# Production, Characterization, and Molecular Phylogenetic Analysis of Phytase from *Aspergillus niger* Isolates of an Indonesia Origin

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## ABSTRACT

This research aimed at analyzing the phytase from fungal isolates SB1, SB2, BS, and WF produced in cornstarch with glucose medium (CS+Glu) as carbon sources and Potatoes dextrose broth (PDB). The activity of phytase from isolates SB1, SB2, BS, and WF produced in CS+Glu medium was 2.97 UmL$^{-1}$, 2.87 UmL$^{-1}$, 3.18 UmL$^{-1}$, and 4.37 UmL$^{-1}$, respectively, while the activity of phytases was 2.07 UmL$^{-1}$, 2.17 UmL$^{-1}$, 2.22 UmL$^{-1}$, and 2.78 UmL$^{-1}$ respectively in PDB medium. The optimal temperature of SB1 and WF phytase was 40°C, while SB2 and BS were 50°C and 60°C, respectively. The optimal pH of SB1 and WF phytase was 5.0, while SB2 and BS phytase were 6.0, and 4.0 respectively. 18S rRNA gene analysis revealed that SB1 was 99% identical to *Aspergillus niger* ANTS (KY825168.1), SB2, BS, and WF were 99% identical to *A. niger* Moriga leaf (MG889596.1). Multiple sequences and phylogenetic analysis of phytase gene showed that phyA_SB1 and phyA_SB2 were 98% homology with *A. ficuum* (AAB26466), 97% with *A. niger* (ADP05107) while phyA_WF was 99% with *A. ochraceoroseus* (PLB29348), 98% with *A. niger* (ADP05105). The deduced proteins contain conserved motifs RHGARYPTD at N-terminal while lacking HD motif at C-terminal. These phytases were in the same cluster with *Aspergillus* sp. phytase A indicating that they belong to Histidine Acid Phosphatases (HAP) family.

## 1. Introduction

Phosphorus is an essential mineral required for growth, development, and many other vital roles in animal metabolism, especially during the formation of macromolecules such as DNA, RNA, energy formation in the form of ATP and bone formation, mainly during the embryo stage (Connelly 2011). Plant-based foods or feedstuff such as cereal grains, oilseeds, some fruits, and vegetables are important sources of phosphorus for monogastric animals like fish, poultry, pig, and human (Connelly 2011). These plant-based foods/feeds contained more than 80% of the total phosphorus stored in phytate form, which is indigestible and unavailable for these animals because of the low level of phytate-degrading enzymes in their digestive tracts (Marlida *et al.* 2010). Phytate is a *myo*-inositol 1, 2, 3, 4, 5, 6 hexayl-dihydrogen phosphate (IP6) which contains a phosphate group at each carbon in the *myo*-inositol ring (Akhmetova *et al.* 2015). The

higher ability to form a complex with minerals and other food nutrients is due to a high negative charge of a phosphate group at each carbon in the *myo*-inositol (Sajidan *et al.* 2004; Li *et al.* 2015). This property makes phytic acid forms a complex with other minerals such as Ca$^{2+}$, Mg$^{2+}$, Zn$^{2+}$, Fe$^{2+}$ and food nutrients such as protein, amino acid, and starch, a process which forms phytate compound hence reducing the nutrient's availability for monogastric animals (Choi and Kim 2017). In this case, the nutritional value of plant-based food or feedstuff decreases, while the unassimilated phytate excretes into the environment causing serious environmental pollution especially in an area with intense livestock keeping and crop productions (Sariyska *et al.* 2014). On the other hand, phytase is *myo*-inositol hexayl-phosphate phosphohydrolase, which catalyzes phosphate release from phytate, forming *myo*-inositol and inorganic phosphate (Greiner and Konietzny 2010). Based on its catalytic mechanism, phytase is classified as Histidine Acid Phosphatases (HAP), Beta Propeller Phytase (BPP), cysteine/Tyrosine-protein like phytase (TPP) or purple acid phytase (PAP); phytase. Based on carbon location

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in the *myo*-inositol ring of phytate where phytase initiate the release of phosphorus as 3-phytase (EC 3.1.3.8), 6-phytase (EC 3.1.3.26) and 5-phytase (EC 3.1.3.72), which initiate dephosphorylation at 3, 6, and 5 respectively of the carbon position in the myo-inositol ring (Singh *et al.* 2011; Sarsan 2013). Phytase releases the phosphate group from phytate, forming various isomers like *myo*-inositol pentakis, tetrakis, tris, bis, and monophosphates (Yi *et al.* 1996). In addition, other mineral ions like calcium, iron, magnesium, zinc or macronutrients such as protein and lipids are released by phytase making them available to monogastric animals (Mittal *et al.* 2013; Vashishth *et al.* 2017). The ability of microbes to degrade phytate and release phosphorus from phytate in the soil is because of their ability to produce phytase, which helps reduce environmental pollutions (Greiner and Konietzny 2006). Thus, to improve animal growth and development, utilization of phytase in animal nutrition to enable monogastric animals to utilize the minerals therein, and at the same time reduce the pollution that could occur due to the disposal of phosphate-containing wastes into the environments.

*Aspergillus niger* has extensively been reported in various literature for its potential metabolites producing abilities. This fungus is eukaryotic, filamentous and appears black or dark brown spore, characters which differentiate it from other filamentous fungi. *A. niger* produces three phytases classified based on their catalytic properties, namely phyA, phyB and phyC or alkaline phytase (Wyss *et al.* 1999).

The phytase production by filamentous fungi has been achieved using different methods such as solid-state fermentation by using solid substrates such as wheat bran, rice bran, and groundnut cakes (Neira-Vielma *et al.* 2018). Other methods include semi-solid state and submerged fermentation using diverse substrates. Given the increased demand for phytase enzymes to mitigate environmental pollution and improve uptake of nutrients by monogastric animals, it is now essential to establish low-cost and locally available substrates that will maximize the production of phytase enzyme cost-effectively from *A. niger*.

Therefore, in this study, we aimed at producing phytase enzyme from *A. niger* by sub-merged fermentation method using a cheap and locally made Cornstarch medium. This paper also reports the biochemical characterization and phylogenetic analysis of the phytase and phytase-producing *A. niger* isolates from Indonesian environments.

## 2. Materials and Methods

### 2.1. Media, and Fungal Isolates

The *A. niger* used in this study were isolated *A. niger* sp. using Potatoes Dextrose Agar (Merck, Germany) from black and white soybeans (*Glycine max* (L.) *Merril*), and Sorghum (*Sorghum bicolor*) as described by (Marlida *et al.* 2010). The *Aspergillus* species isolates with the best phytase producing ability were selected and confirmed based on morphological features on the PDA medium and confirmed by molecular methods.

To produce phytase enzyme, spore suspension was prepared using 2% of tween 80. One ml of 1 x $10^7$/ml spore suspension were grown in sterilized (15 psi, 121°C, 20 minutes) 100 ml of Cornstarch +Glucose medium with the following constituents; 8% Cornstarch (CS); 1% of glucose(Glu); 0.18 g $FeSO_4 \cdot 7H_2O$; 5% of $NH_4NO_3$; 0.5 g KCL; 0.2 g; 0.5 g $MgSO_4 \cdot 7H_2O$; 0.01 g of $MnSO_4 \cdot 4H_2O$; 0.4% Na-phytate and 30 mg/ml of ampicillin as well as in PDB medium containing 0.4% sodium phytate and 30 mg/ml of ampicillin 1% glucose; 0.1% $NH_4NO_3$; 0.05% KCl; 0.04% $MgSO_4 \cdot 7H_2O$; 0.03% $MnSO_4 \cdot 4H_2O$; 0.03% $FeSO_4 \cdot 7H_2O$, pH 5.5. The cultures were incubated in a shaking incubator (150 rpm) at 30°C, for 7 days. Crude phytase enzymes were obtained by centrifugation at 3000xg at 4°C for 5 minutes. The obtained supernatants were used as a source of phytase and were kept at 4°C for further experiments.

Phytase activity assay was determined by measuring the amount of released inorganic phosphates according to Ammonium molybdate methods as described by (Heinonen and Lahti 1981). One unit of enzyme activity was defined as the amount of phytase required to release one μmole of inorganic phosphate from sodium phytate per minute under assay conditions. A calibration curve was prepared using $K_2H_2PHO_4$ (0.2, 0.4, 0.6, 0.8, 1.0, and 1.4 conc/mg-L.

### 2.2. Physio-chemical Characterization

In this assay, different temperatures ranged from 28 to 90°C was used. The enzyme and substrate in 0.1 M of sodium acetate at pH 5.5 were incubated at 28°C, 30°C, 37°C, 40°C, 50°C, 60°C, 70°C, 80°C, 90°C for 30 minutes followed by phytase activity assay. pH was optimized using different buffer solutions at a pH range from 3.0 to 9.0. The phytase enzyme was incubated with Na-phytate prepared in 0.1 M Sodium acetate for pH 3.0-5.0, 0.1 M Tris-HCl buffer for pH 6.0-7.0, and 0.1 M Tris-HCl buffer for pH 8.0-9.0 at 37°C

for 30 minutes, and the liberated inorganic phosphate were measured.

## 2.3. Extraction of Fungal Genomic DNA

Fungal genomic DNA was isolated by taking 100 mg of 5 days mycelium grown in PDB with the conditions as described above. The mycelia were frozen in liquid nitrogen, pulverized to a fine powder using mortar and Pestle. The mycelia were transferred into 1.5 ml Eppendorf tubes. DNA was extracted using Geneaid plant DNA extraction kits (Geneaid, Taiwan) as per manufacturer protocol.

## 2.4. Amplification and Sequencing of 18S rRNA and PhyA Gene

The 18S rRNA gene was amplified using My Taq$^{MT}$ HS red mix (Bioline, Germany) using a pair of primers that targets the 18S rRNA gene. The forward primer PF5'(GGAAGGG [G/A] TGTATTTATTAG-3') and reverse primer- PR5'-(TCCTCTAAATGACCAAGTTTG-3') (Gontia-Mishra and Tiwari 2013) were used to amplify the 18S rRNA gene on Verity 96-well Thermal cycler (Thermo Fisher Scientific, USA). The amplification was performed in 50 µl PCR reaction mixtures, which contained 25 µl PCR mix, 20 mm of forward and reverse primers, 2.25 µl of DNA template, and 18 µl deionized water to bring the final volume to 50 µl. Conditions of amplification were: (i) 95°C for 180 s; (ii) 35 cycles of 95°C for 10 s, 48°C for 10 s and 72°C for 120 s; (iii) 72°C for 600 s.

As per manufacturer protocol, myTaq$^{TM}$HS red master mix (Bioline, Germany) was used to amplify phytase genes from gDNA of the isolates SB1, SB2, BS, and WF. A pair of degenerate primers: PhyAspF, 5'-GACACCGTGGACCAGggntaycartg-3' degeneracy = 16, Tm = 61.0°C and PhyAspR, 5-GCAGATGCCGTGGTCCarngtrttrtt-3' (degeneracy = 32 reaction Tm = 63.0°C) were used to amplify phytase gene as described (Ocampo *et al.* 2012). The PCR reaction conditions were as follows: (i) 95°C for 10 s, ii) 35 cycles of 95°C for 10 s, 41°C for 10 s and 55 for 10 (iii) final extension at 72°C for 10 minutes. Both PCR 18S rRNA and phyA products were subjected to 1.5% (w/v) Agarose (Thermo Fisher Scientific, USA) in 50 ml 1x TAE buffer (Merck, Taufkirchen Germany) and were sequenced.

## 2.5. Sequence and Phylogenetic Analysis

Both forward and reverse DNA sequences of 18S rRNA were assembled using Bioedit software. The obtained sequences were used to perform BLASTn search at National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov). Phylogenetic analysis was performed using Molecular Evolutionary Genetic Analysis software 7 (Kumar *et al.* 2016). A phylogenetic relationship was inferred using the neighbour–joining method (Saitou and Nei 1987) using 500 replications. The evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.* 2004).

The obtained phytase gene sequences were analysed and first converted into protein sequences using the ExPASy Bioinformatics Resources portal (Gasteiger *et al.* 2003). The obtained frame 1-protein sequences were used to perform a BLASTp search at NCBI. Multiple sequence alignments against phytase protein sequences from gene bank were performed using Clustal W to find the conserved nucleotide sequences (Hall 1999). The evolutionary analysis was performed on MEGA7 (Kumar *et al.* 2016), the evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The evolutionary distances were computed using the Poisson correction method with 1,000 replications. The N-glycosylation site's prediction was done using NetNGlyc server (Gupta *et al.* 2002). Furthermore, superfamily and conserved domains were analyzed using InterPro Scan (Jones *et al.* 2014) and NCBI conserved domain Server (Marchler-Bauer *et al.* 2017).

## 3. Results

### 3.1. Isolation, Morphological Characterization of *A. niger*

We selected five isolates that were identified as *Aspergillus* sp. The colonies appeared to proliferate on PDA medium and appeared powdery in texture. After two days of incubation, the colonies appeared white and turned brown to black on the fifth to sixth day as conidia structure formed (Figure 1). Microscopic observations of the colony showed hyaline, septate hyphae, and asexual conidiophores, which appeared long and globosely at the tip as shown in Figure 1.

### 3.2. Phytase Activity, Temperature, and pH Optimization

Phytase activity of isolates SB1, SB2, BS and WF on cornstarch and glucose (CS+Glu) PDB media are shown in Figure 2. The findings show a slight difference in the activity of phytase induced by cornstarch and glucose, which is higher than activity phytase than the activity
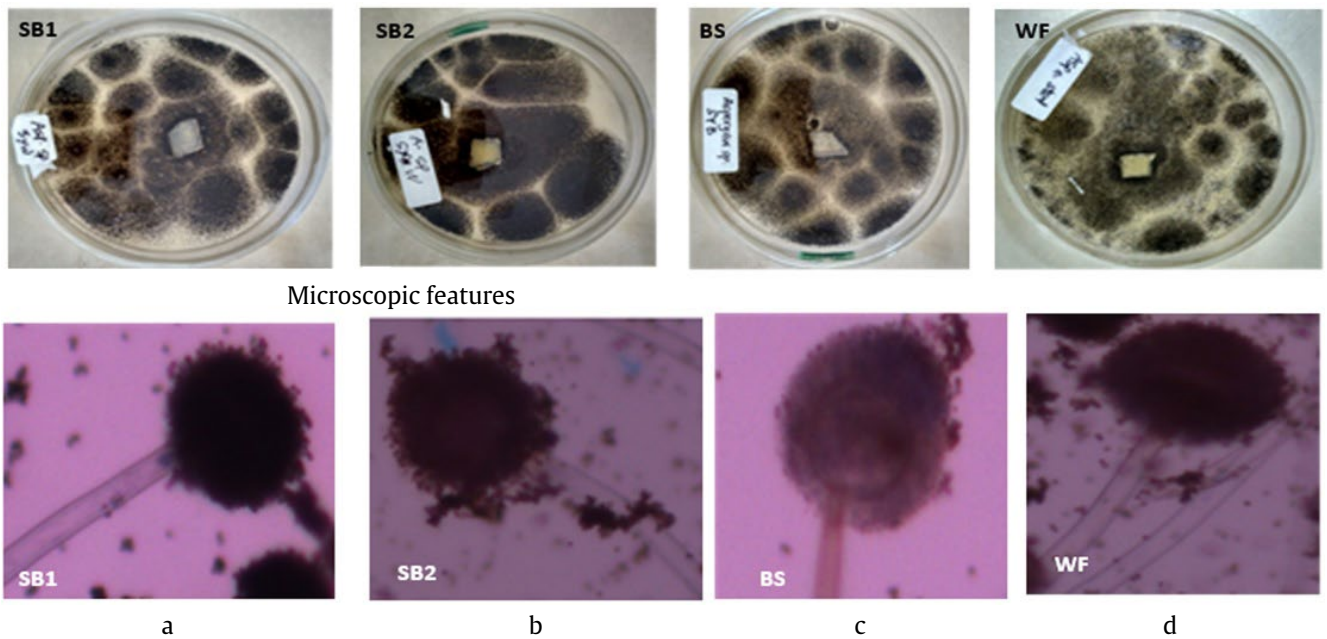
Microscopic features

Figure 1. *A. niger* isolates from three different sources showing asexual conidiophores (a) SB1, (b) SB2, (c) BS, (d) WF
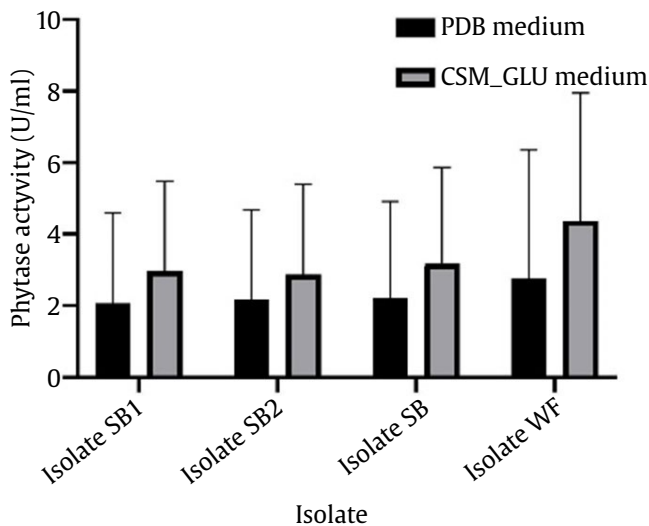


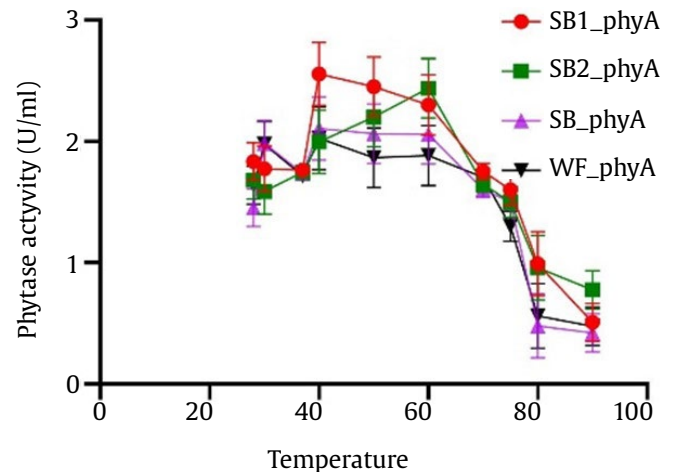Figure 2. Phytase activity from isolates SB1, SB2, BS, and WF



Figure 3. The effect of temperature on the activity phytase from an isolate, SB1 and SB2 isolates from white soybean, BS-isolates from black soybean, WF-isolates from sorghum flour

of phytase in the PDB medium. Isolates WF produced phytase with the highest activity both in medium containing CS+Glu and in PDB with a phytase activity of 4.37 UmL$^{-1}$ and 2.78 UmL$^{-1}$, respectively than other isolates.

Temperature and pH optimization was performed to determine the optimal temperature and pH of phytase. The optimum temperature was determined at 28, 30, 37, 40, 50, 60, 70, 80 and 90°C (Figure 3). The enzymes retained the activity at a temperature range 30 to 60°C but no activity was observed beyond 80°C. The optimal temperature of phytase from isolates SB1 and WF were 40°C, and 50°C for SB2 phytase. On the other hand, the optimal temperature of BS phytase was 60°C as shown in Figure 3. The highest phytase activity for isolates SB1 and WF was observed at pH 5.0 while SB2 and BS phytase were 4.0 and 6.0 respectively, as shown in Figure 4. Phytase enzymes from all isolates were observed to retain the activity at a pH range of 3.0 to 8.0 (Figure 4).

## 3.3. Sequence and Phylogenetic Analysis of 18S rRNA Sequences

The 18S rRNA gene was amplified using a pair of specific primers. The results confirmed the amplification of 1.5 kb PCR products of 18S rRNA

sequences from isolate SB1, SB2, BS, and WF (Figure 5).

Based on similarity search results, the isolate MH041196(SB1) was 99% identical with *A. niger* strain ANTS (Acc no: KY825168.1), isolates MH041197(SB2) and MH041201(WF) showed 99% sequence homology with *A. niger* isolate Moriga leaf (Acc no: MG889592.1) and isolate BS, were 100% identical with *A. niger* Moriga leaf (Acc no: MG889592.1). Phylogenetic analysis based on 18S rRNA indicated that the four isolates formed two clades (100% bootstrap support, isolates SB1, SB2, BS and WF were in the first clade together, showing common ancestors with *A. niger* and *A. awamori*. The first clade separated to form three sub-clade, isolate WF was close to *A. niger* (with 90% bootstrap support),

while isolate SB1, SB2, and BS were closely related to each other and formed another single subclade (90% bootstrap support) which appear small cluster out of *A. niger* subclade shown in Figure 6. The second clade is *A. awamori, A. fumigatus,* and *A. heteromorphous* clusters. The last is clade consists of the outgoing group. The phylogenetic tree shows that all isolates are closer to *A. niger* than to other fungal species like *A. fumigatus* and other fungal sp such as *Acremonium flavum Geosmithia perilla* and *Purpureocillium*, which were used as an outgroup. The phylogenetic tree based on 18S rRNA is as shown in Figure 7. The partial 18S rRNA sequences were deposited in National Center for Biotechnology Information (NCBI) under accession number MH041196, MH041197, MH041198, MH041201.

### 3.4. PCR Amplification and Sequencing of phyA

The partial phytase amplicon with total nucleotide bases of approximately 532,528 and 530 bp isolates SB1, SB2, and WF were. However, no amplification was observed for *A. niger* isolate BS and WF. The failure to amplify the phytase gene in isolate BS could be due to the low quality of the DNA caused by the degradation of the DNA sample The amplified PCR products in 1.5% Agarose gel are as shown in Figure 7. The amplified gene sequences were deposited in the NCBI database under accession number as follows: SB1_phyA (SAMN20866469), and SB2_phyA (SAMN20866470) and WF_phyA(SAMN20866471). All sequences can also be accessed in the Entrez
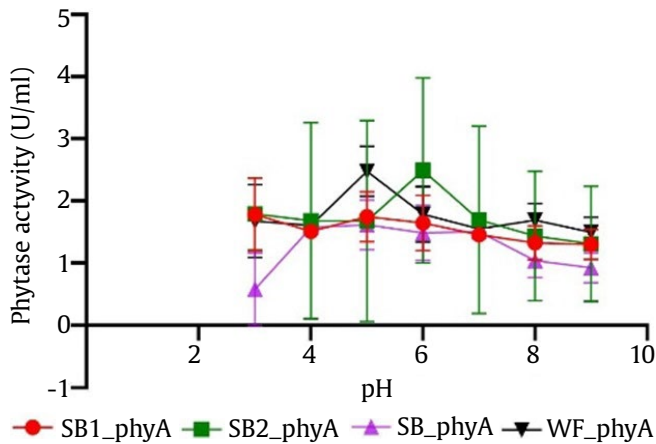


Figure 4. The effect pH on the activity of phytase from isolates SB1 and SB2-isolates from white soybean, BS-isolate from black soybean, WF-isolate from sorghum flour
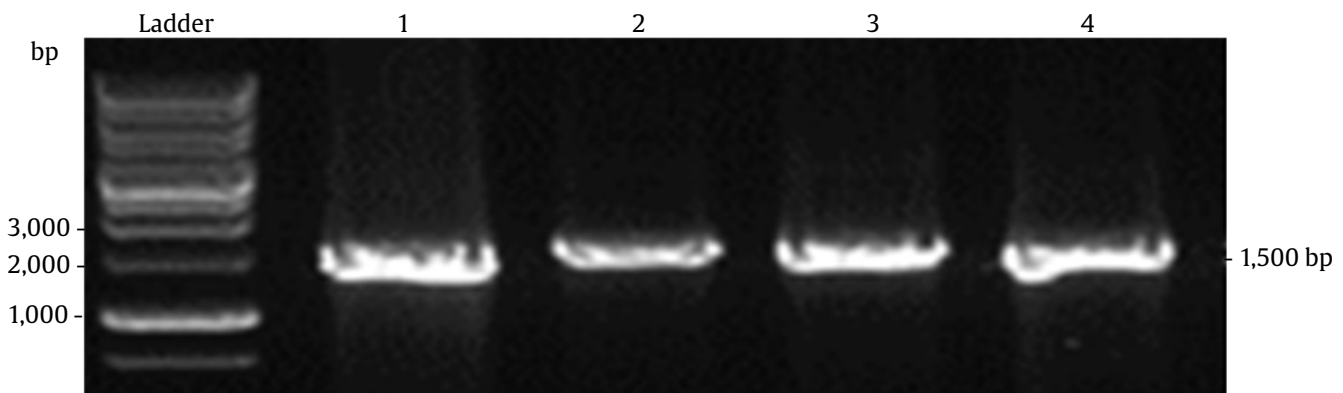


Figure 5. PCR amplification of 18S rRNA gene: 1 kb DNA ladder, 1-isolate SB1, 2-SB2, 3-BS, and 4-WF
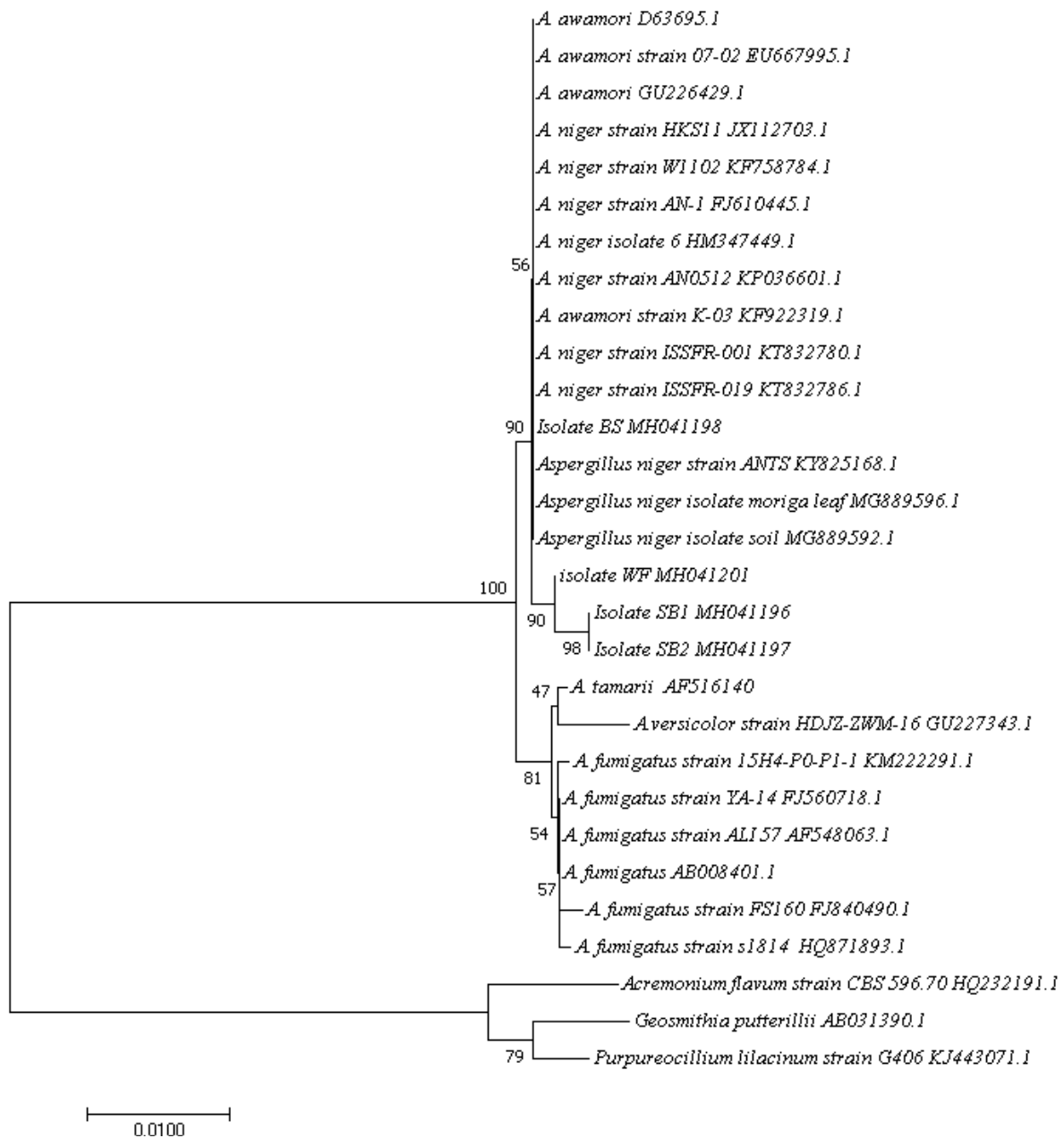
Figure 6. Phylogenetic evolutionary relationship of isolates SB1, SB2, BS, and WF. The number at the branching point indicates bootstrap values
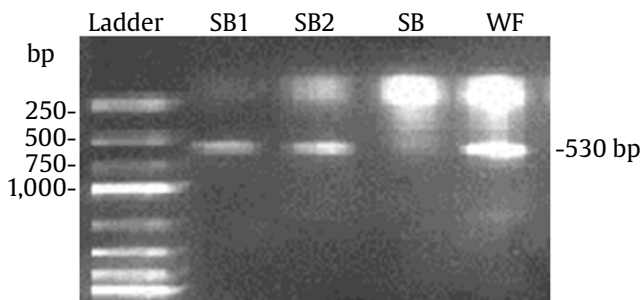


Figure 7. PCR amplification of phyA gene showing Ladder DNA and PhyA gene from isolates SB1, SB2, and WF

Molecular sequence database system under the single Bio-project accession number PRJNA756453.

### 3.5. BLASTp Analysis and Multiple Sequences Alignments

The frame 1 putative amino acid of phytase sequences of SB1 and WF were 177 and 176 amino acids. BLASTp analysis based on protein sequences revealed that the amplified phytase genes are PhyA in which phyA_SB1and SB2 were 98% homology with *A. ficuum* (Acc AAB26466) and 98% homology with *A. niger* phytase A, while phyA_WF was 98% similar with

*A. niger* (ADP05105.1) *A. japonicus* (ACE79228.1), and 99% with *A. ochraceoroseus* (PLB29348.1). In addition, multiple sequence alignments were performed to find a conserved motif in the DNA sequences. The results are as shown in Figure 8.

### 3.6. Phylogenetic Analysis Based on Phytase Protein (PhyA)

The phylogenetic inference of the amino acid sequences of *A. niger* is as shown in Figure 9. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The phylogenetic tree was inferred to find the phylogenetic relationship of the amino acid sequence from this study against 29 amino acid sequences

retrieved from GenBank. The phylogenetic tree of the amino acid sequences, as it can be observed in Figure 9 has three main clusters of phyA, phyB and Alkaline phytase, which seem to share one common ancestor as shown in Figure 9.

### 3.7. Functional Annotation of PhyA-SB1, PhyA-SB2 and PhyA-WF

NCBI domain analysis revealed that the phyA_ and SB2_phyA are members of the Histidine phosphatase superfamily (Accession number: cl11399) having histidine domain (cl11399) is phosphorylated during the reactions. Furthermore, the InterPro scan of the amino acid sequences showed that the proteins are Histidine Phosphatase superfamily, clade-2 (IPR000560).
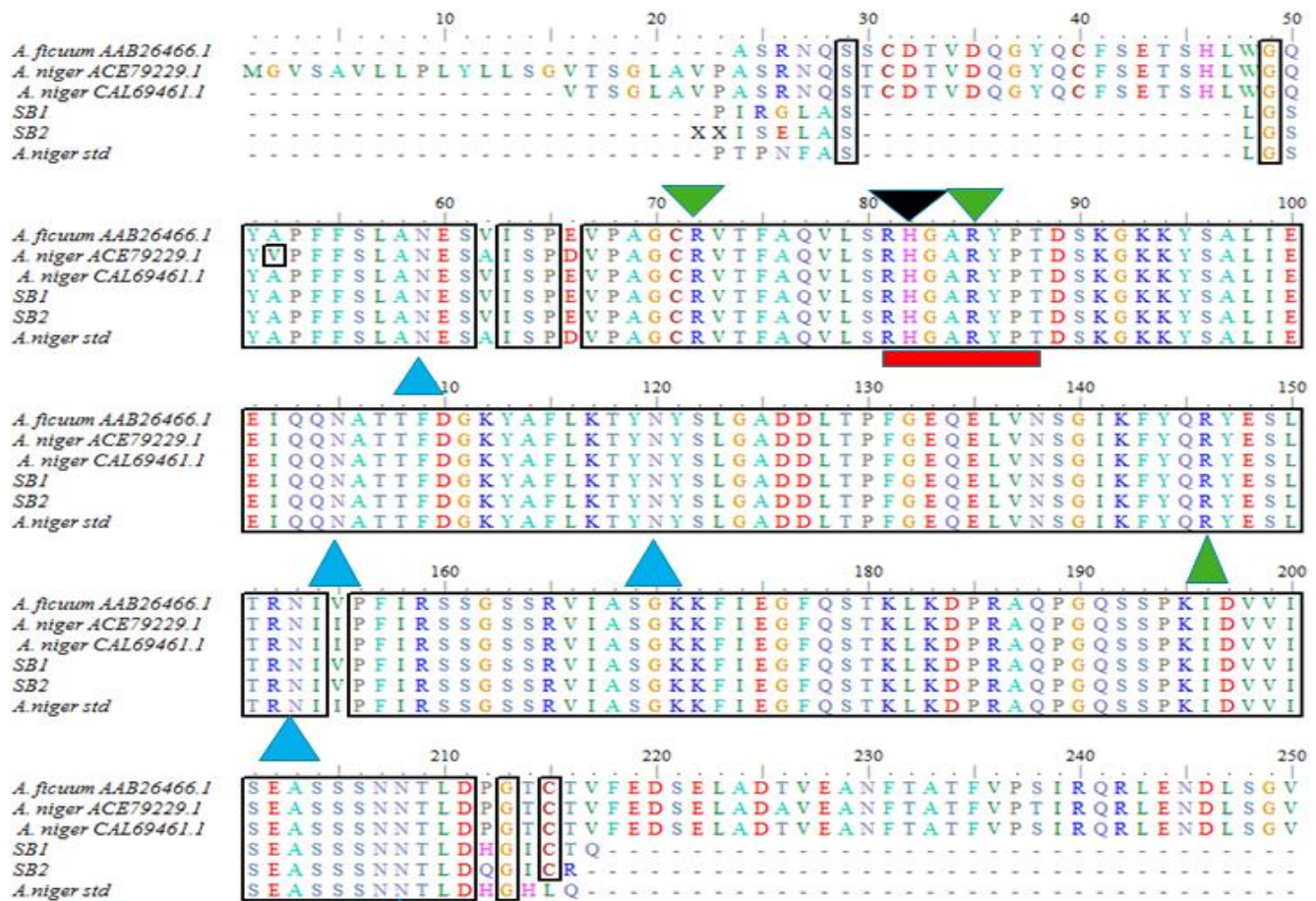


Figure 8. Multiple sequence alignments of phytase showing N-glycosylation sites (blue triangle), R active sites residue that contacts phytate (green triangle), catalytic Histidine (black triangle), and the conserved signature motif of Histidine acid family is marked in red
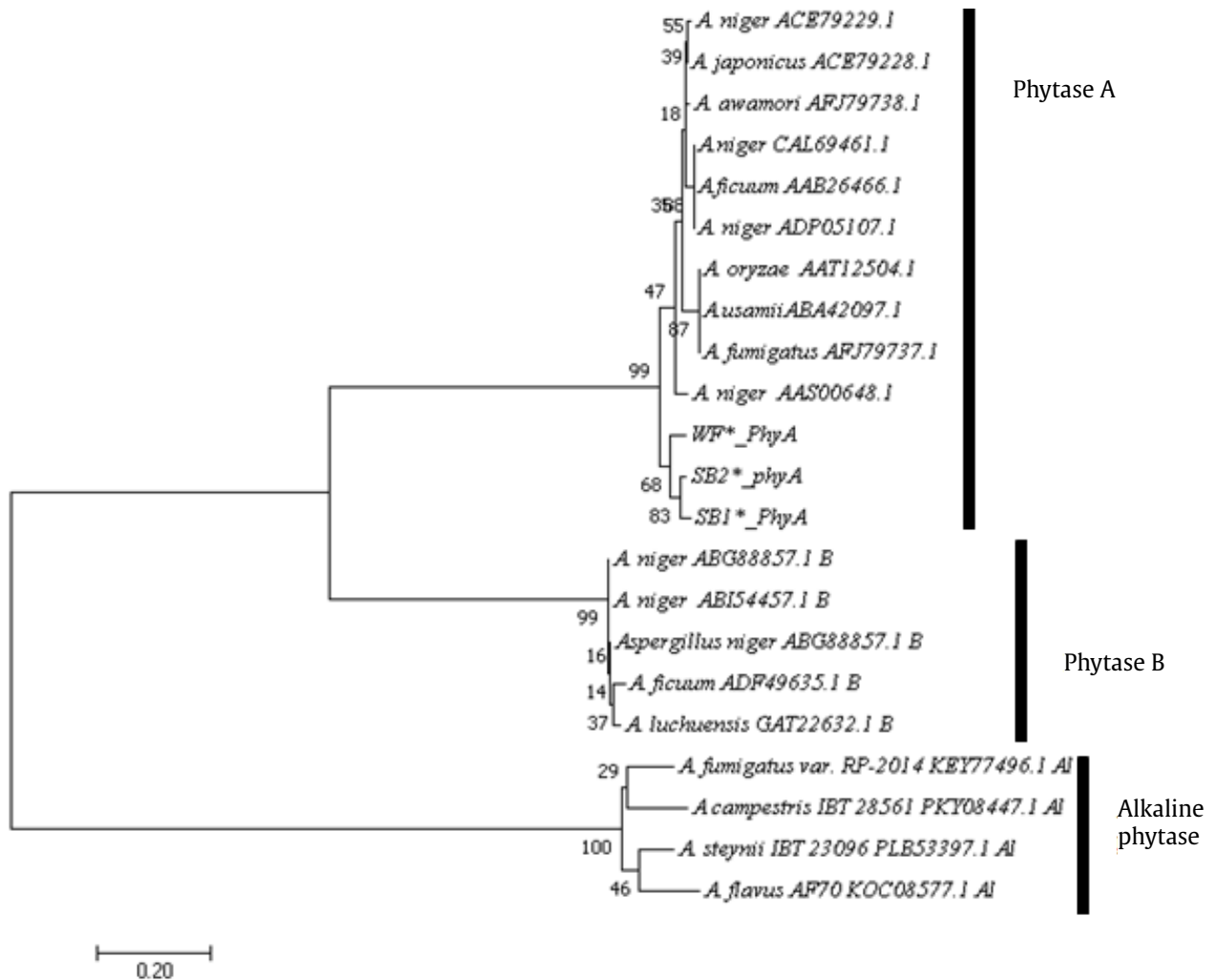
Figure 9. Phylogenetic evolutionary relationship of phytase protein of isolates SB1, SB2, WF. The number at the branching point indicates bootstrap values

## 4. Discussion

A phytase is a group of phosphatase enzymes, which work to improve mineral availability and reduce the environmental pollution caused by an impact of phosphate release in the soil.

This enzyme has been reported from different sources like plants, animals, and the primary source being from a microorganism. Successful phytase production from filamentous fungi depends on several factors, including media components such as carbon, nitrogen, and phosphate, which can either enhance or inhibit its production. In this study, we have produced phytase enzyme from *A. niger* isolates from four isolates SB1 (MH041196), SB2 (MH041197), (MH041198), and WF (MH041201) by using a locally available Cornstarch medium using submerged fermentation method and all isolates showed the ability to produce phytase. Molecular characterization

and phylogenetic analysis confirmed the phylogenetic position of all the phytase produced by these isolates.

The results from this study attempt to discuss the activity of phytase produced in two different media, the first media contained 8% local cornstarch with 1% of glucose. In contrast, the second medium contained only 1% glucose as a carbon source for phytase enzyme production from isolates SB1, SB2, BS, and WF. In comparison, the second medium was made of PDB, phytate, and ampicillin, which were added to inhibit the growth of contaminating bacteria in both media. Accordingly, the results revealed that the optimal phytase production was observed when combined cornstarch was supplemented with 1% glucose which was used as inducing substrate. Comparably, the phytase produced in combined cornstarch and glucose showed the highest extracellular phytase activity (2.97 UmL$^{-1}$, 2.87 UmL$^{-1}$, 3.18 UmL$^{-1}$, and 4.37 UmL$^{-1}$) than phytase produced without cornstarch

or PDB (2.07 UmL$^{-1}$, 2.17 UmL$^{-1}$, 2.22 UmL$^{-1}$, and 2.78) for isolates MH041196, MH041197, MH041198, and MH041201 respectively. Once their activity was compared, the phytase enzyme from isolate MH041201 both using CS+Glu medium and PDB showed a higher phytase activity. These findings agree with the previously reported phytase in various literature. For example, Susana *et al.* 2000 combined cornstarch and glucose to produce phytase from *A. niger* NRRL3135 and *A. niger* 320, and the phytase produced had an activity of 2.808UmL$^{-1}$ and 1.520 UmL$^{-1}$, respectively (Susana *et al.* 2000). However, the phytase activity is relatively lower than the activities of phytase from this study. The activity of phytase reported in this study also agrees with the finding by Vat and Banerjee, who observed the optimal phytase production when the medium contained starch and glucose of  3%  and 1% respectively were used (Vats and Banerjee 2002).

The physio-chemical properties of the phytase produced in our study also agree with the previously reported studies. Phytase from all isolates maintained its activity at 30 to 60°C and seemed to lose activity at a temperature beyond 60°C. The phytase's optimal temperature of 40°C for isolates SB1 and WF is similar to fungal phytase-like phytase from *Penicillium oxalicum* (Kaur *et al.* 2017). The optimal temperature of SB2 and BS phytase of 50°C and 60°C respectively is similar with an optimal temperature of phytase from *A. fumigatus*, which exhibit an optimal temperature of 50°C, and *A. niger* 307 phytase, whose optimal temperature is 55°C and 58°C (Sariyska *et al.* 2014). Likewise, the  *Rhizoctonia* sp. phytase and *Fusarium verticillioides* phytase whose optimal temperatures are 50°C and 60°C respectively, are in good agreement with an optimal temperature of BS phytase (Marlida *et al.* 2010). The phytase from these four isolates seems to have a slightly higher optimal temperature than some of the reported fungal phytase. For instance, the pH from *A. heteromorphus* has optimum temperature at 30°C (Lata *et al.* 2013), but lower than the phytase from *P. chrysonegum* whose optimal temperature ranges between 70-80°C (Nakagi *et al.* 2013). The optimal pH of phytase from all isolates ranged between 4.0 to 6.0 and phytase from all isolate lost activity at pH beyond 6.0. SB1 and WF phytase enzymes' optimal pH was 5.0 while the optimal pH of isolates SB2 and BS was 6.0 and 4.0 respectively. The pH profile of phytase from these fungal isolates agree with the previously reported phytase and are within the optimal pH range of  *Aspergillus* sp. phytase (Wyss *et al.* 1999; Soni *et al.* 2010; Sariyska *et al.* 2014). Nevertheless, SB2 phytase has the highest optimal pH than phytase among all four isolates, similar to *A. hetermorphus* MTCC10685 (Lata *et al.* 2013) and

*Aspergillus fumigatus* (Suresh and Das 2014) phytase. The optimal pH of phytase SB1 and WF are similar with phytase from *A. niger* (Neira-Vielma *et al.* 2018) and *Rhizoctonia* sp. (Marlida *et al.* 2010).

Analysis of the amino acid sequences of an amplified gene aims to identify the conserved motifs and domain of phytase from isolates SB1, SB2 and WF. The presence of conserved signature motif RHGARYPTD at the N-terminal of the amino acid sequences indicates that the phytase from these isolates belongs to Histidine phosphatase (Wyss *et al.* 1999). However, unlike Histidine Acid Phosphatase (HAP), the amino acid sequences lack the conserved HD motif at C-terminal (Gontia-Mishra *et al.* 2014). This could be because the amplified phytase gene sequences were partial and hence sequencing the complete phytase gene from these isolates is required. Moreover, the amino acid sequences from *A. niger* isolate SB1 and SB2 are glycosylated at four different sites at 19 NESV 65 NATT 80 NYSL and 167 NNTL on the contrary, the amino acid sequence of WF phytase only had 3 N-glycosylation sites at 19 NESV, 65 NATT, and 80 NYSL. The number of N-glycosylation in the amino acid sequences of phytase in this study is lower compared to the ten N-glycosylation sites of the normal HAP (Gontia-Mishra *et al.* 2014). Glycosylation is important phytase as it helps to determine ad maintain the structure of Histidine Acid phosphatase. Fungal phytase such as *A. niger* phytase is heavily glycosylated with ten N-glycosylation sites identified as NXS/T (Gontia-Mishra and Tiwari 2013) which influence enzymes molecular weight and protein thermostability (Niño-Gómez *et al.* 2017). Also, the amino acid sequence of phyA_SB1 and SB2 have two conserved cysteine residue at a position 71 and 215 for phyA_SB1 and phyA_SB2 while, phyA_WF phytase has one conserved cysteine at the position 71 the cysteine number which is lower compared with the eight cysteines found in HAP phytase (Gontia-Mishra *et al.* 2014).

Phylogenetic relationship analysis based on the amino acid sequences as represented by a phylogenetic tree in Figure 9 intends to find the phylogenetic position and evolution history of the isolated species and amplified phytase. All isolates are similar to previously reported *A. niger* such as *A. niger* isolates soil (Acc: MG88592.1), *A. niger* isolate Moriga leaf (Acc no: MG889596.1) and *A. awamori* (GU226429.1) as they can be seen to aggregate in a single clade on the phylogenetic tree (Figure 6). The phytase from four isolates formed three main clusters indicating to have similar origin or ancestors. The first main clusters supported by 99% bootstrap consist of *Aspergillus* sp. producing phytase A. Phytase from isolates SB1, SB2 and WF are similar to phytase

A produced by other *Aspergillus* species such as *A. niger* (Acc no: AAS00648.1), *A. fumigatus* (Acc no: AF579737.1), *A. ficcum* (Acc no: AAB26466.1), *A. ausam* (Acc no: ABA42097.1) and other *Aspergillus* species such as *A. japonicas* (Acc no: ACE79228), *A. awamori* (Acc no: AF579738) indicating that the amplified phytase gene are highly similar with phytase A gene from *Aspergillus* species.

To summarize, we have reported and characterized the phytase enzyme from *A. niger* isolates SB1, SB2, and WF. The phytase-producing abilities of these isolates provide a future alternative for phytase production in Indonesia. These isolates produce phytase with properties that make them suitable for use in animal nutrition. The phytase activities observed in this study were even more superior than some of the phytase activities reported previously in the literature. In addition, based on the phytase activity in this study, we suggest that the combined cornstarch and glucose medium could be used as an essential medium for phytase production.

Moreover, the amplified phytase genes from isolates SB1, SB2, and WF are partial, however, they still showed to be highly similar to *Aspergillus* sp. phyA and based on biochemical properties and presence of conserved Histidine motif, indicate that the phytase from isolates SB1, SB2 and WF belongs to a family of Histidine Acid Phosphates (HAP). This study was limited by the time, therefore we recommend further studies that will involve conditions optimization, sophisticated methods to purify the phytase from these isolates. We also recommend amplifying the complete gene and, recombinant DNA technology can be employed to enhance phytase production.

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