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ORIGINAL ARTICLE **OPEN ACCESS**

Isolation, Molecular Identification and Optimization of Phytase Production by *Bacillus firmis* Strain

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ABSTRACT
Phosphorus (P) depletion poses a significant threat to global food security due to the growing world population. It is a vital nutrient for plant growth and development, affecting processes such as photosynthesis, flowering, and root growth. Phytase enzymes play a crucial role in breaking down phytic acid, a common component in plant-based foods, and improving nutrient absorption. Bacterial phytases, known for their catalytic efficiency and substrate specificity, have gained prominence in various applications. In this study, bacterial isolates were screened and characterized for their phytase production capabilities. The most promising isolate, PB07, was identified as Bacillus firmis strain SW01 using 16S rDNA analysis. Optimal conditions for phytase production were determined, including pH (6) and temperature (40℃). The influence of different carbon and nitrogen sources on phytase production was also investigated, with sucrose and yeast extract showing the highest yields. The study emphasizes the significance of bacterial phytases in enhancing nutrient utilization and sustainability in various industries. The findings provide insights into the production and optimization of phytase enzymes, which can aid in addressing the global challenge of phosphorus depletion and improving food security.
Keywords: *Phosphorous, Phytases, Bacillus, 16srDNA*

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INTRODUCTION

Phosphorus (P) is a crucial element whose depletion poses a significant threat to global food security due to the rapidly growing world population. It is the second essential and indispensable nutrient necessary for the growth and development of plants (1, 2) and this depletion may jeopardize the ability to sustain a healthy life. Phosphorus is essential for a range of fundamental processes in plants, including photosynthesis, flowering, fruiting, and maturation. It plays a crucial role in cell division and the development of meristematic tissues, necessitating notably high phosphorus levels. Additionally, phosphorus supports root growth and contributes to nitrogen fixation (3). When phosphorus is deficient in a plant, it often results in characteristics such as slender and weak stems, delayed maturation, irregular flowering, and subpar seed quality. Many plants lacking phosphorus display a purple discoloration in their leaves and stems. Phosphorus is typically found in the soil as myo-inositol hexakisphosphate, known chemically as phytate or phytic acid, in an aggregated form (4). Phytases are enzymes that serve as critical catalysts in the breakdown of phytic acid, a ubiquitous component found in plant-based feed and food sources. Phytases, also known as myo-inositol hexakis phosphate phosphohydrolases, are enzymes specifically designed to hydrolyze phytic acid. Their enzymatic action results in the breakdown of phytate, releasing inorganic phosphate and mitigating the inhibitory effects of phytic acid on nutrient absorption. (5).

A significant portion of phosphorus in most plant-based foods, ranging from 50% to 80%, exists in the form of phytate (6). However, phytic acid, which constitutes phytate, has the adverse effect of reducing the bioavailability of various essential metal ions, including iron (Fe), zinc (Zn), magnesium (Mg), calcium (Ca), and others, thereby diminishing the overall nutritional value of these foods (7). To address this issue, the application of phytase enzymes is essential.

Bacterial phytases are a class of enzymes produced by certain bacterial strains with the remarkable ability to catalyze the hydrolysis of phytic acid, a prominent component in plant-based feed and food. This enzymatic activity results in the release of inorganic phosphate and myo-inositol from phytic acid, rendering it more digestible. The significance of bacterial phytases lies in their potential to improve

nutrient utilization in animal nutrition, reduce environmental phosphorus pollution, and enhance the sustainability of various industries (8). Bacterial phytases have gained prominence due to their economic and industrial applications. While fungal phytases are well-established, bacterial phytases offer advantages such as catalytic efficiency, resistance to proteolysis, and substrate specificity. Bacterial phytases have been extensively studied in various bacterial species, including *Citrobacter, Enterobacter, Pseudomonas, Bacillus,* and *Klebsiella* (9). Fungal phytases, mainly from the *Aspergillus* family, have been industrialized (10). The objective of this study is to investigate the capability of bacteria that produce phytase. This investigation involves the isolation and characterization of these bacteria from soil samples. Additionally, the chosen bacterial isolates will be identified and their phytase production will be optimized under different environmental conditions and nutrient sources.

MATERIAL AND METHODS

SAMPLE COLLECTION

Soil samples were collected from farms and gardens for the isolation and screening of phytase producing organisms.

ISOLATION OF PHYTASE PRODUCING BACTERIA

Bacterial strains were sourced from soil samples collected from various locations, including pulse fields, poultry farms, cattle sheds, and more. Approximately 0.1 g of each sample was suspended in a 0.8% saline solution (5 ml). From this suspension, 0.1 ml was streaked onto a phytase-specific medium. This medium comprised 1.5% glucose, 0.5% $(NH_4)_2SO_4$, 0.01% NaCl, 0.05% KCl, 0.001% $FeSO_4$, 0.01% $MgSO_4.7H_2O$, 0.01% $CaCl_2.2H_2O$, 0.001% MnSO4, and had a pH of 6.5, with the addition of 0.5% calcium phytate. Subsequently, 19 microbial colonies exhibiting the ability to hydrolyze calcium phytate, distinguished by the presence of clear halos around them, were obtained through the re-plating of single colonies on phytase-specific medium agar plates.

SCREENING OF PHYTASE PRODUCING ORGANISMS

The bacterial strains were screened for their ability to produce extracellular phytase, which was conducted using a phytase screening medium comprising the following components: Galactose (2%), Threonine-L (0.5%), Calcium chloride (20mM), Magnesium Chloride (20mM), Sodium phytate (20mM), trace elements (0.1%), Agar (2%), and adjusted to a pH of 6.5. The pure cultures of the isolates were streaked onto plates containing this phytase screening medium, and the plates were then incubated at 30ºC for a duration of 48 hours. Following incubation, the plates were examined for the presence of a clear zone around the bacterial growth as an indicator of phytase production.

SYNTHESIS OF PHYTASE BY BACTERIA

Enzyme production was conducted using the shake flask fermentation method, utilizing a production broth comprised of the following components: Rice bran extract (5%), 1% dextrose, $(NH_4)_2$ SO_4 (0.04g), $MgSO_4.7H_2O$ (0.02g), Casein (1g), KH_2PO_4 (0.05g), K_2HPO_4 (0.04g), with the pH adjusted to 6, and a total volume of 100ml of distilled water. To this production medium, 0.2 ml of calcium chloride from a sterile 2% stock solution was added. A 5% inoculum was introduced into the production medium. Fermentation was carried out in an orbital shaking incubator at 30ºC for a duration of 5-6 days.

EXTRACTION OF CRUDE PHYTASE

The fermentation broth obtained from the production medium was subjected to centrifugation at 10,000g for 15 minutes. This process resulted in the collection of the culture supernatant, which served as the crude enzyme extract. To this culture supernatant, three volumes of cold 95% ethanol were added, and the mixture was kept on ice with agitation for one hour. Subsequently, the precipitated crude extract was separated by centrifugation and then reconstituted in a 0.1M Tris HCl buffer with a pH of 7.0. The resulting extract was utilized for determining phytase activity.

ESTIMATION OF PHYTASE ACTIVITY

Phytase activity was determined by measuring the amount of liberated inorganic phosphate. Estimation was carried out using extracted enzyme. In an assay mixture 1ml of the extract was diluted 1:4 in acetate buffer (pH 5) and incubated at 30ºC for 10 min, with 1ml of 1.5mM sodium phytate in citrate buffer pH 3. The reaction was stopped by adding 1ml of 10% trichloroacetic acid solution (TCA). A blank sample was prepared by mixing 1ml of sodium phytate solution, 1 ml of TCA solution. Thereafter,1ml of distilled water and 5ml Taussky -Shorr (TS) reagent. (Ferrous sulphate / ammonium molybdate) were added to the blank sample and to the enzymatic mixture. After 10 min at room temperature the absorbance at 660 nm was read. One unit (U) of phytase activity was defined as the amount of enzyme that liberates one μ mol of inorganic phosphate per minute under assay conditions (11, 12).

OPTIMIZATION OF CULTURE CONDITIONS FOR PHYTASE PRODUCTION

STABILITY OF TEMPERATURE

In order to investigate the most favorable incubation temperature for achieving maximum phytase production, the flask containing the production medium inoculated with the PB07 was subjected to incubation at a range of temperatures: 20°C, 30°C, 40°C, 50°C, 60°C, and 70°C, over a period of 5 days. All other parameters were maintained at their optimum levels during this experimentation.

STABILITY OF pH

The strain PB07 was used to optimize the initial pH of the production medium, pH values were systematically adjusted to 3, 4, 5, 6, 7, and 8 using either HCl or NaOH. All other parameters were maintained at their optimum levels during this experimentation.

OPTIMIZATION OF NUTRIENT SOURCES

To determine the optimal growth conditions and enhance phytase production, the most promising strain PB07 was introduced into a 50 ml liquid broth. This mixture was then incubated on a shaker at 30°C for a duration of 5 days. Various carbon sources, including 1.5% glucose, maltose, sucrose, fructose, lactose was evaluated. Additionally, different nitrogen sources such as 0.5% yeast extract, ammonium sulfate, sodium nitrate, urea, and ammonium acetate were examined.

IDENTIFICATION OF PHYTASE PRODUCING BACTERIA

The molecular identification of the strain PB07 was done by 16srDNA.The total DNA was extracted from the culture that had been allowed to grow overnight, employing the previously established method as outlined by (13, 14). The PCR reaction mixture was formulated with 30 ng of genomic DNA, 1X buffer, 3mM MgCl2, 200μM of each deoxynucleotide triphosphate, 1U of Taq DNA polymerase and 2.5μM of each Eubacterial universal primer (16s F(27F) 5'-AGAGTTTGATCMTGGCTCAG-3';16s R(1492R) 5'-TACGGYTACCTTGTTACGACTT-3'), as previously documented (15). The thermal cycle program entailed an initial DNA denaturation step at 94°C for 5 minutes, followed by 35 cycles of 1 minute at 94°C, 1 minute at 45°C, and 2 minutes at 72°C. Subsequently, the extension occurred at 72°C for 5 minutes, and the reaction was held at 4°C for 5 minutes, with a thermocycler from Applied Biosystems Vertis being employed. The PCR product was purified through gel extraction using the QIA gel extraction kit from Qiagen in Germany and was further subjected to sequencing using the 27F primer. The partial sequences were subsequently analyzed via BLAST, employing the online option available at www.ncbi.nlm.nih.gov/BLAST (14), providing insights into the identity of the isolates.

RESULTS AND DISCUSSION

ISOLATION AND SCREENING OF PHYTASE PRODUCING BACTERIA

Bacterial strains capable of phytase production were obtained through isolation from a screening medium containing sodium phytate. A total of 19 (PB01-PB19) bacterial strains were isolated based on the presence of a clear halo zone observed on phytase-specific agar medium surrounding their colonies. Among these strains, Strain PB07 emerged as the most promising, exhibiting the ability to generate an approximately 39 mm clear halo zone around its colony. The presence of a zone of hydrolysis surrounding the bacterial colony indicated the positive occurrence of extracellular phytase production, as observed in Fig. 1, which displays the clearance zones around various isolates (16).

PHYTASE SYNTHEIS AND CRUDE EXTRACT EXTRACTION

Dextrose was used as substrate for the production of phytase under submerged fermentation conditions. The phytase was produced on Day 4 showing aggregation in the media. The initial period was considered as the adjustment period, therefore initially the production of phytase was low. Maximum production was achieved during log phase because cells in this phase grow at high rate with time. After this phase production was low due to unbalanced growth. The production of bacteria was carried out in the production medium without addition of agar using shaker flask method. (17)

From the broth the crude was extracted by centrifugation and the supernatant were collected. To that mixture 95% cold ethanol was added and the precipitated crude extract was harvested by centrifugation. The extract thus obtained was used for phytase activity determination.

ESTIMATION OF PHYTASE ACTIVITY

Phytase activity was determined by quantification of the phosphates released from phytase during the enzymatic reaction. Phytase production was measured by using a colorimeter at the absorbance of 660nm, following the release of inorganic phosphate. Each sample was measured in Spectrophotometer and O.D readings were tabulated. Strain PB07 showed maximum phytase activity of 16.8 U/mL on the third day of incubation. The results stated that the production of phytases from the isolated ranged from 0.9-16.8 U/mL (Fig.2). In the process of producing phytase (PB07) through submerged fermentation, rice bran extract served as the substrate. During the initial phase, which was designated as the adjustment period, the

production of phytase was relatively low. The highest production occurred during the log phase because the cells in this phase exhibited rapid growth over time. Subsequently, after this phase, production decreased, primarily due to imbalanced growth (18).

OPTIMIZATION OF CULTURE CONDITIONS FOR PHYTASE PRODUCTION

STABILITY OF pH

The impact of pH on enzyme activity was investigated across a range of pH values from 3 to 8. The findings revealed that the enzyme displayed higher activity levels within the pH range of 3 to 8, reaching its peak efficiency at pH 6. The enzyme exhibited relative activity levels of 15.6 U/ml and 20.7 U/ml at pH values of 5.0 and 6.0 respectively. However, beyond this pH range, both lower and higher pH levels caused a rapid reduction in enzyme activity. These results indicated that the purified phytase demonstrated optimal activity and stability within a slightly acidic pH environment (Fig. 3). The ideal pH range for phytase-producing bacteria, typically falls between 6.0 and 8.0 (8). However, some research indicates that, for the best growth and phytase production, many bacteria tend to thrive and perform optimally at pH levels within the range of 5.0 to 7.0 (19).

STABILITY OF TEMPERATURE

In terms of temperature stability, the profile of the purified phytase was evaluated over a temperature range from 20°C to 70°C using a standard phytase assay. As depicted in Figure 4, the enzyme exhibited higher activity between 35°C and 50°C, with its peak activity observed at 40°C (25.9 U/ml). The research indicated that phytase production exhibited an upward trend as the temperature increased, peaking at 40°C. However, production decreased as the temperature further elevated from 40°C to 70°C. It is worth noting that the optimal temperature range for most microbial phytase production falls between 25°C and 37°C (20).

OPTIMIZATION OF NUTRIENT SOURCES

The choice of carbon sources added to the production process exerts a significant influence on phytase production. When considering various carbon sources such as glucose, maltose, sucrose, glucose, lactose and fructose. The incorporation of sucrose into the production medium yielded the highest phytase activity (26.6 U/ml) in PB07. Following glucose and lactose (23.9 and 22.2 U/ml) showed the next highest phytase activities. The lowest phytase production was observed in media enriched with fructose (refer to Fig. 5).

It's worth mentioning that *Bacillus sp.* and *Enterobacter cloacae* demonstrated their best phytase production when lactose was used as the carbon source (21). Moreover, a previous study (22) highlighted that glucose was conducive to achieving optimal phytase production (12.23 IU/mL) from *Bacillus subtilis* P6.

In the context of phytase production, the production media were supplemented with inorganic sources (sodium nitrate, ammonium acetate, and ammonium sulfate) and organic sources (urea and yeast extract) to investigate the impact of nitrogen sources on phytase yield. Yeast extract as a nitrogen source demonstrated the highest phytase production (28.5 U/ml). Following these, were ammonium acetate urea with 23.6 U/ml and 20.6 U/ml (see Fig. 6). These findings align with a previous study, where the use of alternative nitrogen sources like yeast extract did not significantly differ from the performance where tryptone was used as a nitrogen source (17).

MOLECULAR IDENTIFICATION OF PHYTASE PRODUCING BACTERIA

The molecular identification of highest phytase producing bacterial isolate (PB07) was identified as *Bacillus firmis* strain SW01 (GenBank Accession No. OR150410.1) was done using 16S rDNA. The 16S rDNA gene was amplified by PCR using eubacterial universal primers. The amplicon, measuring approximately 1.5 kilobases, were visualized on a 1% agarose gel and recorded (Fig. 7). Following this, the PCR product was subjected to gel purification utilizing the QIA gel extraction kit from Qiagen, Germany, and subsequently sequenced with the primer 27F. To identify the isolates, the obtained partial sequences were subjected to BLAST analysis, utilizing the online tool accessible at www.ncbi.nlm.nih.gov/BLAST (14). Various bacteria, including *Bacillus spp.* (23), *Klebsiella spp.* (24), *E. coli* (25), and *Mitsuokella spp.* (26), have been documented as producers of phytase. These bacteria exhibit positive responses in plate-clearing assays and display enzymatic activity. The genus Bacillus comprises the most extensive group of extracellular phytase-producing bacteria, and it demonstrates effectiveness in breaking down phytate into mineral components (27).

Fig 1: Zone of clearance of strain PB07

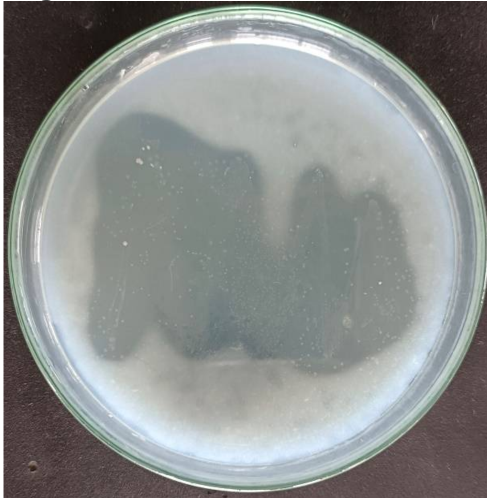


Fig 2: Phytase activity by isolated strains

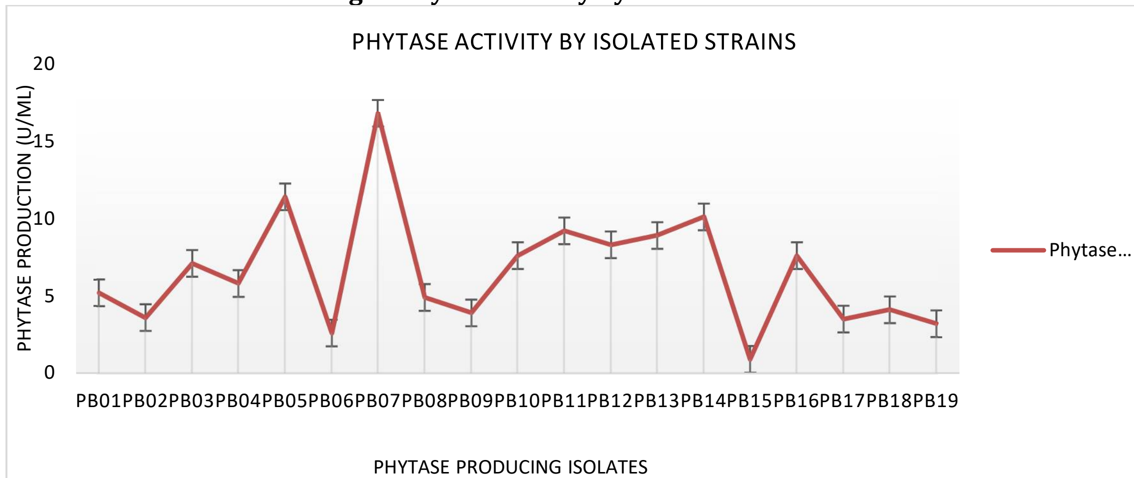


Fig 3: Stability of pH on phytase production

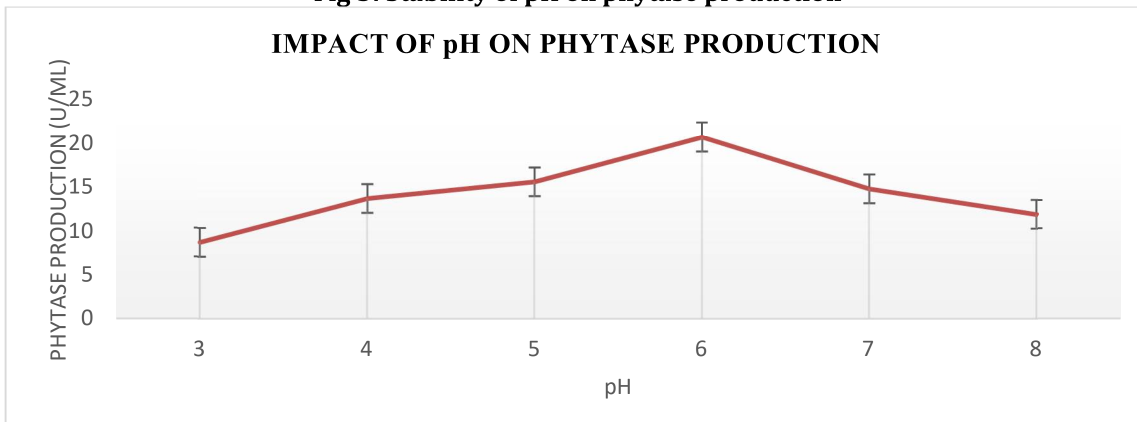


Fig 4: Stability of temperature on phytase production

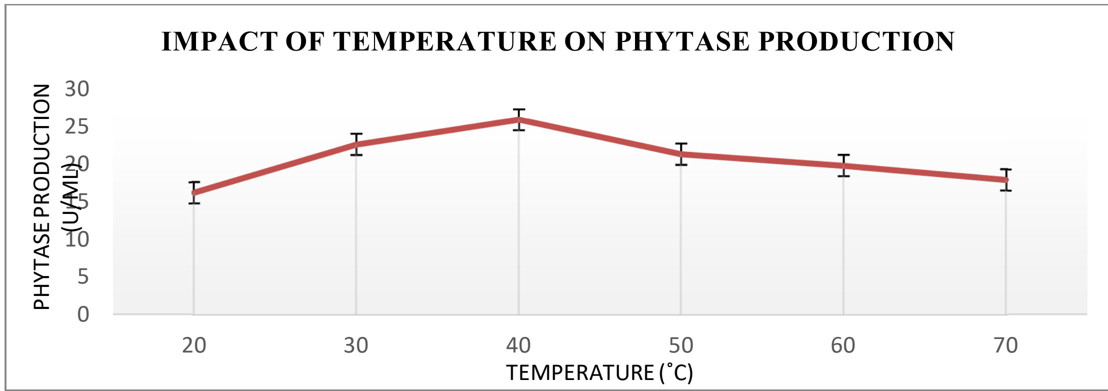


Fig 5: Impact of carbon sources on phytase production

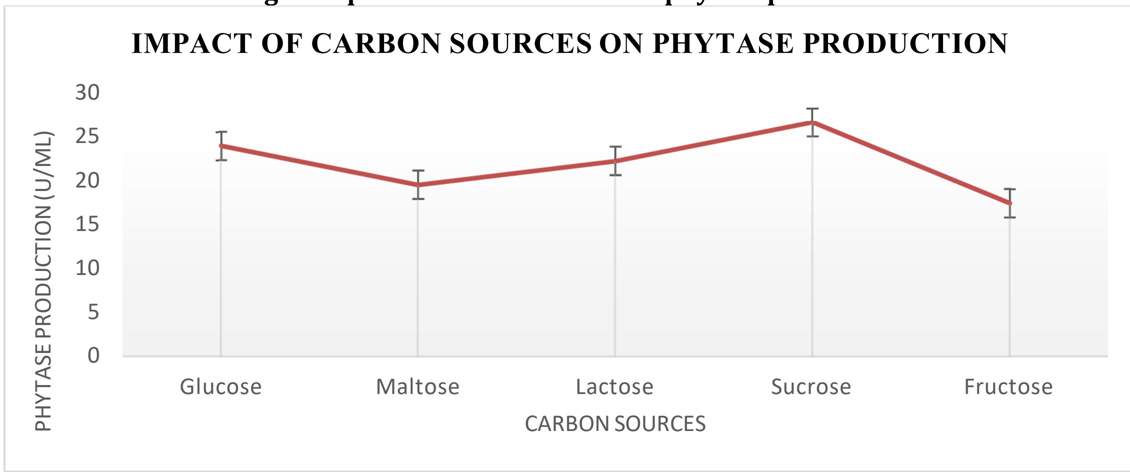


Fig 6: Impact of nitrogen sources on phytase production

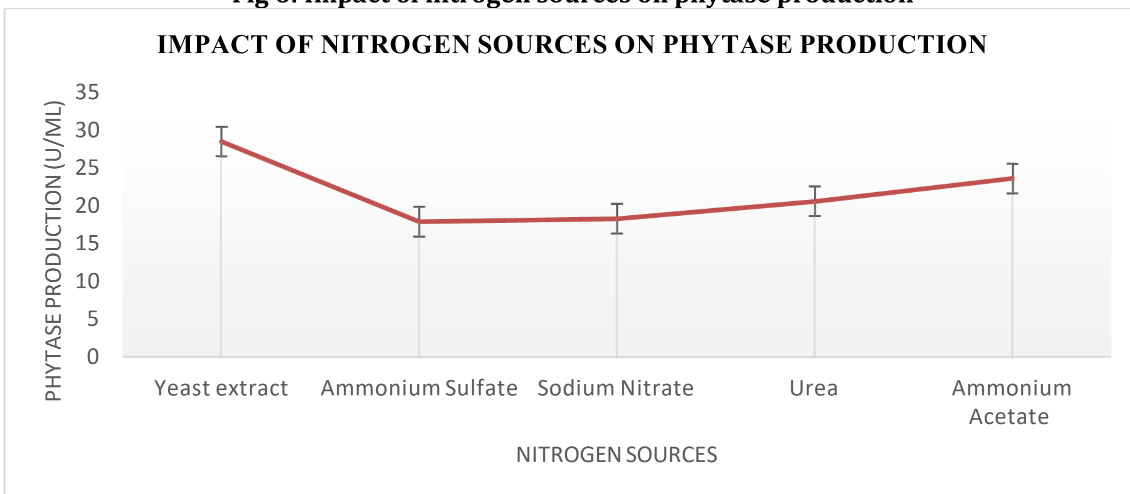
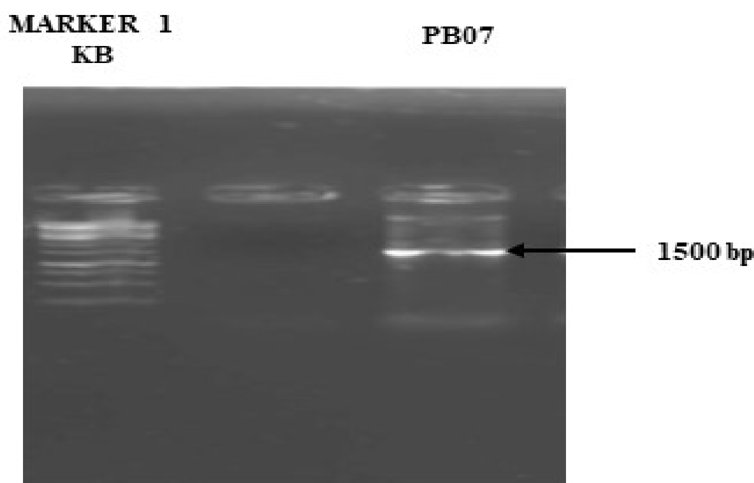


Fig 7: Gel picture showing 16s rDNA amplicon of PB07 (*Bacillus firmis* strain SW01)



CONCLUSION

The study underscores the critical role of phosphorus (P) in global food security and the pivotal contribution of phytase enzymes, particularly bacterial phytases, in mitigating the challenges posed by phosphorus depletion. Phosphorus is an essential nutrient for plant growth and development, affecting various fundamental processes in plants. Bacterial phytases, such as the one identified in this study as *Bacillus firmis* strain SW01, offer significant potential in addressing the adverse effects of phytic acid on nutrient absorption. These enzymes are characterized by their catalytic efficiency, resistance to proteolysis, and specificity towards their substrate. Bacterial phytases have emerged as valuable tools in improving nutrient utilization in animal nutrition, reducing environmental phosphorus pollution, and promoting sustainability in various industries. The optimization of culture conditions, including pH and temperature, demonstrated that this particular phytase reached its peak activity at pH 6 and 40°C. Furthermore, the study highlighted the impact of different carbon and nitrogen sources on phytase production, with sucrose and yeast extract proving to be effective choices. In conclusion, this research provides valuable insights into the production and optimization of bacterial phytase enzymes. These findings have the potential to contribute to the global effort to enhance nutrient absorption, reduce phosphorus depletion, and improve food security. Bacterial phytases offer a promising solution to address these pressing challenges and promote sustainable practices in agriculture and related industries.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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