Utilization of Palm Kernel Cake as a Novel Substrate for Phytase Production by *Aspergillus niger* USM AI1

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The present study was carried out to optimize the cultural conditions of phytase production by *Aspergillus niger* USM AI1 using palm kernel cake (PKC) as the substrate in a solid state fermentation (SSF) system. The optimized cultural conditions of 10 g of PKC with a particle size of ≤0.5 mm, an inoculum size of $1x10^5$ spores/ mL, moisture content of 60% (v/w), and a mixing frequency of once every 48 hours produced 4.91±0.17 U/g dried substrate of phytase and 1.14±0.04 mg glucosamine/ g dried substrate of fungal growth. Maximum growth as well as enzyme production was recorded on the 4th day of cultivation at room temperature (30±2 ºC). The findings indicated that phytase production increased 171.3% after optimization (4.91±0.17 U/g dried substrate) compared to before optimization (1.81±0.16 U/g dried substrate). This study showed that PKC is a potential medium for phytase production in SSF.

Keywords: Phytase; Aspergillus niger; Solid state fermentation; Optimization; Palm kernel cake

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INTRODUCTION

Phytase, which consists of myo-inositol hexakisphosphate 3-phosphorylase (EC 3.1.3.8) and myo-inositol hexakisphosphate 6-phosphorylase (EC 3.1.3.26), belongs to the family of histidine acid phosphatases that catalyze the hydrolytic degradation of phytic acid and its salts (phytates), generally yielding inositol, inositol monophosphate, and inorganic phosphate (Badamchi *et al*. 2013). Interest in the phytase has increased tremendously in the past 20 years, not only because of its wide range of applications in animal and human nutrition, but also in response to concern over phosphorous pollution in the environment (Lei and Porres 2003; Haefner *et al*. 2005). Phytase can be found in plants, microorganisms, and animals (Ocampo *et al*. 2012). However, microorganisms are the most frequently used source of phytase. To date only a handful of commercial phytase products are available on market, and they tend to be costly.

Solid-state fermentation (SSF) is considered an attractive method for the production of industrial enzymes from microorganisms. It is defined as a fermentation process involving solids in the absence or near absence of free water (Krishna 2005; Darah *et al*. 2013). SSF systems are closer to microbial natural habitats, creating conditions for more efficient production of secondary metabolites. The substrates commonly used for phytase production are of plant origin, such as agricultural wastes, which contain large amounts of celluloses and hemicelluloses that could serve as carbon and inducer sources for the production of enzymes (Patil and Dayanand 2006). Furthermore, SSF offers several economical and practical advantages including higher

product concentration, improved product recovery, simple cultivation facilities, reduced wastewater output, lower capital investment, and lower plant operation costs.

Malaysia is one of the main producers of palm oil in the world (Lim *et al*. 2013). Approximately 1.4 million ton per annum of palm kernel cake (PKC) is generated as a by-product of the production of palm kernel oil (MPOP 2013). PKC has been reported not only as protein feed for ruminant, but also as a reservoir of fixed carbon in nature. This resource is particularly attractive as an inexpensive substrate in SSF. Hence, PKC has great potential to be used as substrate to produce phytase in Malaysia.

The objective of this study was to optimize cultural conditions for enhancing phytase production by the locally isolated fungus *Aspergillus niger* USM AI1 using palm kernel cake as the substrate in a SSF system. In a preliminary study, this fungal isolate demonstrated the capacity for producing significant phytase activity.

EXPERIMENTAL

Microorganism and Inoculum Preparation

A. niger USM AI1 was supplied by the Industrial Biotechnology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. The fungal culture was maintained on potato dextrose agar (Merck, Germany) slants at 30 ºC for 4 days aerobically (until sporulation) before storing at 4 ºC until further use. The stored cultures were sub-cultured monthly to maintain viability.

Spore suspensions were prepared using well sporulated cultures. Ten mL of sterilized distilled water were added to the slant. The surface of the fungal colony was gently disturbed with a sterile inoculation needle. The concentration of spore suspension was measured using a haemocytometer (Neubauer, Germany) and adjusted to desire inoculum size by diluting with sterilized distilled water.

Solid State Fermentation and Early Profiling of Phytase Production

PKC was obtained from a local palm oil mill from Prai, Penang. The PKC obtained was dried under the sunlight before use as the substrate in this research. Ten grams of the ≤ 0.5 mm PKC were placed in the 250 mL Erlenmeyer flask. A total of 10 mL of sterile distilled water were added into the flask, the initial pH of the medium was adjusted to pH 7, and the flask was sterilized in an autoclave at 121 °C for 20 min. Sterilized PKC was inoculated with 1.0 mL of inoculum ($1x10^4$ spores/ mL) and the mixture was mixed gently with a sterile spatula to distribute the spores in the substrate. The inoculated flasks were incubated at room temperature (30±2 ºC) for 8 days aerobically. Each day 3 flasks were assayed for phytase activity and fungal growth. All the experiments were performed in triplicate and the values were reported with standard deviations.

Improvement of Physical Parameters

The strategy adopted was to evaluate the effect of an individual physical parameter and incorporate it at optimum level for studying the effect of next parameter. Improving the cultural conditions in a 250 Erlenmeyer flask for maximal phytase production involved various factors. There were physical parameters such as moisture content (v/w: 20%, 40%, 60%, 80%, and 100%); inoculum size of (1×10^4, 1×10^5, 1×10^6, 1×10^7, or 1×10^8 spores/ mL); and mixing frequency (static, or once every 12, 24,

or 48 h [sterile glass rod was used for mixed the entire inoculated substrate in Erlenmeyer flask with approximately 10 circulations]). The cultivations were carried out for 4 days before determining the phytase activity and fungal growth. All of the experiments were performed in triplicate and the values were reported with standard deviations.

Time Course of Phytase Production under Solid State Fermentation

An eight days profile was conducted in a 250 Erlenmeyer flask and the basic conditions of solid-state fermentation was applied. The samples were harvested at 24-h intervals and assayed for phytase activity and fungal growth.

Enzyme Extraction

Crude enzyme (phytase) was extracted by mixing the fermented substrate with a known amount of distilled water containing 0.1% Tween-80 on a rotary shaker at 200 rpm for 1 h at room temperature (Bogar *et al*. 2003). The solid biomass was separated from the mixture by filtration using filter paper (Whatman No. 1, England). The remaining suspension was centrifuged at 10,000 rpm for 10 min, and the supernatant was used as the source of crude enzyme for enzyme assay.

Phytase Assay

Phytase activity was assayed by measuring the inorganic phosphorus released from sodium phytate solution using the method described by Bogar *et al*. (2003). The reaction mixture consisted of 1.0 mL of 0.1M $MgSO_4 \cdot 7H_2O$, 2.4 mL 6.82 mM phytic acid, and 0.6 mL of crude enzyme solution. The reaction was carried out at 55 °C for 60 min. The reaction was stopped by adding 0.5 mL of 10% trichloroacetic acid. A blue colour was developed by the addition of 1.0 mL distilled water, followed by 2.5 mL of freshly prepared Taussky-Schoor reagent. After 2 min, the contents were mixed and the optical density of the reaction mixture was read at 660 nm using a spectrophotometer (Genesys 10 uv, Spectronic unicam, USA). One unit of enzyme activity was defined as the amount of phytase required to release one micromole of phosphorus per minute under the assay conditions. Enzyme yield was expressed as units/gram dried substrate (U/g).

Determination of Fungal Biomass (Glucosamine)

Fungal growth was determined using a method previously described by Swift (1973). The fungal biomasses were carried out by determining the N-acetyl glucosamine released by the acid hydrolysis of the chitin, present in the cell wall of the fungus. Glucosamine released from the chitin by the acid hydrolysis was mixed with 1 mL acetyl acetone reagent and incubated in a boiling water bath for 20 min. After cooling, 6 mL of ethanol was added, followed by the addition of 1 mL of Ehrlich reagent, and incubated at 65 °C for 10 min. After cooling, the glucosamine content was detected spectro-photometrically at 530 nm against the reagent blank. Fungal growth was expressed as mg glucosamine per g of substrate. Glucosamine (Sigma, Germany) was used as a standard.

Scanning Electron Microscope (SEM) Analysis

Two samples (PKC inoculated with *A. niger* USM AI1 and uninoculated PKC) were viewed with an SEM (Leo Supra 50 VP Field Emission SEM, equipped with Oxford INCA 400 energy dispersive X-ray microanalysis system). All the samples were prepared as described previously by Glauert (1980) prior SEM. The samples were placed on Tissue-Tek planchette with double sided sticky tape and placed on a filter paper lined

petri dish. Then few drops of 2% Osmium tetroxide were placed on filter paper. The samples were then plunged into slushy nitrogen (-120 °C) and then freeze dried them. Finally the samples were sputtered with 5 to 10 nm of gold and observed under SEM.

Statistical Analysis

All the experiments were carried out independently (in triplicate). The data represented here are in the form of mean ± SD.

RESULTS AND DISCUSSION

Time Course Profiles of Phytase Production and Fungal Growth before Optimization

The time course of phytase production was studied to determine the optimum incubation time required for maximum phytase production. The phytase activity was initially detected at 24 h and progressively increased with time, from day 0 and peaked at day 4 of cultivation. As shown in Fig. 1, phytase production gradually increased to reach its maximum activity at day 4 of cultivation with about 1.81 ± 0.16 U/g dried substrate and 1.26 ± 0.01 mg glucosamine/g dried substrate. Phytase production and fungal growth dropped drastically after achieving their maximal production. Singh and Satyanarayana (2008) also found maximum phytase production at 5 days of cultivation by a thermophilic fungus, *Sporotrichum thermophile* BJTLR50. However, Howson and Davis (1983) reported the highest extracellular phytase activity from *Aspergillus ficuum* NRRL 3135 after 10 days of cultivation.

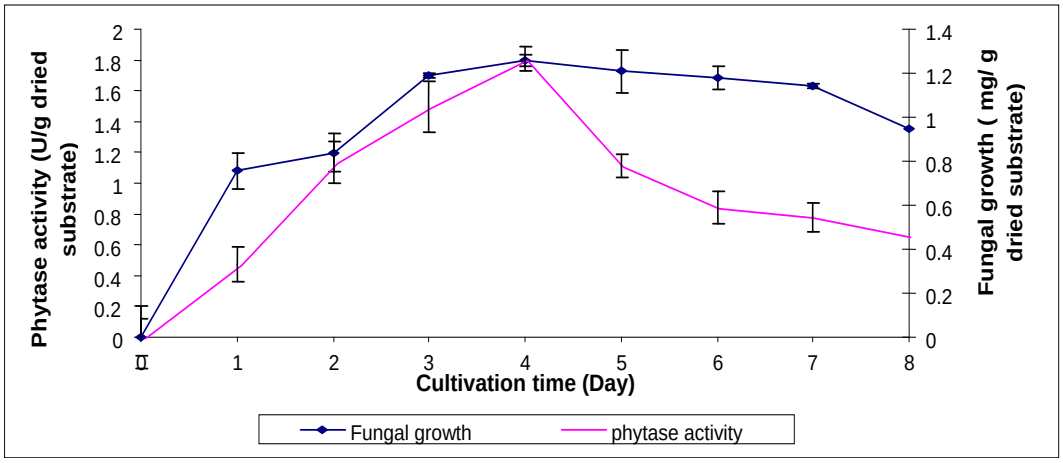

Fig. 1. Time course profile of phytase production and fungal growth before optimization Culture conditions: amount of PKC, 10 g; size of PKC, ≤ 0.5 mm; moisture content, 100%(v/w); initial pH, pH 7, inoculum size, $1x10^4$ spore/mL; temperature, room temperature (30±2 °C); mixing frequency, static

The enzyme yield on prolonged cultivation decreased, which could be due to a reduced nutrient level of the medium or autolysis of the fungal mycelium (Saha 2003). Therefore, it was important to determine the optimal day of cultivation for maximal enzyme production in order to optimize other parameters.

Effects of Inoculum Size

The pattern of phytase production with respect to inoculum size indicated that with a gradual increase in inoculum, phytase production also increased and reached a maximum enzyme production of 2.41 ± 0.20 U/g of dried substrate and fungal growth of 1.12 ± 0.06 mg glucosamine/g dried substrate at an inoculum size of $1x10^5$ spores/mL (Fig. 2). Enzyme production dropped after achieving the maximal production. The inoculum size of 1 x 10^5 spore/mL of spore suspension was incorporated as the optimized level before optimizing the next parameter.

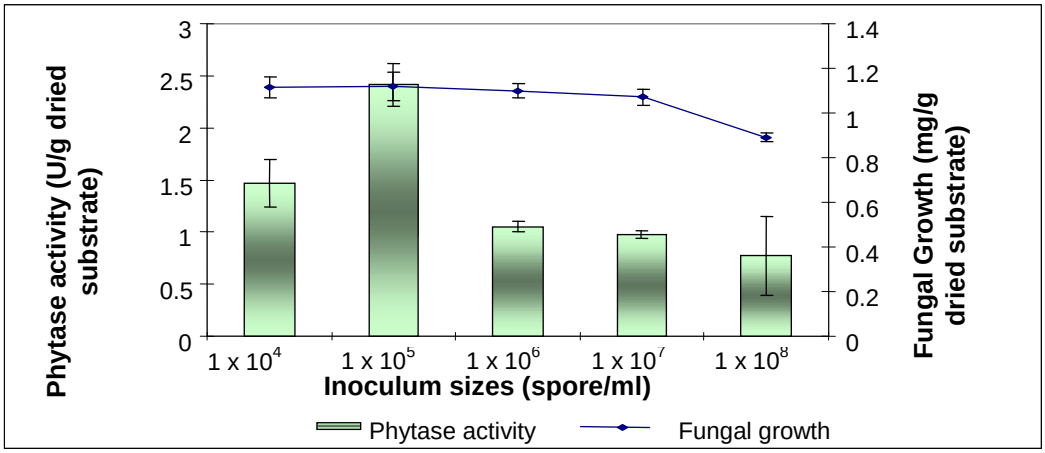

Fig. 2. Effect of inoculum size on phytase production and fungal growth by *Aspergillus niger* USM AI1 in solid state fermentation; Culture conditions: amount of PKC, 10 g; size of PKC, ≤ 0.5 mm; moisture content; 100%(v/w); initial pH, pH 7; inoculum size, ($1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$,$1x10^8$ spores/ mL); temperature, room temperature (30±2 °C); mixing frequency, static; incubation time, 4 days

Higher concentrations of inoculum were inhibitory for phytase production. At higher inoculum levels, enzyme production declined due to competition among the fungal population for the nutrients such as carbon source, leading to an exhaustion of nutrients and resulting in reduced enzyme production. Similar findings were reported with *Mucor racemosus* by Lata *et al*. (2013). Higher inoculum size usually induces cell production and biomass synthesis while shortening the lag phase of fungal growth. Hence, it can reduce the cultivation period. However, increased inoculum size also results in an increased moisture level, which can decrease fungal growth as well as enzyme production (Irfan *et al*. 2012). On the other hand, low inoculum size influences the time duration available for cell proliferation to utilize substrate and produce products (Ramachandran *et al*. 2004).

Effect of Initial Moisture Content

The maximum phytase production was observed at 60% (v/w) moisture content with 2.79 ± 0.05 U/g dried substrate and 1.11 ± 0.03 mg glucosamine/g dried substrate (Fig. 3). Higher or lower moisture contents than the optimum level produced less phytase and fungal growth. A moisture content of 100% (v/w) produced the lowest phytase production with 0.62 ± 0.01 U/g dried substrate and fungal growth of 1.02 ± 0.04 mg glucosamine/g dried substrate. At a moisture content of 20% (v/w), phytase production was 1.69 ± 0.53 U/g dried substrate, whereas the fungal growth was 0.92 ± 0.05 mg glucosamine/ g dried substrate. Based on the results obtained, a moisture content of 60%

was selected, and this was incorporated as the optimized level before optimizing the next parameter.

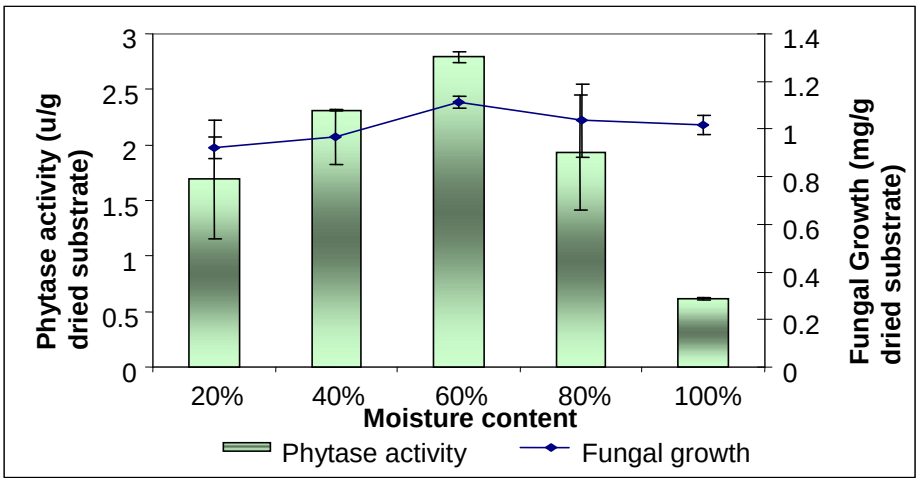

Fig. 3. Effect of initial moisture content on phytase production and fungal growth by *Aspergillus niger* USM AI1 in solid state fermentation; Culture conditions: amount of PKC, 10 g; size of PKC, ≤ 0.5 mm; moisture content, 20%, 40%, 60%, 80%,100% (v/w); initial pH, pH 7; inoculum size, $1x10^5$ spores/ mL; temperature, room temperature (30±2 °C); mixing frequency, static; incubation time, 4 days

In SSF, moisture content has been considered an important fundamental parameter in enzyme production. Most viable cells require more than 80% (v/w) moisture content. The PKC used as substrate in the present study was moistened, and its water holding capacity (1.29 g water/g PKC) is greater than other substrates such as rice husk (0.29 g water/ g rice husk), groundnut cake (0.20 g water/ g groundnut cake) and brewers' dried grains (0.38 g water/ g brewers' dried grains) (Omede *et al*. 2011), thus leading to an optimum moisture content at 60% (v/w). In SSF, water can be present as a thin layer absorbed on the particle substrate surface or be loosely bound within the substrate capillaries (Syarifah *et al*. 2012). The requirement for moisture depends on the type of solid substrate and microbe used; the optimum moisture content differs for each microorganism, even within similar strains (Javed *et al*. 2010). At high moisture contents (> 80% v/w), the PKC porosity is low, which prevents oxygen penetration into the substrate and also facilitates bacterial contamination. On the other hand, low moisture content (40%v/w) inhibits fungal growth due to poor nutrient accessibility (Darah *et al*. 2012).

Effect of Mixing Frequency

Substrate mixing at 48-h intervals showed the highest phytase production of 4.81 ± 0.55 U/g dried substrate and fungal growth of 1.39 ± 0.08 mg glucosamine/g dried substrate compared to the non-mixed control or the 12- or 24-h intervals (Fig. 4). Production of phytase enzyme under static conditions was 3.79 ± 1.18 U/g dried substrate, and the fungal growth was 1.24 ± 0.23 mg glucosamine/g dried substrate. The production of phytase enzyme when the substrate was mixed once every 24 hours was 3.52 ± 1.05 U/g dried substrate, and the fungal growth was 1.22 ± 0.11 mg glucosamine/g dried substrate. The production of phytase enzyme was the lowest when the substrate was mixed at 12-h intervals, with the phytase activity of 1.64 ± 0.60 U/g dried substrate and the fungal growth of 1.11 ± 0.09 mg glucosamine/g dried substrate.

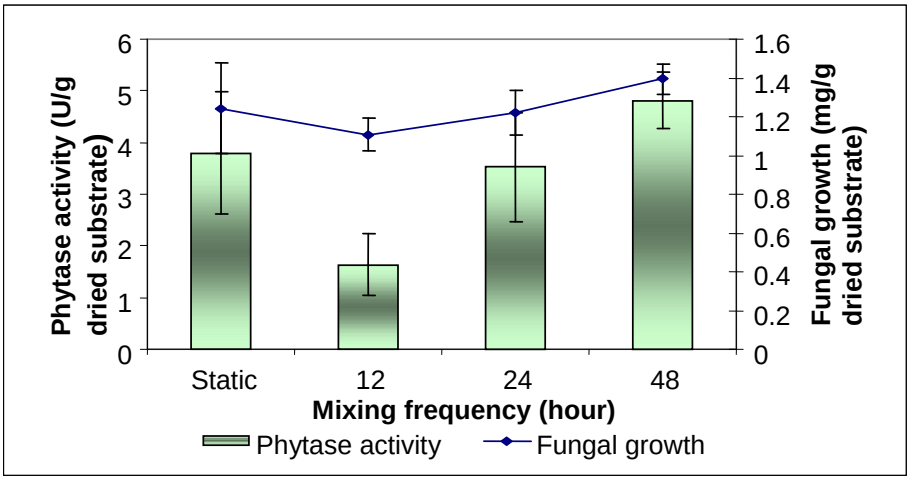

Fig. 4. The effects of mixing frequency on phytase activity and the growth of *Aspergillus niger* USM AI1 in solid state fermentation; Culture conditions: amount of PKC, 10 g; size of PKC, ≤ 0.5 mm; moisture content; 60% (v/w); initial pH, pH 7; inoculum size, $1x10^5$ spores/ mL; temperature, room temperature (30±2°C); mixing frequency, static, 12 h, 24 h, 48 h; incubation time, 4 days

In SSF, inadequate mixing not only compacts the fermented substrate but also results in unequal distribution of fungus, and evolved heat inside the substrate affects the pH and substrate moisture content. Static conditions might not be good for substrates that compact during fermentation, even though the advantage of static fermentation conditions is to avoid fragmentation of fungal mycelia (Darah *et al*. 2013).

The results of this study showed that mixing frequency improved the phytase production. The optimum mixing frequency obtained from the present study was once every 48 h. These variations in optimum level of mixing frequency can be related to the type of microorganism, the level of heat evolution, the quantity of carbon dioxide and other volatile metabolites that must be dissipated, the thickness of substrate bed height, and also the volume of pore space in the substrate (Darah *et al*. 2012). In previous works, it was found that mixing was necessary for some substrate used in SSF where it can influence the fungal growth and enzyme yield (Syarifah *et al*. 2012).

Profile after the Optimization of Physical Parameters

The final time course profiles of phytase production and fungal growth after the improvement of physical parameters [10 g of ≤ 0.5 mm size of PKC substrate, inoculum size of 1 x 10^5 spores/mL, a water content of 60% (v/w), substrate mixed once every 48 h, and an incubation temperature of 30 ± 2 °C (room temperature)] is shown in Fig. 5. The cultivation process in SSF was carried out for 8 days. The results showed that the highest phytase production was achieved on day 4 of cultivation with 4.91 ± 0.17 U/g dried substrate and fungal growth of 1.14 ± 0.04 mg glucosamine/g dried substrate. The production of phytase enzyme started to drop after achieving its maximal production. Phytase activity on day 8 was 4.21 ± 0.16 U/g dried substrate and the fungal growth was 1.46 ± 0.05 mg glucosamine/g dried substrate.

In general, the production of phytase enzyme was increased by 171.3% after physical optimization, compared to before optimization. The SEM micrographs show that the use of PKC as substrate produce phytase was made possible due to fungal degradation. Figure 6a shows the uninoculated PKC as the substrate in SSF carried out in the present study. The surface of PKC was devoid of fungal growth. Figure 6b shows

the inoculated PKC with *A. niger* USM AI1. The inoculated PKC shows the distribution of fungal mycelia and spores on the substrate surface indicating the vigor of the fungus during the degradation process.

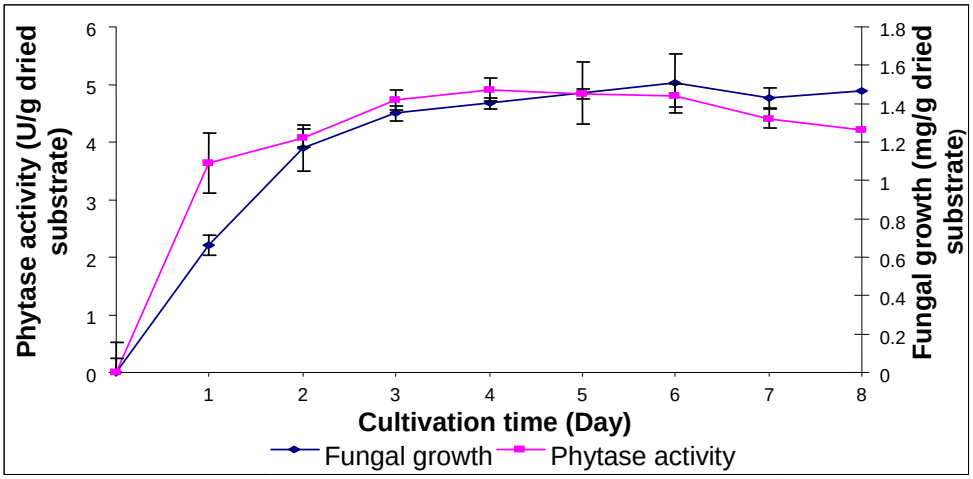

Fig. 5. Time course profiles of phytase production and the growth of *Aspergillus niger* USM AI1 after optimization; Culture conditions: amount of PKC, 10 g; size of PKC, ≤ 0.5 mm; moisture content; 60% (v/w); initial pH, pH 7; inoculum size, $1x10^5$ spores/ mL; temperature, room temperature (30±2 °C); mixing frequency, 48 h

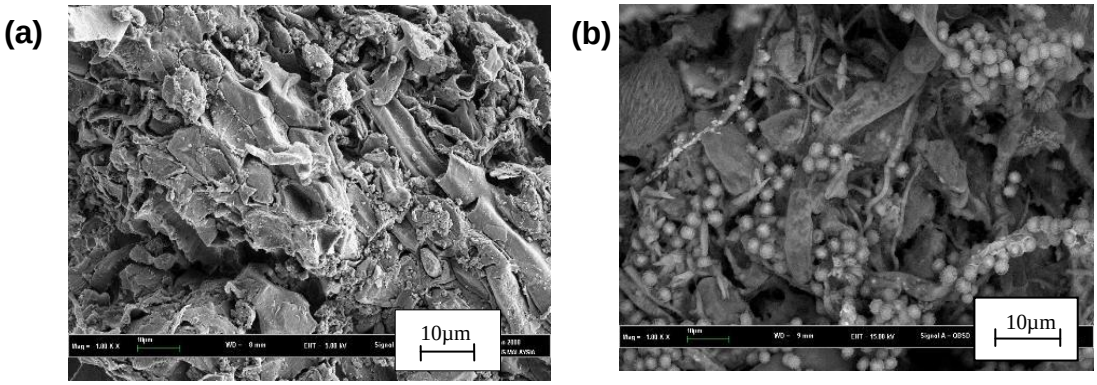

Fig. 6. Texture of the PKC (a) before and (b) after inoculation with *Aspergillus niger* USM AI1 for 4 days at room temperature (30 ± 2 ˚C)

The ideal solid substrate would provide all the necessary nutrients for the micro-organisms. Thus, the selection of an appropriate solid substrate plays an important role in the development of an efficient SSF process. Agro-industrial wastes or residues that are rich with lignocellulolytic materials generally are considered as the best substrates for SSF (Lim *et al*. 2011). The selection of a substrate for enzyme production in SSF depends on several factors such as the cost and availability of the substrate. Furthermore, the solid substrates provided not only nutrients for microbial growth, but also served as a support for the cells and served as anchorage for fungal mycelia to grow (Jacob and Prema 2006). Spier *et al*. (2008) found that phytase production was better in substrates with a low concentration of inorganic phosphate such as citric peels. This is true with PKC because this substrate also contains lower inorganic phosphorous (0.52 to 0.94%) and is high in celluloses and hemicelluloses (Lewal *et al*. 2010). Therefore, the fermented PKC could be a suitable substrate to be formulated as animal feeds especially for broilers, as it contains lower inorganic phosphorous and is high in other nutritional values.

CONCLUSIONS

1. The present work suggests that PKC could be employed as a promising substrate for the production of phytase by *Aspergillus niger* USM AI1.
2. Improvement of cultivation conditions markedly affected the phytase production.
3. Using the optimum cultural conditions, the highest phytase production was achieved with 4.91±0.17 U/g dried substrate.

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