) [8].

Rice husk and rice straw samples pre-treated with xylanase prior to treatment with cellulase, produced higher reducing sugar per 100 g of substrate by 29% to 41%. These findings can be adopted for the extraction of reducing sugars from cellulose and xylan-containing waste materials. The purely enzymatic extraction procedure can be substituted by the harsh and bio-adverse chemical methods [9]. Due to high protein and carbohydrate content, soybean curd residues have been used as the material for the production of lipopeptide antibiotic iturin A. Bacillus subtilis RB14-CS, cultured using soybean curd residues, after 4 days incubation, produced iturin A for about 3,300 mg/kg wet solid material (14 g/kg dry solid material), which is approximately ten fold higher than that in submerged fermentation [10]. Due to the nutrient composition of soybean curd residue and rice straw, we suspect that those materials can be used as a source of phytase, amylase and cellulase production. The objective of this study was to produce phytase, amylase and cellulase on mixed substrate of soybean curd residues and rice straw.

2. Material and Methods

2.1. Fungi Isolation and identification

The isolation of fungi from traditional fermented food was carried out following methods described by [11]. The oncom was obtained from local market in Bogor, West Java, Indonesia. To isolate the fungi from the sample, 1.0 g of sample was diluted in 9 ml sterilized water and vortex-mixed. One-tenth of a ml of successive decimal dilutions was spread on acidified Dichloran Rose Bengal chloramphenicol agar (OXOID, Cat.1076012). This selective medium was used due to the prevention growth of bacteria and the suppression of molds spreading. Plates were incubated for 5 days at room temperature. Strain purification was carried out at least twice by selecting one of each type of fungi colonies and streaking twice for single colony. The plate was incubated at 27°C for 3 days. Representative colonies were selected, purified and maintained on Potato Dextrose Agar (PDA) (OXOID, Cat.CM 0139).

rDNA identification Freshly-grown cells on the Potato Dextrose Broth were used for DNA extraction [12]. PCR amplification of the partial Internal Transcribed Spacer (ITS) ribosomal subunit was performed 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` [13] using GoTaq master mix (Promega, M7122). PCR products were visualized on 2% agarose and then 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) [14].
Morphology Observations. *Aspergillus, Rhizopus* and *Neurospora* were grown on PDA plates at 30°C for 7 days to observe its morphological characteristics and to test its single hyphal growth rate. The determination of the single hyphal growth rate was conducted within 24 h of colony development at 30°C under a microscope. Two photographs of growing fungi on Petri dish were taken. The hyphal growth rates were determined. After sporulation has occurred, the dimensions of the developed perithecia, asci and ascospores were separately observed [15].

**Culture maintenance.** The isolated strains of fungi were grown in PDA (Potato Dextrose Agar) medium for 120 h, at 30°C. These strains were further observed for their ability to produce phytase under submerge culture and solid-state fermentation at various temperatures and media compositions.

2.2. **Fungal strain screening**

The isolated strains of fungi 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 solubilisation from sodium phytate in aqueous media. The strains were grown under agitation at 150 rpm, at 30°C for 96 hours. After 96 hours of incubation time, the fungal biomass was separated by centrifugation at 8000 rpm for 20 minutes and the supernatant was then used for estimation of phytase, amylase, and cellulase production using method described by Liu et al. (1999) [16].

2.3. **Enzyme preparation and assay**

*Inoculum preparation for SS and SmF.* The culture was grown and maintained on potato dextrose agar (PDA) slants. The slants were stored at 4°C and sub-cultured after 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 obtained inoculant contained 4.7 x 10⁷ spores per ml.

*Substrates preparation for SSF.* For the SSF, the media for phytase production was composed of a combination of rice straw powder and soybean curd residue at ration of 0-100% which were obtained from Cibinong Science Centre Rice Filed Trial and local market. Ten grams of the dried mixed substrate taken in a cotton plugged of 250 ml Erlenmeyer flask were supplemented with 6.0 ml of salt solution containing 0.5 % MgSO₄·7H₂O and 0.1 % NaCl. While for the SmF, 10 grams of the dried mixed substrate taken in a cotton plugged of 250 ml Erlenmeyer flask were supplemented with 100 ml of salt solution containing 0.5 % MgSO₄·7H₂O and 0.1 %NaCl.

*Enzyme extraction.* Enzyme extraction was carried out using distilled water with 0.1% of Tween-80. Known quantities of fermented substrates were thoroughly mixed 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 4°C and the clear supernatant was assayed for enzyme activity.

*Phytase assay.* Phytase activity was assayed by measuring the amount of inorganic phosphorus released from sodium phytate solution using the method of Sing et al. (2013)[17]. 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 [18]. An enzyme unit is defined as the amount of enzyme releasing one µMol of equivalent glucose from the substrate per hour at 40°C.

*Cellulase assay.* Cellulase was assayed by adding 0.5 m; of enzyme to 0.5 ml soluble carboxymethyl cellulose (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 [18]. An enzyme unit is defined as the amount of enzyme releasing 1 µMol of equivalent glucose from the substrate per hour at 40°C.
**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 [19]. For this, 0.5 g (dry wt) 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. One ml of solution was mixed with 1 ml acetyl acetone reagent and was incubated in a boiling water bath for 20 min. After cooling, a volume of 6 ml ethanol was poured followed by the addition of 1 ml Ehrlich reagent and the resulting mixture was incubated at 65°C for 10 min. Once the reaction was 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 obtained results were expressed as mg glucosamine per gram dry substrate (gds).

3. Results and Discussion

3.1. Isolation and identification of fungi

Based on morphological observation and molecular analyses, isolates originated from traditional fermented food, were identified as *A. niger*, *R. oryzae* and *N. sitophila*. These isolates produced phytase, amylase and cellulase on submerge fermentation (Table 1).

| No. | Species name   | Phytase (U/ml) | Amylase (U/ml) | Cellulase (Unit/ml) |
|-----|----------------|---------------|---------------|--------------------|
| 1   | *A. niger*     | 11.1          | 13.2          | 8.3                |
| 2   | *R. oryzae*    | 6.5           | 5.6           | 6.9                |
| 3   | *N. sitophila* | 8.3           | 9.6           | 7.5                |

Table 1. Phytase, amylase and cellulase of fungi

Amylase, phytase and cellulase were determined at 96 h incubation.

Under submerge fermentation (SmF), *A. niger*, *R. oryzae* and *N. sitophila* produced phytase enzyme from 6.5 to 11.1 U/ml. The highest phytase, amylase and cellulase production was obtained by *A. niger*. SmF can be used to produce hydrolytic enzymes. But several parameter should be optimized. Contamination problem was less on SmF than that of SSF. But for higher volume of enzymes production, SSF could be more preperable, since it is less expensive than SmF. Therefore hydrolytic enzymes production by fungi is mostly conducted by SSF. Earlier study on production of hydrolytic enzymes is quite intensive. Of 32 microorganisms, isolates from soil and decayed fruits were tested for phytase production. The most active fungal isolates with phytase activity was *A. niger*. The maximum phytase activity occurred at the end of the growth phase [20]. *R. oryzae* produced high phytase on oil cake supplemented with (w/w) mannitol, 2.05%; ammonium sulfate, 2.84% and phosphate, 0.38%) [21]. Earlier study showed that *N. sitophila* not only produced phytase but also proteinase. At which proteinase production increased on substrate containing sugar beet pulp (SBP), wheat bran (WB) and citrus waste (CW) on solid state fermentation (SSF). Optimum conditions for protein enrichment of these lignocellulosic by products by *N. sitophila* under SSF were optimal moisture content for growth on SBP. CW was 75-80% (w/w) whereas for WB, it was 65% (w/w). The protein content of untreated SBP, WB and CW after 5 days SSF on surface was increased from 15, 13 and 7% (w/w) to 30, 30 and 18.2%, respectively [22]. Our finding shows that *N. sitophila* produce 3 hydrolytic enzymes: phytase, amylase and cellulase (Table 1).

3.2. Production of phytase on solid-state fermentation (SSF)

*A. niger*, *R. oryzae* and *N. sitophila* produced higher phytase on solid state fermentation (Table 1 and Figure 1). The highest phytase production was achieved by *N. sitophila* (Figure 1). This is the first report on the ability of *N. sitophila* to produce very high phytase on rice straw powder and soybean curd residues. The activity of phytase produced by *N. crassa* and *N. sitophila* was much higher than
that of produced by *Pichia anomala* [23]. Best formula for phytase production was 30:70 w/w of rice straw powder and Soybean curd residues, respectively.

### 3.3. Production of amylase on solid-state fermentation (SSF)

*A. niger, R. oryzae, and N. sitophila* produced amylase higher on SSF and SmF (Table 1 and Figure 2). The best formula for amylase production was 30:70 w/w of rice straw powder and soybean curd residues, respectively. *A. niger* produced the highest amylase compare to other isolates (Figure 2). Amylase production on submerged fermentation holds tremendous fungal potentiality in high biomass yield of alpha-amylase. This study also shows that amylase could be produced at large amount by *A. niger* using mixed rice straw and soybean curd residue which also implies that this formula could be further developed for hydrolytic enzyme production.

Enzymes production by *A. niger* is affected by pH, temperature and nitrogen sources of the medium for the productivity of *α*-amylase utilizing *Ipomoea batatas*. The maximum activity of *α*-amyase was recorded as 450 U/mg after 7 days of submerged fermentation at pH 7.0 and room temperature 28°C. Among the organic and inorganic nitrogen sources, inorganic sources showed maximum yield in which ammonium nitrate showed high amylase activity of 475 U/mg at the same pH and temperature.

![Figure 1. Phytase activity of A. niger, R. oryzae, and N. sitophila on solid state fermentation at 30°C](image)

Many works have been conducted on *A. niger*. For instance, *A. niger* can be used in industrial process after characterization [24]. Amylase and protease was synthesized by *A. niger* strain UO-1 in media prepared with brewery (BW) and meat (MPW) wastewaters supplemented with different starch concentrations. The highest amylase (70.29 and 60.12 EU/mL) and protease (6.11 and 6.03 EU/mL) production were obtained in the BW and MPW media supplemented with 40 g of starch/L of medium after 88 h of fermentation, respectively. Chemical oxygen demand (COD) appeared affect enzymes production. High COD input reduced enzymes production by more than 92%. High amylase and protease activities were found in the BW medium supplemented with casaminoacids, peptone or yeast extract, but ammonium nitrate and sodium nitrate were also good nitrogen sources for amylase production. The stabilities of amylase and protease were higher at 50°C and pH 4.95 and at 53.4°C and pH 3.87, respectively, but they were highly sensitive at temperatures of 70 °C or higher [25]. These all means that many factors should be optimized for high hydrolytic enzymes production.

The production of amylase by *A. niger* on three cassava whey media in liquid shake culture was compared. The supplemented cassava whey (SCW) medium has exhibited amylase activity of 495 U/ml. Biomass cropped was 1.63 g/l in the SCW medium. Yeast extract employed as a nitrogen
supplement increased biomass yield of *A. niger* to 2.75 g/l with maximum amylase activity of 643 U/ml. Sodium nitrate (NaNO₃) as nitrogen supplement had the lowest biomass yield of 0.77 g/l and amylase activity of 206 U/ml. Thus, yeast extract as nitrogen supplement of cassava whey medium supported maximum production of amylase and biomass of *A. niger* [26].

![Figure 2](image_url)  
*Figure 2.* Amylase activity of *A. niger*, *R. oryzae*, and *N. sitophila* on solid state fermentation at 30°C.

### 3.4. Production of cellulase on solid state fermentation (SSF)

*A. niger*, *R. oryzae*, and *N. sitophila* produced cellulase higher on SSF than SmF (Table 1 and Figure 3). The highest cellulase production was achieved by *A. niger* (Figure 3). Best formula for phytase production was 30:70 w/w of rice straw powder and Soybean curd residue, respectively. Fermentation technique affects cellulase production. SSF cultures produced higher cellulase activities than those in submerged and solid-state cultures (1,768, 1,165, and 1,174 U l⁻¹, respectively). Although SSF-cultures grew less than the other cultures, they produced significantly higher cellulase yields (370, 212, and 217 U g⁻¹ glucose, respectively) and volumetric productivities (24, 16, and 16 U l⁻¹ h⁻¹, respectively).

Likewise, endoglucanase and xylanase activities were higher in SSF-cultures. Under the tested conditions, it seems that fungal attached growth on perlite may favour better enzyme production. Biofilms are efficient systems for cellulase production and may replace solid-state fermentation. SSF holds promise for further optimization and development. The results of this work reveal that fungal biofilms may be implemented for the commercial production of cellulase employing the technology developed for submerged fermentation at high cell densities [27]. Fungal cellulases are well-studied enzymes and are used in various industrial processes. *Trichoderma* cellulase system has been the knowledge of enzymatic depolymerisation of cellulosic material. *Trichoderma* strains can produce substantial amounts of endoglucanase and exoglucanase but extremely low levels of β-glucosidase. This deficiency necessitates screening of fungi for cellulytic potential. A number of indigenously isolated fungi were screened for cellulytic potential.

*A. niger* MS82 was capable of producing moderate to high levels of both endoglucanase and β-glucosidase when grown on different carbon containing natural substrates, for example, grass, corncob, bagasse, and purified celluloses. Furthermore, it was observed that the production of endoglucanase reaches its maximum during exponential phase of growth as opposed to β-glucosidase during the stationary phase. Enzyme production by solid-state fermentation was equally investigated and found to be promising. Highest production of cellulase was achieved at pH 4.0 at 35°C under submerged conditions [28]. *A. niger* KKS, isolated from a farmland near Suwon, was immobilized on Celite and polyurethane foams. Enzyme activities produced by the immobilized cell system in a
bubble column were higher than that of shake-flask culture. The enzyme productivities were twice as high as beta-glucosidase, beta-xylosidase, and xylanase activities obtained in a bubble column were significant when the ground rice straw was used as a substrate [29].

*A. niger* NCIM 548 under same fermentation conditions in submerged fermentation (SmF) and solid state fermentation (SSF) used a central composite face centered design of response surface methodology (RSM). As per statistical design, the optimum conditions for maximum production of pectinase (1.64 U/mL in SmF and 179.83 U/g in SSF) and cellulase (0.36 U/mL in SmF and 10.81 U/g in SSF) were, 126 h, pH 4.6, and carbon source concentration 65 g/L in SmF. By contrast, it was 156 h, pH 4.80, and moisture content 65% in SSF. The response surface modelling was effectively applied to optimize the production of both pectinase and cellulase by *A. niger* under same fermentation conditions. It will result in a cost-effective process in both submerged and solid state fermentation using agro industrial wastes as substrate [30].

![Figure 3. Cellulase activity of *A. niger*, *R. oryzae*, and *N. sitophila* on solid state fermentation at 30°C](image)

Animal feed contains bound phosphorus from 18-88% of total phosphorus content such as phytate. This phytate phosphorus cannot be directly digested by monogastric animals like poultry and pigs due to lack of intrinsic phytase in their gastrointestinal tracts. Phytate behave as an anti-nutrient through chelating various cations such as Ca²⁺, Fe²⁺, Zn²⁺, and Mg²⁺ and thereby reducing their bioavailability. Supplementation of phytase into animal feed will breakdown 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 favourable for phytase production. Development of SSF technology to optimize phytase is furthermore necessary. In addition to temperature, substrate composition influences phytase production. Those parameters will be optimized for future study.

### 4. Conclusion

SSF produced higher phytase than SmF. Maximum phytase production was achieved by *N. sitophila* followed by *A. niger* and *R. oryzae*. Best media formula was 30:70 w/w of rice straw powder and soybean curd residue, respectively. At the same media formulation, the highest amylase was obtained by *A. niger* followed by *R. oryzae* and *N. sitophila*. Meanwhile, cellulose was highly produced by *A. niger* (92.0 U/g) followed by *N. sitophila* and *R. oryzae*. The ability of *A. niger*, *R. oryzae* and *N. sitophila* to produce phytase, amylase and cellulase on mixed substrate containing rice straw powder
and soybean curd residues implies that those isolates are potential for hydrolytic production for poultry feed.

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