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Isolation and Characterization of Potential Phytase-Producing Fungi from Environmental Samples of Antioquia (Colombia)

Aislamiento y Caracterización de Hongos Productores de Fitasa a partir de Muestras Ambientales de Antioquia (Colombia)

Maritza Ocampo Betancur[1]; Luisa Fernanda Patiño Cervantes[2]; Mauricio Marín Montoya[3]; Mauricio Salazar Yepes[4] y Pablo Andrés Gutiérrez Sánchez[5]

***Abstract**. Phytases are enzymes used as feed additive that enhance the phosphorus and mineral uptake in monogastric animals and reduce the level of phosphate excretion in their manure. Due to their easy cultivation and high production of extracellular enzymes, filamentous fungi are one of best sources of phytase for use in the feed industry. Phytase has been found principally in the genera **Aspergillus, Penicillium, Mucor** and **Rhizopus**. In this work, we report the isolation and characterization of environmental fungi producers of phytase with potential use as feed additives. Samples were collected from soils, fruits and cereals in Antioquia (Colombia). A total of 26 fungal strains were isolated and identified using ITS sequencing and morphological analysis. Strains belonged to the following genera: **Penicillium, Aspergillus, Fusarium, Mortierella, Pestalotiopsis, Phoma, Paecilomyces** and **Rigidoporus**. Fifty percent of isolates exhibited halos in phytase screening agar indicating that acidic phytases are common enzymes secreted by environmental fungi. Ten isolates were also able to grow in liquid phytase screening medium revealing their potential use for enzyme production in submerged fermentations. Molecular detection of the **PhyA g**ene from **Aspergillus** was achieved. Partial sequence of the **phyA** gene from one **A. niger** isolate was obtained and analyzed.*

Key words: Molecular methods, phosphate, functional food, *Aspergillus*.

***Resumen**. Las fitasas son enzimas utilizadas como aditivo en productos de alimentación animal, con el fin de mejorar la asimilación de fósforo y minerales en animales monogástricos y disminuir la excreción de fósforo al ambiente. Los hongos filamentosos son una de las mejores fuentes de fitasas debido a su facilidad de cultivo y altos niveles de producción de enzimas extracelulares. Los principales productores de fitasas corresponden a miembros de los géneros **Aspergillus, Penicillium, Mucor** y **Rhizopus**. En este trabajo se reporta el aislamiento y caracterización de hongos ambientales productores de fitasas con aplicación potencial en la industria de alimentación animal. Se obtuvieron e identificaron un total de 26 aislamientos; caracterizados por secuenciación de la región ITS-ADNr y análisis morfológico. Los aislamientos pertenecieron a los siguientes géneros: P**enicillium, Aspergillus, Fusarium, Mortierella, Pestalotiopsis, Phoma, Paecilomyces** y **Rigidoporus**. Se observó la secreción de fitasas en 50% de los aislamientos sugiriendo la ubiquidad de esta enzima en hongos ambientales. Diez aislamientos crecieron eficientemente en medio líquido con fitato como única fuente de fósforo. Estos últimos cumplen con los requisitos para la producción de enzimas mediante fermentación sumergida. Se diseñaron cebadores para la detección molecular del gen **PhyA** en los aislamientos del género **Aspergillus**. Se obtuvo y analizó la secuencia parcial del gen **PhyA** de un aislamiento de **A. niger**.*

Palabras clave: Métodos moleculares, fosfato, alimento funcional, *Aspergillus*.

Phytate (myo-inositol 1,2,3,4,5,6-hexakisphosphate; IP6) is a phosphorylated derivative of myo-inositol important in the storage and retrieval of phosphorus, inositol and ions during plant development and germination (Raboy, 2003). The phosphorus fraction stored as phytate range from 30% in roots and up to 80% in seeds and cereals (Oh *et al*., 2004). Animal feed is primarily made out of oilseed meals, cereal grains and legumes which contain considerable amounts of phytate. Unfortunately, this phosphorous source is unavailable to monogastric animals like pigs and poultry due to the lack of appropriate enzymes (Vats and Banerjee, 2004). To compensate for phosphorus intake, animal feed is supplemented with inorganic

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phosphate, an expensive and scarce mineral. Phytate also acts as a chelator of metal ions preventing the normal intake of calcium, magnesium, zinc and iron (Murry *et al*., 1997). Finally, phosphorus from phytate can end up in manure, giving rise to serious environmental problems such as algal blooms and eutrophication of surface water in areas of intensive farming (Vats and Banerjee, 2004).

Phytases are enzymes used as feed additive that enhance the phosphorus and mineral uptake in monogastric animals and reduces the level of phosphate output in their manure. Degradation of phytate by phytase avoids the necessity to add inorganic phosphate and reduces the amount of phosphorus released to the environment (Haefner *et al*., 2005). Supplementation of swine and poultry diets with microbial phytases can improve significantly the bioavailability of phytate P and reduce P excretion. When added to pig and poultry feed, the normal addition of phosphate can be lowered considerably, and the amount of undigested phytate in the manure of the animals is reduced up to 30-60% (Vats and Banerjee, 2004). Cromwell *et al*. (1993) tested the efficacy of a microbial phytase produced by *Aspergillus niger* in corn-soybean meal or dextrose-corn starch-soybean meal-based diets and were able to prove that phytase is an efficient way of improving the bioavailability of phytate P for pigs. Addition of microbial phytase to diets for growing pigs can increase the apparent absorbability of P by 24% and reduce the amount of phosphate in feces by 35%. More recent studies have shown that addition of phytase improve the protein efficiency ratio of broiler chickens (Kong and Adeola, 2011). Lei *et al*. (2011) have shown that entire substitution of inorganic phosphorus by phytase can significantly increase bone strength, and Ca and P contents in tibia ash in birds. Phytase supplementation improves digestibility and retention of Ca, P and N in starter and growing pigs without any negative effect on pig performance (Columbus *et al*., 2010). Transgenic plants that excrete phytases into soil are also being investigated with a view to increasing the inorganic phosphate available for absorption (George *et al*., 2005).

Phytases are widespread enzymes and can be found in animals, plants, and microorganisms. However, due to their easy cultivation and high production of extracellular enzymes, filamentous fungi are one of best sources of phytase for use in the feed industry. Phytase has been found principally in the genera *Aspergillus, Penicillium*, *Mucor* and *Rhizopus* (Vats and Banerjee, 2004). Other phytase producing strains include *Cladosporium* species, *Myceliophthora thermophila, Emericella nidulans, Talaromyces thermophilus* and *Thermomyces lanuginosus* (Mitchell *et al*., 1997; Pasamontes *et al*., 1997). However, several studies have confirmed *Aspergillus* strains to be the best producers of extracellular phytase (Howson and Davis, 1983; Casey and Walsh, 2003; Vats and Banerjee, 2004; Xiong *et al*., 2004; Zhang *et al*., 2010). In this work, we report the isolation and characterization of environmental fungi producers of phytase from Antioquia with potential use as feed additives.

MATERIALS AND METHODS

Isolation of microorganisms. Fungi were isolated using various substrates like soil, grains cereals and fruits as shown in Table 1. A total of 19 fungal strains from soil were isolated using serial dilutions. Briefly, 1 g of soil from the municipality of Rionegro (Antioquia, Colombia) was dissolved in 9 mL of sterile distilled water and dilutions of 10^{-2}, 10^{-3} and 10^{-4} were plated onto sterilized potato dextrose agar (300 g potato, 20 g dextrose, 20 g agar per liter) containing 100 µg mL^{-1} of ampicillin and incubated at 25 °C in the laboratory for one week. Fungi growing on agar plates were subcultured in fresh PDA medium until pure colonies were observed. Seven environmental isolates from various locations in Medellín (Universidad Nacional de Colombia - Sede Medellín and district of Belen) were collected in moist chambers using 250 mL styrofoam cups with a layer of wet filter paper at the bottom. Grains (rice, wheat and a mix of wheat, barley and oat flakes) and fruits (lemon and orange) were used as substrate. These chambers were left open at the site of collection for one day and then closed and incubated at room temperature. Fungal growth was observed within one week of incubation. Isolation and purification was done on PDA plates as described above. A total of 26 fungal isolates were obtained in pure cultures by single spore transfer onto PDA plates and stored at 4 °C in the laboratory of Industrial Microbiology, Universidad Nacional de Colombia - Sede Medellín for further use. Isolates were preserved by immersing a holepunch of mycelium in 20% sterile glycerol and stored in -80 °C freezer. Isolated fungi were preliminarily identified on the basis of morphological characteristics using updated taxonomical keys (Seifert *et al*., 2011) and MycoBank (Crous *et al*., 2004).

Table 1. Description and identification of fungal isolates used to characterize native phytase producing fungi.

Code (origin)	Substrate	Morphological identification (Genus)	Highest score Blast hit (GenBank accession)	Identity (%)	H	L
MI001 (mu)	PSM agar	*Penicillium*	*Penicillium purpurogenum* (HQ839781)	570/570 (100%)	**-**	**ne**
MI002 (mb)	Orange	*Penicillium*	*Penicillium citrinum* (HM469428)	535/535 (100%)	**+**	**+**
MI003 (mb)	Cereal mix	*Aspergillus*	*Aspergillus flavus* (HQ340106)	568/568 (100%)	**+**	**+**
MI004 (mb)	Lemon	*Fusarium*	*Fusarium oxysporum* (HQ647333)	522/522 (100%)	**-**	**ne**
MI005 (mb)	Orange	*Aspergillus*	*Aspergillus niger* (HQ285563)	550/556 (99%)	**+**	**+**
MI006 (r)	Soil	*Mucor*	ND	ND	**+**	**-**
MI007 (r)	Soil	*Aspergillus*	*Aspergillus flavus* (HQ340103.1)	566/566 (100%)	**+**	**+**
MI008 (r)	Soil	*Mortierella*	*Mortierella* sp. (HQ608143)	593/593 (100%)	**+**	**+**
MI009 (r)	Soil	*Pestalotiopsis*	*Pestalotiopsis* sp. (HQ637299)	493/503 (98%)	**-**	**ne**
MI010 (r)	Soil	*Fusarium*	*Gibberella moniliformis* (HQ637284)	482/482 (100%)	**-**	**ne**
MI011 (r)	Soil	*Eupenicillium*	*Penicillium rolfsii* (HM043803)	565/566 (99%)	**+**	**+**
MI012 (r)	Soil	*Phoma*	*Dothideomycete* sp. (EU680549)	508/508 (100%)	**-**	**ne**
MI013 (r)	Soil	*Phoma*	*Phoma* sp. (HQ630963)	522/524 (99%)	**-**	**ne**
MI014 (r)	Soil	*Fusarium*	*Fusarium oxysporum* (HQ248198)	520/520 (100%)	**-**	**ne**
MI015 (mb)	Rice	*Aspergillus*	*Aspergillus niger* HQ014692)	601/601 (100%)	**+**	**+**
MI016 (r)	Soil	Ni	*Paecilomyces* sp. (GU319995)	584/587 (99%)	**-**	**ne**
MI017 (r)	Soil	*Fusarium*	*Fusarium* sp. (HQ025928)	523/523 (100%)	**-**	**ne**
MI018 (r)	Soil	*Phoma*	*Phoma* sp. (HQ630963)	534/536 (99%),	**+**	**+**
MI019 (r)	Soil	*Phoma*	*Phoma* sp. (HQ130716)	525/526 (99%),	**+**	**-**
MI020 (r)	Soil	*Paecilomyces*	*Paecilomyces carneus* (HQ660442)	575/575 (100%)	**+**	**-**
MI021 (r)	Soil	*Penicillium*	*Penicillium citrinum* (JF793520)	472/475 (99%)	**+**	**+**
MI022 (r)	Soil	*Aspergillus*	*Eurotium amstelodami* (HQ728257)	534/534(100%)	**-**	**ne**
MI023 (r)	Soil	Ni	Uncultured soil fungus (DQ420789)	529/540 (98%)	**-**	**ne**
MI024 (r)	Soil	*Penicillium*	*Penicillium waksmanii* (HQ608108)	473/474 (99%)	**+**	**+**
MI025 (mu)	PSM agar.	Ni	Uncultured fungus (GU721318)	519/519 (100%)	**-**	**ne**
MI026 (r)	Soil	Ni	*Rigidoporus* sp. (AJ537410)	607/609 (99%)	**-**	**ne**

Ni: not identified, only mycelia available for identification; **ne**: not evaluated; Isolates selected as phytase producers are shown in solid background; **H**: halo in PSM agar; **L**: growth in PSM liquid media; **mu**: Medellín at Universidad Nacional de Colombia; **mb**: Medellín at Belen; **r**: Rionegro.

Phytase screening. Screening was carried out on agar plates containing sodium phytate (4 g L^{-1}) as sole phosphorus source (Howson and Davis, 1983). Ten fungi were selected based on their ability to form halos. Positive results from the screening were confirmed by growing the fungi in PSM medium using submerged fermentation. Liter composition of PSM was: 20 g glucose, 4 g sodium phytate, 2 g $CaCl_2$, 5 g NH_4NO_3, 0.5 g KCl, 0.5 g $MgSO_4.7H_2O$, 0.01 g $MnSO_4.H_2O$ and 0.01 g $FeSO_4.7H_2O$. pH was adjusted to 5.5 by drop wise addition of 0.1M HCl. 30 mL aliquots were autoclaved using a Trident Medical horizontal autoclave, at 15 psi, 120 °C for 20 min, in 100 mL Erlenmeyer flasks, inoculated with a holepunch of mycelium and incubated for up to seven days at 30 °C in an Innova 4400 orbital shaker at 150 rpm. A positive control supplemented with an equivalent amount of sodium phosphate and a negative control lacking phosphorous. Preparation of controls was similar to the PSM media described above.

Molecular characterization. 26 isolates were grown on 2% malt extract (ME; 20 g malt extract in 1 L deionised water) for 12 days at room temperature. Mycelium was collected and macerated in liquid nitrogen. DNA was purified using the DNeasy Plant Mini Kit (Qiagen) using the manufacturers protocol. The ITS1, 5.8S and ITS2 regions of the ribosomal RNA operon were amplified using primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3) reported by White *et al*. (1990). The 50 µl polymerase chain reaction (PCR) mixture included 0.2 mM of each dNTP; 0.4 µM of each primer; 2 mM $MgCl_2$; buffer Fermentas 1X; 2.5 U of Taq polymerase (Fementas) and 5-10 ng of DNA template. PCR amplification consisted of an initial denaturation step at 98 °C for 3 min, followed by 35 cycles of 60 s at 95°C, 60 s at 55 °C and 70 s at 72 °C. Final chain elongation was achieved at 72 °C for 10 min. PCR products were separated in 1.5% agarose gel electrophoresis and then purified using Qiagen PCR purification kit for direct sequencing using an ABI PRISM Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, CA). Sequencing results were assembled using CONSED (Gordon *et al*., 1998). Each ITS sequence was used to perform BLASTN searches at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). Preliminary annotation of each isolate was based on the BLAST hit with maximun score to the query sequence. Phylogenetic affinities were determined for fungus considered positive for phytase production by comparison with sequences of related species retrieved from GenBank. Multiple alignments were generated using CLUSTAL (Thompson *et al*., 1994). Phylogenetic relationships were estimated using MEGA program Version 4.0 (Tamura *et al*., 2007). Phylogenetic trees were constructed using the Neighbor-Joining (NJ) algorithm with bootstrap values calculated from 1,000 replicate runs. The Maximum Composite Likelihood model was used to estimate evolutionary distance.

Phytase gene amplification. Due to the importance of *Aspergillus* as a phytase producer, a set of degenerate primers was designed to amplify a fragment of the *PhyA* gene. Primers were designed based on a multiple alignment of the following sequences (Uniprot accession in parentheses): *A. niger* (P34752), *A. fumigatus* (O00092) and *A. terreus* (O00085 and O00100). Sequences were obtained from UniProt, EXPASY (UniProt Consortium, 2008). Primers were obtained with CODEHOP using the protein alignments as input (Staheli *et al*., 2011) and were: PhyAspF, 5'-GACACCGTGGACCAGggntaycartg-3' (degeneracy = 16, Tm = 61.0 °C) and PhyAspR, 5' -GCAGATGCCGTGGTCCarngtrttrtt-3' (degeneracy = 32, Tm=63.0 °C). PCR was carried out in a volume of 10 µL with the same reagent concentration used for the amplification of the ITS regions. The PCR program consisted of an initial denaturation step at 98 °C for 3 min, followed by 35 cycles of 60 s at 95°C, 60 s at 41 °C and 60 s at 72 °C. Final chain elongation was achieved at 72 °C for 8 min. PCR products were separated in 1.5% agarose gel electrophoresis and sequenced as described.

RESULTS AND DISCUSSION

Preliminary screening. A total of 26 fungal strains were isolated and identified using ITS sequencing and morphological analysis (Table 1). Colonial morphology of all strains is shown in Figure 1. According to Blast strains belonged to the following genera: *Penicillium* (5), *Aspergillus* (4), *Fusarium* (3), *Mortierella* (1), *Pestalotiopsis* (1), *Gibberella* (1), *Phoma* (3), *Eurotium* (1), *Paecilomyces* (2), and *Rigidoporus* (1). Sequencing results were not available for isolate MI006. Blast hits for isolates MI023 and MI025 corresponded to uncultured fungus while MI012 was only identified as belonging to class *Dothideomycetes*. Morphological analysis of each isolate confirmed the Blast identification at the genera level for 17 strains (Table 1). Isolates MI010 and MI022 were identified as *Fusarium* sp. and *Aspergillus* sp., respectively. For these isolates Blast results coincided with their teliomorphic states: *Gibberella* and *Eurotium*, respectively. Isolate MI011 was observed and identified as *Eupenicillium*, which corresponds to the teliomorph state of *Penicillium* in the molecular identification. In spite of lacking DNA sequence information for isolate MI006, it was identified morphologically as *Mucor* sp. Morphological identification of isolates MI016, MI023 and MI026 was not possible as neither spores nor reproductive structures were observed.

The most common method for detecting phytase activity relies on the disappearance of precipitated sodium phytate as an indication of enzyme activity. In this assay, fungi secreting phytases produce clear zones on agar media containing sodium or calcium phytate (Shieh and Ware, 1968; Howson and Davis, 1983). According to this test, 50% of 26 isolates produced extracellular phytase (Table 1). Clear zones were observed for isolates belonging to five different genera: *Penicillium* (4), *Aspergillus* (4), *Paecilomyces*

(1), *Mortierella* (1), *Mucor* (1), and *Phoma* (2) (Figure 2). With the exception of MI022, MI023 and MI025, some strains showed significant growth but failed to show clear zones in PSM agar. However, sodium phytate was the only phosphorous source in the media and agarose was used instead of agar to guarantee that trace phosphate was maintained to a minimum. It is possible that some strains use intracellular or/and alkaline phytase, which will not form halos, to utilize this phosphorous source.

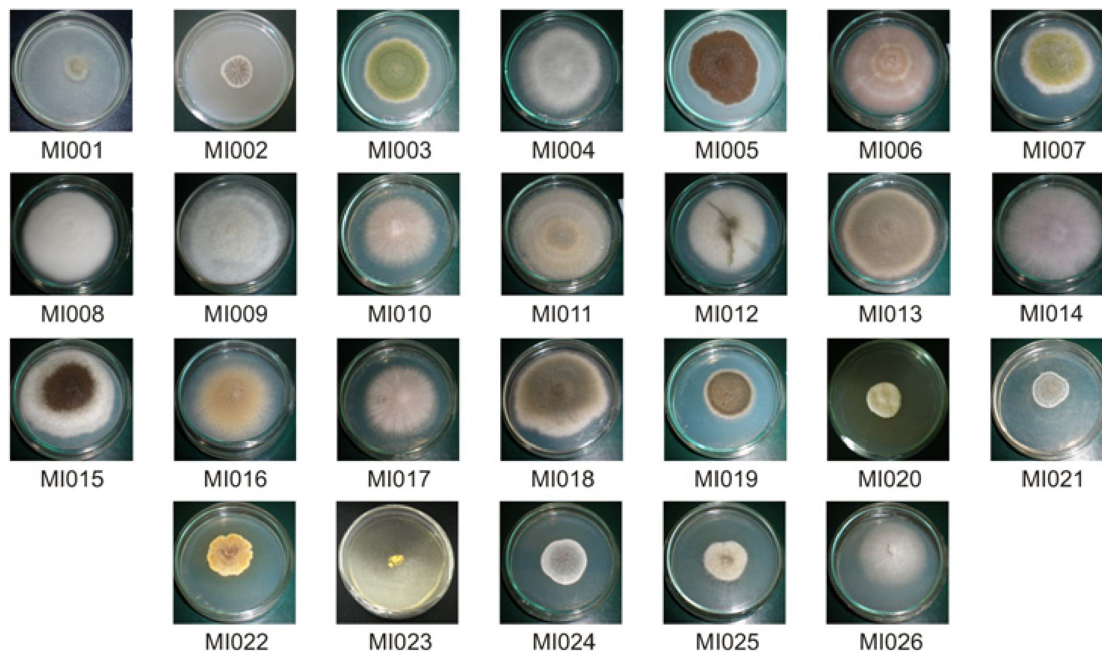

Figure 1. Colonial morphology of fungal isolates used to characterize native phytase producing fungi. See text and Table 1 for details.

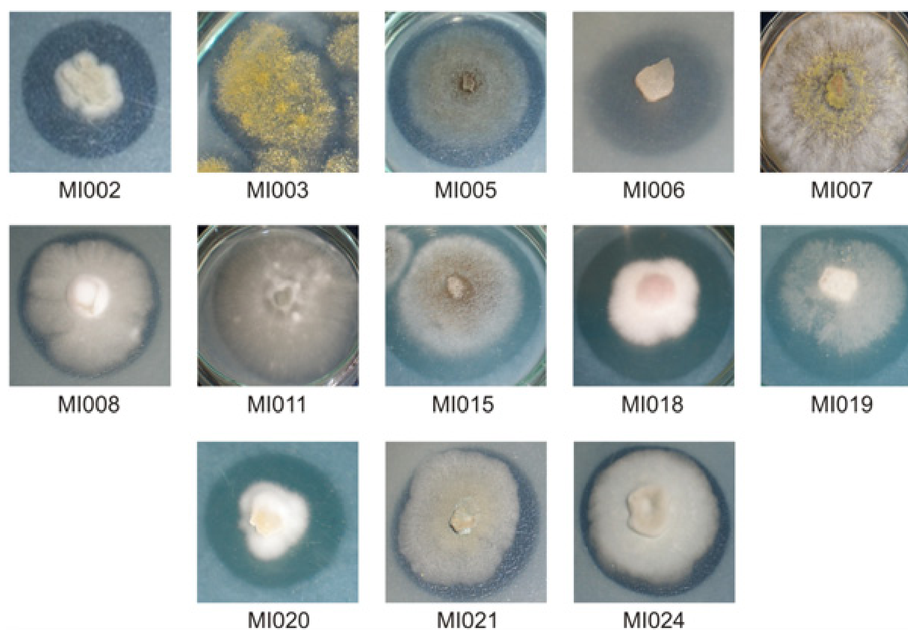

Figure 2. Halo producing strains in PSM agar. See text and Table 1 for details.

Growth in liquid media. It has been shown that PSM can give some false positives with acid producing strains (Bae *et al*., 1999). As useful phytase producers should grow efficiently in liquid media, a second screening was performed. Fungal growth in liquid PSM was compared to a negative control lacking any phosphorous source. According to this criterion, strains MI002, MI003, MI005, MI007, MI008, MI011, MI015, MI018, MI021 and MI024 were chosen as phytase producers of interest (Table 1). Phylogenetic analysis confirmed that these strains belonged to the genera *Phoma, Mortierella, Penicillium* and *Aspergillus*. Morphological features of selected strains are shown in Figure 3. Phytase producing strains.

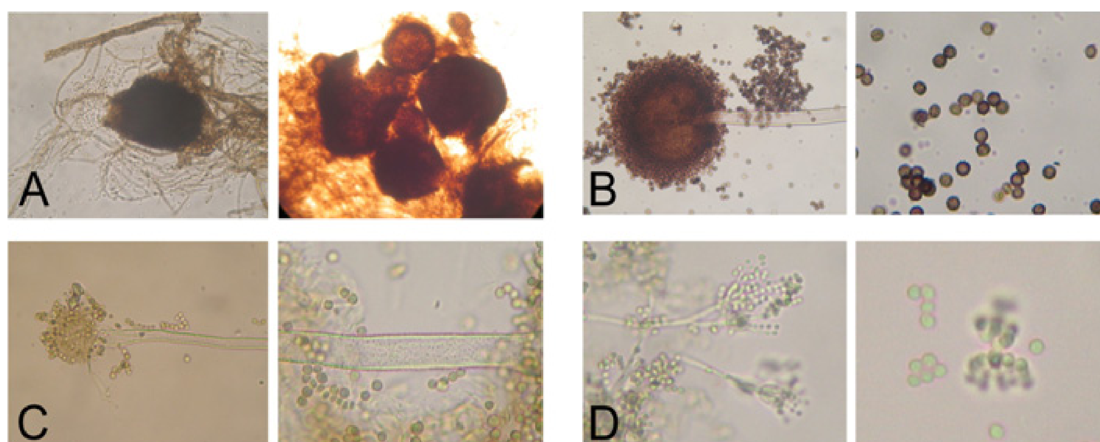

Figure 3. Morphological characteristics of some selected phytase producing fungi. (A) Isolate MI018, *Phoma* sp. (B) Isolate MI015, *Aspergillus niger.* (C) Isolate MI007, A*spergillus oryzae* (D) Isolate MI002, *Penicillium* sp.

Phoma. Isolates MI018 and MI019 formed halos in solid PSM but only MI018 showed significant growth in liquid media. The phylogenetic affinity for these strains is shown in Figure 3A. MI018 is closely related to *Phoma herbarum* and *Phoma macrostoma* (Figure 4A). *P. herbarum* is a ubiquitous saprophyte and versatile producer of many natural products such as gibberellins and haloquinones (Hamayun *et al*., 2009). *P. macrostoma* is a poorly studied fungus originally described by Westendorp in 1852 (Crous *et al*., 2004). Recent reports have shown this species to be a major producer of tyrosol (Tyagunova and Sorensen, 2010). *P. macrostoma* has also been shown to have bioherbicidal effects on weeds (Bailey and Derby, 2001). Interestingly, this is probably the first report suggesting phytase production by members of this genus

Mortierella. *Mortierella* isolate (MI008) produced halos in PSM agar together with significant growth in liquid PSM. Determination of the phylogenetic affinity at the species level was not possible due to the absence of well-curated sequences in GenBank. However, it is clear that MI018 belongs to *Mortierella* and not to the related genus *Umbelopsis* (Figure 4B). *Mortierella* is a fungus that belongs to the class Zygomycetes, commonly found in soil, rhizosphere and plants or animals that remains in contact with soil (Domsch *et al*., 1980; Webster and Weber, 2007). Some species are producers of polyunsaturated lipids of nutritional value such as arachidonic acid and have been used in the production of pharmaceuticals (Dyal and Narine, 2005). *Mortierella* can be isolated on nutrient-poor media and can grow well at 40-42°C (Austwick, 1976). So far there are no reports of phytases produced by *Mortierella*. No genes coding for phytase were found in GenBank either. However there exist some reports of phytase production by fungi of the order Mucorales, such as *Mucor piriformis* (Howson and Davis, 1983) and *Rhizopus oligosporus* (Casey and Walsh, 2004). It has been shown that rhizosphere isolates of *Mortierella* sp. can solubilize rock phosphate and increase the P content of mycorrhizal plants (Osorio and Habte, 2001). Similar results were observed by Zhang *et al*., (2011) who demonstrated that *Mortierella* sp. is an efficient phosphate solubilizing fungus that can significantly affect plant growth. It would not be surprising that phytase is part of the enzymatic pool of this phosphate solubilizing fungus. Genome

sequencing projects of *Mortierella alpina* ATCC 32222 and *M. verticillata* are underway (http://www.ncbi.nlm.nih.gov/sites/genome). These projects will give great insight on the phosphate metabolism of these organisms and could confirm phytase production by this soil fungus.

Figure 4. Phylogenetic affinities of *Phoma* (A) and *Mortierella* (B) isolates. GenBank accession number in parentheses.

Penicillium. *Penicillium* has a prominent industrial use due to its ability to produce large quantities of extracellular enzymes (Webster and Weber, 2007). It comes as no surprise that four out of five *Penicillium* isolates, MI002, MI011, MI021 and MI024, were considered potential phytase producers. Phylogenetic affinities as deduced from NJ analysis are shown in Figure 5A. MI0011 and MI0024 were identified as *P. rolfsii* and *P. decaturense*, respectively. Unfortunately, sequence analysis of strains MI002 and MI021 was not able to resolve their affinity to either *P. citrinum* or *P. griseofulvum*. Identification at the species levels for isolates MI002 and MI021 will require more detailed analysis. *P. rolfsii* is a poorly studied species originally described in 1930 by Thom (Crous *et al.*, 2004). There are no current reports on the biotechnological application of this fungus. *P. decaturense* is a species isolated from wood-decay and recently described by Peterson *et al*. (2004).

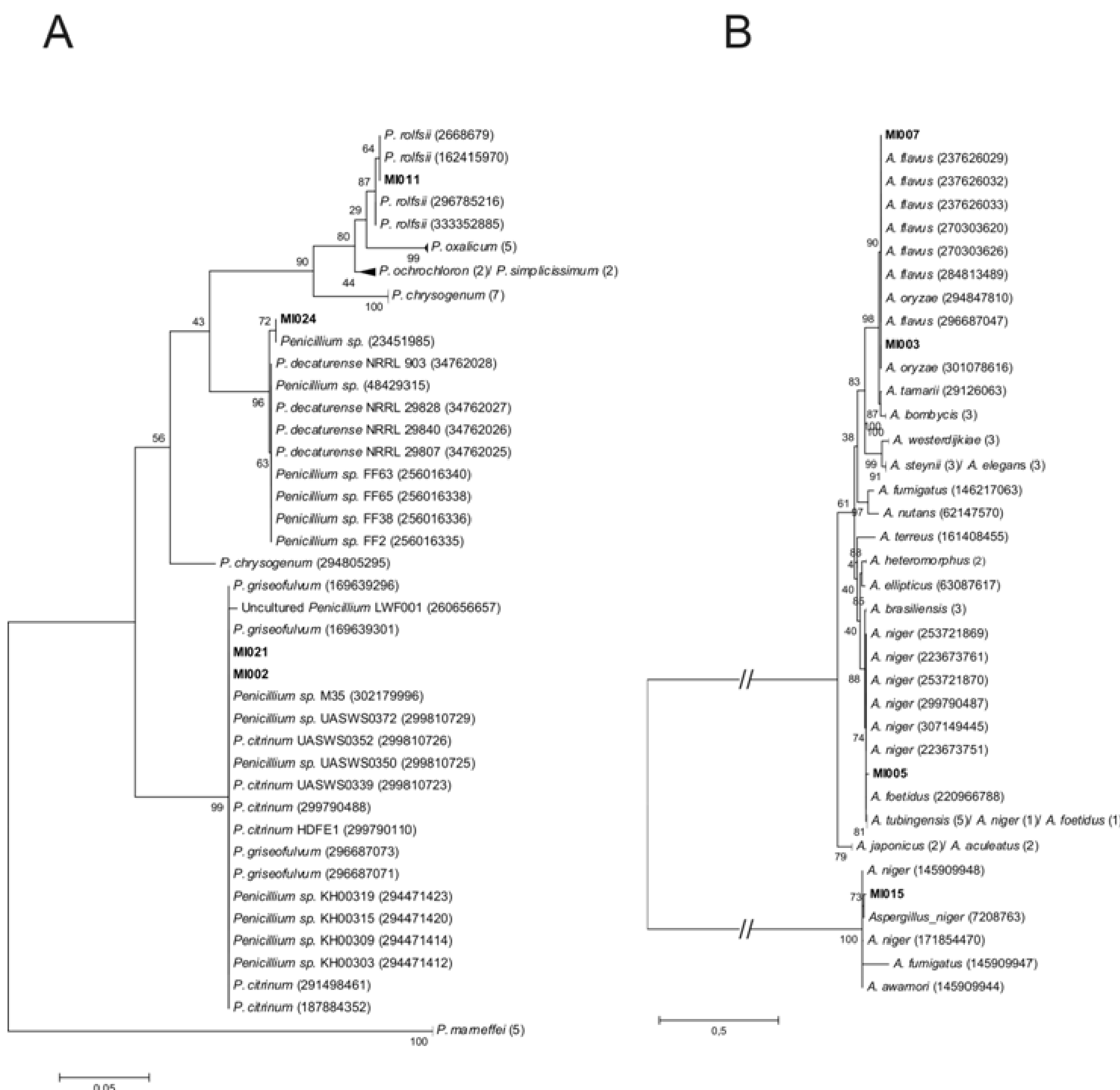

Figure 5. Phylogenetic affinities of *Penicillium* (A) and *Aspergillus* (B) isolates. GenBank accession number in parentheses.

This species is of interest as it can be the source of novel bioactive insecticidal compounds (Zhang *et al*., 2003). *P. citrinum* is a common endophytic fungus of cereal plants like wheat and soybean studied for its production of citrinin and secondary metabolites (Wakiyama *et al*., 2008). *P. griseofulvum* has been associated with blue mold decay in storage apple fruits (Moslem *et al*., 2010), production of metabolites with potential activity against cancer cells (Wang *et al*., 2009) and inhibitors of diacyglycerol acyltransferases (Lee *et al*., 2008). A search for *Penicillium* phytases in GenBank retrieved only six results: *P. oxalicum* (AY071824, HM053476), *P. chrysogenum* (XM_002561048), *P. marneffei* (XM_002148785, XM_002147131) and *Penicillum* sp. Q7 (EF197827). However, none of our isolated species have been studied for phytase production.

The first detailed report on the isolation and characterization of a *Penicillium* phytase dates back to 2000 (Tseng *et al*., 2000). After screening eighty-three isolates from different soil samples the most active fungal isolate with phytase activity was identified as *P. simplicissimum*. The purified enzyme showed an

optimum pH and temperature of pH 4.0 and 55 °C, respectively. This phytase revealed broad substrate specificity and was strongly inhibited by Fe^{2+}, Fe^{3+} and Zn^{2+} (Tseng *et al*., 2000). Lee *et al*. (2007), characterized a novel fungal phytase from *P. oxalicum* PJ3, cloned its gene and studied the expression in *P. pastoris*. The pH optimum of the purified enzyme was 4.5 and an optimal temperature of 55 °C. In a more recent work, Zhao *et al*. (2010) isolated a protease-resistance phytase gene of *Penicillium* sp. and successfully isolated mutants with improved thermal stability and optimal temperature and pH. The authors suggest that these improved properties make these engineered enzymes more suitable to be used as feed additive in the feed industry than the present commercial phytases.

There are few detailed investigations on the application of *Penicillium* phytases. Kozlowski *et al*. (2009), tested the species *P. canescens* phytase in broiler rations with considerably reduced levels of phosphorus and showed that the process of bone mineralization was enhanced by phytase supplementation. Supplementation of pig diets with *Penicillium* phytase increased the apparent total tract digestibility of phosphorus and calcium by 22.6 and 18.3 percent, respectively (Steinera *et al*., 2006). The effect of *P. purpurogenum* phytase in enhancing the mobilization of native unavailable phosphorus was evaluated in arid environments. A significant improvement in plant biomass (30%), root length (21%), P uptake (6%), seed (19%) and straw yield (30%), and P concentration of shoot (15%), root (6%), and seed (33%) resulted from inoculation of *P. purpurogenum* (Yadava and Tarafdara, 2011). In spite of the limited studies available on *Penicillium* phytases, these results suggest interesting applications for this kind of enzymes. It would be interesting to investigate further the biochemical properties of phytases produced by isolates MI002, MI011, MI021 and MI024.

Aspergillus. This genus is the most widely used by industry for the production of enzymes and organic acids (Webster and Weber, 2007). *Aspergillus* phytases have shown a series of desirable features such as thermal stability, substrate specificity and activity at low pH. For this reason the majority of commercial phytases originate from this group (Vats and Banerjee, 2004). In our study, four out of five *Aspergillus* isolates showed potential phytase activity: MI003 (*A. flavus*), MI005 (*A. foetidus*), MI007 (*A. flavus*) and MI015 (*A. niger*). Phylogenetic affinities for the *Aspergillus* isolates as deduced from NJ analysis are shown in Figure 5B. Strains MI003 and MI007 were classified as *A. oryzae* instead of *A. flavus*. However, the former is considered to have resulted from the domestication of *A. flavus*. A comparison of both genomes confirmed this hypothesis and concluded that these two fungi may be ecotypes of the same domestication of *A. flavus*. In nature, *A. flavus* is capable of growing on many nutrient sources. It is predominately a saprophyte and grows on dead plant and animal tissue in the soil. *A. flavus* can infect seeds of corn, peanuts, cotton, and nut trees (Payne *et al*., 2006). *A. foetidus* has been used in the production of enzymes such as xylanase, alpha-galactosidase, amylase and tannase (Michelena and Castillo, 1984; Purohit *et al*., 2006; Liu *et al*., 2007; Valte *et al*., 2010).

There are several published reports of phytase production by species of the *Aspergillus genus*: *A. niger* NRRL 3135, *A. flavus*, *A. terrus*, *A. carneus*, *A. oryzae* and *A. fumigatus* (Shieh and Ware, 1968; Shieh *et al*., 1969; Oh *et al*., 2004). However, the most widely used phytases are isolated from *Aspergillus niger* (Xiong *et al*., 2005), *A. fumigatus* (Pasamontes *et al*., 1997) and *A. ficcum* (Mitchell *et al*., 1997). A database search in GenBank demonstrated the presence of phytase in *A. awamori*, *A. clavatus* NRRL, *A. ficuum*, *A. flavus* NRRL3357, *A. niger* var *awamori*, *A. niger* CBS 513, *A. oryzae*, *Aspergillus* sp. A25 *A. terreus* and *A. usamii* strain N-2418. Using these sequences, a set of degenerate primers was designed in this study to detect *Aspergillus* phytases. PCR amplification gave bands with the expected size (~500 bp) for isolates MI003, MI005 and MI015 (Figure 6A). To confirm that this PCR product corresponded to the phytase gene, the 500 bp from MI0015 was sequenced. A Blastx search of the sequenced amplicon revealed that this sequence shared 99% nucleotide identity with *PhyA* gene from *A. ficuum* (AF537344.1) and 98% with *PhyA* from *A. niger* CBS 513.88 (XM_001401676.2). Multiple alignment of the protein sequence of MI015 phytase revealed its high similarity with *A. ficuum*, *A. niger, A. oryzae* and *A. terreus* phytases (Figure 6B). It is important to note that *A. niger PhyA* is the active ingredient of Natuphos™(BASF animal nutrition), the commercial phytase most commonly used as additive in animal feed (Wodzinski and Ullah, 1996).

There are three main types of phytases in *Aspergillus*: PhyA, PhyB and alkaline phytases. PhyA and PhyB phytase belong to the family of histidine acid phosphatases (HAP) characterized by a RH(G/N)

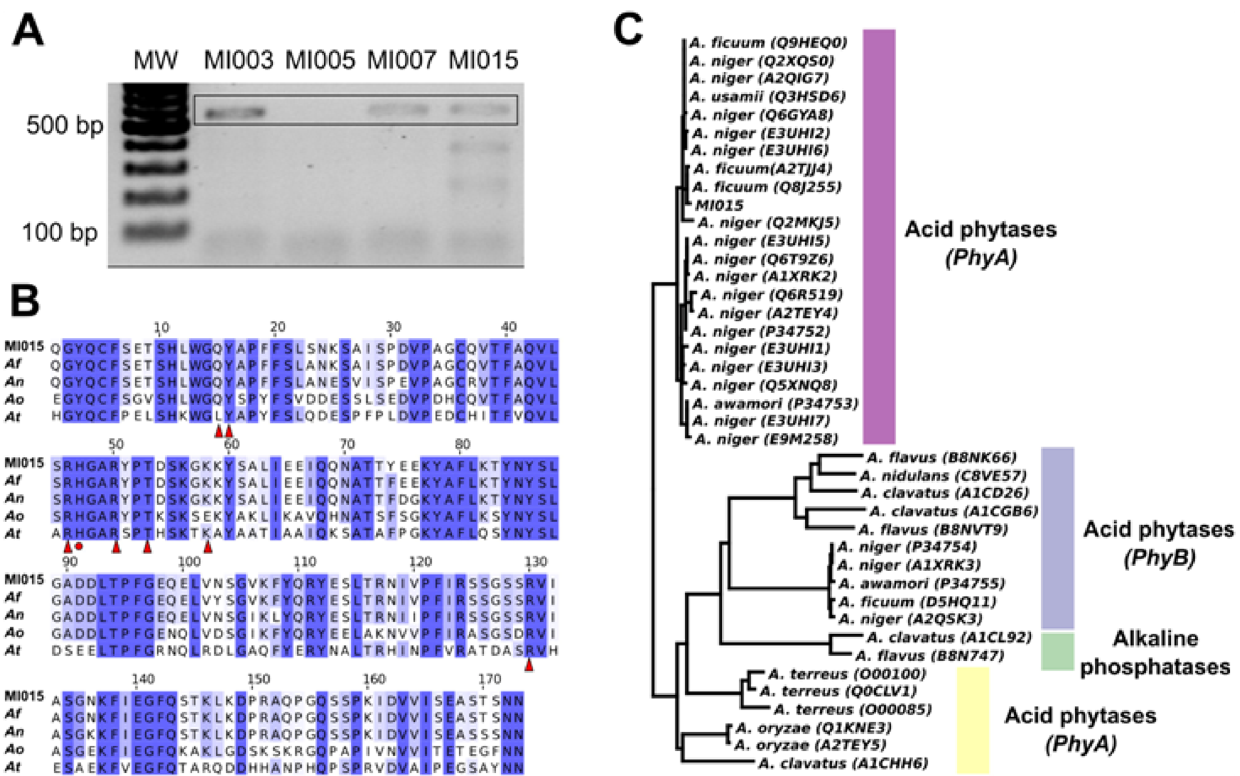

Figure 6. Molecular characterization of the *PhyA* gene from isolate MI015. (A) DNA amplification of the *PhyA* from selected *Aspergillus* isolates. (B) Aminoacid sequence alignment of phytase from MI015 with homologues from *A. ficuum* (Af), *A. niger* (An), *A. oryzae* (Ao) and *A. terreus* (At). Triangles indicate residues that contact phytate. A circle indicates the catalytic histidine. (C) Neighbor joining analysis of phytase MI015 with reference sequences from the genus *Aspergillus*. Swiss-prot accesion code in parentheses.

XRXP motif, unique to this enzyme class (Oh *et al*., 2004). *PhyA* phytases are active in a wide pH range (2.5-5.0) while *PhyB* are capable of hydrolyzing phytate quite efficiently at pH 2.5 but have very little activity at pH 5.0 (Ullah and Phillipy, 1994). HAP can hydrolyze metal-free phytate at acidic pH. Alkaline phytases have an optimum pH of 7.0-8.0 and can hidrolyze phytate in complex with metal cations (Oh, *et al*., 2004). Phylogenetic analysis confirmed the classification of MI015 phytase as a *PhyA* histidine acid phytase (Figure 6C), a desirable condition for future biotechnological applications.

CONCLUSIONS

To our knowledge, this is the first systematic study aimed at the isolation of phytase producing fungi in Colombia. The present investigation suggests that phytase producing fungi can be readily isolated from soils and grains. Fifty percent of all our isolates exhibited halos in phytase screening agar indicating that acidic phytases are a common enzyme secreted by environmental fungi. Ten isolates were also able to grow in liquid phytase screening medium revealing their potential use for enzyme production in submerged fermentations. These results encourage further studies aimed at the isolation and biochemical characterization of native phytase producing fungi as a preliminary step in their use as feed additives and production at industrial scale.

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