Exploring Novel Phytase-Producing *Escherichia coli*: Isolation, Characterization, and Industrial Potential

Running Title: Phytase- Producing *Escherichia coli*

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Abstract
Background and Objectives: Bacterial phytases catalyze the hydrolysis of phytic acid into myo-inositol and phosphoric acid, presenting versatile applications in environmental protection, agriculture, and animal feed industries. This study isolates and assesses the stability of phytase-producing *Escherichia coli* from soil, emphasizing their potential for commercial utilization.
Materials & Methods: Samples were obtained from soil contaminated with animal and poultry feces. Phytase-producing bacteria were isolated on PSM media, and identification was based on biochemical properties, morphological characteristics, and molecular analysis, specifically the 16S rDNA gene.
Results: The optimal time for phytase activity was determined by spectrophotometric measurement of liberated phosphorus in the liquid medium. Various pH levels and temperatures were tested to evaluate enzyme activity and stability. The 16S rDNA gene sequences of the isolates (ZH2 and ZH3) exhibited 99% and 100% homology with *E. coli*. Maximum phytase production occurred between 16-24 hours after incubation. Results indicated that bacterial isolates (ZH2 and ZH3) displayed enzyme activity at pH 3 and 9, and these newly identified strains produced significant amounts of the enzyme at 37 °C.
Conclusion: The study provides comprehensive insights into the potential applications of these newly isolated strains, suggesting their promising role in phytase enzyme production. The adaptability of these strains to diverse pH conditions and their activity at 37°C positions them as valuable candidates for applications in animal feed supplementation and environmental management.
Keywords: *Escherichia coli*, Phytase, Optimization, Characterization

Introduction:
Phytic acid, also known as myo-inositol hexakis phosphate or phytate, along with its derivatives, represents the predominant form of phosphorus in soil, cereals, oils, and legumes. Phosphorus stands out as a crucial essential mineral nutrient for both animal growth and crop production (1). Phytases, a group of enzymes, have gained significant attention in recent years due to their diverse applications in nutrition, environmental preservation, and human health. These enzymes catalyze the hydrolysis of phytic acid into myo-inositol and phosphoric acid (2).
The absence of the phytase enzyme in animals restricts the utilization of phosphate from phytate, giving rise to potential anti-nutritional challenges for both humans and animals. Phytate acts as an effective cation chelator, leading to substantial disruptions in protein absorption by forming complexes with dietary proteins and subsequently diminishing their accessibility (3). Additionally, undigested phytate and unabsorbed inorganic phosphorus contribute to ecological issues such as severe phosphorus pollution, culminating in eutrophication, particularly in regions with intensive livestock production (1).
The supplementation of commercialized phytase presents a viable solution to mitigate the anti-nutritional properties associated with phytate in animal feedstuffs (4). Given the industrial significance of phytase, the quest for an optimal enzyme has captivated researchers' interest. Phytase enzymes have been successfully isolated from microorganisms spanning various taxa (5). The biotechnological applications of phytase-producing bacteria underscore the need to discover and isolate novel bacterial strains capable of producing phytase, coupled with the optimization of enzyme production. Our primary objectives encompass the isolation of phytase-producing bacteria, characterization of pH activity profiles, and assessment of temperature stability.
Materials and Methods

Screening and Culturing Conditions: Soil samples, obtained from areas contaminated with livestock and poultry feces, were cultured in Peptone Water medium enriched with Yeast extract for screening phytase-producing bacteria. Subsequently, suspensions were inoculated onto phytase screening medium composed of 1.5% glucose, 0.5% (NH4)2SO4, 0.05% KCl, 0.01% MgSO4.7H2O, 0.01% NaCl, 0.01% CaCl2.2H2O, 0.001% FeSO4, 0.001% MnSO4, pH 6.5, with 0.5% sodium phytate (Sigma), and incubated for 48 hours at 37 °C (7). Following incubation, colonies were selected based on morphology and subjected to biochemical tests for genus identification (8).

Phytase Activity Assay: Eleven selected colonies were cultured in PSM broth at 180 rpm for 24 hours in a rotary shaker. After centrifugation at 4000 rpm for 10 minutes at 37°C, 0.1 ml of the cell suspension or supernatant containing enzyme solution was incubated for 10 minutes with 0.3 ml sodium phytate. The reaction was halted by adding 0.4 ml of 5% trichloroacetic acid. Free phosphate was measured at 700 nm using colorimetric quantification with a color reagent prepared by combining 1.5% (w/v) ammonium molybdate, 3.2% (v/v) sulfuric acid, and 7.2% (w/v) ferrous sulfate solution (9).

Measurement of Phytase Activity at Different Time Points: To determine the optimal time for phytase synthesis, strains were cultured in PSM at 180 rpm for 4 days. Colorimetric quantification at 700 nm was employed to measure free phosphorus released during phosphate hydrolysis (8).

Measurement of Phytase Activity at Different pH Levels: Phytase activity was measured at pH levels of 3, 5, 7, and 9 for 72 hours. Enzyme activity was calculated after 24 hours of incubation, as previously described (10).

Estimation of Growth: Growth curves were determined by cultivating overnight cultures in PSM, sampling 500 ml of each culture at 2-hour intervals over 78 hours of incubation, and monitoring cell growth spectrophotometrically at 600 nm (9).

PCR Amplification and 16S rRNA Sequencing: Genomic DNA extraction was performed using the boiling method. PCR amplification of 16S rRNA was carried out in a 25 µl PCR master mixture, including 2 µL of template, 10.5 µL of 2X PCR master mix, and 1 µL each of forward (5-AGTTTGATCCTGGCTCAG-3) and reverse (5-GGC/TACCTTGTTACGACTT-3) primers. The cycling program comprised denaturation at 94 °C for 5 min, followed by 45 s at 94°C, 45 s at 54°C, and 90 s at 72°C, with a final extension step at 72°C for 10 min. Amplification products were confirmed by electrophoresis in 1.5% (w/v) agarose gel. DNA sequencing was conducted on both strands through Pishgam Biotech Co, Iran.

Analysis and Phylogenetic Tree Construction: The obtained product sequences were analyzed, and the phylogenetic tree was constructed by comparing 16S rRNA sequences with other strains using MEGA 7 software and the maximum likelihood method (11).

Results

Screening and Isolation of Phytase-Producing Bacteria: Among the strains capable of growth on the PSM plate, 10 phytase-producing bacteria were selected for further investigation based on their morphology on the PSM and gram staining (gram-negative). The screening process involved liquid culture containing sodium phytate, from which three superior phytase-producing bacteria, designated as ZH1, ZH2, and ZH3, were selected based on their phytase activity (Table 1). Considering cultural, biochemical, and morphological characteristics (Table 1), these isolates were identified as Escherichia sp.

Table 1. Morphological and biochemical tests for identification of *Escherichia* genus.

Test	**Result**		
	ZH1	**ZH2**	**ZH3**
Shape	Rods	Rods	Rods
Gram staining	-	-	-
Motility	+	+	+
Catalase	-	+	+
Indol Production	+	+	+
Oxidase	-	-	-
MR/VP	+/-	+/-	+/-

Identification of Phytase-Producing Bacteria: PCR amplification of the 16S rRNA gene using universal primers confirmed the presence of the 16S rRNA gene in the isolated strains. The PCR products of the 16S rRNA gene are depicted in Figure 1. Sequence analysis against the NCBI database revealed a 99% and 100% homology to *E. coli* entries in the Gene Bank. Phylogenetic tree analysis using the maximum likelihood (ML) technique in MEGA7 software (Figure 2) further confirmed a robust relationship of the isolated strains to *E. coli*, showing 99% and 100% homology.

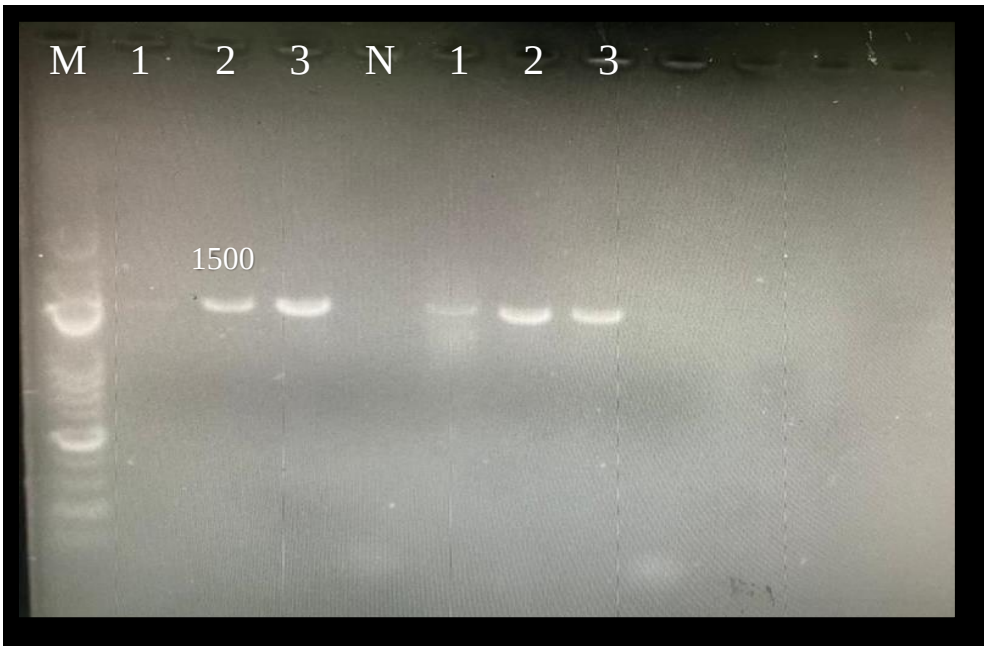

Figure 1. 1.5% agarose gel electrophoresis of the PCR products of *16S rRNA* for genomic DNA extracted from isolated strains. Lane M: GeneRulerTM 100 bp plus DNA ladder; lane 1-3: amplified fragments of *16S rRNA* genes in the ZH1, ZH 2 and ZH3 respectively; lane N: negative control.

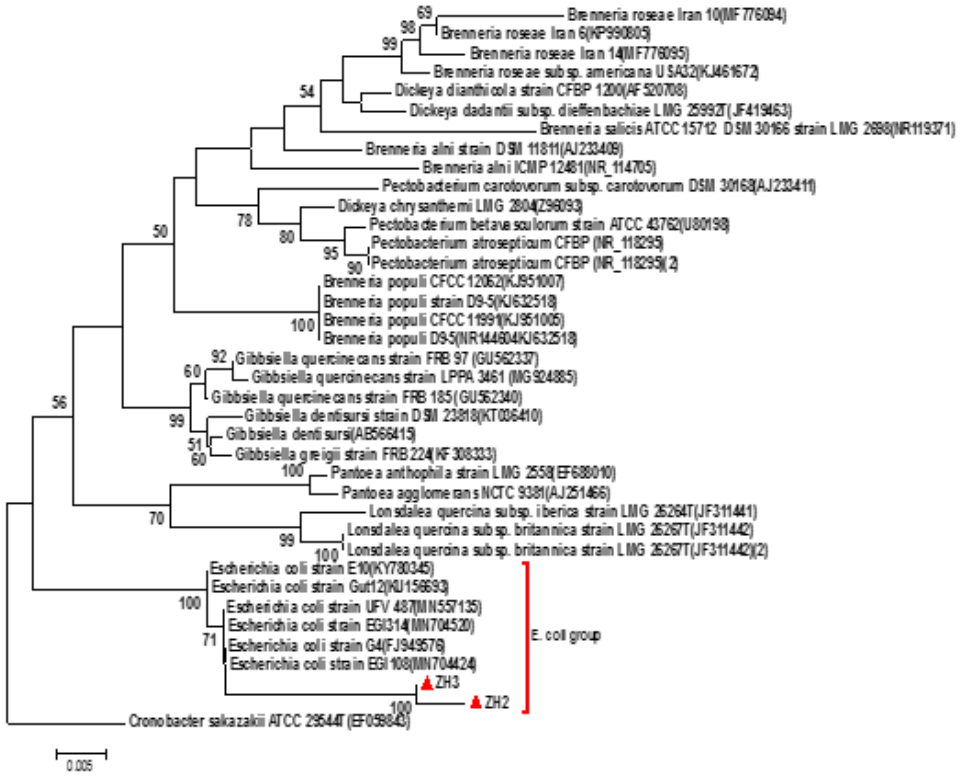

Figure 2: the phylogenic tree made by the maximum likelihood method showing the position of ZH2 and ZH3

Identification of the Optimal Time for Phytase Production: Culturing the three isolates in liquid medium containing sodium phytate at 37°C for 72 hours allowed for the spectrophotometric assay of phytase activity at 8-hour intervals using the Molbidate-Blue method. Data indicated that the optimal time for enzyme production was at 18 hours after incubation, with a significant decline observed after 40 hours of cultivation (Figure 3).

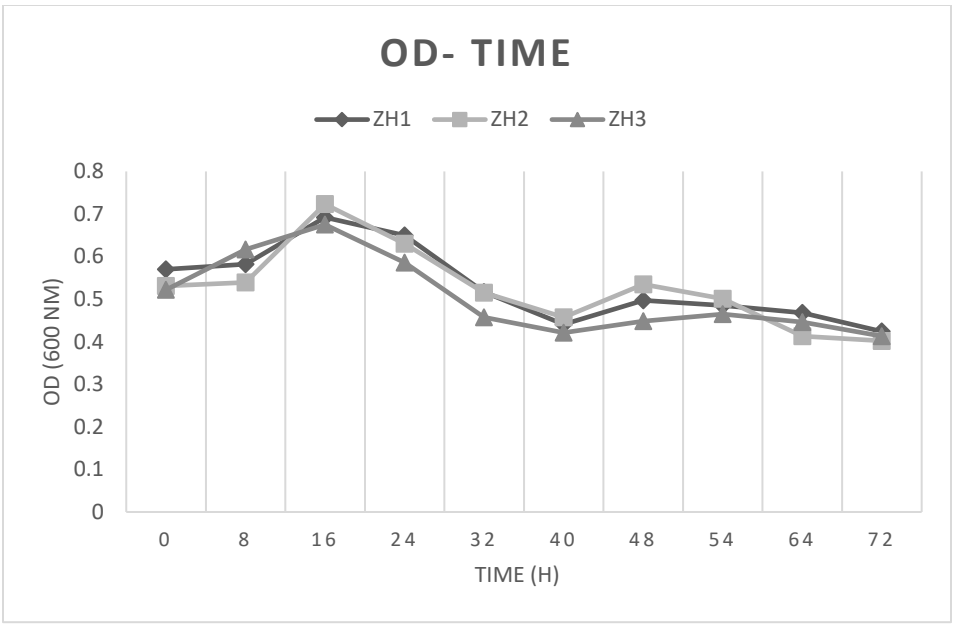

Figure 3. The best time for phytase production in different strains.

Growth Curve: To assess the growth curve and time course of phytase production, the strains were cultured in PSM broth at 37°C for 76 hours. Monitoring bacterial growth at 600 nm at 2-hour intervals revealed similar trend changes in all three strains. As depicted in Figure 4, a rapid increase occurred within the first few minutes, reaching a steady rate up to 42 hours, suggesting entry into the stationary phase after 8 hours. Phytase activity initiated during the stationary phase.

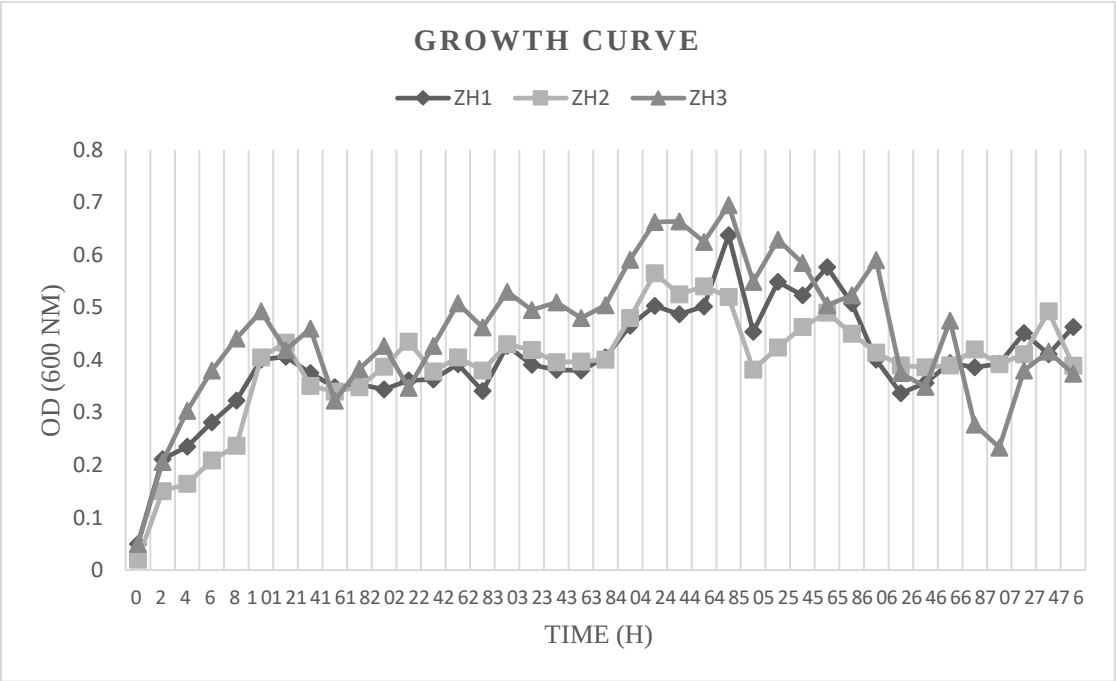

Figure 4. Growth curve for ZH1, ZH2 and ZH3 strais

Enzyme Activity at Different Temperatures: Phytase production was analyzed by incubating liquid medium containing sodium phytate at different temperatures (37, 45, 55°C). The optimal temperature for enzyme activity was determined to be approximately 37°C at pH 7.0. Results demonstrated a sharp decrease in phytase production at higher temperatures, with notable thermostability observed at 55°C (Figure 5).

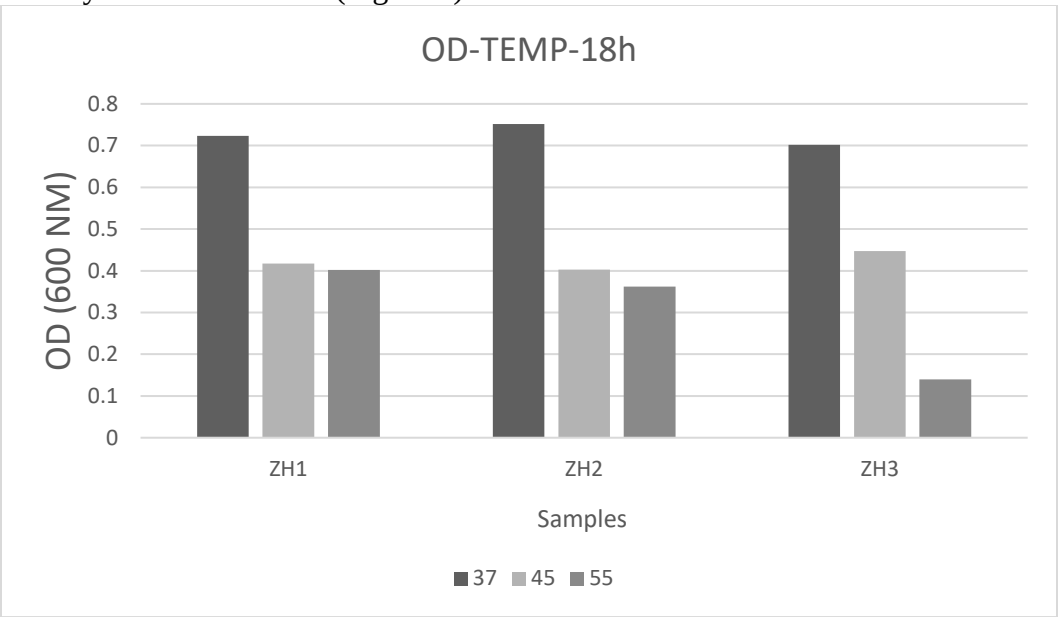

Figure 5. Effect of different temperature on phytase activity of ZH1, ZH2 and ZH3.

Enzyme Activity at Different pH: The pH activity profile of the three isolated strains was assessed in PSM at different pH levels (3, 5, 7, and 9) (Figure 6). Phytase production in the ZH1 strain reached maximum activity after 24 hours of incubation at pH 7.0 and 3.0. ZH2 exhibited optimal phytase activity at 24 hours after cultivation, displaying similar activity across different pH levels, indicating activity in both acidic and basic conditions. Phytase from ZH3 exhibited activity at pH 3.0 and 9.0, with a slight drop in stability at pH 5.0. Overall, phytase activity diminished after 24 hours, highlighting the stability of ZH2 and ZH3 phytases across a pH range of 3.0 to 9.0 (Figure 6).

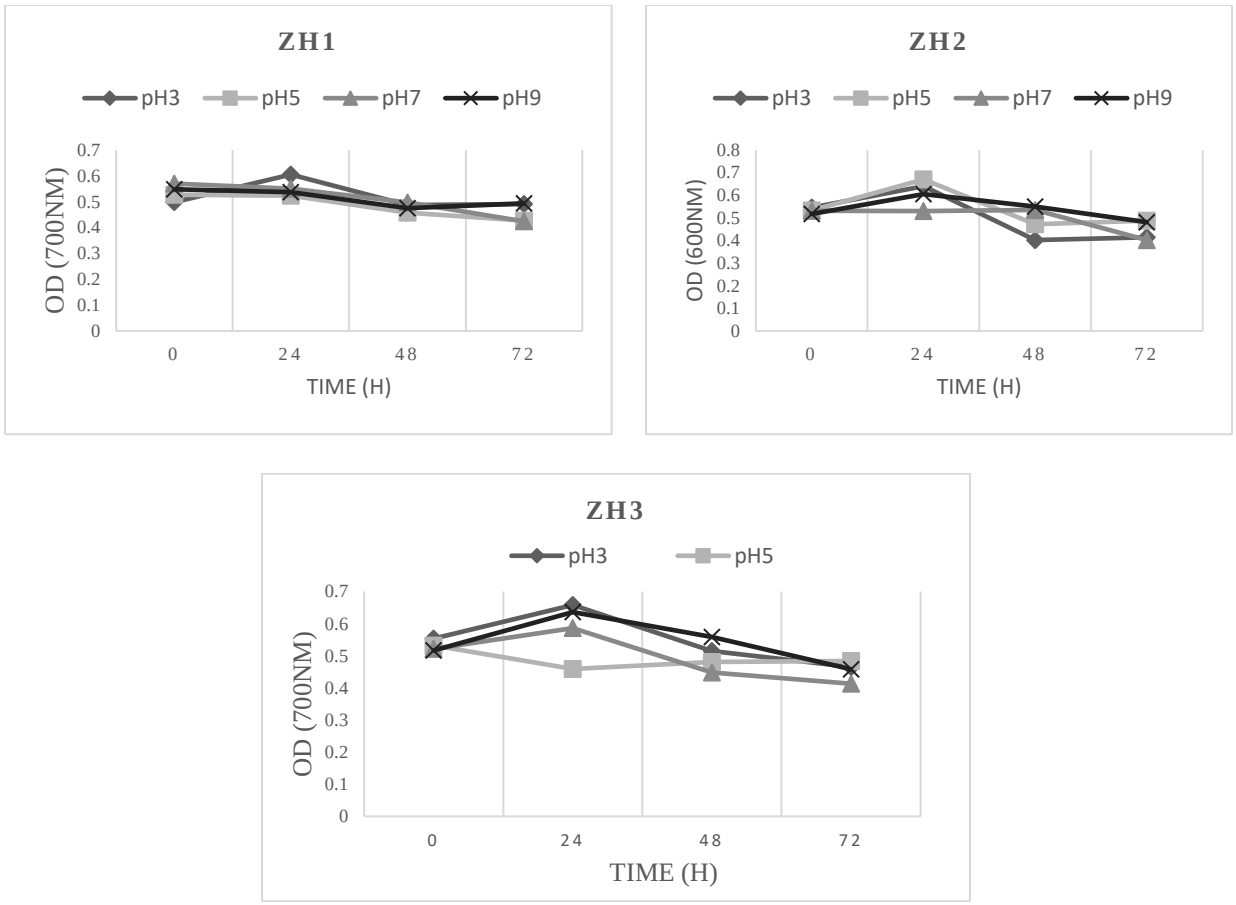

Figure 6. Effects of different pH on activity and stability of ZH1, ZH2 and ZH3 phytase.

Discussion

The enzyme phytase, a crucial catalyst in various industrial applications, is produced by diverse soil microorganisms (12). The imperative need to cultivate potent phytase-producing bacteria and enhance conditions for phytase production underscores the significance of our study's objectives. Our findings reveal that the peak phytase activity in bacteria occurred between 16 to 18 hours of incubation. Moreover, the growth curves of the phytase-producing bacteria indicate a substantial increase in enzyme activity during the stationary phase, reaching its maximum after 48 hours of cultivation. This correlation between phytase production and cell growth aligns with similar observations in previous studies, such as the maximum specific activity of phytase in *Bacillus subtilis* (natto), *Bacillus sp*. KHU-10, and *E. coli* coinciding with the stationary phase (9, 13), suggesting a nutrient-driven response (14).

Given the influence of physical parameters on enzyme formation, our study focused on optimizing conditions that play a crucial role in enhancing phytase production. The optimal temperature for phytase production was determined to be 37°C, with a decline in enzyme activity noted at higher temperatures (45 and 55°C). This aligns with the consensus that many phytase-producing microorganisms exhibit optimal activity in the range of 25 to 37°C (15). Notably, the temperature preferences for various *Bacillus* species have been reported at 37°C (14), while other studies found optimal temperatures at 60°C for *B. subtilis* (natto) N-77 (10), 70°C for *Bacillus amyloliquefaciens* DS11 (16), and *E. coli* WC7 (17).

The pH stability of ZH1, ZH2, and ZH3 phytases was assessed across different pH levels. ZH1 phytase exhibited optimal activity at pH 3 and 7, consistent with similar findings in *Bacillus licheniformis* (pH 7.5) (18) and *Bacillus sp*. DS11 (pH 7.0) (19). In contrast, ZH2 and ZH3 phytases demonstrated optimal function at pH 9 and 3, aligning with reports on Pseudomonas sp. (pH 7.0) (21) and variations in *Bacillus sp*. phytases (20, 21). Moreover, the adaptability of ZH2 and ZH3 phytases to a wide pH range (3.0–9.0) positions them as valuable in diverse environments. Acidic phytases, as demonstrated by ZH2 and ZH3, are particularly relevant for liberating mineral phosphate from animal feed (10).

Conclusion

This study comprehensively assessed physical parameters for enhanced phytase production by newly isolated strains (ZH1, ZH2, and ZH3). Notably, the adaptability of these strains to acidic and alkaline pH, coupled with their activity at 37°C, positions them as promising candidates for phytase production. Considering the acidic environment of the animal stomach, the application of phytase enzymes in animal feed supplementation could enhance the bioavailability of phosphorus compounds in food. Furthermore, phytase's ability to reduce phosphorus contamination in regions with intensive animal production underscores its potential environmental benefits. These findings contribute valuable insights into the optimization of phytase production, offering avenues for future applications in agriculture, animal nutrition, and environmental management.

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Conflict of Interest

The authors declare that there is no conflict of interest.

References

1- Chen C-C, Wu P-H, Huang C-T, Cheng K-J. A Pichia pastoris fermentation strategy for enhancing the heterologous expression of an *E. coli* phytase. Enzyme Microb Technol 2004; 35(4): 315-20.
2- Sharma N, Angural S, Rana M, et al. Phytase producing lactic acid bacteria: Cell factories for enhancing micronutrient bioavailability of phytate rich foods. Trends Food Sci Technol 2020; 96: 1-12.
3- Andrabi ST, Bhat B, Gupta M, Bajaj BK. Phytase-producing potential and other functional attributes of lactic acid bacteria isolates for prospective probiotic applications. Probiot Antimicrob Proteins 2016; 8: 121-9.
4- Romano N, Kumar V. Phytase in animal feed. In: Polaina J, MacCabe AP, editors. Industrial enzymes. Amsterdam: Elsevier; 2007. p. 73-88.
5- Lei XG, Porres JM, Mullaney EJ, Brinch-Pedersen H. Phytase: source, structure and application. In: Polaina J, MacCabe AP, editors. Industrial enzymes. Amsterdam: Springer; 2007. p. 505-29.
6- Jorquera M, Martinez O, Maruyama F, et al. Current and future biotechnological applications of bacterial phytases and phytaseproducing bacteria. Microbes Environ 2008; 23(3): 182-91.
7- Parhamfar M, Badoei-Dalfard A, Khaleghi M, Hassanshahian M. Purification and characterization of an acidic, thermophilic phytase from a newly isolated *Geobacillus stearothermophilus* strain DM12. Prog Biol Sci 2015; 5(1): 61-73.
8- Demirkan E, Baygın E, Usta A. Screening of phytate hydrolysis Bacillus sp. isolated from soil and optimization of the certain nutritional and physical parameters on the production of phytase. Turk J Biochem 2014; 39(2): 208-14.

9- Greiner R, Konietzny U, Jany KD. Purification and characterization of two phytases from *Escherichia coli*. Arch Biochem Biophys 1993; 303(1): 107-13.
10- Badoei-Dalfard A, Parhamfar M, Karami Z. Characterization of a thermostable, acidic-phytase from a newly isolated *Bacillus tequilensis* Dm018; medium optimization by response surface methodology. Catal Lett 2019; 149(11): 2961-72.
11- Parhamfar M, Badoei-Dalfard A, Parhamfar M, Fahimi Rad S. Purification and Characterization of an Extracellular Phosphatase Enzyme From Bacillus spp. J Cell Mol Res 2016; 8(2): 90-7.
12- Oh BC, Choi WC, Park S, Kim YO, Oh TK. Biochemical properties and substrate specificities of alkaline and histidine acid phytases. Appl Microbiol Biotechnol 2004; 63(4): 362-72.
13- Shimizu M. Purification and characterization of phytase from *Bacillus suhtilis* (natto) N–77. Biosci Biotechnol Biochem 1992; 56(8): 1266-9.
14- Choi YM, Noh DO, Cho SH, et al. Isolation of a phytase-producing *Bacillus sp*. KHU-10 and its phytase production. J Microbiol Biotechnol 1999; 9(2): 223-6.
15- Sasirekha B, Bedashree T, Champa K. Optimization and partial purification of extracellular phytase from Pseudomonas aeruginosa p6. Eur J Exp Biol 2012; 2(1): 95-104.
16- Vasudevan UM, Jaiswal AK, Krishna S, Pandey A. Thermostable phytase in feed and fuel industries. Bioresour Technol 2019; 278: 400-7.
17- Choi WC, Oh BC, Kim HK, Lee ES. Medium optimization for phytase production by recombinant *Escherichia coli* using statistical experimental design. J Microbiol Biotechnol 2002; 12(3): 490-6.
18- Wang Q, Fu SJ, Sun JY, Weng XY. Characterization of a thermostable alkaline phytase from *Bacillus licheniformis* ZJ-6 in Pichia pastoris. World J Microbiol Biotechnol 2011; 27(5): 1247-53.
19- Oh TK, Kim HK, Bae KS, et al. DS11 (KCTC 0231BP), novel Bacillus sp. strain and novel phytase produced by it. Google Patents; 2001.
20- Shamna K, Rajamanikandan K, Mukesh Kumar D, et al. Extracellular production of phytases by a native *Bacillus subtilis* strain. Ann Biol Res 2012; 3: 979-87.
21- Kim YH, Gwon MN, Yang SY, et al. Isolation of phytase-producing Pseudomonas sp. and optimization of its phytase production. J Microbiol Biotechnol 2002; 12(2): 279-85.