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Antimicrobial activity of phytase extracted from a thermophilic fungus, *Rhizomucor pusillus*

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Abstract: This study aimed to extract and characterize phytase from *Rhizomucor pusillus*, a thermophilic fungus isolated from various soil samples, and investigate its antimicrobial activity. Six different fungal strains were isolated from soil samples at Mansoura University using standard media. These strains were then characterized and screened to determine whether they could produce phytase through submerged fermentation in a 250 ml flask with 1 g of wheat bran and one hundred milliliters of basal medium with the subsequent composition (g/L): 0.5 KCl, 0.1 MnSO4, 0.5 NaCl, 0.2 MgSO4.7H2O, 0.0075 CaCl.6H2O, 1.0 yeast extract, pH (5.0), and autoclaved for 30 minutes at 121 º C. After cooling, the mixture was inoculated with colonies. Following inoculation, the contents of each flask were combined. At 37º C, the flasks were incubated. To get rid of the debris, the flask contents were centrifuged. The phytase activity was measured using the crude supernatant. When thermophilic fungal strains were used to screen the isolated fungi for phytase production, *Rhizomucor pusillus* showed the highest activity. The second day of high Activity was ascertained through the measurement of optical density and the enzyme activity during the incubation period at pH 5.0 and 37ºC. *Rhizomucor pusillus* can be further utilized for the commercial synthesis of phytase as it was isolated from soil samples exhibiting notable phytase activity. Phytase is an antibacterial agent that works well.

Key words: *Rhizomucor pusillus*; phytase, Optimization; Purification

1.Introduction:

Phytases, also known as Phosphohydrolases of myoinositol (1,2,3,4,5,6) hexakisphosphate have garnered significant interest from researchers and industry practitioners in the fields of biotechnology, nutrition, and environmental conservation. The most prevalent inositol phosphate in nature, Hexakisphosphate (phytate) (myoinositol 1,2,3,4,5,6), can be gradually dephosphorylated by phosphomonoesterases, of which phytoses are a subgroup. They have been discovered in certain animal tissues, plants, and microbes [1]. Phytases are even present in plant seeds and microorganisms in a variety of forms [2–14]. and these might show distinct phytate dephosphorylation stereospecificity, be controlled differently, be targeted to distinct locations both inside and outside the generating cell, and consequently serve distinct physiological roles. While the capacity phytases to hydrolyze phytate is widely recognized based on *in vitro* research. experiments, little is known about their activity in vivo. As a result, some of the enzymes that are currently categorized given that phytases might not genuinely be engaged in the in vivo degradation of phytate but rather serve entirely different purposes. The only

enzymes known to take part in the breakdown of phytate to release minerals, phosphate, and myoinositol for growth and development plant during germination are the germination-inducible phytases found in plant seeds [15]. Since phosphate starvation is the catalyst for the formation of extracellular phytases in molds and yeast, these enzymatic hydrolyze substances that are organically phosphorylated, including phytate, in order to supply cell with extracellular phosphate. Consequently, these enzymatic substances are nonspecific phosphatases with phytate-degrading capabilities. Other enzymes with phytate-degrading activity have mostly hypothetical roles in vivo. Because of its previously mentioned ability to supply phosphate to the cell, it has also been suggested that it plays a part in bacterial pathogenesis or the stress response [16–20].

Phytases are a broad class of enzymes with a variety of shapes, dimensions, as well as catalytic functions. Phytases can be called purple acid phytases PAPhy, cysteine phytases (CPhy), β-propeller phytases (BPPhy), or histidine acid phytases (HAPhy) depending on the catalytic mechanism [21–22]. Phytases have been further classified into acid and alkaline phytases based on their pH optimum. Additionally, they are classified as 3-phytases (EC 3.1.3.38), 6-phytases (EC 3.1.3.26), and 5-phytases (EC 3.1.3.72) according to the carbon in the phytate myo-inositol ring where dephosphorylation starts. Most of the phytases that have been identified so far are members of the histidine acid phosphatases subfamily and function best without a co-factor. Microorganisms, plants, and animals have all been found to contain them [1, 23–25]. The α/β-domain of histidine acid phosphatases is conserved, while the α-domain is variable in their structures [26–27]. The interface where the two domains meet is where you can find the active site. Variations in the α-domain have been linked to variations in substrate binding. Additionally, the suggested structures offer molecular details regarding substrate binding and the catalytic mechanism. The sequence motif RH(G/N) XRXP, which is highly conserved, which is thought to be the phosphate acceptor site close to the N-terminus, is shared by histidine acid phosphatases [28–30]. Not all histidine acid phosphatases, though, have the ability to react with phytate. The hydrolysis product orthophosphate, Zn^{2+}, fluoride, molybdate, wolframate, and vanadate were the most powerful inhibitors of histidine acid phytases [1]. It is unclear if metal ions form metal ion–phytate complexes that are poorly soluble or bind to the enzyme to modify phytase activity. When Fe^{2+} or Fe^{3+} are added in order to test mixtures, a precipitate appears, which indicates that the observed decrease in the rate of dephosphorylation is caused by a decrease in the concentration of active substrate as a result of the formation of poorly soluble iron phytate [6]. Histidine acid phytases are competitively inhibited by the well-known acid phosphatase inhibitor fluoride, which has an inhibitor constant of 0.1 to 0.5 mM. Furthermore, it was discovered that orthophosphate, the hydrolysis product, and its structural analogues wolframate, molybdate, and vanadate were competitive inhibitors of the enzymatic degradation of phytate. These transition metal oxo-anions have been proposed to elicit their inhibitory actions via complex formation the transition state resembling the geometry trigonal bipyramidal. [32]

It has also been reported that the substrate phytate inhibits several histidine acid phytases alongside the phosphate product of hydrolysis. For the soybean enzyme [33], the minimum phytate concentration required to impede activity of phytase is 20 mM, while for the enzyme found in maize roots [8], it is 300μM. The charge caused by phosphate groups may have an impact on the immediate surroundings of the enzyme's catalytic domain at high substrate concentrations. This may prevent the enzyme-substrate complex from converting in order to an enzyme and product, though it's possible that inhibition from the formation of phytase-phytate complexes that are poorly soluble could also cause this. When calculating phytase activity using the standard in vitro assay, substrate inhibition should be taken into account because different phytases may exhibit varying degrees of activity reduction at the assay's substrate concentration. The amino

acid motifs typical of histidine acid phosphatase have only been found in one alkaline phytase to date [34].

This enzyme was found in lily pollen, is not inhibited by fluoride, and needs Ca^{2+} for complete catalytic activity [3,34]. Pollen from the cat's tail (Typha latifolia L.) and several legumes [36–38] were also found to contain plant alkaline phosphatases, whose level of activity has increased in the presence of Ca^{2+}. Regretfully, there is a lack of sequence data to validate the existence of histidine acid phosphatases' signature motifs and no cloning of the corresponding genes. There is no similarity between the amino acid sequences of β-propeller phytases and any other phosphatase that is currently known [39–41]. There is even the absence of HD and RH(G/N)-XRXP are potential active site motifs, which are present in histidine acid phosphatases. Phytases with β-propellers were first identified in Bacillus species [39, 42–44]. β-propeller phytases have been recently found in the aquatic bacterium *Shewanella oneidensis* [45] and the plant pathogen Xanthomonas oryzae [20]. Additionally, it has been suggested Phytases with β-propeller protein sequences are identified widely distributed in aquatic environments [45–46]. Each protein molecule of β-propeller phytases contains six calcium-binding sites and an architecture with six folding propeller blades [47]. By connecting distant the binding of three calcium ions to high affinity calcium-binding sites, and loop segments in the amino acid sequence leads to a significant increase in thermal stability. The enzyme's catalytic activity is activated when three more Low-affinity calcium-binding sites located at the top of the molecule are occupied by calcium ions. This occurs because the extremely polarized cleft becomes a more conducive environment for binding of phytate. According to kinetic studies, phytases with β-propellers are capable of hydrolyzing calcium phytate at pH values between 7.0 and 8.0 [48]. β-propeller phytases exhibit no decrease in activity when exposed to fluoride, in contrast to histidine acid phytases [39, 42-45 and 49-50].

It was reported that the RH(G/N) XRXP motif is absent from two additional classes of phytases [51–52]. In an acidic environment, members of both classes demonstrate their maximum catalytic activity. In a soybean seedling (Glycine max L. Merr.), in the cotyledons that was germinating, the initial metal-containing binuclear phytase was discovered [51]. After the soybean phytase gene was cloned, Characterization of the gene product was discovered that the enzyme shared motif features with a wide variety of phosphoesterases, including purple acid phosphatases. There are representatives of purple acid phosphatases in bacteria, fungi, plants, and mammals [53]. and have Fe (III)–Me (II) binuclear centers, where Me can be either Fe, Mn, or Zn. There have also been reports of purple acid phosphatases with phytase activity in barley (Hordeum vulgare L.) [55], wheat (Triticum aestivum L.) [10, 55-56], and Medicago tranculata L. [54]. Purple acid phosphatases that exhibit phytase activity seem to be specific to plants thus far.

An anaerobic ruminal bacterium called *Selenomonas ruminantium* has been shown to produce a different class of phytase [52–57]. For enzymatic activity, this enzyme does not require a co-factor. tyrosine phosphatases of proteins that are members of the cysteine phosphatase group are thought to have a distant relation to phytase. Along with other significant similarities, *S. ruminantium* phytase and cysteine phosphatases share the active site motif HCXXGXXR(T/S). Protein tyrosine phosphatases are the only ones with an active site loop which serves as a substrate-binding compartment. The fully phosphorylated inositol group of phytate can be accommodated in this pocket because *S. ruminantium* phytase has a wider and deeper pocket [52]. Similar to histidine acid phytases, *S. ruminantium* phytase's enzymatic phosphorylation of phytate is inhibited when metal cations are present. The ability of cations of iron, copper, zinc, and mercury to create complexes with phytate was found to be responsible for their inhibitory effect, while lead cations' stimulatory effect is still unknown [58]. It was recently reported that the anaerobic

bacteria *Selenomonas lacticifex* [59], *S. ruminantium* subsp. *Lactilytica* [60], and *Megasphaera elsdenii* [61] contain tyrosine phosphatase protein -like phytases. Phytases that resemble protein tyrosine phosphatase seem to be limited to anaerobic bacteria thus far.

2.Materials and methods:

Collection of soil samples and isolation of thermophilic fungi:

In this study, the fungi used were isolated from soil samples collected from different areas of Mansoura University. Soil fungal strains were isolated by using the dilution plating technique [62]. The modified in submerged fermentation in a 250 ml flask with one gram of wheat bran in it and 100 ml basal medium of the following composition (g/L): KCl, 0.5; $MnSO_4$, 0.1; $MgSO_4.7H_2O$,0.2; NaCl, 0.5; $CaCl.6H_2O$,0.0075; Yeast extract,1; pH (5.0).

Microscopic identification of the isolated fungi:

In Czapeck Dox agar medium the resulting fungal colonies were sub cultured for identification and then each pure culture was identified and characterized microscopical and morphological characteristics, colour of colony and sporulation. Examination was done as described by [63], making use of needle mount preparation. Using a needle and a drop of alcohol, the fragment of the culture's sporing surface was removed and placed on a sterile glass slide. After adding a drop of lactophenol to stain the fragment and carefully applying a cover slip to air bubbles preventing, under a light microscope the preparation was inspected. Whenever feasible, the isolated, purified fungi were identified down to the species level. The following generally recognized keys for the identification of various isolated fungi were used to help identify fungal genera and species. [64 - 65].

Phytase production by submerged fermentation:

Using modified in submerged fermentation in a 250 ml flask containing 1 g wheat bran and 100 ml basal medium of the following composition (g/L): KCl, 0.5; $MnSO_4$, 0.1; NaCl, 0.5; $MgSO_4.7H_2O$,0.2; $CaCl.6H_2O$,0.0075; Yeast extract,1; pH (5.0) then autoclaved at 121 º C for 30 min, cooled and inoculated with a colony. Content of each flask was mixed after inoculation. The flasks were incubated at 37º C. The contents of the flasks were centrifuged to remove debris. The crude supernatant was used for assaying the phytase activity by filtering through filter paper Whatman No. 1, the cultures were collected. For the purpose of estimating enzyme activity, As a basic enzyme, the culture filtrate was employed.

Assay of phytase activity:

Phytase activity was measured in an assay mixture containing 600µl substrate solution, (0.2% (w/v) sodium phytate and sodium acetate (0.1 M, pH 5.0) and 150µl from suitably enzyme. After 30 min of incubation at 37 ºC by adding 750µl of 5% (w/v) trichloroacetic acid, the reaction was stopped [66]. The amount of liberated phosphate ions was measured by combining 750 µl of the assay mixture with 750 µl of daily preparation made by combining four volumes of 2.5% (w/v) ammonium molybdate solution in 5.5% (v/v) sulfuric acid and one volume of 2.5% (w/v) ferrous sulfate solution. The production absorbance of phosphomolybdate, or the liberated inorganic phosphate (Pi), was measured spectrophotometrically at 700 nm. [67]. The amount of phytase enzyme that liberates 1.0 µmole of inorganic phosphate (Pi) per minute was defined as one unit of activity. [68]. By applying the same conditions to standard phosphate solutions without the addition of phytase, a standard curve (utilizing KH2PO4) was created.

Microbial susceptibility testing:

Agar well diffusion method:

A volume of the microbial inoculum is applied to the whole surface of the agar plate to inoculate it. Subsequently, a 9 mm diameter hole is aseptically punched using a sterile cork borer or tip, and 100 µL of the desired concentration of sample is added to the well. After that, agar plates are incubated in the appropriate

environment for the test microorganism. The microbial strain under investigation is prevented from growing as the antimicrobial agent diffuses throughout the agar medium [69].

Bacillus cereus, Bacillus subtilis, Enterobacter cloacae, Salmonella typhi, Klebsiella pneumoniae, Staphylococcus aureus, and Staph. epidermidis were the strains of bacteria that were tested.

Determination of Minimum Inhibitory Concentration (MIC):

In different concentrations of bacterial suspension, Serial dilutions, in nutrient broth medium were used to determine MIC. The control contained only inoculated broth and incubated for 24 h at 37 °C. The MIC end point is the lowest concentration of bacterial suspension where no visible growth is seen in the tubes. To confirm the MIC value the visual turbidity of the tubes was noted, both before and after incubation and O.D was measured at 600 nm to confirm the result [70]. The tested bacterial strain was *Bacillus cereus.*

3. Results and Discussion

Isolation and screening of microorganisms

Six distinct fungal cultures were isolated from the soil sample. The cultures were identified morphologically as *Aspergillus fumigatus*, *Penicillium chrysogenum*, *Penicillium digitatum*, *Penicillium purpurogenum*, *Rhizomucor miehei* and *Rhizomucor pusillus* figure (1).

phytase enzyme activity from thermophilic fungal strains show the highest activity for *Rhizomucor pusillus* with optical Density 0.981 and activity 2.112E-02 U/ml. The lowest activity for *Rhizomucor miehei* with optical density 0.901 and activity 1.940E-02 U/ml as shown in Table1.

Table (1): Phytase enzyme activities from thermophilic fungal strains

Fungal strains	OD	Activity U/mL
Aspergillus Fumigatus	0.977	2.103E-02
Penicillium crysogenum	0.969	2.086E-02
Penicillium digtatum	0.925	1.991E-02
Penicillium purpurogenum	0.958	2.062E-02
Rhizomucor miehei	0.901	1.940E-02
Rhizomucor pusillus	**0.981**	**2.112E-02**

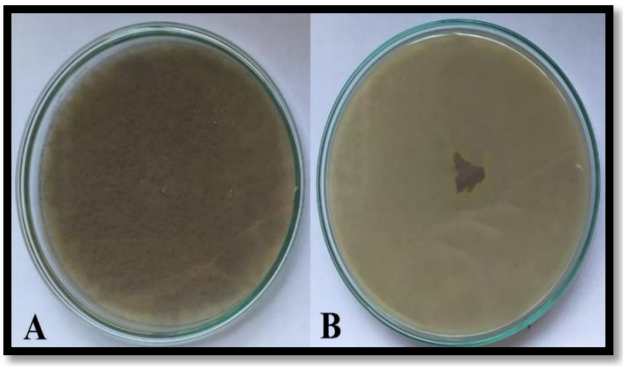

Figure (1): The isolated thermophilic fungal cultures of. *Rhizomucor pusillus* from the forward (A) and backward (B).

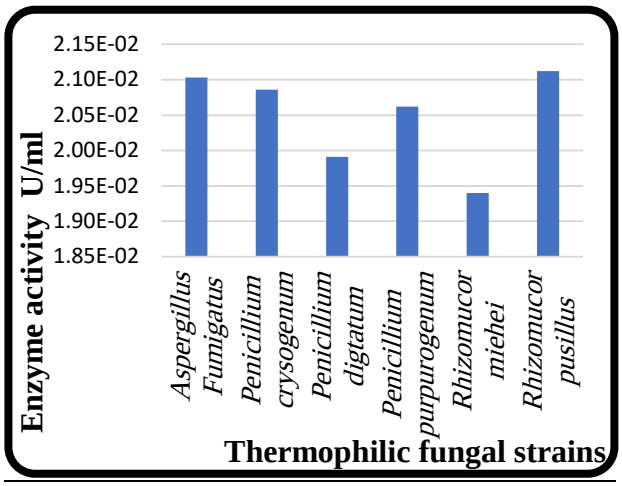

Figure (2): Phytase enzyme activities from thermophilic fungal strains

Submerged fermentation:

Based on a one-variable-at-a-time approach, independent variables (pH of medium, temperature and incubation period) were chosen for optimization. The enzyme activity and the optical Density in period incubation time at pH 5.0 and temperature 37°C defined the second day with high activity 2.841E-03 U/ml shown in Table 2. The optical Density and the enzyme activity at deferent pH degree defined the pH 5.0 with high activity at 37° C equal 2.841E-03 U/ml shown in Table 3. The optical Density and the enzyme activity at deferent incubation temperatures degree defined the 40°C with high activity equal 2.885E-03 U/ml shown in Table 4.

Table (2): Optical Density and enzyme activity in period incubation time at pH 5.0 and temperature 37°C

Incubation Time	O. D	Enzyme Activity
0 day	0.096	2.067E-03
1st day	0.112	2.411E-03
2nd Day	**0.132**	**2.841E-03**
3rd Day	0.108	2.325E-03
4th Day	0.106	2.282E-03

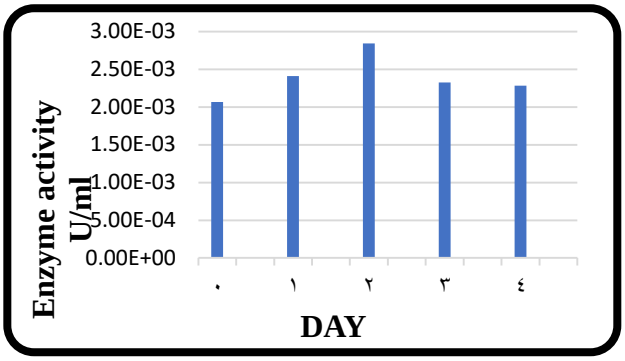

Figure (3): Optical Density and enzyme activity in period incubation time at pH 5.0 and temperature 37°C

Table 3: Optical density and enzyme activity at deferent pH degree to defined at 37°C

pH	O. D	Enzyme Activity
4	0.128	2.755E-03
5	**0.132**	**2.841E-03**
6	0.092	1.980E-03
7	0.067	1.442E-03
8	0.100	2.153E-03

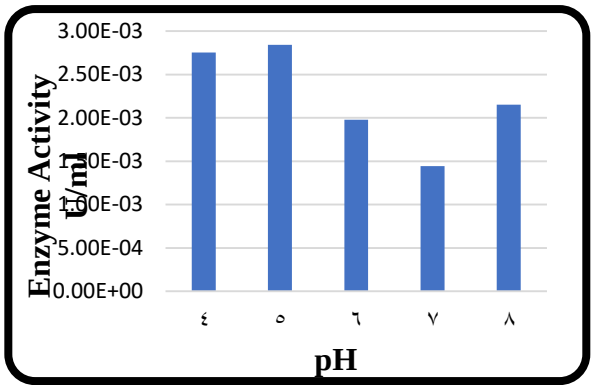

Figure 4: Optical density and enzyme activity at deferent pH degree at 37°C

Table (4): Optical density and enzyme activity at deferent incubation temperatures degree

Incubation temp. (° C)	O. D	Enzyme Activity
10	0.059	1.270E-03
20	0.077	1.658E-03
30	0.121	2.605E-03
40	**0.134**	**2.885E-03**
50	0.128	2.755E-03

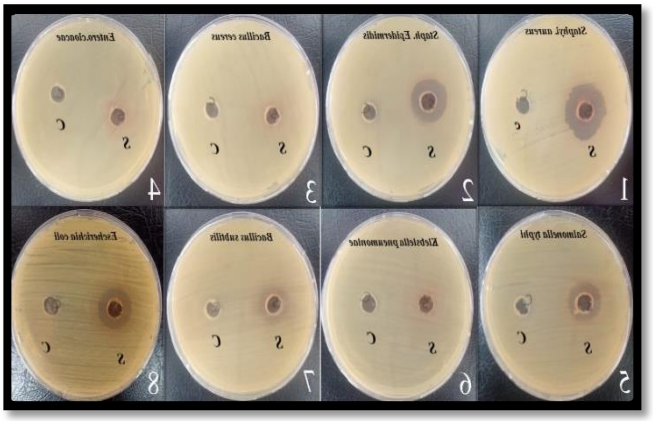

Figure (5): The optical density and the enzyme activity at deferent incubation temperatures degree

The antimicrobial test by disc diffusion assay of phytase extracted from *Rhizomucor pusillus* agaist 8 different pathogenic bacteria with Tetracycline as +ve control showed significance inhibition zones (mm) against *Bacillus cereus*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Staph. epidermidis* as shown in Figure 6 and Table 5. So it has been chosen to carry out next experiments.

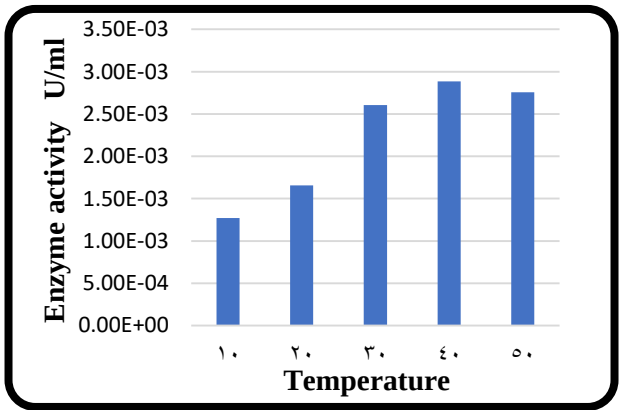

Figure 6: Antimicrobial activity of phytase extracted from Rhizomucor pusillus against 1.

Staphylococcus aureus, 2. Staph. Epidermidis, 3. Escherichia coli, 4. Salmonella typhi, 5. Bacillus subtilis, 6. Bacillus cereus, 7. Enterobacter cloacae and 8. Klebsiella pneumoniae.

Table 5: Antimicrobial activity of phytase extracted from *Rhizomucor pusillus* against different bacterial species

Bacterial species	Diameter of clear zone by phytase extraction (mm)	Control
1. ***Bacillus cereus***	-ve	-ve
2. ***Bacillus subtilis***	15.75	-ve
3. ***Enterobacter cloacae***	-ve	-ve
4. ***Escherichia coli***	18.4	-ve
5. ***Klebsiella pneumoniae***	-ve	-ve
6. ***Salmonella typhi***	17.05	-ve
7.***Staphylococcus aureus***	19.1	-ve
8. ***Staph. epidermidis***	19.0	-ve

The MIC value was taken as the minimum concentration of phytase extracted from *Rhizomucor pusillus* against bacterial growths of *Staphylococcus aureus* at which no microbial growth was observed.

The MIC of phytase was 0.20 g/mL against bacterial growths of *Staphylococcus aureus*. The tubes turbidity was noted after incubation to confirm the MIC value. The effective interaction between flavonoids and phenolics (phytochemicals) and a murine component of bacterial cell wall and chitin monomer 2-acetamido-2-deoxy-beta-D-glucopyranose, respectively, is demonstrated. To determine the cytotoxicity of the extracts and the ideal dosage of therapeutic formulations, more validation research is required.

Conclusions:

This study has demonstrated that soils are a rich source of phytase-producing fungi, with *Rhizomucor pusillus* isolated from Egyptian soil having the capacity to produce large amounts of the enzyme. phytase could be purified in just two steps while maintaining high enzyme activity. This enzyme has many excellent qualities that make it highly valuable to be used as a potent antimicrobial and anticancer agent, Moreover, based on the findings of this investigation, the isolated *Rhizomucor pusillus* holds promise as a potential source of phytase.

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