Vol.58, n.5: pp. 711-717, September-October 2015
http://dx.doi.org/10.1590/S1516-89132015050173
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Phytase-Producing Bacteria from Extreme Regions in Indonesia

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ABSTRACT

In this study, 154 isolates capable of producing extracellular phytate-degrading activity were isolated from four soil samples from volcanic areas in Central Java, Indonesia. Six strains with high phytate-degrading activity were selected for strain identification and characterization of the corresponding phytate-degrading enzyme. Blast analysis of 16S rRNA gene sequences revealed high similarities for all the six isolates to reference sequences belonging to the genus Bacillus. *Isolates MS5, MC6, D10 and D16 showed 99% sequence identity to* B. cereus, *while isolate MC8 exhibited 99% sequence identity to* B. aryabhatti *and D6 99% sequence identity to* B. psychrotolerans. *The crude extracellular phytase preparations from the isolates showed following optimal conditions for phytate dephosphorylation: pH 4.0 and 50°C (isolate D10), pH 5.0 and 60°C (isolate MC6, and isolate MS5), pH 6.0 and 50°C (isolate D16) and pH 6.0 and 60°C (isolate D6) and pH 6.0 and 40°C (isolate MC8). Zn^{2+} and Fe^{3+} strongly inhibited phytate dephosphorylation with all phytase preparations studied. In the presence of Ca^{2+}, an increase in phytase activity of 10-15% was obtained.*

Key words: 16S rDNA, *Bacillus* sp.; bacterial phytase; phytate; phytate-degrading enzyme

INTRODUCTION

Phytate [*myo*-inositol (1,2,3,4,5,6) hexakisphosphate] is the major storage form of phosphate in plant seeds and grains (Konietzny and Greiner 2002). Therefore, phosphate is predominately organically bound in plant-based food and feed. It was reported that about 30% of the phosphate present in plant-based feed exist in its free form and the remaining 70% in form of phytate (Kembhavi 2005). Due to is high negative charge under physiological conditions, phytate forms strong complexes with cations such as proteins, amino acids, minerals and trace elements, and thus renders them less available for absorption (Weaver and Kannan 2001). In addition, monogaster are not capable of dephosphorylating phytate during the digestion due to the lack of significant amounts of endogenous phytate-degrading activity in their stomach and small intestine (Iqbal et al. 1994). To ensure the optimal growth of animals such as pigs and poultry, animal feed must be supplemented with inorganic phosphate. However, rock phosphate is a non-renewable natural resource and it might become scarce in the near future (Abelson 1999; Ashley et al. 2011). Furthermore, the phytate reaching the large intestine of monogaster is either

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dephosphorylated by the intestinal microflora or excreted via the feces and dephosphorylated by soil and water microorganisms. Therefore, high amounts of phosphate are released into the environment in the areas of intense life-stock management. The resulting pollution of ground and surface waters causes algae blooming, a decrease in oxygen levels, and death of aquatic animals (Shin et al. 2001).

Phytases are enzymes catalyzing the stepwise release of phosphate residues from the *myo*-inositol ring of phytate (Jorquera et al. 2008). These enzymes are ubiquitous in nature and have been reported from more than 2000 microbial soil isolates. Phytases are a diverse group of enzymes that encompass a range of sizes, structures and catalytic mechanisms. Based on the catalytic mechanism, phytases can be referred to as histidine acid phytases, β--propeller phytases, cysteine phytases or purple acid phytases (Greiner and Konietzny 2006). Depending on their pH optimum, phytases have been divided into acid and alkaline phytases, and based on the carbon in the *myo*-inositol ring of phytate at which dephosphorylation is initiated into 3-phytases (E.C. 3.1.3.8), 6-phytases (E.C. 3.1.3.26) and 5-phytases (E.C. 3.1.3.72).

Phytases were originally proposed as an animal feed additive to enhance the value of plant material in animal feed by liberating phosphate (Mitchell et al. 1997). Phytase is present in about 75% of all the diets for simple-stomach animals and its market volume exceeds US$350 million annually (Shivange et al. 2012). The current global phytase market has been estimated to account for more than 60% of the total enzyme market. The increase in economic pressure and increased concern over the environmental impact of life-stock production, have paved the way for the economic success of phytases as an animal feed additive. Phytases used for animal feed application differ in their enzymatic properties such as pH profile, stability under stomach conditions, temperature stability, kinetic constants, and substrate specificity. 'Ideal' phytases for animal feed applications should fulfil a series of quality criteria. They should be effective in releasing phytate phosphate in the digestive tract, stable to resist inactivation by heat during the feed processing and storage as well as cheap to produce.

Thermal stability is a particularly important issue since feed pelleting is commonly performed at temperatures between 60 and 95°C. Although phytase inclusion using an after-spray apparatus for pelleted diets and/or chemical coating of phytase may help bypass or overcome the heat destruction of the enzyme, thermostable phytases would no doubt be better candidates for feed supplements. Thus, the aim of the study was to isolate phytase-producing bacteria from volcanic areas in Central Java, Indonesia.

MATERIAL AND METHODS

Soil sampling

Four days after eruption, two samples of volcanic ash were taken from Selo on the north side of Mount Merapi in Central Java, Indonesia, and from Cangkringan Yogyakarta on the south side of the mountain. Two samples of mud and water were taken from Sikidang crater on Dieng Mountain, also in Central Java. During the collection, the temperature of the samples was 60°C.

Isolation of phytate-degrading bacteria

One gram of sample was suspended in 10 mL of 1% sodium chloride solution. The suspensions were diluted 10^2 to 10^6 fold and each dilution (10 µL) was plated onto LB plates containing (g L^{-1}) 10 tryptone, 5.0 yeast extract, 10 NaCl, and 15 agar for the isolation of phytate-degrading bacteria. The pH value of the media was adjusted to pH 7.0. Petri dishes were incubated at 37˚C for 16 hours. Thereafter, single colonies were grown in liquid LB supplemented with Na-phytate (4 g L^{-1}) at 37°C for 16 h. After pelleting the bacteria cells by centrifugation at 3,500*g* for 5 min, phytase activity was determined in the supernatants.

Standard phytase activity assay

The spectrophotometric assay was carried out in a total volume of 150 µL at 37˚C for 60 min. The reaction mixture consisted of 125 µL 0.1 M sodium acetate buffer (pH 5.0) containing 5 mM sodium phytate and 25 µL of the enzyme preparation. The liberated P_i was measured using a modified ammonium molybdate method (Heinonen and Lahti 1981). The reaction was terminated by the addition of 750 µL of a freshly prepared solution consisting of acetone, 2.5 M sulfuric acid and 10 mM ammonium molybdate (2:1:1 v/v). Thereafter, 100 µL of 1 M of citric acid were added. Absorbance was determined at

355 nm. A calibration curve was prepared from 5 to 600 mM phosphate. Phytate-degrading activity (U) was defined as the amount of enzyme that released 1 µmol phosphate per min.

To study the pH optimum of the phytate-degrading enzymes, the following buffers were used in the above described standard phytase activity assay: pH 3.0-6.0, 0.1 M sodium acetate-acetic acid; pH 6.0-7.0, 0.1 M Tris-acetic acid; pH 7.0-9.0, 0.1 M Tris-HCl.

The temperature profiles of the phytate-degrading enzymes were determined in the temperature range from 30 to 90°C using the standard phytase activity assay. The effect of cations on enzyme activity was investigated by pre-incubating the compounds with phytate-degrading enzymes for 15 min at 37°C before the standard phytase activity assay was performed. The following cations and potential inhibitors were used in a concentration of 1.0 mM and 0.1 mM: Mg^{2+}, Ca^{2+}, Fe^{3+}, and Zn^{2+}.

Identification of phytase producing bacteria

The bacterial strains were cultured at 37˚C for 16 h in LB supplemented with 0.4% Na-phytate. Then the cells were pelleted by centrifugation at 3,500*g* for 10 min. Extraction of the DNA was performed using the Wizard® Genomic DNA Purification Kit (Promega) according to the instructions of the manufacturer using 5'-GAGAGTTTGATCCTGG CTCAG-3' as a forward and 5'- CTGTTTGCTCCC CACGCTTTC-3' as a reversed primer for the amplification of the 16S rDNA as described by Damiani et al. (1996). The PCR reaction mixtures contained 2.0 µL of a dNTPs mixture (1.25 mM each), 1.0 µL of each primer (20 mM), 2.5 µL of DNA, 1.0 µL of Taq polymerase and sterile deionized water to bring the final volume to 100 µL. The mixtures were denatured at 95°C for 4 min. The PCR temperature profile consisted of 30 cycles of 60 s denaturation at 95°C, 45 s annealing at 50°C and 90 s primer extension at 72°C followed by a final extension at 72°C for 10 min. A negative control was included to eliminate the possibility of reagent contamination. PCR products were analysed using agarose (0.8%) gel electrophoresis and visualized using ethidium bromide. The identity of the bacteria detected by the 16S rRNA PCR was revealed by sequencing of the PCR products (1st Base Singapore, Singapore) and comparison of these sequences to the Genbank database using the BLAST program available at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Cluster analysis was performed using the Multiple Clustal Alignment software from Clustal W (www.ebi.ac.uk/tool/msa/clustalW).

RESULTS

Screening for phytate-degrading enzyme producing bacteria

One hundred thirty four single colonies were obtained from the two soil samples taken from Dieng Mountain (D1-D134) and 10 single colonies, each from the soil samples taken from Selo (MS1-MS10) and Cangkringan Yogyakarta (MC1-MC10). The isolates derived from Merapi Mountain exhibited an extracellular phytate-degrading activity between 77.6 and 107.1 mU mL^{-1} (Fig. 1).

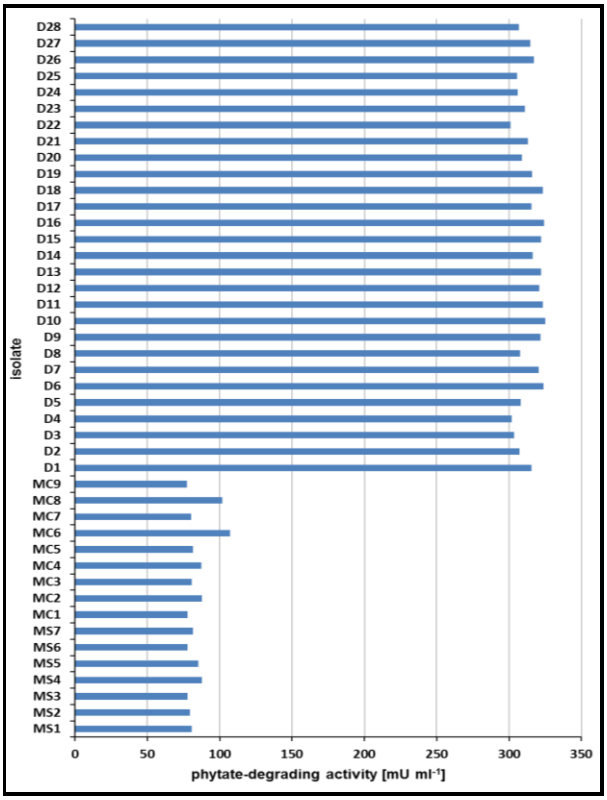

Figure 1 - Extracellular phytate-degrading activity of the positive isolates derived from Merapi Mountain north side (MS), Merapi Mountain south side (MC) and Dieng Mountain (D) after fermentation for 16 hrs at 37°C in liquid LB supplemented with sodium phytate.

No significant difference in the extracellular phytate-degrading activity was observed for the isolates from the Selo (MS1-MS7), or the

Cangkringan Yogyakarta (MC1-MC9) soil sample. The isolates derived from Dieng Mountain (D1-D28), however, showed a significant higher extracellular phytate-degrading activity (300.8 to 323.9 mU mL^{-1}) compared to the isolates from Merapi Mountain.

Identification of phytate-degrading enzyme producing bacteria

Blast analysis of the partial 16S rRNA gene sequences six isolates (MS5, MC6, MC8, D6, D10 and D16) showed high similarities to the reference sequences to the genus *Bacillus*. Isolates MS5, MC6, D10 and D16 showed 99% sequence identity to *B. cereus*, while isolate MC8 exhibited 99% sequence identity to *B. aryabhatti* and D6 99% sequence identity to *B. psychrotolerans*.

Neighbor-joining phylogenetic analysis of the sequence data revealed that the all the six isolates were located in three distinct clusters (Fig. 2). Group 1 consisted of the closely related isolates MS5 and MC6 as well as *B. cereus*, whereas group 2 comprised the isolate D6 and MC8 as well as *B. aryabhattai*. Group 3 consisted of the closely related isolates D10 and D16 as well as two uncultured bacteria clones.

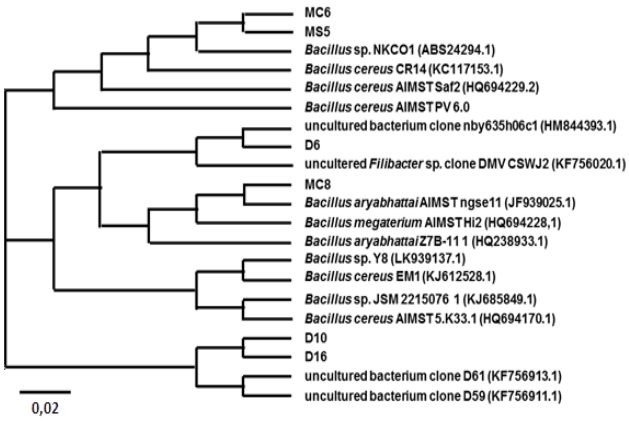

Figure 2 - ClustalW phylogenetic tree based on 16S rRNA multiple sequence alignment of phytase-producing bacteria.

Properties of the phytate-degrading enzymes

Optimal conditions for phytate dephosphorylation were determined using the crude extracellular phytase preparations from the isolates. The pH optimum of the phytase preparation of isolate D10 was 4.0, which was 5.0 for the isolates MC6 and MS5 and 6.0 for the isolates D6, D16 and MC8 (Fig. 3). For all the isolates, the pH profile for phytate dephosphorylation was broad, pointing to the presence for more than one phytate-degrading enzyme in the crude enzyme preparations. Isolate MC 8 showed temperature optima at 40°C for its phytase preparation, which was 50°C for the phytase preparation from the isolates D10 and D16 and 60°C for the isolates D6, MS5 and MC6 (Fig. 4).

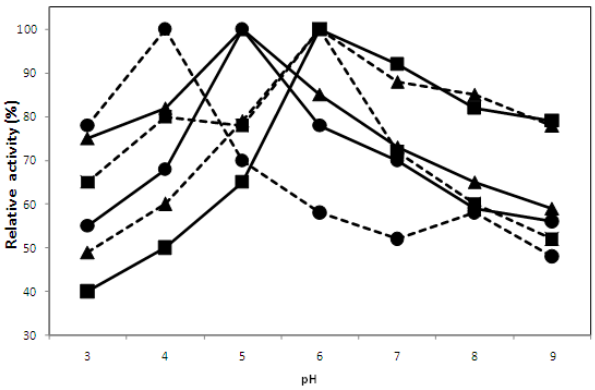

Figure 3 - pH Profile of phytate dephosphorylation by the crude phytase preparations of isolate D6 (▲), D10 (●), D16 (■), MS5 (●), MC6 (■), and MC8 (▲).

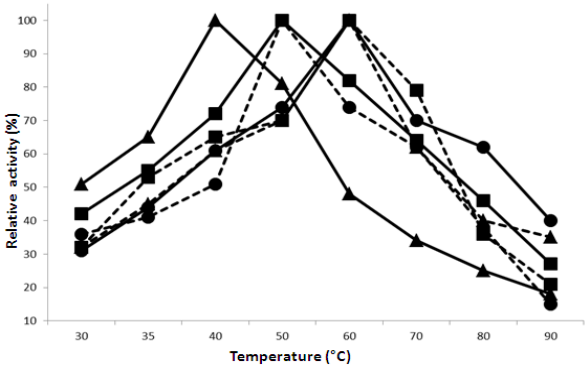

Figure 4 - Temperature profile of phytate dephosphorylation by the crude phytase preparations of isolate D6 (▲), D10 (●), D16 (■), MS5 (●), MC6 (■), and MC8 (▲).

Results on the study of the effect of cations showed that 0.1 mM and 1.0 mM Mg^{2+} did not affect phytase activity at all. Zn^{2+} and Fe^{3+}, however, were strong inhibitors of phytate dephosphorylation with all phytase preparations studied. In the presence of 0.1 mM Zn^{2+}, the reduction of phytase activity was 12-17%. A 22-28% reduction was observed in the presence of 1

mM Zn^{2+}. A concentration of 0.1 mM Fe^{3+} resulted in a decrease in phytase activity of 18-24%, whereas a 32-36% reduction was observed in the presence of 1 mM Fe^{3+}. In the presence of 0.1 mM and 1.0 mM Ca^{2+} an increase in phytase activity of 10-15% was determined (Table 1).

Table 1 - Effects of potential inhibitors on phytase activity.

phytase isolate	residual activity [%]							
	Mg^{2+}		Zn^{2+}		Fe^{3+}		Ca^{2+}	
	0.1 mM	1.0 mM	0.1 mM	1.0 mM	0.1 mM	1.0 mM	0.1 mM	1.0 mM
D6	no effect	no effect	84.2	73.3	78.1	65.4	114.2	114.6
D10	no effect	no effect	85.4	73.4	70.8	67.6	109.8	110.1
D16	no effect	no effect	83.7	72.8	77.6	65.2	114.4	114.9
MS5	no effect	no effect	88.3	77.9	82.1	68.4	111.3	111.4
MC6	no effect	no effect	86.7	77.1	81.3	67.1	110.7	110.9
MC8	no effect	no effect	82.9	71.7	76.3	63.9	114.7	115.3

The data are mean values of three independent experiments.

DISCUSSION

Bacillus sp. has been identified as the predominant producer of extracellular phytate-degrading activity in the soil samples of different origin (Kerovuo et al. 1998; Kim et al. 1998; Choi et al. 2001; Park 2001; Joseph and Raj 2007; Gulati et al. 2007; Anis Sobirin et al. 2009; Shamna et al. 2012; El-Toukhy et al. 2013; Kumar et al. 2013; Singh et al. 2013; Ushasri et al. 2013; Demirkan et al. 2014; Jain and Chauhan 2014). To our knowledge, *B. aryabhattai*, however, has been found for the first time as a phytase producer. The ability of the different *Bacillus* strains investigated to produce extracellular phytate-degrading activity was dependent on the strain itself as well as the culture conditions used. The results obtained in this study (77.6 - 323.9 mU mL^{-1}) were in good agreement with those already reported for *Bacillus* sp. KHU-10 (60 mU mL^{-1}) (Choi 2001), *B. laevolacticus* (158 - 283 mU mL^{-1}) (Gulati et al 2007) and *B. subtilis* DR6 (129 - 378 mU mL^{-1}) (Singh et al. 2013). Other studies reported significantly higher extracellular phytase activities such as 720 U mL^{-1} (*B. subtilis* MJA) (El-Toukhy et al. 2013), 20 - 150 U mL^{-1} (*B. subtilis*) (Powar and Jagannathan 1982), 55 - 88 U mL^{-1} (*B. subtilis*) (Shamna et al. 2012), and 39 - 348 U mL^{-1} (*B. cereus* and *B. subtilis*) (Anis Sobirin et al. 2009). The differences in phytase production might be explained by the difference in the phytase expression levels among the *Bacillus* strains studied and in the application of optimised culture conditions in respect to phytase production. Histidine acid phytases, β--propeller phytases and cysteine phytases have been identified in bacteria (Greiner and Konietzny 2006). The majority of the so far known phytases belong to the subfamily of histidine acid phosphatases and do not need any co-factor for optimal activity. Currently, all the phytases used for the animal feed application belong to the class of histidine acid phytases. Cysteine phytases have been reported from the anaerobic ruminal bacteria (Chu et al. 2004) and these enzymes also do not need any co-factor for enzymatic activity. The amino acid sequences of ß-propeller phytases exhibit no homology to the sequences of any other known phosphatase (Kerovuo et al. 1998; Kim et al. 1998; Ha et al. 2000). Initially, ß-propeller phytases were reported from *Bacillus* species (Kerovuo et al. 1998; Kim et al. 1998; Choi et al. 2001; Tye et al. 2002), but protein sequence identity suggested that ß-propeller phytases were widespread in the aquatic environment (Cheng and Lim 2006). In contrast to the other two phytase subfamilies, ß-propeller phytases need calcium ions for optimal stability and enzymatic activity (Powar and Jagannathan 1982; Shimizu 1992; Kerovuo et al. 1998; Kim et al. 1998; Choi et al. 2001; Oh et al. 2001; Park 2001; Shin et al. 2001; Gulati et al. 2007; Anis Sobirin et al. 2009; El-Toukhy et al. 2013; Kumar et al. 2013; Yu and Chen 2013; Jain and Chauhan 2014). Maximum phytase activity of ß-propeller phytases occurs in the pH range of 6.5 to 8.0 (Choi et al. 2001; Gulati et al. 2007; Jain and Chauhan 2014; Kerovuo et al. 1998; Kim et al. 1998; Oh et al. 2001; Park 2001; Powar and Jagannathan 1982; Shimizu 1992; Yu and Chen 2013). All the six *Bacillus* strain included in this study exhibited a broad pH profile. Interestingly, for three of the strains investigated, a significant higher phytate-degrading activity under acidic compared to neutral conditions was observed. The presence of

more than one phytate-degrading enzyme in the *Bacillus* strains might explain this observation. This conclusion was further confirmed by the relatively high temperature optimum at pH 5.0 in the absence of Ca^{2+}. Optimal temperature for phytate dephosphorylationy was at 40, 50 and 60°C. In the absence of calcium ions in the assay buffer, temperature optima around 40°C were reported for *Bacillus* phytases (Powar and Jagannathan 1982; Choi et al. 2001; Park 2001; El Thouky et al. 2013), whereas higher temperature optima (60 - 70°C) were found in the presence of 1 - 5 mM Ca^{2+} (Kerovuo et al. 1998; Kim et al. 1998; Park 2001; Choi et al. 2001, Gulati et al. 2007; Jain and Chauhan 2014). The *Bacillus* phytases investigated in this study exhibited an increased phytase activity in the presence of Ca^{2+} and decreased phytase activity in the presence of Zn^{2+} and Fe^{2+}. Mg^{2+} did not show any effect on enzymatic activity. This was in accordance with already reported data on phytases (Konietzny and Greiner 2002).

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Received: April 19, 2015;
Accepted: June 16, 2015.