

## Phytic Acid Quantification In Agroindustrial Residues And Their Potential As Inducers For Phytase Production In Solid State Fermentation

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### Abstract

Phytases have been one of the focal enzymes for animal nutrition and human health during the past two decades. In order to study typical agroindustrial residues used in animal feed as potential inducers for phytase production in Solid State Fermentation (SSF), a methodology to quantify phytic acid in solids was implemented. The agroindustrial residues used for this study were: agave bagasse, sugarcane bagasse, coffee husk, corn husk, cottonseed meal, wheat straw and wheat bran. Bartlett (1959) technique for phytic acid extraction and Haug-Lantzsch (1983) for phytic acid quantification were implemented. Three different acids were assayed for phytic acid extraction (3% HCl, 5% H<sub>2</sub>SO<sub>4</sub> and 10% trichloroacetic acid) using 1:20 (w/v) ratio for 1 h at 30°C. The acid showing the best extraction results was used for further studies to improve the phytic acid extraction conditions. Three acid concentrations (0.2M, 0.6M and 1M); temperatures (25°C, 50°C, and 100°C) and weigh/volume ratios (1:5, 1:10 and 1:20) were tested. Solid state fermentations were carried out for 8 days, pH 6.5, 60% moisture and 30°C, using polyurethane as inert support and a laboratory strain identified as *Aspergillus niger*. Only, three agroindustrial residues (corn husk, cottonseed meal and wheat bran) showed a considerable phytic acid content (approximately 0.5%) using 3% HCl for extraction. The best conditions for extracting phytic acid were: 0.2M of HCl, 25°C and 1:20 ratio for the three agroindustrial residues, finding 0.57 %, 0.45 % and 0.67 % of phytic acid for corn husk, cottonseed meal and wheat bran, respectively. The three extracts obtained were assayed as inducers for phytase production in SSF. The maximum phytase activity reached was 0.39±0.09 U/gDM (grams of dry matter) at 24 h of fermentation using a wheat bran extract as inducer, followed by corn husk extract with 0.35±0.01 U/gDM at 24 h of fermentation; cottonseed meal extract used as SSF inducer, does not present significant phytase activity. It was possible to implement a quantification methodology for phytic acid in agroindustrial residues and the extracts obtained proved their potential as inducers in phytase production.

(Keywords: Phytic Acid, Agroindustrial Residues, Phytase)

### Introduction

Phytic acid (PA) (myo-inositol hexa-phosphoric acid, IP<sub>6</sub>) is the most abundant phosphorus storage form in seeds, legumes and cereals, it may account for more than 60-90% of the total phosphorus (P) [1]. In these plants, phytate is approximately between 1% and 2% of the total plant weight. In terms of location, phytic acid in legumes and seeds is uniformly distributed in the cotyledon and associated to protein structures [2]. In cereals is found predominantly in the external aleurone layer.

Phytate chelates mineral cations as well as proteins and amino acids, is therefore considered to be an antinutritional factor [3]. Animal food is elaborated using cereals, seeds and legumes as raw material, therefore monogastric or single stomach species cannot utilize P in phytic acid because they do not possess sufficient levels of phytic acid-degrading enzymes in their digestive tract. Traditionally, the limited bioavailability of plant phosphate was compensated by adding inorganic feed phosphates in animal

rations. This led to increasing phosphate contamination known as eutrophication. To solve this problem phytic acid-degrading enzymes are added, known as phytases.

Phytases are hydrolases from phosphatases family with phosphomonoesterase activity [4], capable of hydrolyze phytic acid producing orthophosphate, partial hydrolyzed phosphate esters and releasing myo-inositol [5-10]. Phytases can be classified in order to their regioselectivity in 3 (from microbial) and 6 (from plants) phytases [10]. Microbial phytases are used for commercial applications, obtained generally from fungus due to their biochemical properties. Fungal phytases can be produced by solid state fermentation (SSF) due to its potential compared to submerged fermentation (SmF), obtaining less contamination, cheaper process, using agroindustrial residues as substrate-support [11].

Researches has been focused in phytase production from agroindustrial residues rich in phytic acid, but phytic acid extracts from row material have not been used as inducers.

Analytical determination of phytate levels in feed raw materials is considered "primitive", since most methods for photometrical determination of phytate are based on indirect measurements (inositol phosphate) or establishment of stoichiometric ratio between phytate and some cations easy to determine with a direct tool. Several methods have been published for phytate quantification. Most of them (Bartlett, 1959) with acidic extraction carried out with HCl, H<sub>2</sub>SO<sub>4</sub>, or trichloroacetic acid with different concentrations and extraction times, followed by precipitation with Fe<sup>3+</sup> [12]. Unprecipitated ferric ions are determined by spectrophotometry. The difference between initial and remaining ferric ion concentration is then used to calculate phytate concentration. Acid extraction is preferable, because raw materials such as wheat might contain high levels of endogenous phytase, which could degrade phytate if allowed. A well-known photometrical phytate method is the protocol developed by Haug and Lantzsch that uses 2,2'-bipyridine as a complexing chromogenic agent to quantify ferric ions [13].

In the present study phytic acid extracts were obtained from agroindustrial residues: agave bagasse (AB), sugarcane bagasse (SB), coffe husk (CFH), corn husk (CH), cottonseed meal (CM), wheat straw (WS) and wheat bran (WB). The Haug-Lantzsch modified method was used for quantification. Subsequently phytic acid extracts were tested as inducers for phytase production by solid state fermentation.

## Methods and materials

### Phytic acid extraction

Agave bagasse, sugarcane bagasse, coffe husk, corn husk, cottonseed meal, wheat straw and wheat bran, were ground and sieved on 40 net. Phytic acid extraction was performed according to Bartlett (1959) [12]: 20 mL of diluted acid (3% HCl, 5% H<sub>2</sub>SO<sub>4</sub> and 10% trichloroacetic acid) were added to one gram of raw material (dry) and incubated for 1 h at 30°C and 200 rpm. Supernatant was recovered by centrifugation (4000 rpm, 15 min) and used for phytic acid quantification.

The acid showing the best extraction results was used for further studies to improve the phytic acid extraction conditions. Three concentrations (0.2M, 0.6M and 1M); temperatures (25°C, 50°C, and 100°C) and weigh/volume ratios (1:5, 1:10 and 1:20) were tested.

### Phytic acid quantification

Phytic acid quantification was implemented according to Haug-Lantzsch (1983) with modifications [13]. 0.5 mL of sample (supernatant for the extraction process) were added to 1 mL of ferric solution (0.2 g of ferric ammonium sulfate dissolved in 100 mL of concentrated HCL graduated with water to 1000 mL). The mixture was heated to boiling for 30 min and then cooled with ice for 10 min. 0.5 mL of the solution were added to 1 mL 2,2'-bipyridine solution (1g 2,2'-bipyridine plus 1 mL of thioglycolic acid and graduated with water to 100 mL). Absorbance of the mixture was read at 519 nm. The same procedure was applied to construct a calibration curve using phytic acid (0-1 g/L) as standard.

### Solid State Fermentation

*Aspergillus niger*, a strain indentified by molecular techniques in our laboratory, was 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. Polyurethane was used as inert support, 0.8 g were moistened with 0.12 mL of impregnation media: phytic acid extract, mineral salt solution ((g/L): sucrose 5, MnSO<sub>4</sub>

0.1, MgSO<sub>4</sub> 0.5, Fe<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub> 0.1, KCl 0.5, yeast extract 2) and inoculum (3X10<sup>7</sup> spores/gDM). Fermentation was carried out in column reactors for 8 days, pH 6.5, 60% moisture and 30°C.

#### Enzyme extraction

Enzyme extraction from 1.4 g of fermented solids was carried out by adding 3.6 mL of distilled water, containing 0.1% (v/v) Tween-80, and shaken at 200 rpm for 1 h at room temperature. Fermented solid was then centrifuged at 5,000rpm for 20 min at 4°C. The supernatant was collected and used for enzyme assay.

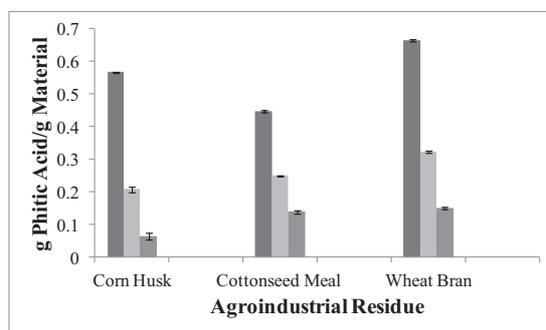
#### Enzyme Assay

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 temperature and pH 5. Enzyme yield was expressed as U/gDM (gram dry matter).

## Results

### Phytic acid content

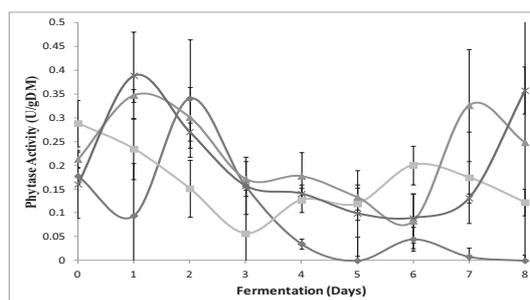
Corn husk, cottonseed meal and wheat bran showed a considerable phytic acid content (approximately 0.5%), while in the rest of agroindustrial residues tested, phytic acid was undetectable. The best phytic acid extraction was reached with 3% of HCl. In the other hand, when H<sub>2</sub>SO<sub>4</sub> and trichloroacetic acid were used, low phytic acid extraction was achieved. This result differs from those of Hatzack (2008) [1], that found trichloroacetic acid as the best acid for phytic acid extraction. Further studies to improve phytic acid extraction were carried out. The best conditions for extracting phytic acid were: 0.2M of HCl, 25°C and 1:20 ratio (residue:acid) for the three agroindustrial residues tested, finding 0.57% (567 mg PA/gM), 0.45% (447 mg PA/gM) and 0.67% (665 mg PA/gM) of phytic acid for corn husk, cottonseed meal and wheat bran, respectively. Phytic acid extraction reached in wheat bran was 10-fold greater than that reported previously (58.39 mg PA/gM) by Hatzack (2008) [1]. Proposed modifications to Bartlett (1959) [12] technique were promising for phytic acid extraction in solid agroindustrial residues, finding higher phytic acid extraction.



**Figure 1.** Ratio (residue/acid) (w/v) for phytic acid extraction in agroindustrial residues. Extractions were carried out with 0.2M of HCl at 25°C, for 1 hour at 200rpm. Where ■ is 1:20 ratio; ▒ is 1:10 ratio; ■ is 1:5 ratio.

### Phytase production in SSF

Three phytic acid extracts (corn husk, cottonseed meal and wheat bran) were assayed as inducers for phytase production in SSF using polyurethane as inert support. The maximum phytase activity reached was 0.39±0.09 U/gDM at 24 h of fermentation using a wheat bran extract as inducer, followed by corn husk extract with 0.35±0.01 U/gDM at 24 h of fermentation. Cottonseed meal extract used as SSF inducer, does not induce a significant phytase activity. Phytase production reached was 6-fold higher than the obtained for Ríos (2008) [14] (0.06 U/gDM) in SSF with a collection strain of *Aspergillus ficuum* DSM 932, this might be due to fermentation conditions (mineral media) used by the author, 20 g of glucose as carbon source, which is reported to repress phytase production [15]. However the phytase activity in this study was lower compared to phytase activity reported by Gunashree (2008), for a collection strain of *Aspergillus niger* CFR335 (66 U/gDM) [6]. The higher activity is probably due to the different phytase activity technique used for quantification, besides the authors improved the conditions for phytase production. Phytic acid extracts proved their potential as inducers for phytase production in SSF.



**Figure 2.** Effect of phytic acid extracts on phytase production by *Aspergillus niger* in SSF. Where ●, phytic acid; ■, cottonseed meal; ▲, corn husk; and ✕, wheat bran.

## Conclusion

It was possible to improve a phytic acid extraction and quantification methodology to be used in agroindustrial residues. The extracts obtained proved their potential as inducers in phytase production by SSF using polyurethane as inert support.

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