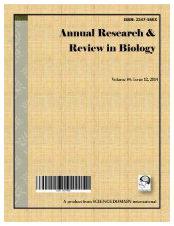

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Cloning and Sequence Comparison of Phytase (*Phy*) Genes from *Aspergillus niger* and *Bacillus atrophaeus*

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Authors' contributions

This work was carried out in collaboration between all authors. Author FAR designed the study, supervised the work and wrote the first draft. Authors BMAAZ and DAI carried out the experimental work, data analysis and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To clone phytase genes from prokaryotic and eukaryotic microorganisms and provide partial sequence comparisons between the two systems in relative to their protein functional domains.
Study Design: Gene cloning of *Phy* genes from *Bacillus atrophaeus* and *Aspergillus niger strain 103* and analysis of their amino acid sequence.
Methodology: Genomic DNA was isolated from microbial prokaryotic and eukaryotic *Bacillus atrophaeus* and *Aspergillus niger strain 103,* respectively. Their phytase genes were isolated and cloned, sequenced and translated into amino acid sequences. Their genes were analyzed and compared with their homologues that are available on the GenBank database using appropriated bioinformatics tools.
Results: This study reports the successful cloning of phytase genes from *Bacillus atrophaeus* and *Aspergillus niger strain 103* and the partial characterization and comparison of their sequences in

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relative to conserved domains that may affect their enzymatic activities. Both genes possess the required phytase activity domains even though no significant homology exists between the bacterial and fungal *Phy* genes.
Conclusion: Phytase genes possess a wide spectrum of hydrolyzing enzyme structures that support the required ability of the enzyme to degrade phytate.

Keywords: Aspergillus niger; Bacillus atrophaeus; myo-inositol hexakisphosphate phosphohydrolases; PhytA gene; Phytase; phytate.

1. INTRODUCTION

Phytic acid (IP6)-(*myo*-inositol-1,2,3,4,5,6-hexakisdihydrogenphosphate) is the storage compound of phosphorus that represent 60-90% of total phosphate in seeds [1]. Phytate is formed during the ripening period and accumulate into dense protein storage vacuoles called globoids that are also called aleurone particles [2]. Phytate is considered an anti-nutritive agent as it is characterized by the ability to chelate positively charged multivalent cations, especially Fe^{+2}, Zn^{+2}, Mg^{+2} and Ca^{+2} [3], thus reducing the availability of these compelled minerals. Phytate can also complex with proteins and starch that are associated with a decrease in their availability [4]. On the contrary, of anti-nutritive effect of phytate, it is suggested to have an important nutrient value for human health. Phytate is now considered an antioxidant agent, anticancer and anticalcification with important roles in various other human conditions [5].

Phytases are phosphatase enzymes that are widely distributed in plants, microorganisms and in animal tissues. Phytase enzymes are high molecular weight proteins, with the majority of the characterized phytate-degrading enzymes having molecular masses between 40 and 70 kDa and behave like monomeric proteins, even though some phytases are composed of multiple subunits [6,7].

Phytase classification is dependent on variable issues. Phytic acid is hydrolyzed by the enzyme Phytase, which belongs to a group of phosphatase enzymes (*myo*-inositol hexa kisphosphate phosphor hydrolase) that catalyze the hydrolysis of *myo*-inositol hexakisphosphate (phytic acid) to inorganic mono phosphate and lower *myo*inositol phosphates, and in some cases to free *myo*-inositol. Despite that all Phytases hydrolyze P from phytic acid in the same mechanisms, they don't necessarily share the same structure [8]. The Enzyme Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB) (http://www.chem.qmul.ac.uk/iubmb/) distinguishes three types of Phytases: (i) 3-Phytase (EC 3.1.3.8), (ii) 4-Phytase (EC 3.1.3.26) and (iii) 5-Phytase (EC 3.1.3.72). This classification is based on the first phosphate group attacked by the enzyme. Other broadly categorized phytases are also used based on their optimum pH; acidic phytases (pH optimum: 3.0-5.5) and alkaline phytases (pH optimum: 7.0-8.0)[9]. Phytases also have been categorized according to their catalytic mechanism into histidine acid phosphatases (HAPs), purple acid phosphatases (PAPs), β-propeller phytases (BPPs) or cysteine phytases [10] and a protein tyrosine phosphatase (PTP) that is related to inositol polyphosphatases (IPPases).

The 3-Phytase is typical for microorganisms, whereas 4- and 5-Phytases are common in plants.

It is well documented that the amino acid sequences of many fungal Phytase enzymes possess high homology with their corresponding fungal *Aspergillus niger* NRRL 3135 (*PhytA*) phytase. For example, a Phytase cloned from *A. niger* var. awamori has over 97% identity to the *PhytA*, while this homology does not exist between the *PhytA* and the bacterial Phytase [11]. Less homologies, however, were documented for the *A. niger* NRRL 3135 Phytase with those from *A. fumigatus (*65%), *A. terrus (*62%), *A. nidulans (*62%), and *Myceliophthora thermophile* (46%). The PhyB from *A. niger* NRRL 3135 shows 99% identity to the corresponding protein from *A. niger* var. awamori. Surprisingly, two Phytases (PhytA and PhyB) from *A. niger* NRRL 3135 share only 25% homology.

Phytases from prokaryotic microorganisms, such as the bacterial Phytase from *E. coli* and from animals, such as that of the rat hepatic MIPP, does not exhibit any sequence similarity to the fungal *A. niger* NRRL 3135 Phytase. Despite their distant similarity, they do share a highly conserved sequence motif - RHG - that is found at the active sites of acid phosphatases [12,13]. Furthermore, they contain a remote C-terminal motif with histidine and aspartic acid residues that probably take part in the enzyme catalytic activity. Therefore, these Phytases are said to form the Phytase subfamily of histidine acid phosphatases [14].

Most of Phytase amino acid sequences share the highly conserved sequence motif RHGxRxP that is considered to be the P acceptor site near the protein N terminus. This is in addition to the C-terminus conserved HD-motif where the aspartate, which is assumed to be the proton donor for the substrate leaving group [11].

On the other hand, the PhytI and PhytII *Zea mays* plant Phytases are only practically identical and do not show any homology to other Phytases and phosphatases. Despite the low sequence homology between these two plant Phytases and the fungal PhytA, they possess a region of 33 amino acids with high similarity to *A. niger* NRRL 3135 Phytase and believed to be the acceptor site for phosphate [15]. Furthermore, a 72% identity exist between the *B. amyloliquefaciens* Phytase [16] and an open reading frame in the genome sequence of the *Bacillus subtilis* [17], even though it was not homologous to any phosphatases or Phytases. In a similar situation, the Phytase identified from the *Enterobacter* sp. 4 was found not to possess any homology to any other Phytases or histidine acid phosphatases, with the exception of 30-38% homology to low molecular weight acid phosphatases from *Chryseobacterium meningosepticum* and *Streptococcus equisimilis*. It was observed that certain lysine and tryptophan residues appeared to be conserved.

This study aimed at cloning the *phy* genes from prokaryotic and eukaryotic microorganisms and provides partial characterization for their amino acid sequences, particularly in relative to their functional domains.

2. MATERIALS AND METHODS

2.1 Genomic Extraction from *Bacillus atrophaeus* and *Aspergillus niger*

Bacterial cells of *B. atrophaeus* were kindly donated by Dr. Sameer Bargouthi (AL-Quds University) and spores of an isolated Malaysian *A. niger* 103 strain were kindly donated by Ali Hashlamoon (University of Malaysia). Genomic extractions were done according to standard protocols using commercially available kits. For *A. niger*, mRNA was isolated and cDNA synthesized according to standard protocols.

2.2 Amplification and Visualization of *phy* genes from *B. atrophaeus* and *A. niger*

To amplify the *Phy* genes from *B. atrophaeus* and *A. niger*, the following primers were used based on the *phy* gene sequences available on NCBI accession number (Table 1).

The gene was amplified by PCR using thermostable *Taq* DNA polymerase (Hy Labs/HTD0078). The reaction mixture consisted of 14.9 µl ultrapure water, 2.5µl 10X reaction buffer (HyLabs/ HTD0078), 2.5 µl $MgSO_4$ (Hy Labs/ HTD0078), 2 µl dNTPs (Sigma/DNTp10-1KT), 1.0 µl forward and 1.0 µl reverse primers, and 1.0 µl of the template DNA with total volume of the reaction was 25 µl. PCR amplification was carried out with a thermal cycler (Cat# 2720, Applied Biosystems), under the following conditions: initial denaturation at 94°C for 2 min, 25 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 2 min, followed by a final extension at 72°C for 15 min. Amplified PCR reaction (25 µl) was mixed with 2 ul 6X loading buffer (0.25% (w/v) bromophenol blue (Fluka/ 417639/1), 0.25% (w/v) xylene cyanol FF (Amresco/1897B066), 30% glycerol (Amresco/0176B017) [18], and then loaded onto a 1% (w/v) agarose (Seakem/ 5080021) gel containing 1X TBE buffer (1L of 5X stock contained 54 g Tris-base (Promega/ H5131), 27.5 g boric acid (Sigma/078k0037), 20 ml of 0.5 M EDTA (Alfa aesar/ 10122546), pH 8.0)[18], stained with ethidium bromide (EtBr) (Hy Labs). Gel electrophoresis was carried out in 1X TBE buffer at 90 V. Ultraviolet light emitted from a transilluminator was used to visualize the band corresponding to the expected size of the gene. The DNA was purified from the PCR product using the AccuPrep® PCR Purification Kit (Bioneer, K-3035) following the same protocol.

2.3 Cloning of *phy* genes in pGEM® -T Easy Vector

The *phy* gene was cloned into the pGEM® -T Easy vector (Promega/A1360) cloning vector. The ligation reaction was prepared and mixed by pipetting according to standard cloning procedures [19] then the ligation reaction was incubated overnight at 4°C. The ligation reaction was transformed into DH5α competent cells by

Table 1. Primers used to amplify *Phy* genes from *B. atrophaeus* and *A. niger*

	Primer sequence	Accession No.
B. atrophaeus		
PhyF′	5'- GCATACCTTATGACTGCTGCT-3	*CP002207.1
PhyR′	5'- GCTTTTTGCTGCCTTATGTTCC-3'	*CP002207.1
A. niger		
PhyF′	5`-ATGGGTGTCTCTGCCGTTCTAC-3`	AB022700.1
PhyR′	5`- CTAAGCGAAACACTCCCCCC-3`	AB022700.1

** NCBI accession number: CP002207.1 [position 1733322-1734479]*

heat shock method. After that, the transformed cell culture was plated onto labeled agar plates containing ampicillin, Isopropyl β-D-1 Thiogalactopyranoside (IPTG) and X-Gal then sealed and incubated overnight at 37°C. Cloning in pGEM® -T Easy vector was presented in white positive colonies and further verified by PCR and sequencing test in the Heredity Lab at Bethlehem University sequencing facility. Sequence comparisons were carried out using online and NCBI sources (http://www.ncbi.nlm.nih.gov/).

3. RESULTS AND DISCUSSION

For *B. atrophaeus*, the extracted genomic material was used as a template in PCR reaction for *phy* gene amplification. The PCR reaction was carried out using specific forward and reverse primers (Table 1), which were designed based on the *phy* sequences available on the GenBank. The PCR product was loaded on 1% agarose gel, detected by UV light before documented using a digital camera. PCR reaction results showed a highly specific band that was visualized from the extracted *B. atrophaeus* genomic material and matches the expected size of *phy* gene available on the GenBank. The *phy* gene size was 1134 bp and the amplicon has a size of 1156 bp because the primers included bases upstream and downstream the gene (Fig. 1).

As for *A. niger,* the PCR reaction was loaded on agarose gel as above. PCR reaction results showed a highly specific band that was slightly larger than the expected size of a *phytA* gene on the NCBI. The *PhytA* gene amplicon appeared on the gel at approximately 1500 bp (Fig. 2). Phytases are not necessarily identical in *Aspergillus* sp., and can accommodate some variations among different strains.

The *phy* genes were then cloned into pGEM®-T-Easy cloning vector and transformed into DH5α competent cells using heat shock transformation method. The transformed cells were plated onto agar plates containing ampicillin, X-gal and IPTG to detect the positive clones, which were confirmed by sequencing of the PCR amplicons. A full sequence of *phy* genes for both *B. atrophaeus* and A. *niger* were obtained by sequencing. The total length of the sequenced *B. atrophaeus* gene was 1134 bp which was blasted using the NCBI Blastin nucleotides website (http://www.ncbi.nlm.nih.gov/). The results confirmed that our cloned gene was related to 3-phytase of *B. atrophaeus* gene**.** The full sequence for *A. niger* that was cloned from the genomic DNA from start to stop codon was 1507bp and it contained two exons; 1-44 and from 148-1507. The total length of the coding sequence is 1404 bp, which lies in the range of Phytases from the *A. niger* available on the public domain.

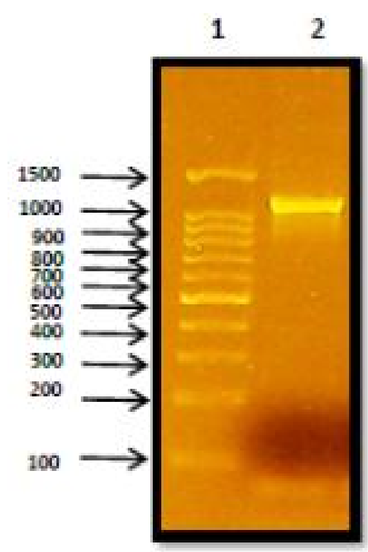

Fig. 1. Agarose gel photo of *phy* gene amplified from *B. atrophaeus*.
Phy gene was amplified by PCR and loaded on 1% agarose gel, stained with EtBr and visualized using gel documentation system. Lane 1 contains 100 bp DNA ladder (Promega/G5711) and Lane 2: shows the amplified phytase gene. The band expected size was 1156 bp and matches with ladder.

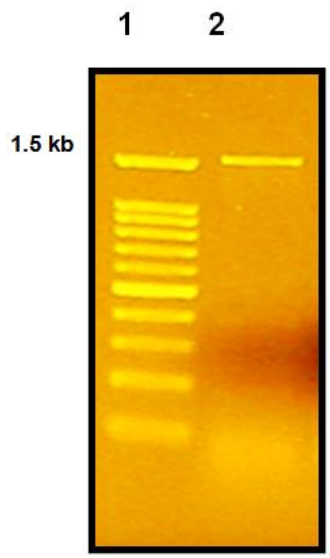

Fig. 2. Agarose gel photo showing the *PhytA* gene size of *A. niger*

The PhytA gene was amplified by PCR and loaded on 1% agarose gel, stained with EtBr and visualized using gel documentation system (right lane). Lane 1 is showing 1.5 kb DNA ladder (Promega/ G5711) and Lane 2 shows the amplified gene. The band size is approximately 1500 bp, which matches the expected size of 1513 bp for the gene, but approximately 100 nucleotide larger than its mRNA

The obtained sequence of the *B. atrophaeus* was translated using Expasy translation tool (http://www.expasy.ch/tools/dna.html) (Fig. 3) and blasted against protein database at NCBI using protein Basic Local Alignment Search Tool (BLAST) and the result proved that the protein was the *3-phytase* enzyme, with 98% identity with *B. atrophaeus* phytase *phy* WP_010788969.1. Moreover, this protein showed 72% identity with *B. subtilis* phytase and 71% with *B. amyloliquefaciens* phytase when specifying the search to be from protein database PDB, which enforces that the cloned gene has a conserved functional domain related to phytase superfamily and closely related to bacillus phytases and distant from other phytate degrading enzymes (data not shown).

For the *A. niger*, similarly the *phy* gene coding sequence was aligned against the GenBank AB022700.1 cds (*PhytA* gene) sequence that was used to generate the primers. The coding sequence showed 22 nucleotide differences between the *A. niger* 103 strain and the *A. niger* AB022700.1 cds. The AB022700.1 cds (*PhytA* gene) sequence is published at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/), and when compared with our 103 strain sequence, most nucleotide variation appeared to be at the 3rd letter in the codon, which means there is a small chance that the amino acid could be different. To look at the protein level, the coding sequence of 103 strain was translated using Expasy translation tool (http://www.expasy.ch/tools/dna.html) (Fig. 4).

The resulted protein sequence composed of 466 amino acids was blasted against protein database at NCBI using protein Basic Local Alignment Search Tool (BLAST) and the result proved that the protein was the phytA enzyme, with a protein match of 99% with the *A. niger* AB022700.1 mRNA. Amino acid sequence alignment of 103 strain with PhytA shows only 6 amino acid differences, mostly are conserved. The differences in amino acids between 103 strain and *A. niger* AB022700.1 PhytA enzyme are: ^{18}Phe/Ser; ^{72}Gln/His; ^{76}Thr/Ala; ^{111}Glu/Gly; ^{137}Leu/Val; ^{451}Arg/Lys (Fig. 5).

M T A AA G L LL T S L S F S A P L A A K Q V P S H N N H F T V K A S A E T K P V A S G D D A A
D N P A I W V N E K R P E K S K L I T T N K K A G L V V Y D L D G K E I N S Y Q F G K L N N V D
L R Y D F P L N G K K A D I AAA S N R T D G K N S I E I Y S F D G E K G E L E S I T D P K H P I S
T G I A E V Y G F S L Y H S Q K T G K F Y A L V T G K Q G E F E Q Y E I A D N G K G Y V T G K K
V R Q F K L N S Q T E G V A A D D E Y G H I Y I A E E D A A I W K F S A E P N G G T Q G SI I D R
A D G K H L T S D I E G L T I Y Y A P D G K G Y I M A S S Q G N N S Y A I Y E R Q G S N K Y I A
N F E I T D G E K I D G T S D T D G I D V I G F G L G A K Y P N G I F I A Q D G K N T E N G Q A V
N Q N F K I V P W E R I A K P I G A A L D V K K Q A DP R R L K D R S G T

Fig. 3. The amino acid sequence translated from the cloned *Phy* gene of *B. atrophaeus*

The DNA sequence was translated using Expasy translation tool (http://www.expasy.ch/tools/dna.html) and blasted against protein database at NCBI using protein Basic Local Alignment Search Tool (BLAST) and the result proved that the protein was the 3-phytase enzyme

M G V S A V L L P L Y L L S G V T F G L A V P A S R N Q S T C D T V D Q G Y Q C F S E T S H L
W G Q Y A P F F S L A N K S A I S P D V P A G C Q V T F T Q V L S R H G A R Y P T D S K G K K
Y S A L I E E I Q Q N A T T F E EK Y A F L K T Y N Y S L G A D D L T P F G E Q E L L N S G V K F
Y Q R Y E S L T R N I V P F I R S S G S S R V I A S G N K F I E G F Q S T K L K D P R A Q P G Q
S S P K I D V V I S E A S T S N N T L D P G T C T V F E D S E L A D D I E A N F T A T F V P S IR
Q R L E N D L S G V S L T D T E V T Y L M D M C S F D T I S T S T V D T K L S P F C D L F T H E
E W I N Y D Y L Q S L N K Y Y G H G A G N P L G P T Q G V G Y A N E L I A R L T H S P V H D D
T S S N H T L D S N P A T F P L N S T L Y A D F S H D N G I I SI L F A L G L Y N G T K P L S S T T
A E N I T Q T D G F S S A W T V P F A S R MY V E M M Q C Q S E Q E P L V R V L V N D R V V P
L H G C P V D A L G R C T R D S F V R G L S F A R S G G D W G E C F A

Fig. 4. The amino acid sequence translated from the cloned *Phy* gene of *A. niger*

The DNA sequence was translated using Expasy translation tool (http://www.expasy.ch/tools/dna.html) and blasted against protein database at NCBI using protein Basic Local Alignment Search Tool (BLAST) and the result proved that the protein was the 3-phytase enzyme

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PhytA 103 aa translated   MGVSAVLLPLYLLSGVTFGLAVPASRNQSTCDTVDQGYQC    40
PhytA GenBank NCBI        MGVSAVLLPLYLLSGVTSGLAVPASRNQSTCDTVDQGYQC    40
Consensus                 mgvsavllplyllsgvt glavpasrnqstcdtvdqgyqc

PhytA 103 aa translated   FSETSHLWGQYAPFFSLANKSAISPDVPAGCQVTFTQVLS    80
PhytA GenBank NCBI        FSETSHLWGQYAPFFSLANKSAISPDVPAGCHVTFAQVLS    80
Consensus                 fsetshlwgqyapffslanksaispdvpagc vtf qvls

PhytA 103 aa translated   RHGARYPTDSKGKKYSALIEEIQQNATTFEEKYAFLKTYN   120
PhytA GenBank NCBI        RHGARYPTDSKGKKYSALIEEIQQNATTFEGKYAFLKTYN   120
Consensus                 rhgaryptdskgkkysalieeiqqnattfe kyaflktyn

PhytA 103 aa translated   YSLGADDLTPFGEQELLNSGVKFYQRYESLTRNIVPFIRS   160
PhytA GenBank NCBI        YSLGADDLTPFGEQELVNSGVKFYQRYESLTRNIVPFIRS   160
Consensus                 yslgaddltpfgeqel nsgvkfyqryesltrnivpfirs

PhytA 103 aa translated   SGSSRVIASGNKFIEGFQSTKLKDPRAQPGQSSPKIDVVI   200
PhytA GenBank NCBI        SGSSRVIASGNKFIEGFQSTKLKDPRAQPGQSSPKIDVVI   200
Consensus                 sgssrviasgnkfiegfqstklkdpraqpgqsspkidvvi

PhytA 103 aa translated   SEASTSNNTLDPGTCTVFEDSELADDIEANFTATFVPSIR   240
PhytA GenBank NCBI        SEASTSNNTLDPGTCTVFEDSELADDIEANFTATFVPSIR   240
Consensus                 seastsnntldpgtctvfedseladdieanftatfvpsir

PhytA 103 aa translated   QRLENDLSGVSLTDTEVTYLMDMCSFDTISTSTVDTKLSP   280
PhytA GenBank NCBI        QRLENDLSGVSLTDTEVTYLMDMCSFDTISTSTVDTKLSP   280
Consensus                 qrlendlsgvsltdtevtylmdmcsfdtiststvdtklsp

PhytA 103 aa translated   FCDLFTHEEWINYDYLQSLNKYYGHGAGNPLGPTQGVGYA   320
PhytA GenBank NCBI        FCDLFTHEEWINYDYLQSLNKYYGHGAGNPLGPTQGVGYA   320
Consensus                 fcdlftheewinydylqslnkyyghgagnplgptqgvgya

PhytA 103 aa translated   NELIARLTHSPVHDDTSSNHTLDSNPATFPLNSTLYADFS   360
PhytA GenBank NCBI        NELIARLTHSPVHDDTSSNHTLDSNPATFPLNSTLYADFS   360
Consensus                 neliarlthspvhddtssnhtldsnpatfplnstlyadfs

PhytA 103 aa translated   HDNGIISILFALGLYNGTKPLSSTTAENITQTDGFSSAWT   400
PhytA GenBank NCBI        HDNGIISILFALGLYNGTKPLSSTTAENITQTDGFSSAWT   400
Consensus                 hdngiisilfalglyngtkplssttaenitqtdgfssawt

PhytA 103 aa translated   VPFASRMYVEMMQCQSEQEPLVRVLVNDRVVPLHGCPVDA   440
PhytA GenBank NCBI        VPFASRMYVEMMQCQSEQEPLVRVLVNDRVVPLHGCPVDA   440
Consensus                 vpfasrmyvemmqcqseqeplvrvlvndrvvplhgcpvda

PhytA 103 aa translated   LGRCTRDSFVRGLSFARSGGDWGECF                 466
PhytA GenBank NCBI        LGRCTRDSFVKGLSFARSGGDWGECF                 466
Consensus                 lgrctrdsfv glsfarsggdwgecf
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Fig. 5. Amino acid sequence alignment of *A. niger* 103 and PyhtA enzymes. Note that there are only 6 amino acid differences from the published *A. niger* AB022700.1 mRNA among the 466 amino acids of the enzyme. These amino acids are: F for S; Q for H; T for A; E for G; L for V; R for K.

This indicates that a functional Phytase was cloned and it contained two conserved domains; Histidine phosphatase domain and conserved amino acid belong to the Mucin-like glycoprotein.

The Histidine phosphatase domain contains a His residue which is a subject for phosphorylation during the reaction and is found in several functional proteins, mostly phosphatases [20]. These proteins include cofactor-dependent and cofactor-independent phosphoglycerate mutases (dPGM, and BPGM, respectively), fructose-2, 6-bisphosphatase (F26BP) ase, histidine acid phosphatases and Phytases [20]. They are involved in several critical functions in metabolism, signaling, and regulation.

The Mucin-like glycoproteins resemble vertebrate mucins. The protein consists of three regions. The N and C termini are quite conserved in these proteins, whereas the central region is not, but it contains a large number of threonine residues which can be glycosylated [20].

4. CONCLUSION

Although prokaryotic and eukaryotic phytases possess enzymatic activity and have the required functional domains, they do not show significant sequence homology, an indication of the wide spectrum of hydrolyzing enzyme structures.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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