Phytase Production By *Aspergillus niger* And *Aspergillus terreus* In Solid State Fermentation Using Wheat Bran

Nova, J.I.[1]; Alcantar, X.L.[1]; Rodríguez, J.A.[1]; Mateos, J.C.[1]; Camacho, R.M.[1*]

[1]Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, A. C., Avenida Normalistas #800, Colonia Colinas de la Normal, 44270 Guadalajara, Jalisco, México. E-mail: * rcamacho@ciatej.net.mx

Abstract

Phytases have been one of the focal enzymes for animal nutrition during the past two decades. In this work, the use of wheat bran as substrate for phytase production in Solid State Fermentation (SSF) was studied. In order to find the best fermentation conditions for phytase production using a laboratory strain indentified as *Aspergillus niger,* three different pretreatments (1: wheat bran without wash and autoclaved, 2: wheat bran washed and autoclaved, and 3: wheat bran without wash and UV exposed for 2h); and two different impregnation media (A (g/L): sucrose 5, $MnSO_4$ 0.1, $MgSO_4$ 0.5, $Fe_2(SO_4)_2$ 0.1, KCl 0.5, yeast extract 2; and B (g/L): glucose 5, starch 5, $MnSO_4$ 0.1, $MgSO_4$ 0.5, $Fe_2(SO_4)_2$ 0.1, KCl 0.5, $CaCl_2$ 0.5, NH_4NO_3 2) were tested. Fermentations (pH 6.5, 60% moisture at 30°C) were carried out for 12 days in 15 mL tubes containing 2.5 g of solid culture media (SCM). Subsequently, the best pretreatment and impregnation media were used to investigate the phytase production in column reactors containing 25 g of the formulated SCM, using two strains identified as *Aspergillus niger* and *Aspergillus terreus*. The fermentations (pH 6.5, 60% moisture at 30°C) were monitored for 8 days. The best fermentation conditions found using 15 mL tubes as reactor were: wheat bran without wash and UV exposed for 2h and culture media A; reaching a maximum phytase activity of 1.91±0.25 U/gdm (gram of dry matter) at 192h of fermentation. The other wheat bran pretreatments as well as culture media B do not present significant phytase activity. Maximum phytase activity reached using column reactors were: 4.2±0.71 U/gdm at 96h of culture for *Aspergillus niger* and 0.49±0.005 U/gdm at 96h for *Aspergillus terreus*. The use of 15 mL tubes fermentation system; show to be an easy and practical way to assay different fermentation conditions at the same time, obtaining rapid and reliable results. UV sterilization is an excellent process for using wheat bran as substrate for phytase production. Moreover the column reactors fermentation increased significantly the phytase productivity. The fermentation conditions obtained in this research can be used to find the best phytase producing strain for future experiments.

(Keywords: Phytase, Solid State Fermentation, Wheat Bran.)

Introduction

Phytic acid (myo-inositolhexaphosphate) is a polyanionic chelating factor that form complexes with divalent cations of great nutritional importance (Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+} y Mn^{2+}) [1-3], as well as protein and amino acids [4]. Phytic acid is an energy source and phosphorus store commonly found in cereals, legumes and oilseeds [5-7], these raw materials are used for animal feed production. Monogastric animals have a low content of phytate degrading enzymes [3-6], therefore their nutrition is altered due to the decrease of available nutrients in food.

Enzymes that hydrolyze phytic acid are called phytases, which are a type of hydrolases from the phosphatases family, which posses phosphomonoesterase activity [6], they are capable of hydrolyze phytic acid producing orthophosphate releasing myo-inositol and a minor concentration of partial hydrolyzed phosphate esters [1, 2, 4, 7-9]. Phytases can be classified in order to their regioselectivity in 3 (from microbial) and 6 (from plants) phytases, pH optima (alkaline or acid phytases) and catalytic mechanisms (histidine acid phosphatases, ß-propeller phytase, cysteine phosphatases or purple acid phosphatases) [10, 11].

The industrial demand for phytases with high specific activity and stability under high temperature conditions for feed pellet production and under acidic conditions in the stomach of monogastric animals continues to stimulate the search for new enzyme sources [12]. Phytases are found naturally in plants and microorganisms, particularly fungi. Most of scientific work has been focused on *Aspergillus* spp. phytases due to their biochemical properties found. *Aspergillus niger* PhyA was the first well characterized and commercialized phytase, with two optimal pH at 2.5 and 5.0–5.5, an optimal temperature at 55–60°C, and high affinity for phytic acid [13]. *Aspergillus fumigatus* phytase shares a 66% sequence similarity with *A. niger* PhyA phytase, but displays better thermotolerance [14].

Filamentous fungi require phosphorus to carry out oxidative phosphorylation, where the energy is obtained in NADH, $FADH_2$ and ATP form, which is used for metabolic regulation and signal transduction pathways [15]. Thus, the use of a culture media containing phytic acid as sole phosphorus source may induce phytase production [16].

An alternative for phytase production is solid state fermentation (SSF), it offers several advantages over submerged fermentation (SmF), as hiperglycosylated proteins, making them more stable, higher yields and productivities [16-18].

Wheat bran is one of the agroindustrial residues most used in phytase production by SSF, due to their high phytic acid concentration (1-2%) compared to other residues [16]. It is important to give an adequate pretreatment to the residue in order to be used for phytase production avoiding contamination and preventing phytic acid hydrolysis [17].

In the present study, Filamentous fungi (164) belonging to CIATEJ collection, isolated from different agroindustrial wastes and unconventional environments (mescal, cocoa fermentation, coffee pulp, etc.), were grown in a media using phytic acid as sole source of carbon and phosphorus in order to find phytase producing candidates. A screening method using 15 mL tubes fermentation system was performed to assay different fermentation conditions at the same time, obtaining rapid and reliable results. Three different pretreatments for wheat bran and two different impregnation media where studied. Phyatse production was studied by solid state fermentation using two laboratory strains *Aspergillus niger* and *Aspergillus terreus*.

Methods and materials

Microorganisms and Inoculum Preparation

Aspergillus niger and *Aspergillus terreus* selected from the CIATEJ collection, were identified by molecular techniques. These strains were cultivated in a medium using wheat bran as substrate, for 5 days at 30°C. Spores were collected with 50 mL of sterile distilled water containing 0.01% Tween-80.

Inductor Substrate-Support Preparation

Wheat bran was used as substrate-support for solid fermentation, with three different pretreatments: 1; wheat bran without wash and autoclaved, 2; wheat bran washed and autoclaved, and 3; wheat bran without wash and UV exposed for 2h, in order to study the pretreatment that favored phytase production.

Impregnation Media

Two different impregnation media were used; A (g/L): sucrose 5, $MnSO_4$ 0.1, $MgSO_4$ 0.5, $Fe_2(SO_4)_2$ 0.1, KCl 0.5, yeast extract 2; and B (g/L): glucose 5, starch 5, $MnSO_4$ 0.1, $MgSO_4$ 0.5, $Fe_2(SO_4)_2$ 0.1, KCl 0.5, $CaCl_2$ 0.5, NH_4NO_3 2), these media are commonly used for phytase production.

Solid State Fermentation

Fermentations were carried out with 2.5 g (15 ml tubes) and 12 g (column reactors) of wheat bran moistened with the corresponding impregnation media, cultures were incubated for 12 days at pH 6.5, 60% moisture and 30°C, an inoculum of $3X10^7$ spores/gdm was used.

Enzyme Extraction

Enzyme extraction was carried out adding 5 mL (for 15 mL tubes) or 50 mL (for column reactors) of distilled water containing 0.1% (v/v) Tween-80 at the fermented solids. The mixture was shaken at 200 rpm for 1 h at room temperature. Solid fermented was centrifuged at 5,000rpm for 20 min at 4°C. The supernatant was collected and used for enzyme assay.

Enzyme Assay

Phytase activity was assayed according to Harland and Harland. One international unit of phytase was defined as the amount of enzyme required for releasing 1 μmol of inorganic phosphorus per minute at 37°C and pH 5. Enzyme yield was expressed as U/gdm.

Results

Effect of wheat bran pretreatments and impregnation media on phytase production

Three different pretreatments for wheat bran and two impregnation media were tested in order to improve phytase production, it was observed that pretreatment 3 (wheat bran without wash and UV exposed for 2 h) and impregnation media A favored phytase production (Figure 1-A). In these conditions *Aspergillus niger,* known as a phytase producer, reached a maximum phytase activity of 1.91±0.25 U/gdm at 192 h of fermentation. The other wheat bran pretreatments as well as culture media B do not present significant phytase activity. Although these results were lower than the results found by Gunashree (2008) [2], reporting 66 U/gdm in SFF with a collection strain *Aspergillus niger* CFR335. This might be caused by different fermentation conditions, however, the study conducted in 15 mL tubes helped to find factors that favor phytase production in a shorter time.

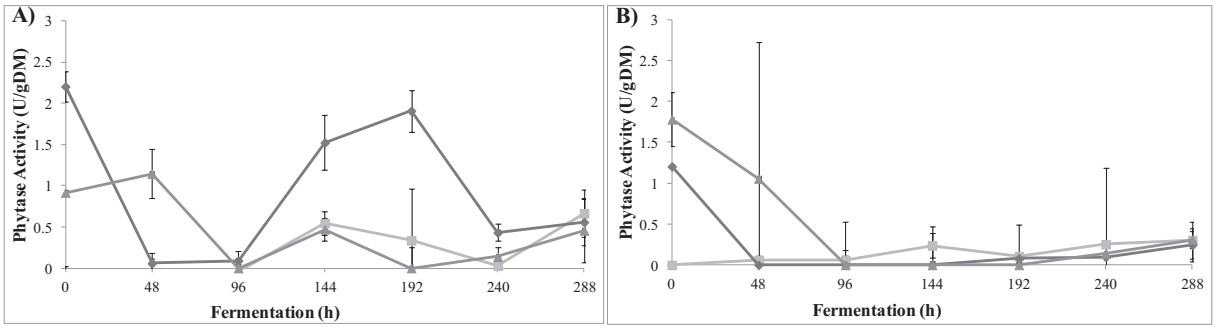

Figure 1. *Aspergillus niger* fermentation using the three different pretreatments: 1 ( ); wheat bran without wash and autoclaved, 2 ( ); wheat bran washed and autoclaved, and 3 ( ); wheat bran without wash and UV exposed for 2h; and the two impregnation media: A) medium A and B) medium B.

Phytase production in column Reactors

Solid state fermentation in column reactors is known to be a good fermentative system in order to perform studies at laboratory scale [18]. Maximum phytase activity reached using column reactors were: 4.2±0.71 U/gdm at 96 h of culture for *Aspergillus niger* and 0.49±0.005 U/gdm at 96 h for *Aspergillus terreus* (Figure 2). Compared to phytase activity obtained in 15 mL tubes, for *Aspergillus niger* activity was 2-fold higher and was reached in a shorter time.

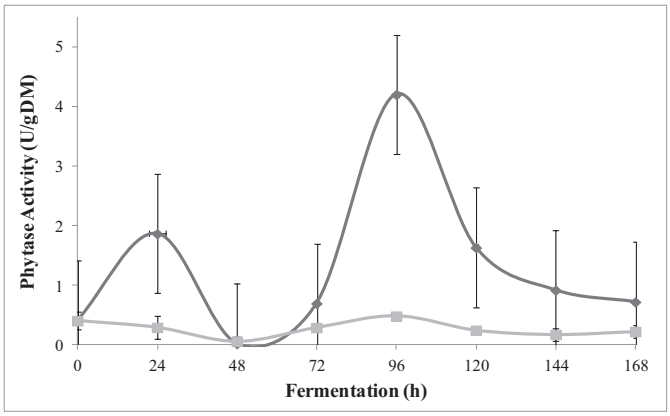

Figure 2. Phytse production in column reactors by *Aspergillus niger* ( ) and *Aspergillus terreus* ( ) using wheat bran as substrate-support and impregnation media A.

Conclusion

The use of 15 mL tubes fermentation system; show to be an easy and practical way to assay different fermentation conditions at the same time, obtaining rapid and reliable results. UV sterilization is an excellent process for using wheat bran as substrate for phytase production. Moreover the column reactors fermentation increased significantly the phytase productivity. The fermentation conditions obtained in this research can be used to find the best phytase producing strain for future experiments.

References

1. Singh, B. and T. Satyanarayana, Phytase production by a thermophilic mould Sporotrichum thermophile in solid state fermentation and its potential applications. Bioresource Technology, 2008. 99(8): p. 2824-2830.
2. Gunashree, B. and G. Venkateswaran, Effect of different cultural conditions for phytase production by Aspergillus niger CFR 335 in submerged and solid-state fermentations. Journal of Industrial Microbiology & Biotechnology, 2008. 35(12): p. 1587-1596.
3. Zhang, G.Q., et al., Purification, characterization, and cloning of a novel phytase with low pH optimum and strong proteolysis resistance from Aspergillus ficuum NTG-23. Bioresource Technology, 2010. 101(11): p. 4125-4131.
4. Haefner, S., et al., Biotechnological production and applications of phytases. Applied Microbiology and Biotechnology, 2005. 68(5): p. 588-597.
5. Singh, B. and T. Satyanarayana, Characterization of a HAP-phytase from a thermophilic mould Sporotrichum thermophile. Bioresource Technology, 2009. 100(6): p. 2046-2051.
6. Guimaraes, L.H.S., et al., Screening of filamentous fungi for production of enzymes of biotechnological interest. 2006.
7. Gulati, H., B. Chadha, and H. Saini, Production of feed enzymes (phytase and plant cell wall hydrolyzing enzymes) by Mucor indicus MTCC 6333: Purification and characterization of phytase. Folia Microbiologica, 2007. 52(5): p. 491-497.
8. Frontela, C., ROS, Gaspar and MARTINEZ, Carmen, Application of phytases as functional ingredient in foods. . scielo, 2008. 58: p. 215-220.
9. Bei, J., et al., Structure-based fragment shuffling of two fungal phytases for combination of desirable properties. Journal of Biotechnology, 2009. 139(2): p. 186-193.
10. Polaina, J. and A.P. MacCabe, Industrial enzymes : structure, function and applications. 2007, Dordrecht: Springer.
11. Oh, B.C., et al., Biochemical properties and substrate specificities of alkaline and histidine acid phytases. Applied Microbiology and Biotechnology, 2004. 63(4): p. 362-372.
12. Fu, D., et al., A highly pH-stable phytase from Yersinia kristeensenii: Cloning, expression, and characterization. Enzyme and Microbial Technology, 2008. 42(6): p. 499-505.
13. Han, Y., D.B. Wilson, and X.G. Lei, Expression of an Aspergillus niger phytase gene (phyA) in Saccharomyces cerevisiae. Vol. 65. 1999. 1915-8.
14. Wyss, M., et al., Biophysical Characterization of Fungal Phytases (myo-Inositol Hexakisphosphate Phosphohydrolases): Molecular Size, Glycosylation Pattern, and Engineering of Proteolytic Resistance. Applied and Environmental Microbiology, 1999. 65(2): p. 359-366.
15. Kostrewa, D., et al., Crystal structure of Aspergillus niger pH 2.5 acid phosphatase at 2.4 Ã… resolution. Journal of Molecular Biology, 1999. 288(5): p. 965-974.

16. Afinah, S., Yazid, A. M., Anis Shobirin, M. H. and Shuhaimi, M., Review Article Phytase: application in food industry. International Food Research Journal, 2010. 17: p. 13-21.
17. Spier, M.R.G., R.; Rodriguez-León, J. A.; Woiciechowski, A. L.; Ashok Pandey; Soccol, V. T.; Soccol, C. R., Phytase production using citric pulp and other residues of the agroindustry in SSF by fungal isolates. Food Technology and Biotechnology, 2008. 46: p. 178-182.
18. Bogar, B., et al., Optimization of phytase production by solid substrate fermentation. Journal of Industrial Microbiology & Biotechnology, 2003. 30(3): p. 183-189.