Effect of culture conditions on phytase production by *Aspergillus ficuum* in solid-state fermentation using rice bran as substrate

Wien Kusharyoto*, Martha Sari, Asep Muhamad Ridwanuloh
Research Center for Biotechnology − Indonesian Institute of Sciences (LIPI)
Cibinong Science Center, Jalan Raya Bogor Km. 46, Cibinong−Bogor 16911
Phone: 021-8754587 Fax.: 021-8754588 E-mail: wien.kyoto@gmail.com

ABSTRACTS

Phytic acid is an antinutritional factor that forms 1–2% of most of the seeds and their co-products representing more than 60% of their total phosphorus. Monogastric and agastric animals are unable to utilize phytate phosphorus either due to lack of or insufficient amount of phytate degrading enzymes. Phytases (*myo*-inositol hexakisphosphate-phosphohydrolase) are a special class of phosphatases that catalyze the hydrolysis of phytic acid in a stepwise manner to lower inositol phosphates, *myo*-inositol and inorganic phosphate. Phytases are found naturally in plants and microorganisms and a sizeable number of phytases have been purified and characterized from various fungi, yeasts and bacteria. The present investigation involves studies on the effect of moisture content, pH value and different media ingredients such as carbon, nitrogen, and surfactants on the production of phytase by the fungus *Aspergillus ficuum* DSM 932 in solid-state fermentation (SSF) using rice bran as substrate. The production of phytase by SSF was favored, when the fungus was grown at a moisture content of 60% and pH 7.0, resulted in a phytase activity of 5.2 units/g dry substrate. There was a 20% increase in phytase yield in the presence of sucrose in SSF medium, while glucose and fructose were not effective in enhancing the phytase activity when used individually. Yeast extract was found to be a favorable nitrogen source for phytase production by SSF, which resulted in a 20% increase in phytase activity. There was no significant effect in increasing phytase production with the use of either soy peptone or tryptic soy as nitrogen source. Approximately 30% inhibition in phytase activity was shown in the presence of the surfactant Tween-80 or Triton X-100 in the SSF. By supplementing rice bran with sucrose and yeast extract, and performing the SSF in tray bioreactors, a phytase activity of 6.76 units/g dry substrate could be obtained.

Keywords: phytase, solid-state fermentation, *Aspergillus ficuum*, nutritional factors, rice bran

***Corresponding author**

INTRODUCTION

One of the major problems associated with the use of plant products in animal feed is the presence of anti-nutritional factors, such as phytic acid. Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate) is the major phosphorus storage compound in plant seeds and can account for up to 80 percent of total phosphorus. Soluble inorganic and cellular phosphorus (phosphorus bound in nucleic acids, phosphorylated proteins, phospholipids, phosphor-sugars) represents the remaining phosphorus. Because of the high density of negatively charged phosphate groups, phytate chelates with mineral cations such as Ca^{2+},

Cu^{2+}, Fe^{2+}, Mg^{2+} and Zn^{2+}, and forms poorly soluble complexes.

Apart from minerals, phytate also forms complexes with proteins and amino acids. The amino group present on the side chain of the amino acids, such as in lysine, is one of functional groups involved in protein-phytate interaction, thereby decreasing the digestibility of proteins. These salts of phytic acid are known as phytins or phytate and their availability/digestibility to monogastric or agastric animals like poultry, swine, most aquatic animals and human, is very limited due to the lack of intestinal phytase (Baruah *et al*. 2004). They are released into the environment in the form of phytate phosphorus which in turn are acted upon by microorganisms that release the phosphorus leading to pollution like in the form of algal bloom (Gabriel *et al*., 2007).

Phytases (*myo*-inositol hexakis phosphate phosphohydrolase) are a special class of phosphatases that catalyze the hydrolysis of phytic acid in a stepwise manner to lower inositol phosphates, *myo*-inositol and inorganic phosphate. Since monogastric and agastric animals do not have phytases in their gastrointestinal tracts and cannot digest phytate-phosphorus, it is necessary to supplement inorganic phosphorus in their diets to meet their nutrient requirement for phosphorus. This represents a significant expense in animal feeding. Besides, inorganic phosphorus is non-renewable, and the easily accessible inorganic phosphorus on earth may be exhausted in 80 years at the current rate of extraction (Abelson, 1999).

Low-phytate plants (Spencer *et al*., 2000) and phytase-transgenic plants (Ullah *et al*., 2002) or animals (Golovan *et al*., 2001) have been developed to cope with the nutritional and environmental problems associated with phytate-phosphorus. Although these approaches represent remarkable scientific advances, there are limitations in their practical application. In contrast, microbial phytases have become a widely accepted and highly effective tool for animal industry to improve feed phytate-phosphorus bioavailability to animals and to comply with environmental laws restricting phosphorus excretion in animal waste. Numerous animal experiments have shown that adding phytase to feed at 500 to 1,000 units kg^{-1} may replace inorganic-phosphorus supplements for pigs and poultry and reduce their phosphorus excretion by approximately 50% (Lei *et al*., 2001; Augspurger *et al*., 2003).

Phytases have a wide distribution in plants, microorganisms, and in some animal tissues. Although phytases from several plant and microbial species have been characterized (Vohra and Satyanarayana, 2003), commercial production currently focuses on the soil fungus Aspergillus (Greiner, 2004). However, the drawback to the widespread application of phytase as a feed supplement is the current high price of commercial phytase (Papagianni *et al*., 1999). The enzyme is currently produced by conventional submerged fermentation (SmF), a more expensive high technology process. An economical alternative for enzyme production and application would be solid state fermentation (SSF) (Pandey *et al*. 1999; Pandey *et al.* 2001; Mitchell *et al*. 2006).

The present article deals with the studies on the effect of moisture content, pH value, and media ingredients, such as carbon, nitrogen, and surfactants on phytase production by *Aspergilus ficuum* DSM 932 in a solid-state fermentation (SSF) process using rice bran as substrate. In an SSF by filamentous fungi, the fungal fermentation product will not only contains phytase, but also accessory enzymes, cellulases, hemicellulases, pectinases, amylases, fungal protein and organic acids that can increase feed digestibility and access to phytin in plant cells.

MATERIALS AND METHODS

Fungus and Inoculum Preparation

Aspergillus ficuum DSM 932 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). The fungus was grown and maintained on potato dextrose agar (PDA) slants. Viable spores from 6-day-old fully sporulated slants were harvested by washing with 0.1% Tween-80 containing water, and the spore suspension was adjusted to approximately 10^6 cfu (colony forming units on PDA plates) per milliliter for inoculation.

Solid State Fermentation in Erlenmeyer Flaks

Air-dried substrate (10 g) was placed in 500 ml cotton-plugged Erlenmeyer flasks, and sterilized at 121°C for 20 min. After cooling to room temperature the substrate was supplemented with 5 ml salt solution containing (g/l): NH_4NO_3, 23; $MgSO_4 \cdot 7 H_2O$, 4.6 and NaCl, 4.6. Different moisture levels were adjusted with sterile deionized water. Afterwards the wet substrate was inoculated with 1 ml spore suspension of the respective fungus at 10^6 cfu/ml, and mixed well. The flasks were incubated for 4 days at 27 ± 2°C in a static horizontal position. All experiments were carried out in triplicate. The results shown are average values.

Solid State Fermentation in Trays

Air-dried rice bran (200 g) was spread on an aluminum tray (170×130×80 mm) to about 3 cm thick, and sterilized at 121°C for 60 min. The cooled substrate was wetted with 100 ml of a solution containing (g/l): NH_4NO_3, 23; $MgSO_4 \cdot 7 H_2O$, 4.6; NaCl, 4.6; 1% (x/v) sucrose and 0.5% (w/v) yeast extract. The substrate was inoculated with 20 ml spore suspension of *A. ficuum* DSM 932 containing 10^6 cfu/ml. The moisture content of the inoculated substrate was adjusted to 60% level with sterile deionized water. The inoculated substrate was covered with two layers of filter paper, and incubated for 4 days at 27 ± 2°C in a chamber. The trays were ventilated twice each day for a few minutes.

Effect of different nutritional ingredients

Effect of carbon sources

Studies on the effect of various carbon compounds in SSF for phytase production was performed by supplementing the rice bran medium with the carbon sources such as glucose, fructose and sucrose at 1% w/v. The medium without additional carbon source served as control.

Effect of nitrogen sources

Various nitrogenous compounds, such as yeast extract, triptic soy or peptone, were incorporated in place of ammonium nitrate at 0.5% w/v in SSF medium, respectively. Their effect on the production of phytase by *A. ficuum* DSM 932 was determined. The medium with ammonium nitrate as nitrogen source served as control.

Effect of surfactants

In the ammount of 0.25% w/v, Tween-80 and Triton X-100 was added to the SSF media, respectively. The medium without any addition of surfactant was used as control.

Analytical methods

Enzyme activities were determined from the culture extracts. SSF samples were extracted with water containing 0.1% Tween-80 (10 ml water/g dry substrate), by shaking them for 1 h at 200 rpm at room temperature (25°C). The suspension was centrifuged (10,000 x *g*, 10 min) and the supernatant, designated as the crude enzyme preparation, was then stored at 4°C until assays were performed.

Alpha-amylase activity

Alpha-amylase activity was determined based on the released reducing sugars (maltose). The reaction mixture consisted of 1.25 ml 1% soluble starch solution, 0.25 ml 0.1 M sodium acetate buffer (pH 5.0), 0.25 ml distilled water and 0.25 ml appropriately diluted crude enzyme solution. After 10 min incubation at 50°C, the liberated reducing sugars (maltose equivalent) were estimated by BCA (bicinchoninic acid) assay (Kenealy and Jeffries, 2003). One unit of alpha-amylase was defined as the amount of enzyme releasing 1 µmol glucose equivalent per minute under the assay conditions.

Phytase activity

Phytase activity was assayed by measuring the inorganic phosphorus released from sodium phytate solution using the method described previously (Harland and Harland, 1980). The assay was initiated by mixing 1 mL of diluted (1:10) crude enzyme with 0.5 mL of sodium acetate (0.2 M) buffer of pH 5.5 and 0.5 mL of sodium phytate (15 mM). The reaction mixture was incubated at 40 °C in a water bath for 45 min. The reaction was terminated by the addition of 2 mL of 15% trichloroacetic acid. Assay mixture of 0.5 mL was then mixed with 4 mL of 2:1:1 v/v of acetone, 10 mM ammonium molybdate, 2.5 M sulfuric acid (AAM solution) and 0.4 mL of 1 M citric acid solution. The amount of free phosphate released was determined spectrophotometrically at 655 nm. A standard graph was plotted using potassium dihydrogen phosphate with working concentration ranging from 30 to 360 µM. One unit of phytase was defined as the amount of enzyme releasing 1 µmol inorganic phosphate per minute under the assay conditions.

Phosphatase activity

Phosphatase activity against 4-nitrophenyl phosphate was determined by incubating 1 ml of properly diluted crude enzyme solution with an equal volume of 0.64% 4-nitrophenyl phosphate disodium salt dissolved in 0.2 M sodium acetate buffer (pH 5.2) (Pasamontes *et al*., 1997). After 15 min of incubation at 37°C the reaction was terminated by cooling on ice. The color developed by the liberated p-nitrophenol was measured spectrophotometrically at 420 nm. One unit of phosphatase was defined as the amount of enzyme releasing 1 µmol p-nitrophenol per minute under the assay conditions.

Xylanase activity

Xylanase activity was assayed using a 1% solution of xylan from birchwood as substrate. The release of reducing sugars in 10 min at 50°C was measured as xylose by using BCA assay (Kenealy and Jeffries, 2003). One unit of xylanase was defined as the amount of enzyme releasing 1 µmol xylose equivalent per minute under the assay conditions.

Statistical analysis

Data are presented as means ± SD (standard deviation). Comparisons between different factors used in the production of phytase by *A. ficuum* DSM 932 in solid-state fermentations were performed with the analysis of variance (ANOVA) using XLSTAT statistical package for Microsoft Excel 2003. Values are considered significant at $P < 0.05$

RESULTS AND DISCUSSION

Effect of moisture content on phytase activity of *Aspergillus ficuum* DSM 932

Moisture content of the medium in SSF is very important for the growth of microorganisms, production of enzymes and for enzyme activity. The effect of moisture content on phytase production by *A. ficuum* DSM 932 was studied at pH 7.0 for three different moisture levels (Fig. 1). The fungus showed the highest phytase activity when it grew at moisture content

of 60% compared to other moisture contents. The phytase activity obtained at this moisture content was 5.30 ± 0.39 units/g ds. The phosphatase activity was also higher at moisture content of 60% compared to 50%, but there was no significant difference in phosphatase activity obtained at moisture content of 60% and 70%. For further experiments, moisture content of 60% in SSF was used in phytase production by the fungus.

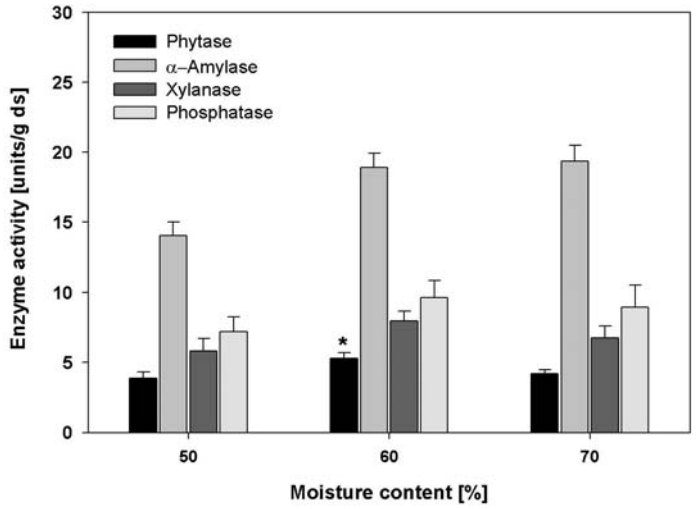

Fig. 1 Effect of moisture contents on the production of phytase and other hydrolytic enzymes by *A. ficuum* DSM 932 in solid-state fermentation. *Asterisk* indicates significant difference ($P < 0.05$) in phytase production as compared to other moisture contents.

Effect of pH value on phytase activity of *Aspergillus ficuum* DSM 932

The pH value of the medium in SSF plays a significant role on the growth of microorganisms, production of enzymes and for enzyme activity. The effect of pH value on phytase production by *A. ficuum* DSM 932 was studied at moisture content of 60% for three different initial pH values (Fig. 2). A maximum phytase production was obtained in SSF at initial pH 7.0 compared to other initial pH values. At initial pH 7.0 and moisture content of 60% a phytase activity of 5.22 ± 0.32 units/g ds could be obtained. The phosphatase activity was also at maximum when the fungus was grown at initial pH 7.0, however, there was no significant different between phosphatase activity obtained in SSF at pH 6.0 and 7.0. In contrary, the amylase activity was higher in SSF at initial pH 5.0 than at other pH values, although the difference was not significant.

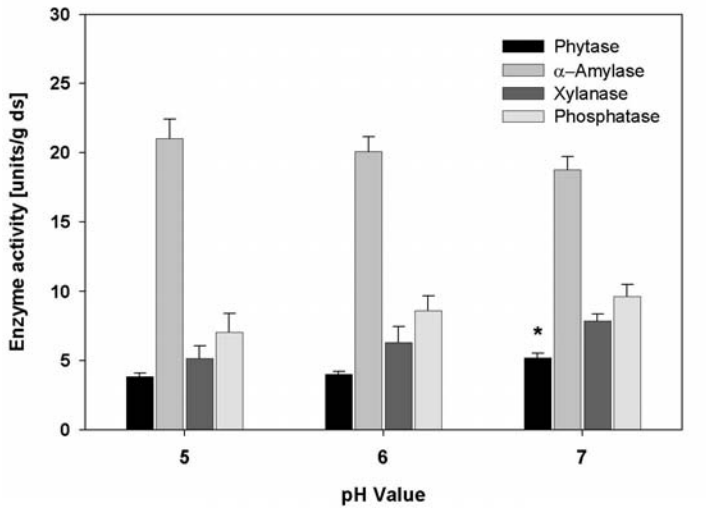

Fig. 2 Effect of initial pH value on the production of phytase and other hydrolytic enzymes by *A. ficuum* DSM 932 in solid-state fermentation. *Asterisk* indicates significant difference ($P < 0.05$) in phytase production as compared to other initial pH values.

Effect of carbon sources on phytase activity of *Aspergillus ficuum* DSM 932

The effect of additional carbon sources during SSF on phytase activity has been studied using glucose, fructose and sucrose at the concentration of 1% (w/v) (Fig. 3). The SSF medium with additional carbon sources serving as control showed activity of 5.17 ± 0.21 units/g ds (Fig. 3). Glucose and fructose were not effective in enhancing the phytase activity when used individually. There was no significant effect in phytase production with the addition of glucose to the medium, while fructose, on the other hand, reduced the phytase activity slightly. In combined form as sucrose, the phytase activity was higher when compared to the control medium. It could be suggested that α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside linkage in sucrose makes the carbon atoms more available to the fungus. The phytase activity obtained with sucrose as additional carbon source in SSF was 6.26 ± 0.30 units/g ds, which corresponds to a 20% increase in phytase activity compared to control.

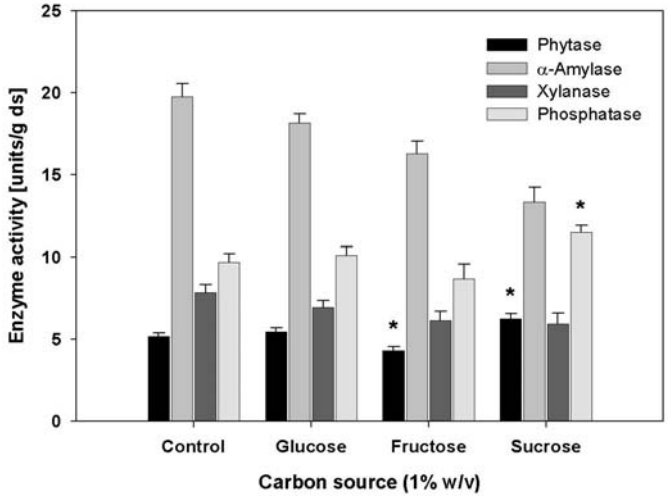

Fig. 3 Effect of different carbon sources on the production of phytase and other hydrolytic enzymes by *A. ficuum* DSM 932 in solid-state fermentation. *Asterisks* indicate significant difference ($P < 0.05$) in enzyme production as compared to the control

Effect of nitrogen sources on phytase activity of *Aspergillus ficuum* DSM 932

The effect of nitrogen sources in place of ammonium nitrat in SSF on phytase activity has been studied using yeast extract, soy peptone and tryptic soy at the concentration of 0.5% (w/v) (Fig. 4). The SSF medium with ammonium nitrate serving as control showed activity of 5.17 ± 0.21 units/g ds. The addition of soy peptone and tryptic soy in SSF had no effect on phytase activity. The SSF medium with yeast extract as carbon source showed increased phytase activity of 6.37 ± 0.25 units/g ds, which was similar to 1.2-fold higher activity than the control. This is in accordance with previous report where the significant role of nitrogen in growth and phytase production in *Aspergillus niger* van Teigham was studied and a maximum phytase production was obtained with bio-peptone followed by ammonium nitrate and ammonium sulfate (Vats and Banerjee, 2004).

Organic forms of nitrogen such as 1% peptone along with 1% yeast extract have been used extensively for the production of phytase in *Aerobacter aerogenes* and *Klebsiella aerogenes* (Jareonkitmongkol *et al*., 1997). However, there was no enhancement in the phytase activity with peptone in SSF in the present study. It could be thus stated that an appropriate concentration of yeast extract can be used as a suitable nitrogen source for improved phytase activity by *A. ficuum* DSM 932 in solid-state fermentation.

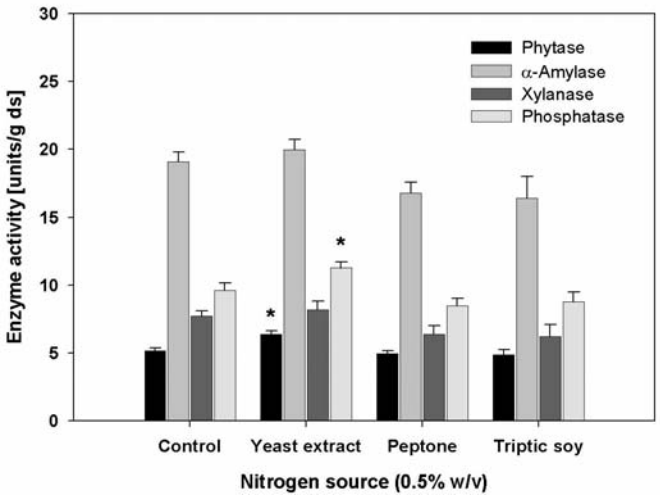

Fig. 4 Effect of different nitrogen sources on the production of phytase and other hydrolytic enzymes by *A. ficuum* DSM 932 in solid-state fermentation. *Asterisks* indicate significant difference ($P < 0.05$) in enzyme production as compared to the control

Effect of surfactants on phytase activity of *Aspergillus ficuum* DSM 932

Several researchers have shown that the incorporation of surfactants induced the formation of smaller pellets in the liquid medium and hence higher yield of phytase (Al-Asheh and Duvnjak, 1994; Nampoothiri *et al*., 2004). Surfactants are surface active agents having a number of applications that include lowering of surface and interfacial tensions, wetting and penetration actions, flocculating actions and microbial growth enhancement (Mullaney *et al*., 2000).

To investigate the effect of surfactants on phytase production, Tween-80 and Triton X-100 were added at a concentration of 0.25% (w/v) into the SSF medium, respectively. The results indicated that there was approximately 30% inhibition in the phytase activity in the presence of either Tween-80 or Triton X-100 (Fig. 5). Different results have been reported on the effect of surfactants on phytase production by SSF. Higher yield of phytase by *Aspergillus niger* NCIM 563 with 0.5% Triton X-100 has been reported (Mandvihala and Khire, 2000), whereas a 50% inhibition in the phytase activity has been demonstrated in SSF by *A. niger* CFR 335 in the presence of Tween-20, Triton X-100 or EDTA (Gunashree and Venkateswaran, 2008).

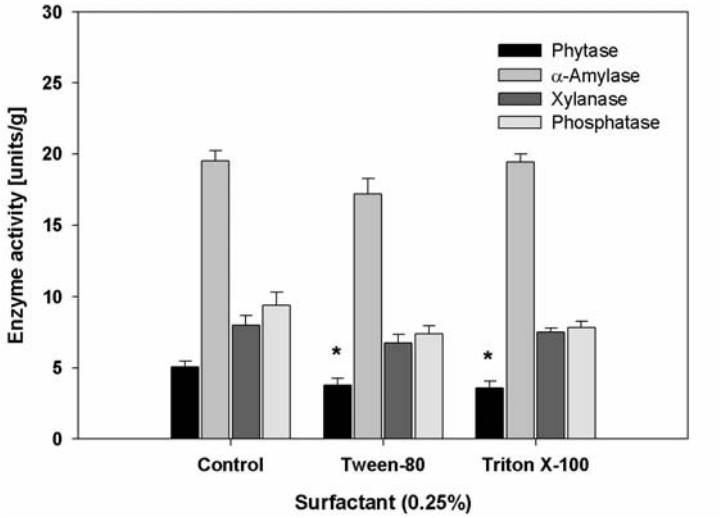

Fig. 5 Effect of different surfactants on the production of phytase and other hydrolytic enzymes by *A. ficuum* DSM 932 in solid-state fermentation. *Asterisks* indicate significant difference ($P < 0.05$) in phytase production as compared to the control

Solid-state fermentation by *Aspergillus ficuum* DSM 932 in trays bioreactor

The kinetics of phytase production by laboratory scale SSF on the optimized rice bran medium is shown in Fig. 6. Phytase activity reached 7.24 ± 0.30 units/g ds in 4 days, accompanied by significant activities of phosphatase and other hydrolytic enzymes such as alpha-amylase and xylanase (not shown), necessary for the efficient degradation of phytin in plant cells.

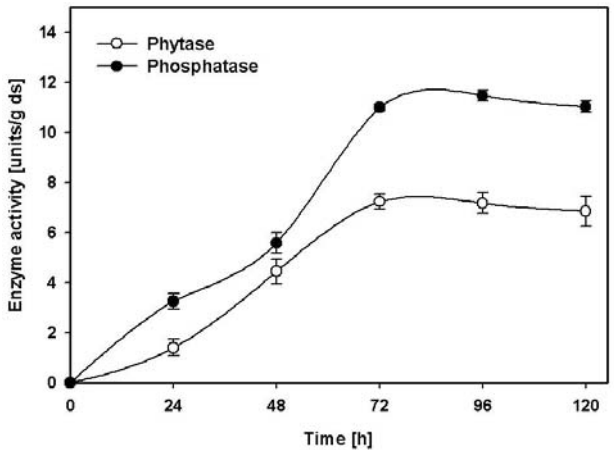

Fig. 6 Kinetics of solid substrate fermentation by *A. ficuum* DSM 932 in 500 ml flasks. Fermentation conditions: 10 g of air-dried rice bran were supplemented with salt medium, 1% (w/v) sucrose and 0.5% (w/v) yeast extract, moisture content of 60%, initial pH 7.0 and temperature of 27 ± 2°C.

In an attempt to approximate industrial conditions for phytase production, SSF was conducted in stationary tray bioreactors. The phytase production by *A. ficuum* DSM 932 was performed using rice bran supplemented with salt medium (without ammonium nitrate), 1% (w/v) sucrose and 0.5% (w/v) yeast extract at a moisture content of 60%, initial pH 7.0 and temperature of 27 ± 2°C. The kinetics of enzymes production (Fig. 7) shows that a maximum phytase activity of 6.90 ± 0.36 units/g ds could be obtained in 5 days, accompanied by a significant activity of phosphatase.

Considering the fact that the product of this fermentation is an enriched feed ingredient that contains phytase and other hydrolytic enzymes such as amylase, xylanase and phosphatase, enzymes preparation from such fermentation can be easily mixed with other ingredients in feed in order to increase feed digestibility and access to phytin in plant cells.

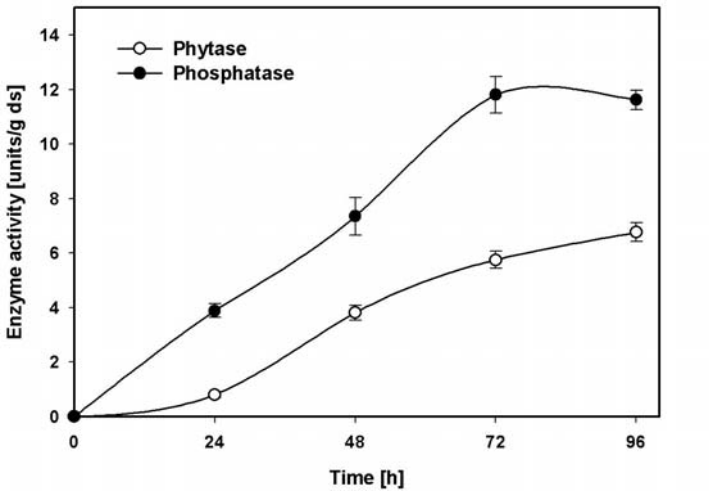

Fig. 7 Kinetics of solid substrate fermentation by *A. ficuum* DSM 932 in a tray bioreactor. Fermentation conditions: 120 g of air-dried rice bran were supplemented with salt medium, 1% (w/v) sucrose and 0.5% (w/v) yeast extract, moisture content of 60%, initial pH 7.0 and temperature of 27 ± 2°C.

CONCLUSIONS

In conclusion, sucrose was found to be a suitable carbon source instead of glucose and fructose in solid-state fermentation for phytase production by *Aspergillus ficuum* DSM 932. Yeast extract was shown to enhance the phytase activity, whereas incorporation of surfactans Tween-80 and Triton X-100 showed an inhibition effect on phytase production. Further experiments are necessary in order to increase the production of phytase by *A. ficuum* DSM 932, thus to make its application as feed supplement economically more feasible. Further attemps to increase phytase production by the fungus can for instance be conducted using more appropriate substrates and other additional ingredients for solid-state fermentation. Incorporation of statistical experimental designs for the optimization of phytase production, such as Plackett-Burman and surface response methodology will be of advantage in such optimization process. Despite considerable economic interest and large number of phytase producing microorganisms, low yield and high cost for production are the limiting factors in using this enzyme in animal diet. Thus, there is an ongoing interrest to obtain significant titers of phytases with an ultimate aim to produce the enzyme at cost effective level and establish the suitability for its industrial application.

ACKNOWLEDGEMENT

This research was funded by a grant from DIPA 2009 of Southeast Asian Regional Centre for Tropical Biology (SEAMEO BIOTROP).

REFERENCE

Abelson P. H. (1999) A potential phosphate crisis. *Science* 283: 2015

Al-Asheh S., Duvnjak Z. (1994) The effect of surfactants on the phytase production and the reduction of the phytic acid content in canola meal by *Aspergillus carbonarius* during a solid state fermentation process. *Biotechnol. Lett.* 16:183–188.

Augspurger, N. R., Webel D.M., Lei X.G., Baker D.H. (2003) Efficacy of an *E. coli* phytase expressed in yeast for releasing phytate-bound phosphorus in young chicks and pigs. *J. Anim. Sci*. 81: 474–483.

Baruah K., Sahu N.P., Pal A.K., Debnath D. (2004) Dietary Phytase: An ideal approach for a cost effective and low-polluting aquafeed. NAGA, *WorldFish Center Quarterly* 27: 15–19

Gabriel U.U., Akinrotimi O.A., Anyanwu P.E., Bekibele D.O. Onunkwo D.N. (2007) The role of dietary phytase in formulation of least cost and less polluting fish feed for sustainable aquaculture development in Nigeria. *Afr. J. Agric. Res*. 2: 279–286

Golovan, S.P., Meidinger R.G., Ajakaiye A., et al. (2001) Pigs expressing salivary phytase produce low phosphorous manure. *Nat. Biotechnol*. 19: 741–745

Greiner R. (2004) Purification and Properties of a Phytate-degrading Enzyme from *Pantoea agglomerans*. *Prot. J*. 23: 567–576

Gunashree B.S., Venkateswaran G. (2008) Effect of different cultural conditions for phytase production by *Aspergillus niger* CFR 335 in submerged and solid-state fermentations. *J. Ind. Microbiol. Biotechnol.* 35: 1587–1596.

Harland B.F., Harland J. (1980) Fermentative reduction of phytate in rye, white, and whole wheat breads. *Cereal Chem*. 57: 226–229

Jareonkitmongkol S., Ohya M., Watanabe R., Takagi H., Nakamori S. (1997) Partial purification of phytase from a soil isolate bacterium, *Klebsiella oxytoca* MO-3. *J. Ferment. Bioeng.* 83(4): 393–394.

Kenealy W.R., Jeffries T.W. (2003) Rapid 2,2'-bicinchoninic-based xylanase assay compatible with high throughput screening. *Biotechnol. Lett*. 25: 1619–1623

Lei, X. G., Stahl C.H. (2001) Biotechnological development of effective phytases for mineral nutrition and environmental protection. *Appl. Microbiol. Biotechnol*. 57: 474–481.

Mitchell D.A., Krieger N., Stuart D.M., Pandey A. (2000) New developments in solid-state fermentation: II. Rational approaches to design, operation and scale-up of bioreactors. *Process Biochem*. 35: 1211–1225

Mullaney E.J., Daly C., Ullah A.B. (2000) Advances in phytase research. *Adv. Appl. Microbiol*. 47: 157–199.

Nampoothiri K.M., Tomes G.J., Krishnan R., Szakacs G., Nagy V., Soccol C.R. et al (2004) Thermostable phytase production by *Thermoascus aurantiacus* in submerged fermentation. *Appl. Biochem. Biotechnol.* 118: 205–214.

Pandey A., Selvakumar P., Soccol C.R., Nigam P. (1999) Solid state fermentation for the production of industrial enzymes. *Curr. Sci.* 77: 149–162

Pandey A., Szakacs G., Soccol C.R., Rodriguez-Leon J.A., Soccol V.T. (2001) Production, purification and properties of microbial phytases. *Bioresour. Technol*. 77: 203–214

Papagianni M., Nokes S.E., Filer K. (1999) Production of phytase by *Aspergillus niger* in submerged and solid-state fermentation. *Process. Biochem*. 35: 397–402

Pasamontes L., Haiker M., Wyss M., Tessier M., van Loon A.P.G.M. (1997) Gene cloning, purification, and characterization of a heat-stable phytase from the fungus *Aspergillus fumigatus*. *Appl. Environ. Microbiol*. 63: 1696–1700

Spencer J.D., Allee G.L., Sauber T.E. (2000) Phosphorus bioavailability and digestibility of normal and genetically modified low phytate corn for pigs. *J. Anim. Sci*. 78: 675–681

Ullah A.H.J., Sethumadavan K., Mullaney E.J., Ziegelhoffer T., Austin-Phillips S. (2002) Cloned and expressed fungal phyA gene in alfalfa produces a stable phytase. *Biochem. Biophys. Res. Commun.* 290: 1343–1348

Vats P., Banerjee U.C. (2004) Production studies and catalytic properties of phytases (*myo*-inositol hexakis phosphate phosphor hydrolases): an overview. *Enzyme Microb. Technol.* 35: 3–14.

Vohra A., Satyanarayana T. (2003) Phytases: microbial sources, production, purification, and potential biotechnological applications. *Crit. Rev. Biotechnol*. 23: 29–60