Phytase activity of phytase-producing bacteria isolated from mangrove sediment

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Abstract. Through the role of phytase, some soil bacteria can mineralize insoluble organic P such as phytate (myoinositol hexakisphosphate). Phytase is a secreted enzyme possessing the ability to hydrolyze phytate into phosphate esters and inorganic P for plants absorption. This study aimed to isolate phytase-producing bacteria from mangrove sediment and examine the influence of nutrients (source of carbon, nitrogen, phosphorus) and physical conditions (temperature, pH, NaCl tolerance) on maximum phytase production. The presence of phytase activity was determined by examining the individual colonies for the formation of a clear zone. Furthermore, the isolates were screened qualitatively and quantitatively using solid and liquid phytase screening medium (PSM) containing sodium Phytate as substrates. The result showed that a total of 48 isolates have the potential to produce phytase with a production range of 1.11 - 14.83U/mL. The isolate F15 as Bacillus altitudinis was found to produce the highest phytase after 72 hours of incubation, was selected for further analysis. This strain resulted in optimal phytase levels at 35°C and a pH of 6.5 in physical parameters, tolerated 5% NaCl in the presence of lactose and tryptone, which served as carbon and nitrogen sources, respectively.

Keywords: Phytase-producing Bacteria, Phytase, mangrove.

1. Introduction
Mangrove forest is an ecosystem located in the transition of the sea and fresh water at the intertidal zone. It functions as a boundary for terrestrial and marine ecosystems as well as a breeding ground for various groups of plants, animals, and microorganisms [1,2]. This is a productive ecosystem due to various groups of microorganisms are found living freely around mangrove roots and sediments. Its productivity is majorly influenced by the important bacteria in the cycle of carbon, nitrogen, sulfur, and phosphorus, which participate in various processes of decomposition and mineralization of organic material [3,4].

Mangrove microorganisms provide various enzymatic activities and catalyze various biochemical reactions [2]. Furthermore, various hydrolytic enzymes such as amylase, nuclease, phosphatase, and protease are produced by several genera of bacteria isolated from rhizosphere and mangrove sediments. Phytase (Myo-inositol hexakisphosphate phosphohydrolases) belongs to the class of phosphatase enzymes that are capable of hydrolyzing phosphates containing organic molecules such as phytate and then releasing inorganic phosphorus in a stepwise reaction [5,6]. They are obtained from various sources including plants, animals, and microorganisms. Bacteria, which are one of the most important sources of phytase production. Several genera of phytase-producing bacteria have been isolated from mangrove
ecosystems, including *Bacillus*, *Enterobacter*, *Pseudomonas*, *Klebsiella*, *Proteus*, *Lysinibacillus*, and *Staphylococcus* [7,8,9].

Most phosphoruses are not available to mangrove plants in their sediments on interstitial waters. Besides inorganic phosphate, about 30 – 80% of total soil phosphorus is present in organic form. One of the main forms of organic P which is present about 50%-60% in the soil is the phytic acid as phytate (salt of phytic acid). Moreover, phytic acid is a source of organic P that is not available to plants, this is because phosphorus forms complexes with cations or absorbs into various soil components [10].

The presence of phytase-producing bacteria in the mangrove ecosystem is very important for the breakdown of organic P, which increases the availability of phosphorus and it is useful as plant nutrients [11]. Therefore, it plays an important role in the P cycle in soil [12].

However, there is little information about the presence of phytase-producing bacteria and their characterization from the topical mangrove ecosystem. This study aims to explore the potential of phytase-producing bacteria which includes their isolation and characterization from a tropical mangrove ecosystem. Furthermore, the selected isolates are identified and optimized for phytase production in various physical conditions and nutrient sources.

2. Materials and Methods

2.1. Isolation of phytase producing bacteria

Phytase-producing bacteria were isolated from mangrove sediment at 9 locations of mangrove forest in Segara Anakan, Cilacap, Central Java, Indonesia. A gram of soil from each sample was dissolved in 0.85% sterile NaCl to make serial dilution up to 10⁻³. Afterward, 0.2 ml of the sample solution (dilutions 10⁻³ and 10⁻⁵) was placed in sterile Petri dish, and the phytase screening media (PSM) agar solution (50°C) containing (1.5% glucose, 0.5% (NH₄)2SO₄, 0.01% NaCl, 0.05% KCl, 0.001% FeSO₄, 0.01% MgSO₄.7H₂O, 0.01% CaCl₂.2H₂O, 0.001% MnSO₄, pH 6. with 0.5% sodium phytate) agar solution (50°C) was poured on top, incubated for 7 days at room temperature [13]. The phytase-producing bacteria that grow are characterized by the presence of a clear zone around the colony. The selected isolates were then stored on LB agar media tilted for further analysis.

2.2. Quantitative analysis of phytase activity

The isolates were inoculated on LB media, incubated on a shaker at 150 rpm for 24 hours at room temperature. Also, 3% culture was inoculated into liquid PSM, incubated on a shaker at 150 rpm for 1-5 days. The culture of each isolate was then centrifuged at 10,000 rpm at 4°C for 10 minutes. Furthermore, phytase activity was measured from the supernatant obtained as a source of extracellular phytase.

Enzyme activity was carried out using the ammonium molybdate method by measuring the amount of inorganic phosphate released during the enzymatic reaction [14]. A total of 0.1 ml of the sample supernatant was mixed with 0.5% calcium phytate and dissolved in sodium acetate buffer (0.1M, pH 5.5) as a substrate. The reactant mixture was incubated at 45°C for 30 minutes, it was then stopped by adding 5% trichloroacetic acid. Afterward, a 160μl dye reagent consisting of 10N H2SO4, 10% ammonium molybdate, and 5% FeSO4 was added. In the case of blanks without samples, the color reagent was added before incubation while the substrate solution was added after. After incubation at 45°C, it was left for 30 minutes. Absorbance is the amount of free phosphate released, and the resulting color is measured using a spectrophotometer with a wavelength of 660nm. Furthermore, enzyme activity is expressed in international units (U), where one unit of phytase enzyme activity is defined as 1 mole of inorganic phosphate produced per ml in 1 minute [15].

2.3. Identification

Based on the highest phytase activity, isolate F15 was selected for further identification and analysis. The phytase-producing bacteria were identified by the PCR amplification method at 16 S rDNA using Primers 27 F: 5’ - AGA GTT TGA TCC TGG CTC AG - 3’ and Primary 1492 R: 5’ - GGT TAC CTT GTT ACG ACT T - 3’ [16]. The amplified PCR products were then sequenced by an automated DNA
sequencer (ABI PRISM 3130 Genetic Analyzer) with the Sanger method. The obtained sequences were analyzed using BLAST with genomic data registered at the NCBI/National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/).

2.4 Optimization on phytase production

Enzyme production is influenced by the growing conditions of the culture and the composition of the nutrient medium. Optimization of the components of the medium with various parameters, including temperature, pH, various nitrogen, carbon, and phosphates sources, different NaCl concentration on the phytase screening medium (PSM) broth was determined. All experiments were carried out in triplicate. For finding the optimum time on growth and phytase production, the Phytase screening broth medium was inoculated with selected isolate and incubated at 120 rpm for 120 h. The culture was harvested at an interval of 24, 48, 72, 96, and 120 hours.

Optimization of growth condition for maximum phytase production was carried out by inoculating the selected bacterial isolates in the phytase production medium for 72 h under different conditions: temperatures (25, 30, 35, 40, 45, 50) °C; pH (3.5, 4.5, 5.5, 6.5, 7.5, and 8.5); carbon sources such as sucrose, maltose, lactose, dextrose and glucose (control); nitrogen sources including tryptone, peptone, yeast extract and beef extract, ammonium chloride and ammonium sulfate (control); P sources such as Ca₃PO₄, KH₂PO₄, NaH₂PO₄ and concentrations of NaCl (1, 2, 3, 4, 5, 6%)

2.5. Statistical Analysis

Duncan’s Multiple Range Test (DMRT) at P < 0.05 level was conducted to compare the significant differences between the treatments using SPPS software version 16 and presented as the means ± standard deviation (SD), with three replications.

3. Results and Discussion

3.1. Isolation of phytase producing bacteria and Identification

A total of 48 out of the 68 bacteria isolated from mangrove sediments were phytase-producing bacteria. This is determined based on the presence of a clear zone around the colonies in solid PSM. Furthermore, these 48 isolates were tested quantitatively for phytase activity in liquid PSM. Results presented in Fig. 1 revealed different isolates had varying phytase production ranging from 1.11 to 14.83 U/mL.

This phytase production lower than phytase production of Bacillus licheniformis, Bacillus amyloliquefaciens, and Xanthomonas isolated from mangrove ≥ 100 U/mL in [17], varied from 70 to 160 U/mL of Bacillus sp. EBD 9-1 [18]. Another study found that phytase production from Proteus sp., RK 24 and Raoultella terrigenia, RK 33 were 0.261 U/mL and 0.876 U/mL respectively [19].

![Figure 1. Phytase production by phytase-producing bacteria.](image-url)
3.2. Identification
The screening results showed that the F-15 isolate produced the highest phytase and was identified based on the analysis of 16 S rRNA sequencing as *Bacillus altitudinis* strain F-15 (GenBank Accession No. MN845151.1). The sequences showed 99.61% similarity with the existing sequence in the NCBI database.

Furthermore, some bacteria (genus *Bacillus*) were isolated from the mangrove ecosystem such as *Bacillus circulans*, *B. licheniformis*, *B. pantothenicus*, and *Bacillus* sp. [7]; *B. licheniformis*, *B. amyloliquefaciens* [17]; *Bacillus* sp. [8]. The finding of these studies was consistent with [20], which isolated *Bacillus* sp strain LA12 and showed phytase activity. [21] also reported the presence of phytase activity from *Bacillus subtilis*.

Bacteria from the genus *Bacillus* is the largest extracellular phytase-producing bacteria and is effective in mineralizing phytate. Furthermore, the application of *Bacillus* as a biofertilizer in agriculture as well as in other fields has been studied extensively [22, 23].

3.3. Optimization of growth media

3.3.1. The influence of incubation time of phytase production
The effect of the incubation period on phytase production by *Bacillus altitudinis* is illustrated in Fig. 2. The result revealed that the production of phytase occurred at 24 hours and the stationary growth phase was reached at 72 hours of incubation, thereafter decreased at 96 and 120 hours. Maximum cell growth (biomass), as well as phytase production, was 0.27 and 15.63 U/mL respectively. A similar result has been reported that phytase production of *Bacillus* sp was recorded in 56-72 h of incubation period [18].

The incubation time of bacteria will differ from one another, this depends on several factors such as the isolate and the source of isolation, the availability of nutrients in the medium, and culture conditions. Another researcher [24] found that the maximum phytase production of *Enterobacter* and *Serratia* species occurred at 48 h of growth. The maximum phytase production has been detected after 44 h of incubation from *Bacillus subtilis* P6 [25]. It was obtained that maximum production of phytase at 48 h was shown by *Achromobacter* sp [26].

![Figure 2. Phytase production by *Bacillus altitudinis.*](image)

3.3.2. The influence of temperature on phytase production.
Cell growth and the production of phytase enzymes are strongly influenced by physical factors such as temperature. Therefore, to obtain the optimal temperature for phytase production, bacterial cultures were incubated at 25-50 °C. Then, the maximum phytase production was achieved at a temperature of 35 °C. The study showed that phytase production increased with increasing temperature and reached its maximum production at 35 °C, then decreased at 40 °C to 50 °C. However, at the highest temperature of
50 °C, phytase activity showed the lowest results (Fig.3). This is consistent with the report of [18] that the highest phytase production from Bacillus sp. EBD 9-1 was obtained at 35 °C.

It was also reported that most microbial phytase production is achieved at the optimum temperature between 25-37 °C [27]. This is also in line with the reports of [28] that some microorganisms showed the best phytase activity at 37 °C. Another result was found by [29] who was isolated Bacillus subtilis which has maximum phytase activity at 32 °C. Maximum phytase production was obtained at the optimum temperature of 37°C respectively for Bacillus badius, B. subtilis, B. ginsengi humi M2.11, and Klebsiella sp. [30, 31, 32, 33]. In contrast, some studies reported that phytase-producing bacteria showed maximum activity at a temperature of 40-60 °C. This implies that the optimal temperature for Bacillus sp. SP-46 is 40 °C [34]; Bacillus subtilis DR6 grows well at 50 °C [35]; the optimal temperature of B. licheniformis is 55 °C [7]; Bacillus sp strain LA12 has an optimum temperature at 60 °C [20].

![Figure 3. Phytase production by Bacillus altitudinis.](image)

3.3.3. The influence of pH on phytase production.
Phytase activity is also influenced by pH and the optimum value varies depending on the origin of the isolate. Our experiment showed that the highest phytase activity was obtained in bacterial cultures incubated at pH 6.5. Furthermore, phytase production increased with increasing pH from 3.5 to 6.5 pH, then the activity decreased from 7.5 to 8.5 pH (Fig.4). This result is consistent with [30] that the optimal phytase production of Bacillus badius was obtained at pH 6.5.

Optimum pH for phytase-producing bacteria, especially for the genus Bacillus and Enterobacter is ranging from 6.0 to 8.0 [36, 6]. However, other studies found that for optimal growth and production of phytase, most bacteria prefer pH in the range of pH 5.0-7.0 [37] and pH 6.5 and 7.5 [38, 39]. It was obtained that Bacillus sp DR6 isolates reached maximum production at pH 5.5 [35] and [31] reported that phytase production was at pH 5 to 6.

![Figure 4. Phytase production by Bacillus altitudinis](image)
3.3.4. The influence of N sources on phytase production

Phytase production media was added with inorganic (ammonium chloride and ammonium sulfate) and organic (peptone, tryptone, beef extract, and yeast extract) sources of C to determine the effect of nitrogen sources on phytase production. The use of tryptone as a source of N produces the highest phytase and is not significantly different from yeast extract. Followed by peptone, beef extract, ammonium chloride, and ammonium sulfate. However, the addition of ammonium sulfate as a control showed the lowest phytase results (Fig.5).

These results are consistent with [30] that the phytase production of B. badius PHY06 grown on media enriched with tryptone showed significantly higher yields than other N sources. Furthermore, the use of other nitrogen sources such as yeast extract was not significantly different from tryptone.

It was reported that Bacillus subtilis produced higher phytase when grown on media containing yeast extract [29]. This result is consistent with [40, 35, 41], for Bacillus subtilis 168, Bacillus subtilis DR6 and other phytase-producing bacteria, respectively, which achieved optimal phytase production with the addition of yeast extract. The results showed that the use of organic nitrogen sources produces higher phytase than inorganic. However, [42] found that the bacterial phytase activity of Enterobacter cloacae was optimal when given inorganic nitrogen.

![Figure 5. Phytase production by Bacillus altitudinis.](image)

3.3.5. The influence of C sources on phytase production

Nutrient sources such as suitable carbon added to the production have a strong influence on phytase production. Among the carbon sources (dextrose, maltose, sucrose, glucose, and lactose) used, the addition of lactose to the production medium resulted in the highest phytase activity of Bacillus altitudinis. Followed by glucose, sucrose. The lowest phytase results were obtained in media enriched with maltose (Fig.6).

Bacillus sp. EBD and Enterobacter cloacae showed the best phytase production with the addition of lactose as a C source [18,42]. Furthermore, [25] noted that glucose resulted in optimal phytase production (12.23 IU/mL) from Bacillus subtilis P6.
3.3.6. The influence of P sources on phytase production
According to [43], the use of inorganic phosphate in phytase media has a suppressive or inducers effect on phytase production in different microorganisms.

In the present study, the addition of inorganic phosphate, namely Ca$_3$(PO$_4$)$_2$ (22.63 U/mL), KH$_2$PO$_4$ (21.19 U/mL) and NaH$_2$PO$_4$ (17.65 U/mL) had a positive effect on phytase production compared to the basal media without inorganic P (15.62 U/mL/see Fig. 2). The results showed that the use of tricalcium phosphate as a source of P produces higher phytase than KH$_2$PO$_4$ and the lowest with the addition of NaH$_2$PO$_4$ (Fig. 7).

It was reported that the positive result of phytase production of Pseudomonas AP-MSU 2 was achieved with the use of sodium dihydrogen orthophosphate, whereas disodium hydrogen orthophosphate, calcium hydrogen orthophosphate and bismuth phosphate had a negative effect [43]. Furthermore, [25] noted that phytase production from Bacillus subtilis P6 could be stimulated in media enriched with K$_2$HPO$_4$.

![Figure 6. Phytase production by Bacillus altitudinis.](image)

3.3.7. The influence of NaCl concentration on phytase production
The effect of NaCl on phytase production was tested by adding NaCl with a concentration of 1-6% to the phytase production medium. The results showed that Bacillus altitudinis is a halotolerant bacterium and can survive and produce phytase in 5% NaCl media production (Fig. 8). Furthermore, the phytase activity of several bacterial species growing at same and lower concentrations was shown by Bacillus sp S1 and S2 [44], which was tolerant to 5% NaCl, Pseudomonas AP-MSU persisted at 3% [43]. Some
bacteria such as *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, and *Xanthomonas* were found to be optimum at 3% NaCl [17]. Halotolerant bacteria, namely *Bacillus pantothenticus* was successfully isolated and it produce phytase in production media with 10% NaCl added [7]. Also, [45] isolated saline-tolerant *Bacillus amyloliquefaciens* US573 which showed phytase activity at 20 g/l concentration of NaCl

![Figure 8. Phytase production by *Bacillus altitudinis*.](image)

4. Conclusion
A total of 48 bacteria was isolated from mangrove sediments showed their ability to produce phytase enzymes. The selected isolate for this study, *Bacillus altitudinis*, is a halotolerant bacterium that can grow and produce phytase at a concentration of 5% NaCl. Furthermore, the maximum phytase production was obtained on the 72nd hours of the incubation period at 35 °C and 6.5 pH. Conclusively, the use of lactose, tryptone, and tricalcium phosphate as sources of C, N, and P, respectively, showed maximum phytase results.

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