International Journal of Design & Nature and Ecodynamics
Vol. 20, No. 12, December, 2025, pp. 2943-2948
Journal homepage: http://iieta.org/journals/ijdne

Isolation, Characterization, and Feed Application of a Halotolerant Phytase-Producing *Streptomyces* sp. from Indonesian Mangrove Sediments

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https://doi.org/10.18280/ijdne.201218

Received: 10 November 2025
Revised: 14 December 2025
Accepted: 23 December 2025
Available online: 31 December 2025

Keywords:
phytase, actinobacteria, mangrove litter, Streptomyces griseorubens, poultry feed

ABSTRACT

Phytase is a key enzyme for improving phosphorus availability in poultry feed by hydrolyzing phytic acid. This study screened actinobacteria isolated from mangrove litter in Segara Anakan, Cilacap, for phytase production and evaluated their application in poultry feed ingredients. Twelve actinobacterial isolates exhibited phytase activity, with isolate K-2C showing the highest activity (3.42 U mL^{-1} under optimal conditions). Molecular identification based on the 16S rRNA gene revealed 99.02% similarity of K-2C to *Streptomyces griseorubens* NBRC 12780. Maximum phytase production was achieved after two days of incubation, with optimal activity at pH 7.0 and 50°C. In vitro application of K-2C phytase to rice husk, corn flour, soybean meal, and pollard significantly increased inorganic phosphorus release after 12 h of incubation, with phosphorus levels rising by 28.6–61.3% compared to controls. The highest phosphorus release was observed in rice husk, while corn flour showed the lowest response. These findings demonstrate that actinobacteria from mangrove litter are a promising indigenous source of phytase for reducing phytic acid and enhancing phosphorus availability in poultry feed.

1. INTRODUCTION

Poultry farming is one of the fastest-growing agricultural sectors globally, driven by increasing demand for affordable and high-quality animal protein such as eggs and meat [1]. The productivity of poultry is closely linked to feed quality and nutrient bioavailability. Poultry diets are predominantly composed of plant-derived ingredients, including corn, bran, and soybean meal. However, a major limitation of these feed materials is the high content of phytic acid, the principal storage form of phosphorus in plant seeds, which monogastric animals such as poultry cannot efficiently digest.

Phytic acid (myo-inositol hexakisphosphate) acts as an anti-nutritional factor by chelating essential minerals (e.g., Ca^{2+}, Mg^{2+}, Fe^{2+}, and Zn^{2+}) and forming complexes with proteins and carbohydrates, thereby reducing nutrient digestibility and bioavailability [2, 3]. Undigested phytic acid-bound phosphorus is excreted in feces and can contribute to environmental problems such as eutrophication when released into surface waters [4].

Phytase (myo-inositol hexakisphosphate phosphohydrolase) catalyzes the stepwise hydrolysis of phytic acid into lower inositol phosphates and inorganic phosphorus, thereby enhancing phosphorus availability and reducing anti-nutritional effects in poultry feed [2]. Supplementation of phytase in poultry diets has been widely reported to improve growth performance, nutrient digestibility, feed efficiency, and environmental sustainability [1, 4-6].

Phytases are derived from plants, animals, and microorganisms; however, microbial phytases are the most commercially valuable due to their higher activity, scalability, and stability. Fungal phytases (e.g., *Aspergillus niger* and *Peniophora lycii*) are widely used industrially but often exhibit limitations such as narrow pH optima and reduced thermostability. In contrast, bacterial phytases tend to show broader pH tolerance, higher thermal stability, and greater adaptability to industrial processes [2, 7].

Among bacterial sources, actinobacteria have attracted increasing attention due to their metabolic diversity and ability to produce robust extracellular enzymes. Several actinobacterial genera, including *Amycolatopsis*, *Arthrobacter*, *Streptomyces*, and *Nocardiopsis*, have been reported to produce phytase or solubilize phosphate efficiently [8-10]. Notably, *Amycolatopsis vancoresmycina* S-12 produces an extracellular phytase that is stable under acidic conditions and elevated temperatures, highlighting the industrial potential of actinobacterial phytases [8].

Mangrove ecosystems represent unique and underexplored

habitats characterized by high organic matter, salinity fluctuations, and microbial diversity. Mangrove litter from Segara Anakan, Cilacap, has previously been reported as a rich source of actinobacteria producing hydrolytic enzymes such as amylase, protease, lipase, and urease [11]. However, the potential of actinobacteria from this ecosystem as a source of phytase has not yet been systematically investigated.

This study therefore aimed to: (1) isolate and screen phytase-producing actinobacteria from mangrove litter in Segara Anakan, Cilacap; (2) identify and characterize the most promising phytase-producing isolate; (3) optimize phytase production and activity conditions; and (4) evaluate the efficacy of the crude phytase in hydrolyzing phytic acid in common poultry feed ingredients in vitro.

2. MATERIAL AND METHODS

2.1 Sample collection and pretreatment

Mangrove litter samples were collected from the Segara Anakan mangrove area, Cilacap, Central Java, Indonesia (7°43′–7°46′ S; 108°46′–108°49′ E). Litter was collected from the surface layer (0–10 cm depth) beneath mangrove stands using sterile gloves and placed into sterile polyethylene bags. Samples were transported to the laboratory in a cooled container and processed within 24 h. Prior to isolation, litter samples were air-dried at room temperature for 48 h, ground, and pretreated by heating at 55°C for 10 min to reduce fast-growing non-actinobacterial contaminants [12].

2.2 Isolation and maintenance of actinobacteria

Twelve actinobacterial isolates were obtained and maintained on starch casein nitrate (SCN) agar. The SCN medium was composed of (g L^{-1}): starch 10.0, casein 0.3, KNO_3 2.0, K_2HPO_4 2.0, $MgSO_4 \cdot 7H_2O$ 0.05, NaCl 2.0, $FeSO_4 \cdot 7H_2O$ 0.01, and Bacto agar 15.0. Nystatin (100 µg mL^{-1}) was added to suppress fungal growth [12]. The cultures were incubated at room temperature (28–30°C) for seven days prior to use.

2.3 Screening of phytase activity

Each isolate was inoculated into 50 mL of liquid SCN medium containing nystatin (100 µg mL^{-1}) and incubated on an orbital shaker (150 rpm) at room temperature. Samples were collected daily for five days and centrifuged at 10,000 rpm for 10 min to separate mycelium and supernatant. Preliminary screening of phytase activity was conducted using the colorimetric method of Purwati et al. [13], based on phosphomolybdate complex formation measured at 415 nm. This method was used exclusively for relative screening to select the most active isolate.

2.4 Identification of actinobacteria

Molecular identification was performed based on 16S rRNA gene sequencing following Asnani et al. [14]. Genomic DNA was extracted using the Presto™ Mini gDNA Bacteria Kit (Geneaid®). PCR amplification employed primers 27F and 1492R using KAPA Taq Extra HotStart ReadyMix®. Sequencing was performed bidirectionally, and species identification was achieved using BLAST nucleotide analysis.

2.5 Determination of growth curve

Ten agar plugs (Ø 5 mm) from actively growing cultures were inoculated into liquid SCN medium and incubated for 14 days. Cultures were harvested daily, centrifuged, and the mycelial dry weight was determined after drying at 60°C to constant weight. The exponential growth phase was used to determine the optimal inoculum age.

2.6 Determination of the production curve

A 10% (v/v) inoculum was transferred into SCN production medium supplemented with 1% (w/v) bran husk. Cultures were incubated for 0–7 days under shaking conditions. Phytase activity was quantified using the Engelen et al. [15] method, and the incubation time yielding maximum activity was selected for enzyme production.

2.7 Production and characterization of phytase

Phytase was produced under optimal incubation conditions determined from growth and production curves. Crude enzyme was obtained by filtration and centrifugation. Enzyme characterization was performed by varying pH (5.0-8.5) and temperature (35-60°C) to determine optimal activity conditions.

2.8 Determination of phytase activity and protein concentration

Quantitative phytase activity throughout production, characterization, and application experiments was determined exclusively using the Engelen et al. [15] method, based on iron sulfate–molybdate complex formation measured at 700 nm. One unit (U) of phytase activity was defined as the amount of enzyme releasing 1 µmol of inorganic phosphate per minute under assay conditions. Protein concentration of crude enzyme extracts was determined using the Bradford method, allowing calculation of specific activity (U mg^{-1} protein).

2.9 Application of phytase in feed ingredients

Crude phytase (1 mL, equivalent to 3.42 U mL^{-1}) was mixed with 1 g of feed ingredient (rice husk, corn flour, soybean meal, or pollard), corresponding to an application rate of 3.42 U g^{-1} feed. This dosage was selected based on preliminary optimization and commonly reported in vitro phytase application ranges [15]. Mixtures were incubated for 0, 12, and 24 h prior to centrifugation and phosphate analysis.

3. RESULT AND DISCUSSION

3.1 Screening and identification of phytase-producing actinobacteria

The screening of potential phytase-producing actinobacteria was conducted using sodium phytate as a substrate in sodium acetate buffer, followed by colorimetric detection of released inorganic phosphate. The results demonstrated that all twelve actinobacterial isolates exhibited detectable phytase activity, indicating that mangrove litter is a rich ecological niche for

phytase-producing microorganisms. However, the magnitude of enzyme activity varied considerably among isolates.

Quantitative screening results (Figure 1) showed that five isolates—K-2C, K-3E, P-6B, P-7C, and P-7E—displayed substantially higher relative phytase activity compared to the remaining isolates. Importantly, Figure 1 presents the relative phytase activity of all five isolates, thereby demonstrating the comprehensiveness of the screening process and confirming the superior performance of isolate K-2C. Among all tested isolates, K-2C consistently exhibited the highest relative activity, reaching approximately 717% relative activity, whereas the other high-performing isolates ranged between 400–600%.

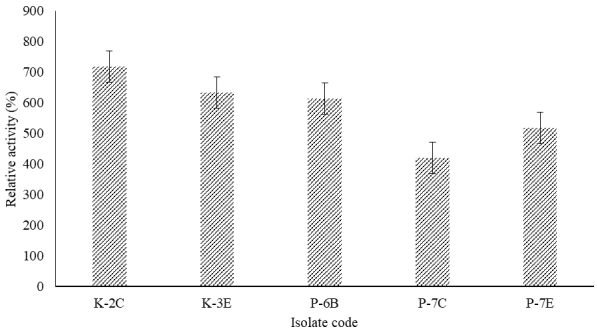

Figure 1. Relative activity of phytase from K-2C, K-3E, P-6B, P-7C, and P-7E isolates

It should be emphasized that the data presented in Figure 1 represent relative screening activity, used solely to identify the most promising isolate. Absolute phytase activity values (U mL^{-1}) were subsequently determined using the Engelen method during production and characterization stages. Thus, the statement that "K-2C exhibited the highest activity" refers specifically to its relative superiority during initial screening, not absolute enzyme units.

Molecular identification based on 16S rRNA gene sequencing revealed that isolate K-2C shared 99.02% sequence similarity with *Streptomyces griseorubens* strain NBRC 12780. To strengthen species-level identification beyond percentage similarity, a phylogenetic tree was constructed using the maximum likelihood (Figure 2). The phylogenetic analysis showed that the query sample K-2C clusters closely with the type strain of *S. griseorubens* and was clearly separated from other closely related *Streptomyces* species. This clustering pattern supports the assignment of isolate K-2C to *S. griseorubens*.

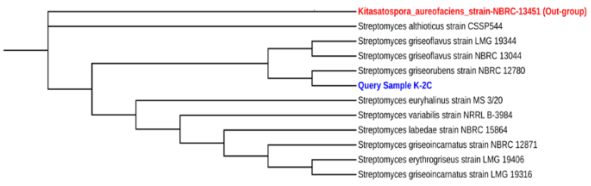

Figure 2. Phylogenetic tree indicating isolate K-2C is most closely related to the *Streptomyces griseoflavus* lineage

The genus *Streptomyces* is widely recognized as a key group of phosphate-solubilizing actinobacteria [16]. Several studies have reported *S. griseorubens* as an efficient solubilizer of phosphate and potassium, including strains BC3 and BC10 [17]. Soumare et al. [10] identified *S. griseorubens* as one of the most efficient phosphate-solubilizing actinobacteria, capable of enhancing phosphorus availability through mineralization and enzymatic hydrolysis. Beyond phytase production, *S. griseorubens* is also known for producing cellulase, xylanase [18], chitinase with biocontrol activity [19], and various antibacterial and anticancer compounds [20]. These multifunctional traits highlight the ecological and biotechnological relevance of this species.

3.2 Growth characteristics of *S. griseorubens* K-2C

The growth curve of *S. griseorubens* K-2C (Figure 3) was evaluated to determine the optimal physiological phase for enzyme production. Microbial growth was monitored based on mycelial dry weight over a 14-day incubation period. The results revealed a typical actinobacterial growth pattern, consisting of lag, exponential, stationary, and death phases.

During the initial lag phase (day 2–4), biomass accumulation was minimal, as indicated by a relatively constant growth rate ($\mu \approx 0.45–0.46$), reflecting cellular adaptation to the growth medium (Figure 3). The duration of the lag phase is influenced by inoculum size, physiological state of the cells, and nutrient availability [9]. Following adaptation, isolate K-2C entered the exponential growth phase starting on day 6 and reached a maximum growth on day 10, as evidenced by the highest growth rate value ($\mu \approx 0.49$). This maximum growth rate indicates robust cell proliferation under the experimental conditions before entering the stationary phase.

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The stationary phase occurred between days 10 and 12, during which biomass accumulation plateaued due to nutrient depletion and accumulation of metabolic byproducts. Actinobacteria are known to maintain metabolic activity during this phase, often producing secondary metabolites, including extracellular enzymes. The death phase was observed on day 14, characterized by a gradual decline in viable biomass due to prolonged nutrient limitation and toxic metabolite accumulation.

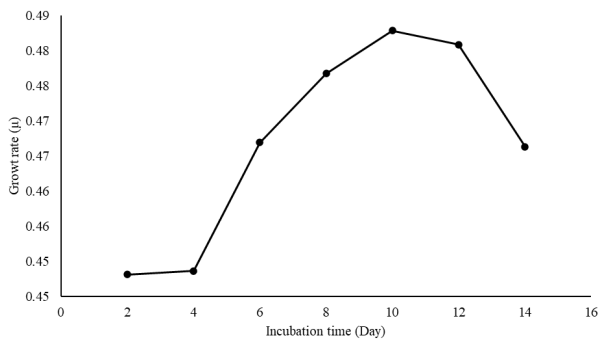

Figure 3. Growth curve of *S. griseorubens* K-2C based on growth rate (μ) over a 14-day incubation period

These findings indicate that isolate K-2C exhibits relatively slow but stable growth, which is typical of *Streptomyces* species. The identification of the exponential phase at day 8 was critical for selecting the appropriate inoculum age for phytase production.

3.3 Phytase production profile of K-2C

The phytase production curve was evaluated to determine the optimal incubation time for maximum enzyme synthesis. Enzyme production was conducted using rice bran and sodium phytate as substrates, enabling the assessment of phytase production under conditions simulating both complex and pure phytate sources.

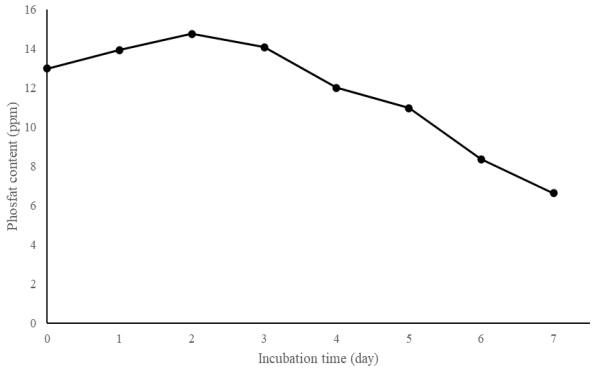

Figure 4. Production curve of phytase activity based on phosphate production from K-2C

The results showed that phytase production increased rapidly during the early incubation period and reached a maximum on day 2, producing phosphate of approximately 15 ppm (Figure 4). After reaching this peak, phytase production gradually declined with prolonged incubation time.

The decrease in phytase content after day 2 may be associated with enzyme degradation, nutrient depletion, feedback inhibition, or reduced metabolic activity as the culture entered the stationary phase.

Based on these results, a 2-day incubation period was selected as the optimal production time. This relatively short production cycle is advantageous from an industrial perspective, as it reduces fermentation time and associated costs.

3.4 Characterization of phytase from *S. griseorubens* K-2C

Characterization of crude phytase from isolate K-2C was conducted to evaluate the effects of incubation temperature and pH on enzyme activity, as indicated by the release of inorganic phosphate. The results showed that phytase activity increased with temperature and reached an optimum at 50°C, producing approximately 2.9 ppm of soluble phosphate, before declining at higher temperatures (Figure 5).

Similarly, phytase activity was strongly influenced by pH, with the highest phosphate release observed at pH 7.0, reaching approximately 7.2 ppm, followed by a sharp decrease under more alkaline conditions. These results indicate that the phytase from *S. griseorubens* K-2C exhibits maximal catalytic performance under neutral pH and moderately high temperature conditions.

Enzyme activity increased with temperature up to the optimum due to enhanced kinetic energy and increased frequency of enzyme–substrate collisions. However, activity declined sharply above 50°C, likely due to thermal denaturation and conformational changes in the enzyme's active site. Similarly, phytase activity was strongly influenced by pH, with maximal activity at neutral pH. Deviations from this optimum resulted in reduced activity, reflecting alterations in enzyme ionization state and substrate binding affinity.

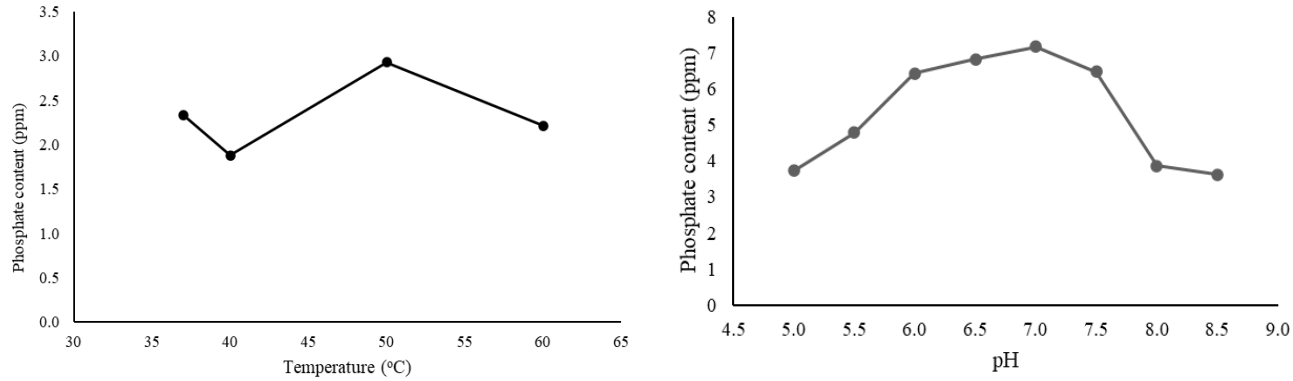

Figure 5. The characterization of K-2C phytase is contingent upon the variables of incubation temperature and pH

The neutral pH optimum (pH 7.0) of *S. griseorubens* K-2C phytase distinguishes it from many commercial fungal phytases, which typically exhibit optimal activity under acidic conditions (pH 4.5–5.5). This characteristic may offer significant advantages, particularly for applications in animal feed, where phytase activity in the small intestine—characterized by near-neutral pH—is highly desirable. Neutral phytases may therefore exhibit improved in vivo efficiency without requiring acidification of feed or reliance on gastric conditions.

Moreover, because isolate K-2C originates from a mangrove ecosystem, which is frequently exposed to salinity fluctuations, anaerobic stress, and metal-rich sediments, it is plausible that its phytase possesses enhanced stability under stressful environmental conditions. Mangrove-derived enzymes have been reported to exhibit salt tolerance and resistance to metal ions, making them attractive candidates for industrial applications. Although salt and metal ion tolerance were not evaluated in this study, these properties warrant further investigation.

3.5 Application of phytase in poultry feed ingredients

The application of crude phytase from *S. griseorubens* K-2C was evaluated using four poultry feed ingredients: rice husk, corn flour, soybean meal, and pollard. Phytase treatment was performed under optimal conditions (50°C, pH 7.0), and soluble phosphorus release was measured after 0, 12, and 24 h of incubation.

The results demonstrated that phytase application significantly increased soluble phosphorus levels in all tested feed ingredients (Figure 6). After 24 h of incubation, soluble phosphorus increased by 5.96 ppm in rice husk, 1.56 ppm in corn flour, 4.08 ppm in soybean meal, and 2.23 ppm in pollard. Rice husk exhibited the highest increase, while corn flour showed the lowest response.

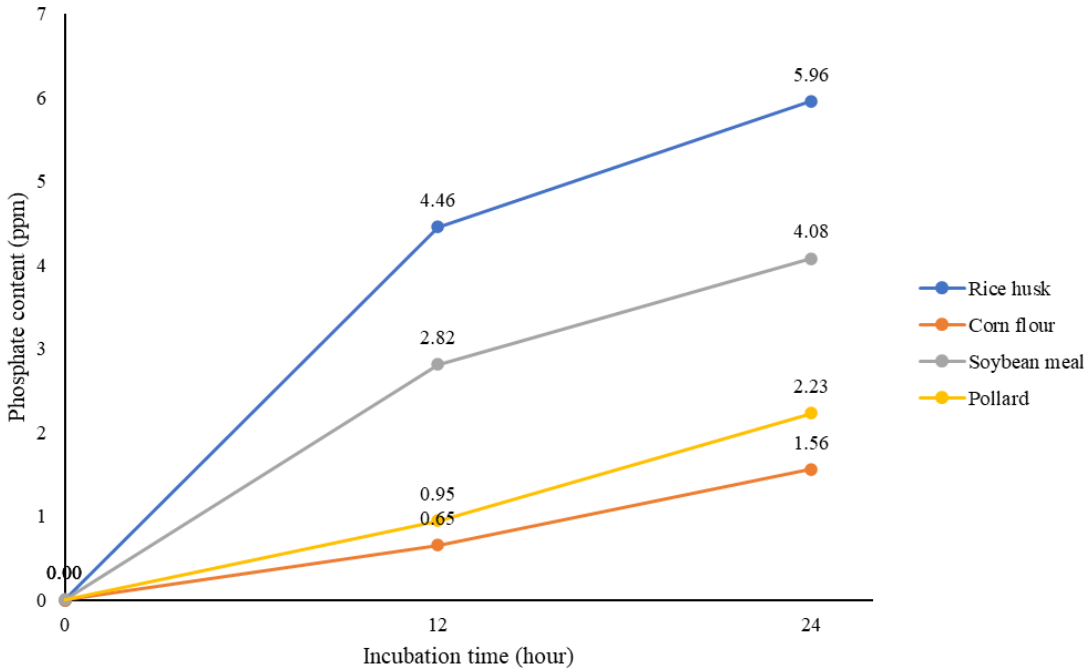

Figure 6. Application of phytase in poultry feed ingredients

Statistical analysis using ANOVA indicated that feed ingredient type and incubation time had a significant effect ($P < 0.01$) on soluble phosphorus content. However, no significant interaction ($P > 0.05$) was observed between feed ingredient type and incubation time, suggesting that phytase efficacy was consistently influenced by time across different substrates.

Phytate is widely recognized as an antinutritional factor due to its strong chelation of essential minerals such as phosphorus, calcium, zinc, magnesium, and iron, thereby reducing nutrient bioavailability [3]. The observed increase in soluble phosphorus confirms the functional effectiveness of K-2C phytase in degrading phytate and releasing bioavailable phosphate.

When compared with other actinomycete-derived phytases reported in the literature, the phytase from *S. griseorubens* K-2C demonstrates comparable activity levels, a favorable neutral pH optimum, and effective phytate hydrolysis in complex feed matrices. These characteristics highlight its potential as a promising alternative to conventional fungal phytases, particularly for applications requiring neutral pH activity.

4. CONCLUSIONS

The results of this study demonstrate that the mangrove ecosystem of Segara Anakan, Cilacap, represents a promising source of indigenous actinobacteria with phytase-producing potential. Among the twelve isolates screened, isolate K-2C, identified as *Streptomyces griseorubens* based on 16S rRNA gene analysis, exhibited the highest phytase activity and was selected for further characterization. The growth profile of K-2C indicated a clear exponential phase at day 8, while phytase production reached its maximum on the second day of incubation.

Characterization of the crude phytase revealed that the enzyme displayed optimal activity at a temperature of 50°C and a neutral pH of 7.0, conditions that resulted in the highest release of soluble phosphate. Application of the phytase to poultry feed ingredients demonstrated effective phytate hydrolysis, with rice bran showing the greatest increase in soluble phosphorus, followed by soybean meal, pollard, and corn flour. These findings indicate that phytase derived from indigenous mangrove actinobacteria is effective in improving phosphorus availability in plant-based feed ingredients.

Overall, this study highlights the biotechnological potential of *S. griseorubens* K-2C as a phytase producer and supports the use of phytase-based enzymatic approaches as a sustainable strategy to reduce phytic acid content in poultry feed. Further studies focusing on enzyme stability, scale-up production, and comparative performance with commercial phytases are recommended to support its industrial application.

ACKNOWLEDGMENT

The authors gratefully acknowledged the Ministry of Education, Culture, Research, and Technology of the Republic

of Indonesia for the Research Grant with contract No. T/1281/UN23.18/PT.01.03/2022 through the Directorate of Research and Community Service.

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