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Research Article

Characterisation of Plant Growth Promoting Actinobacteria from Sungai Wain Protected Forest, East Kalimantan

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ABSTRACT

The excessive use of chemical-based fertilisers, herbicides, and pesticides in Indonesian agriculture has resulted in negative environmental impacts. As a sustainable alternative, the application of biofertilisers remains uncommon despite their proven ecological benefits. A group of microorganisms known as Plant Growth Promoting Rhizobacteria (PGPR) that plays a crucial role in enhancing plant growth. Among them, actinobacteria are gram-positive bacteria that involved in ecological processes such as organic matter decomposition, nutrient cycle, plant growth promotion and plant pathogen's suppression. This study aims to investigate the potency of actinobacteria isolated from Sungai Wain Protected Forest, East Kalimantan as plant growth-promoting agents. A total of 96 actinobacteria isolates were revived from glycerol stock and subjected to primary screening through qualitative assays to assess phosphate solubilisation activity, nitrogen fixation, and *Indole-3-Acetic Acid* (IAA) production. Twenty-eight isolates demonstrated positive result among three PGP traits were subsequently selected for secondary screening, involving quantitative determination of IAA production and phosphate solubilisation through colorimetric assay. K22S-63 was identified as the most promising candidate, exhibiting the highest IAA production and phosphate solubilisation values of 43.80 and 41.51 µg mL^{-1}, respectively. Molecular analysis was performed by amplifying PGPR-associated genes using PCR. The targeted genes included *iaaM*, *phoD*, *PKS*, and *NRPS*. Phylogenetic analysis based on 16S rRNA gene sequencing revealed that K22S-63 shared 99.65 % similarity with *Peterkaempfera griseoplana*. These findings suggested that K22S-63 holds potential as bioinoculants with promising capabilities as plant growth promoter and biocontrol agents.

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INTRODUCTION

Indonesia is one of the most biodiverse countries and exhibits a high level of microbial diversity, particularly within its soil ecosystems. Data provided by Ritung et al. (2015), revealed that Indonesia encompasses approximately 144.47 million hectares of dry land, representing 76.20 % of the total land area in Indonesia. A significant 82 % of the dry lands in Indonesia is classified as suboptimal, characterised by low productivity and diminished soil fertility. In response to the challenges, farmers in Indonesia frequently rely on the use of chemical fertilisers, herbicides, and pesticides due to their high effectiveness, affordability, and accessibility. The utilisation of biofertilisers remains uncommon despite their potential benefits for sustainable agriculture. The overuse of chemical fertiliser has long-term impacts on the soil, such as disruption of soil ecosystem and reduction in biodiversity, which could contribute to land degradation. One of the strategies is the utilisation of biofertiliser that can enhance soil fertility by microbial activity and nutrient cycling, leading to an increase in agricultural productivity.

Actinobacteria are gram-positive bacteria belonging to the order *Actinomycetales* and phylum *Actinobacteria* that are widely distributed in various habitats and ecosystems, including marine and terrestrial environments (Meenakshi at al. 2024). Actinobacteria play a major role in ecological processes, including biodegradation and humus formation, by facilitating the breakdown of complex polymers such as the cellulose and lignin (Bhatti et al. 2017). Actinobacteria are widely recognised as plant growth promoting rhizobacteria (PGPR) due to their ability to enhance plant growth and productivity through both direct and indirect mechanisms. These mechanisms involve the provision of essential nutrients, secondary metabolites and bioactive compounds productions that support plant development (Katz & Baltz 2016). The utilisation of plant growth-promoting Actinobacteria (PGPA) as a biofertiliser inoculant continuously and gradually offers a promising sustainable alternatives to enhance food security and agricultural stability in response to minimising the reliance on chemical fertiliser, which potentially harms the environment when excessively applied over time. Gangola (2018) assessed the impact of pesticides on soil health and their biodegradation using indigenous microorganism, and the result showed that a consortium of *Bacillus* strains efficiently degraded pesticides such as cypermethrin (99 %), imidacloprid (99 %), fipronil (95 %), and chlorsulfuron (93 %) within 15 days compared to only 7 % degradation in the control.

A deep understanding of Actinobacteria potency as PGPR is essential for advancing their application as biofertiliser. Comprehensive and collaborative research across various fields is necessary to identify and to scrutinise the potential strain and explore their capabilities as PGPR. This study evaluated actinobacteria from the unspoiled Sungai Wain Protected Forest, East Kalimantan, focusing on their plant growth-promoting abilities, including phosphate solubilisation, IAA production, and nitrogen fixation. Further molecular analysis using PCR-based gene amplification was conducted to detect the presence of functional genes associated with PGPR activity, including *iaaM*, *phoD*, PKS and NRPS. The selected strain with the highest PGPR activity were further identified using 16S rRNA sequence analysis. The findings of this study are expected to provide valuable references and knowledge for other researchers in related fields, especially for the development of Actinobacteria-based biofertiliser for the agriculture sector.

MATERIALS AND METHODS

Sample Collection, Preservation and Rejuvenation

Soil sample were collected from Sungai Wain Protected Forest, Balikpapan City, East Kalimantan, Indonesia. A total of nine soil samples, coded as K22S,

were obtained and preserved in glycerol stock at -80 °C at the Indonesian Culture Collection (InaCC) BRIN. In this study, ninety-six Actinobacteria isolates were revived from glycerol stock and grown in Yeast Starch Agar (YSA) media. Each sample was inoculated aseptically using quadrant streak plate method and incubated for 48 hours at room temperature (27 °C).

Primary Screening of PGPR Activity

Phosphate Solubilization Assay

Primary Screening of phosphate solubilisation activity of 96 Actinobacteria were carried out using plate assay based on modified method of Widawati et al. (2022). Pure culture samples were inoculated on Pikovskaya Agar Media (pH 7) containing tricalcium phosphate. Plates were divided into four quadrants as replication and isolates were inoculated on the centre of each quadrant with spot inoculation technique and incubated at 27 °C for 10 days. The clear zone that presence around the isolate is indicates the positive result of phosphate solubilisation ability. The solubilisation index was measured in day 10 by calculating the diameters of clear zone and colony as follows:

$$\text{Solubilisation Index (SI)} = (\text{Halo zone diameter} + \text{Colony Diameter})/(\text{Colony Diameter})$$

Qualitative and quantitative classification of phosphate solubilising activity into high, moderate, and low categories were determined through statistical analysis using SPSS Statistics (IBM SPSS Statistics Version 29.0.2.0). Classification thresholds for each activity level were defined according data distribution.

Indole Acetic Acid (IAA) Production

The initial screening of IAA production was tested using Tryptic Soy Agar (TSA) media supplemented with 200 ppm L-tryptophan as precursor (Widawati et al. 2022). The plate was divided into eight quadrants, each sample was grown on the quadrant with two replications and incubated at 27 °C for 48 hours in dark condition. After incubation time, colonies were dropped with a Salkowski reagent (250 mL H_2SO_4, 249 mL distilled water and 1 mL of 0.5 M $FeCl_3$) and stored in dark condition for 30 min. The isolate discoloration into pink indicates the IAA production of Actinobacteria.

Nitrogen-Fixing Activity

The nitrogen fixation test was performed qualitatively based on Widawati et al. (2022). method with modification using New Fabio (NFb) semi solid media. Actinobacteria was inoculated into 5 mL NFb semi solid media in reaction tube using inoculation needle aseptically, isolates were incubated at 27 ° C. Media was observed for 7 days, positive result was shown with discoloration into blue on the media. The isolates were grouped into high, moderate of low nitrogen-fixing potential based on the intensity of the colour.

Secondary Screening of PGPR Activity

Phosphate Soluble Content

The isolates that showed a positive result in primary screening were determined quantitatively using spectrophotometric molybdenum blue method based on Widawati et al. (2022) with modification. Each isolate was inoculated into a 100 mL erlenmeyer containing 50 mL Pikovskaya liquid medium incubated in incubator shaker at 30 °C and shaken at 160 rpm for 7 days; non-inoculated media was used as negative control. After 7 days, 20 mL of each culture was filtered using filter paper Whatman number 42 and centrifuged at 10,000 rpm for 15 min. Furthermore, 5 ml of supernatant was collected into reaction tube and mixed with 0.5 mL reagent solution, vortex for 30 seconds

and incubated at room temperature for 30 min, the samples colour was changed to blue. 200 µL of each sample was placed into 96-well plate with 3 replicates and the optical density (OD) was measured by spectrophotometer (Varioskan™ LUX *multimode Microplate reader*), at 693 nm. The estimation of phosphate soluble content was determined by comparing the sample absorbance within the phosphate standard curve.

IAA Production Assay

The isolates were determined quantitatively with the colorimetric assay based on Widawati et al. (2022), each isolate was inoculated into 100 mL Erlenmeyer covered with aluminium foil, containing 25 mL Tryptic Soy Broth (TSB) media supplemented with 200 ppm L-Tryptophan and incubated in incubator shaker at 30 °C and shaken at 160 rpm for 4 days in dark condition, non-bacterial media was used as a negative control. After incubation time, the culture was centrifuged at 10,000 rpm for 10 minutes at 4 °C. Furthermore, 1 mL supernatant was placed into reaction tube and mixed with 4 mL Salkowski reagent, the tube was incubated at room temperature for 30 minutes in dark condition. Each isolate was placed into 96-well plate with 3 replicates and measured with spectrophotometer (Varioskan™ LUX *multimode Microplate reader*), at 435 nm. The estimation of IAA production was determined by comparing the sample absorbance within IAA standard curve. The concentration of IAA produced by isolate was statistically analysis using SPSS Statistics (IBM SPSS Statistics Version 29.0.2.0), and isolates were group into high, moderate, and low IAA producers based on the distribution pattern of the measured values.

Morphological Characterisation of Potential Actinobacteria

Actinobacteria isolate that exhibited positive result and highest value across all PGP properties was subjected to morphological characterisation and molecular identification based on 16S rRNA gene analysis. The morphological characterisation of potential isolate was carried out by observed the actinobacteria morphology in YSA media and the micro-morphology were observed using Scanning Electron Microscopy (SEM) (JSM-IT200 JEOL, Japan), observed at voltage of 10kV and magnifications of 25,000 ×.

DNA Extraction, PGPR Genes Detection and Molecular Identification of Potential Actinobacteria

DNA extraction of potential actinobacteria was carried out using Quick-DNA™ High Molecular Weight MagBead Kit Zymo (Zymo Research, CA, USA) based on the protocol with modification.

Crude DNA was stored at –20 °C, the quality and quantity of DNA were measured using Varioskan Lux Micro Plate Reader. DNA amplification of genes encoded PGPR activity and 16S rRNA gene was performed using primers presented in Table 1. The 25 µL PCR reaction consisted 12.5 of µL MyTaqTM HS Red Mix 2x (Bioline, Germany), 1 µL of forward primer (10 pmol), 1 µL of reverse primer (10 pmol), 1 µL of DNA template, and 9.5 µL of Nuclease-Free Water (NFW). PCR Products were furthermore visualized qualitatively using electrophoresis, 2.5 µL of PCR product was injected into 1 % gel electrophoresis with Florosafe DNA Staining (1st BASE, Malaysia) in 1x TAE buffer pH 8.0. The electrophoresis was performed at 100 volts for 25 minutes and visualized on Gel DocTM EZ Imaginer (Bio-Rad). The PCR product of 16S rRNA gene was sequenced and analysed using BioEdit and EZBioCLoud (https://www.ezbiocloud.net). The isolate sequence was aligned using Clustal W, and the phylogenetic tree was constructed using MEGA11 based on neighbour-joining program using 1000 replicates of bootstrap.

Table 1. List of primer sequences used for molecular analysis in this study.

Gene	Primer	Sequence (5'-3')	Size (bp)	Reference
16S rRNA	27F	AGAGTTTGATCCTGGCTCAG	1500	Xu et al. 2016
	1492R	TACGGCTACCTTGTTACGACTT		
	920 F	AAACTCAAATGAATTGACGG		Nurkanto & Agusta 2015
	920 R	CCGTCAATTCATTTGAGTTT		
	520 F	GTGCCAGCAGCCGCGG		
	520 R	ACCGCGGCTGCTGGC		
phoD	ALPSF730	CAGTGGGACGACCACGAGGT	350	Amri et al. 2022
	ALPSR1101	GAGGCCGATCGGCATGTCG		
nifH	PoL-F GC	CGCCCGCCGCGCGCGGCGGGCGGGG CGGGGGCAC- GGGGGGTGCGAYCCSAARGCBGACTC	500	Koirala et al. 2025
	PoL-R	ATSGCCATCATYTCRCCGGA		
*iaa*M	*iaa*M F	ATGACGTCCACCGTGCCCAACGCG	150	Passari et al. 2016
	*iaa*M R	CTAGTCCTCGGGGAGTTCCACGGG		
PKS	PKS F	CGCGCGCATGTACTGGACNGGNGAYYT	480	Amos et al. 2015
	PKS R	GGAGTGGCCGCCCARNYBRAARAA		
NRPS	NRPS F	GGCAACGCCTACCACATGCANGGNYT	350	
	NRPS R	GGTCCGCGGGACGTARTCNARRTC		

RESULTS AND DISCUSSION

Rejuvenation of Actinobacteria Isolates

Bulk soil samples were collected from nine separate sites within the Sungai Wain Protected Forest for this study. A total of ninety-six actinobacteria isolates coded K22S were successfully revived from glycerol stock were subsequently tested in a primary screening for PGP (plant growth-promoting) activities. All isolates exhibit distinct morphological colonies, with a representative subset presented in Figure 1. Cultivation on Yeast Starch Agar (YSA) medium revealed notable phenotypic variation among the isolates, particularly in shape, surface, elevation, pigmentation, margin, and aerial mycelial development. The morphological features commonly used as preliminary taxonomic indicators within Actinobacteria.

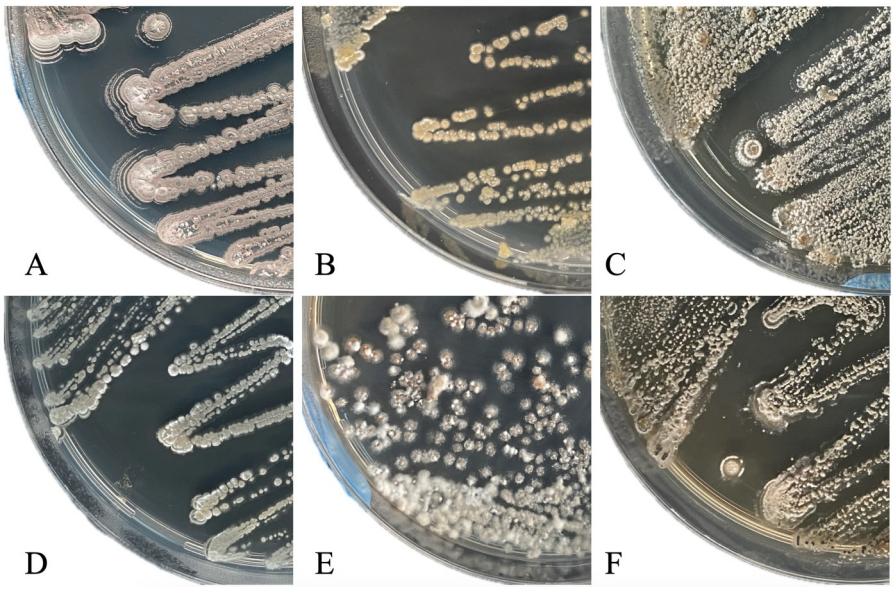

Figure 1. Morphology of actinomycetes colony grown in YSA media. (A) K22S-23; (B) K22S-20; (C) K22S-41; (D) K22S-58; (E) K22-84; (F) K22S-88.

Primary Screening of PGPR Activity

Phosphate Solubilization Assay

Ninety-six actinobacteria isolates were screened qualitatively to assess its PGP activity focusing on phosphate solubilization, IAA production and nitrogen fixation. The qualitative result of actinobacteria isolates on their Plant-Growth Promoting (PGP) ability were showed in Table 2.

Table 2. Qualitative result of actinomycetes isolates towards their PGP activity.

PGP Activity	Total of Isolate			
	High (+++)	Moderate (++)	Low (+)	Total
Phosphate solubilising	3	20	46	96
IAA Production	1	17	29	96
Nitrogen Fixation	16	17	31	96

The actinobacteria isolates were grouped into negative (-), low (+), moderate (++) and high (+++) based on their activity. Actinobacteria isolates were tested qualitatively for phosphate solubilization activity using plate assay in Pikovskaya media containing tricalcium phosphate. The formation of halo-zone around the colony indicates the phosphate solubilisation activity of actinobacteria

The Phosphate Solubilization Index (PSI) was measured in day 10 by calculating the diameters of halo-zone and colony. A total 69 isolates were exhibited the ability to solubilise the phosphate representing 71.88 % of all screened isolates, as indicated by halo-zone formation around the colony after ten days of incubation with PSI values ranging between 1.00 ± 0.00 to 9.31 ± 0.70 showed by K22S-84 and K22S-65, respectively. The grown actinobacteria isolates without halo-zone formation were categorised as negative; a total 27 out of 96 Actinobacteria isolates were showed negative result. The selected strains were further tested to confirm their activity to soluble the phosphate.

Fatmawati et al. (2019) reported that actinobacteria isolated from rhizosphere have an ability to dissolve phosphate, strain ASR 49 had the highest phosphate solubilizing activity as determined by qualitative assessment with PSI value of 2.62. The halo-zone around colony formed due to the secretion of organic acid and polysaccharides, as well as enzyme production by the actinobacteria isolates. Microbial phosphate solubilization generally occurs through multiple pathways, including the acidification mechanism that facilitated by the production of organic acid such as citric, gluconic, lactic, malic and oxalic acid. Another mechanism involves the reduction in pH level of the medium and the chelation of metal ions associated with insoluble phosphorus (Al, Fe, Ca), that facilitating the release of phosphorus (Boubekri et al. 2021).

IAA Production Assay

Auxins are a group of molecules that function as signals regulating physiological processes during plant-microorganism interactions. Auxins produced by microorganisms modify environmental auxin levels to an optimal range, resulting in increased lateral or secondary root growth and expansion of the root surface area, thereby enhancing plant growth and nutrient uptake (Olanrewaju et al. 2021). Each actinobacteria isolates were screened qualitatively using TSA media supplemented with 200 ppm L-tryptophan as precursor. Tang et al. (2023) stated that IAA biosynthesis by microorganism classified into tryptophan-independent and tryptophan-dependent pathway based on tryptophan utilization as key metabolic precursor due to their ability as the most densed amino acid among other amino acids.

IAA production of Actinobacteria isolate was indicated by discolourating the colony into pink after addition of Salkowski reagent and incubated in dark condition. Pink colour formation of Actinobacteria colony due to the reduction of Fe^{3+} to Fe^{2+}, by the interaction between IAA produced by Actinobacteria and iron (Fe) contained in Salkowski reagent (Etesami & Glick 2024). Out of 96 Actinobacteria isolates, 47 isolates known to has ability to produce IAA based on plate assay in TSA media, representing 48.97 % of all screened Actinobacteria isolates. The result of assay was classified into four group based on their colour change of Actinobacteria colonies: no discoloration (-);

whitish to yellowish (+); yellowish to pinkish (++); pinkish to purplish (+++). Twenty-eight isolates categorised as low producer of IAA, while 17 isolates were moderate and one isolates was identified as a high IAA producer, as presented in Table 2.

Nitrogen Fixing Activity

Nitrogen fixing activity of Actinobacteria isolates were evaluated qualitatively using Nfb media following method by Widawati et al. (2022). Out of 96 isolates evaluated for their nitrogen-fixing ability, a total of 64 isolates exhibited positive result representing 66.67 % of total screened Actinobacteria. In particular, 16 actinobacteria isolates (16.67 %) were classified in the high category; 17 (17.71 %) and 31 isolates (32.29 %) were categorised as moderate and low nitrogen-fixers, respectively. The colour change in media reflected the ability of actinobacteria to assimilate nitrogen in the form of nitrate and ammonia (Suleiman et al. 2019).

The results of the nitrogen fixation assay were categorized the positive results into three groups based on the degree of discoloration: light blue as low activity (+), medium blue as moderate (++), and deep blue as high activity (+++). Further test is required to validate the ability of actinobacteria through biochemical and molecular approaches. In this study, only the Actinobacteria isolates that demonstrated the capacity to solubilise phosphate, produce IAA, and fix nitrogen were further evaluated quantitatively.

Nitrogen gas (N_2) is an essential component for plant, but plants cannot directly utilize it due to the stability of its covalent bonds. Certain microorganisms possess the ability to convert atmospheric nitrogen (N_2) into inorganic compounds that plants can readily absorbed. This biochemical process, known as nitrogen fixation, enables the transformation of atmospheric nitrogen into assimilable forms. However, this current study aims to detect the ability of actinobacteria to fix the atmosphere nitrogen. Further test is required to validate the ability of actinobacteria through biochemical and molecular approaches. The genus Frankia represents a first group of actinobacteria recognised for their enzymatic capabilities to facilitate the conversion of atmospheric nitrogen into ammonia compounds (NH_3). In the preceding decade, it has been established that additional varieties of actinobacteria exhibiting nitrogen-fixing activities belonging to the genus Streptomyces, *Mycobacterium*, *Microbacterium*, *Agromyces*, *Mincromonaspora* and *Corynebacterium* (Silva et al. 2022).

Secondary Screening of PGPR Activity in Actinobacteria

Phosphate Soluble Content

Out of 96 Actinobacteria isolates that were screened qualitatively, 28 actinobacteria isolates exhibited all three PGP traits and were subsequently evaluated to quantitative assessment regarding their ability in phosphate solubilisation and IAA production. Additionally, 28 potential isolates were further examined for their ability to solubilize the phosphate. P soluble content resulted from this study refers to the measured concentration of soluble phosphate (P) released by actinobacteria. P soluble concentration determined as actual amount of P that converted into soluble form and directly available for utilisation by plants.

Figure 2 shows the actinobacteria isolates after addition of reagent, the intensity of the blue coloration indicates the highest phosphate solubilisation content. Statistical analysis shows strong correlation between actinobacteria activity and phosphate solubilisation, as indicated by significance values of <0.05. The quantitative assay revealed that K22S-78 had the highest level of phosphate solubilization, followed by K22S-63, K22S-88 with the concentration values of 69.29 ± 2.39, 41.51 ± 1.09 and 37.13 ± 0.98 µg mL^{-1}, respec-

tively. Compared to Solubilisation Index (SI), K22S-78 and K22S-63, which possess the highest P soluble content, exhibit low solubilisation activity as determined by qualitative method, with PSI values of 2.02 ± 0.20 and 1.72 ± 0.09, respectively.

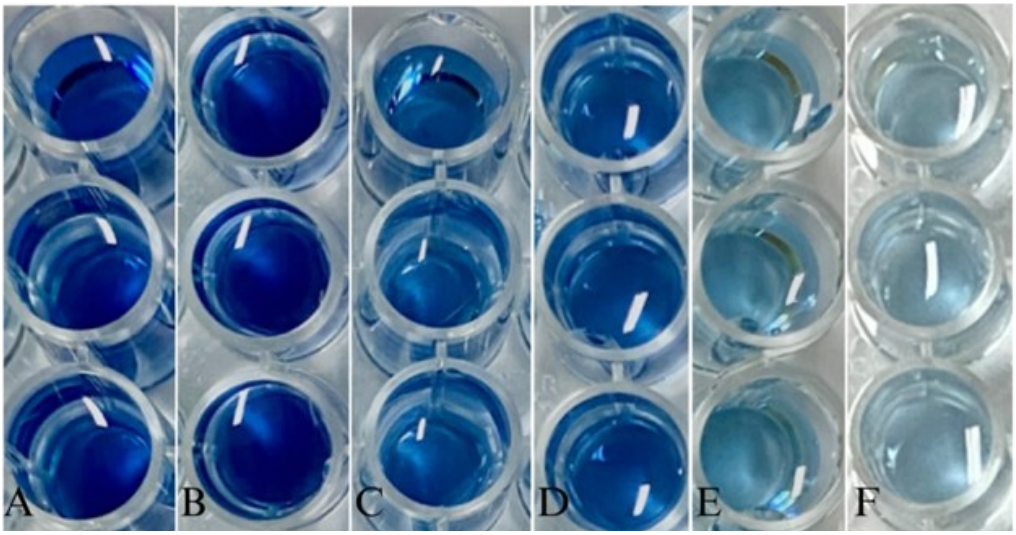

Figure 2. Quantitative assay of phosphate solubilisation activity in potential isolates. (A) K22S-78; (B) K22S-63; (C) K22S-88; (D) K22S-98; (E) K22S-41; (F) K22S-10.

Similarly, K22S-88, which has the third highest P soluble content, was categorised as moderate phosphate solubilizer according to qualitative method, with PSI value of 4.55 ± 0.05. Table 3 shows K22S-84 and K22S-97, which demonstrated the highest solubilisation index qualitatively, also shows quite high potential for phosphate solubilisation based on quantitative method, with P soluble values of 20.44 ± 0.27 and 37.04 ± 0.98 µg mL^{-1}, respectively.

The comparative analysis between qualitative and quantitative method revealed that both approaches did not exhibit the same pattern. Figure 3 illustrates the regression curve correlating the qualitative (PSI) and quantitative (colourimetric) methods, the result revealing an absence of a significant relationship, as indicated by a p-value exceeding 0.5 %. ($r = 0.0261$). This present study is in line with study observed Walpola et al. (2020) result showed the different pattern in phosphate solubilization in qualitative and quantitative method.

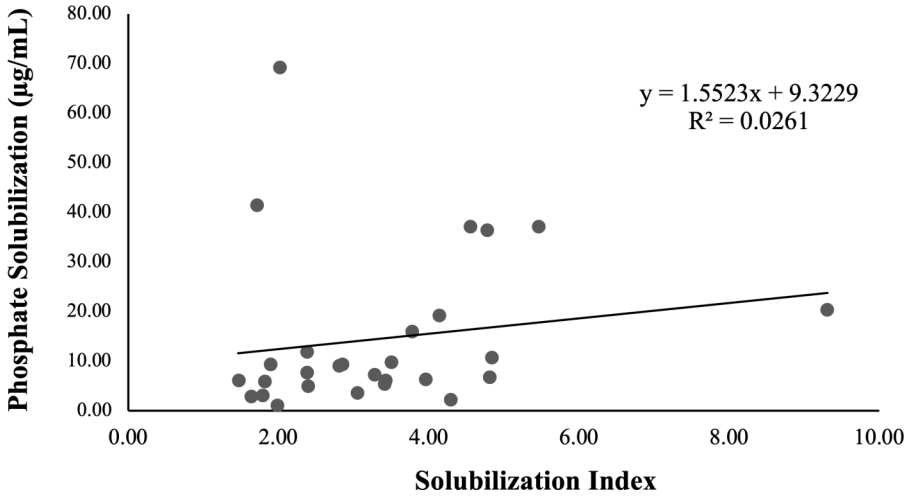

Figure 3. Comparative analysis between qualitative and quantitative method of phosphate solubilizing activity of potential actinomycetes isolates after seven days of incubation.

Table 3. Phosphate solubilization, IAA production and N-Fixation of potential isolates.

Isolate Code	Phosphate Solubilization		IAA Production (µg mL^{-1})	N Fixation
	SI	P Soluble (µg mL^{-1})		
K22S-009	4.81 ± 0.13^{l}	6.86 ± 0.06^{efg}	19.88 ± 0.55^{jk}	+
K22S-010	1.99 ± 0.13^{abc}	1.15 ± 0.70^{a}	14.02 ± 0.12^{e}	+
K22S-011	2.40 ± 0.24^{cd}	4.97 ± 0.16^{d}	18.56 ± 0.41^{h}	+
K22S-012	4.84 ± 0.73^{l}	10.66 ± 0.65^{ij}	20.34 ± 0.40^{kl}	+
K22S-013	2.38 ± 0.39^{bcd}	11.77 ± 0.71^{j}	14.08 ± 0.02^{e}	+
K22S-015	3.25 ± 0.14^{efgh}	7.64 ± 0.45^{g}	15.27 ± 1.15^{f}	+++
K22S-023	1.90 ± 0.09^{abc}	9.35 ± 0.17^{h}	8.08 ± 0.53^{b}	+
K22S-024	1.64 ± 0.13^{a}	2.93 ± 0.27^{bc}	15.21 ± 0.30^{f}	+
K22S-030	3.78 ± 0.48^{hij}	16.02 ± 0.58^{k}	9.79 ± 0.29^{c}	+++
K22S-034	1.46 ± 0.06^{a}	6.07 ± 0.15^{def}	16.18 ± 0.06^{g}	++
K22S-036	3.96 ± 0.69^{ij}	6.24 ± 0.11^{def}	18.72 ± 0.72 ^{hi}	++
K22S-037	3.42 ± 0.14^{fghi}	5.45 ± 0.75^{d}	15.47 ± 0.18^{fg}	+++
K22S-041	4.30 ± 0.13^{jkl}	2.25 ± 0.69^{ab}	40.37 ± 0.29^{p}	+++
K22S-043	3.43 ± 0.06^{fghi}	6.14 ± 0.15^{def}	5.85 ± 0.09^{a}	+
K22S-046	4.14 ± 0.59^{jk}	19.18 ± 0.47^{l}	20.81 ± 0.25^{l}	+++
K22S-049	3.05 ± 0.08^{efg}	3.62 ± 0.74^{c}	8.67 ± 0.58^{b}	+
K22S-052	2.85 ± 0.09^{def}	9.26 ± 0.51^{h}	13.12 ± 0.11^{d}	++
K22S-056	2.81 ± 0.25^{de}	9.11 ± 0.66^{h}	19.40 ± 0.36^{ij}	++
K22S-058	1.79 ± 0.01^{ab}	3.08 ± 0.15^{bc}	28.55 ± 0.54^{o}	++
K22S-063	1.72 ± 0.09^{a}	41.51 ± 1.09^{o}	43.80 ± 0.45^{q}	++
K22S-073	4.78 ± 0.19^{l}	36.49 ± 0.56^{n}	12.75 ± 0.05^{d}	+
K22S-078	2.02 ± 0.20^{abc}	69.29 ± 2.39^{p}	19.81 ± 0.36^{jk}	++
K22S-083	3.51 ± 0.25^{ghi}	9.73 ± 1.23^{hi}	24.77 ± 0.83^{m}	+
K22S-084	9.31 ± 0.70^{n}	20.44 ± 0.27^{m}	14.95 ± 0.38^{f}	+
K22S-088	4.55 ± 0.05^{kl}	37.13 ± 0.98^{n}	26.56 ± 0.60^{n}	++
K22S-094	3.27 ± 0.00^{efgh}	7.29 ± 0.19^{fg}	27.98 ± 0.68^{o}	+
K22S-097	5.47 ± 0.50^{m}	37.04 ± 0.93^{n}	18.07 ± 0.17^{h}	+
K22S-098	1.81 ± 0.06^{abc}	5.91 ± 0.27^{de}	12.23 ± 0.23^{d}	+++

Walpola et al. (2020) conducted the comparative evaluation of qualitative and quantitative phosphate solubilisation methods and found no correlation between the two approaches. Isolate PSB 10 which possessed the lowest solubilisation index (0.61), has classified as the high phosphate solubiliser using quantitative method (873.87 µg mL^{-1}). However, this result found that qualitative assay not consistently correlate with quantitative methos, indicating the fundamental methodological differences between two approaches. The discrepancies may be attributed to several factors, including inoculation technique, incubation time, different type and diffusion rates of different organic acid in microorganism, which affect the halo formation in qualitative assay. Qualitative assay provides the rapid screening of phosphate solubilisation potential that are useful for rapid preliminary screening of phosphate solubilising ability, while the quantitative assay performed in liquid media offer more precise evaluation by estimated the actual concentration of solubilized phosphate (Amri et al. 2023). Therefore, both of qualitative and quantitative method could be conducted in parallel for more reliable result to improve efficiency and to identify the most potential microorganism isolate as phosphate solubilisers.

IAA Production

In the present study, twenty-eight of actinobacteria isolates were evaluated quantitatively to identify the most potential isolate to produce the IAA using TSB media supplemented with 200 ppm tryptophan as precursor. Tabel 3. showed that all isolates possess the ability to synthesize IAA, with concentration values ranging from 5.85 ± 0.09 to 43.80 ± 0.45 µg mL^{-1}. K22S-63 exhibited the highest IAA concentration, followed by K22S-41 with respective val-

ues of 43.80 ± 0.45 and 40.37 ± 0.29 µg mL^{-1}, significantly different among other strain ($p<0.05$) (Figure 4).

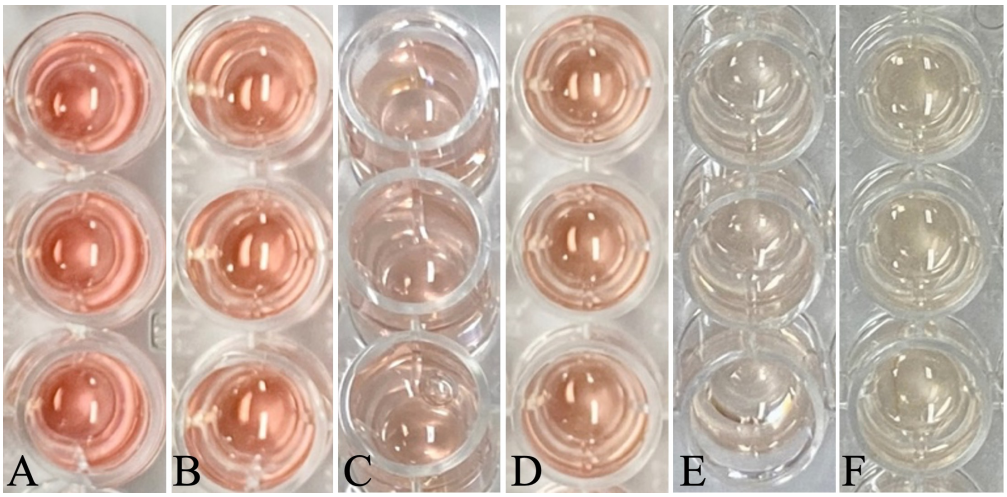

Figure 4. Quantitative assay of IAA production activity in potential isolates after Salkowski reagent addition. A. K22S-63; B. K22S-41; C. K22S-58; D. K22S-94; E. K22S-30; F. K22S-43.

In contrast, the lowest IAA concentration was exhibited by K22S-43 with value of 5.85 ± 0.09 µg mL^{-1}. Anwar et al. (2016) conducted a screening of PGP traits in Actinobacteria and reported that, among 96 Actinobacteria isolates obtained from wheat and tomato rhizospheric soils, six isolates exhibited the highest ability to produce IAA, as determined by colorimetric test using Salkowski reagent with IAA concentration values ranging from 10 to 79.5 µg mL^{-1}. Strain WA-3 which exhibit the highest IAA production, was closely related to *Streptomyces nobilis*. This strain was empirically shown to significantly enhance Wheat (*Triticum aestivum*) growth parameters, including shoot length (65 %), root length (81 %), plant fresh weight (84 %), dry weight (85 %), number of leaves (27 %), and number of roots (30 %), compared to control plants.

The presence of IAA was determined by appearance of pink colour after addition of salkowski reagent. IAA produced by Actinobacteria isolates make a complex with tris-(indole-3-acetate)-iron-(III) which formed pink color. This reaction indicates the actinobacteria ability to metabolise tryptophan as precursor into IAA or other indole compounds. Tang et al. (2023), stated that IAA is not the only indole produced by microorganism, hence the biosynthetic pathway of IAA production classified into several groups; indole-3-butyric acid (IBA), indole-3-propionis acid (IPA), indole-3-pyruvic acid (IPyA), indole -3-acetamide (IAM), indole-3-acetonitrile (IAN), indole-3-lactic acid (ILA) dan indole-3-acetaldoximme (IAAId). Salkowski reagent used for IAA production assessment cannot be able to detect the IAA specifically, but other indole derivates and analog compound. Hence, overestimation of IAA values could be detected with Salkowski reagent (Beatriz et al. 2024).

However, despite its limitation, salkowski reagent continues to be extensively used to detect the IAA production to determining plant growth promoting actinobacteria due to low level of difficulty, cost effectiveness, time efficiency and minimal equipment requirements. As demonstrated in Table 3. K22S-63 isolate exhibited the highest activity in both P solubilization and IAA production as determined through quantitative assessment with concentration values of 41.51 ± 1.09 µg mL^{-1} and 43.80 ± 0.45 µg mL^{-1} respectively, statistical analysis revealed that K22S-63 is significantly different with a p-value < 0.05. Given its potential as plant growth promoter, K22S-63 was further subjected to molecular identification.

Morphological Characterisation of Potential Actinobacteria

The morphological characterisation of potential actinobacteria isolate were further analyzed based on colony growth and Scanning Electron Microscopy (SEM) observations. Figure 5 shows morphological characterization of K22S-63 in YSA medium. Based on the Inter-Society Color Council National Bureau of Standards (ISCC-NBS) color chart, the mycelium in 4-7 days of incubation forms greyish-pink color. Additionally, the spores exhibit light-greyish olive color after 7-10 days of incubation on YSA medium.

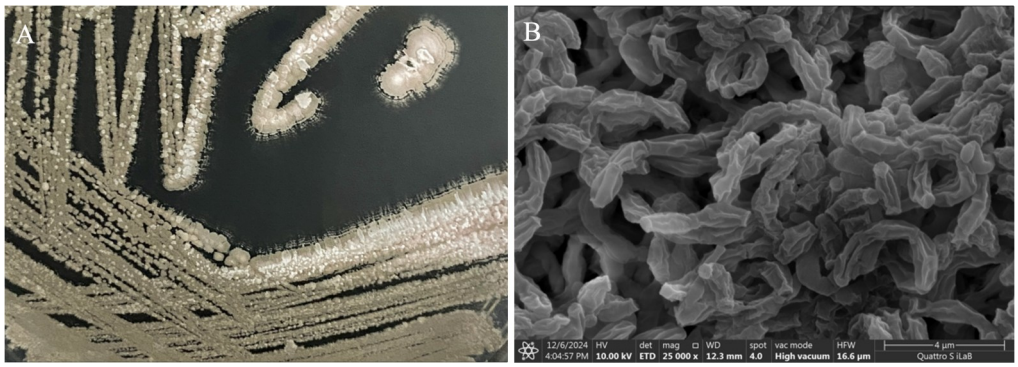

Figure 5. Morphology characterisation of K22S-63 from Sungai Wain Protected Forest. A. YSA media. B. Scanning Electron Microscopy (SEM).

Additionally, the SEM visualization (Figure 5) reveal that K22S-63 exhibit a straight monopodial mycelium, the filament surface appears rough, with an average hyphae diameter approximately 0.5 to 0.7 µm. The spore chains exhibit a poly-sporus structure appeared in *retinaculiaperti* type, characterized by primitive open loops shaped, which consist of cylindrical spore with parallel rugose surface measuring 0.5 thickness and 1µm length.

PGPR Genes Detection of Potential Actinobacteria

Among the 96 isolates, we selected K22S-63 as the promising isolate for promoting plant growth. To validate the plant growth promoting potential of K22S-63, PCR amplification was performed to detect the presence of functional genes including PKS, NRPS, *IaaM*, *NifH* and *PhoD*. The gene specific primers were used and the presence of target genes was confirmed by the amplification of DNA fragments corresponding to the expected product size, as visualized through agarose gel electrophoresis.

The result of PCR amplification confirmed the presence of PKS, NRPS, *IaaM* and *phoD* genes in the isolates, indicating its potential for secondary metabolite production, phytohormone synthesis and phosphate solubilisation. Differently, the *nifH* gene which associates with nitrogen fixation, appeared absence and was not detected based on PCR amplification method. The *phoD* gene encodes alkaline phosphatase D enzyme, which is involve inorganic phosphate mineralisation through hydrolysis of phosphate-ester bonds present in the soil. The biosynthesis of alkaline phosphatase in microorganism was encoded by three types of genes, including *phoA*, *phoX*, and *phoD*. According to Fraser et al. (2015), the *phoD* gene is highly abundant in soil ecosystem and is expressed in environment with phosphate deficiency. Moreover, this type of gene particularly widespread in terrestrial ecosystems, as well as among in archaea and bacteria (Ragot et al. 2017). In this study, amplification of *phoD* gene was generated an amplicon of approximately 300 base pairs, aligning closely with the expected product size range of 371-400 bp reported by Amri et al. (2022). The presence of *phoD* gene in K22S-63, was confirmed through secondary screening and supported its potential for phosphate solubilizing capacity via enzymatic mechanism, particularly through the action of alkaline phosphatase.

Given the significant IAA production observed in K22S-63, further molecular analysis was conducted to confirm the presence of *iaaM* gene. PCR amplification followed by visualisation through agarose gel electrophoresis, successfully detected the *iaaM* gene in K22S-63. This finding is consistent with the result of secondary screening, which shows that K22S-63 exhibited the highest IAA production value among other potential isolates. *iaaM* gene is one of key gene encodes tryptophan monooxygenase which involved in biosynthetic of IAA through IAM pathway (Tang et al. 2023). The result obtained is in line with the study of Passari et al. (2016), that confirms that *iaaM* gene was detected at a size of 150 bp. The presence of *iaaM* gene was demonstrate the ability of K22S-63 to produce the IAA compound, in accordance with the result of both qualitative and quantitative method.

Contrary, the result indicated the absence of the *nifH* gene in K22S-63; however, the qualitative nitrogen-fixing activity test yielded a positive result. This may be attributed to the primer used, which targeting only one gene (*nifH*). The detection of a single gene in nitrogen-fixing activity has a limitation if assessed by only one gene, because nitrogenase has three structural genes (*nifH*, *nifD*, *nifK*). The detection of more than one gene would provide more accurate evidence of nitrogenase in actinobacteria isolates (Mahyarudin et al. 2015). Gaby et al. (2017) conducted a comprehensive evaluation of the *nifH* encode gene, revealing that PolF/PolR primers has the less amount of bias of all primer tested, but has the lowest coverage value which is only amplify 25 % of the 23.847 *nifH* gene sequence tested. Additionally, each microorganism may exhibit the variance in DNA sequence due to single nucleotide polymorphisms (SNPs), which contribute the genetic variation. when SNP occur within the primer binding site, they can prevent DNA amplification as the primer may fail to bind properly.

This study involved the detection of PKS and NRPS genes, which are gene clusters associated with the synthesis of non-ribosomal peptides and polyketide active compounds. Secondary metabolites are involved in bioactivities including antimicrobial, antifungal, antiparasitic, antitumor, and immunosuppressive functions. The *Streptomyces* genus is recognized for its production of secondary metabolites that serve as important resources in the pharmaceutical industry. The amplification of PKS and NRPS genes in the K22S-63 resulted in amplicon sizes of approximately 500 bp and 350 bp, respectively. These results align with the expected fragment sizes reported by Amos et al. (2015). The identification of both genes suggests that the K22S-63 isolate possesses the potential to function as an antimicrobial agent and is capable of producing antimicrobial compounds.

Molecular Identification of Potential Actinobacteria Based on 16S rRNA Gene

The phylogenetic analysis of K22S-63 was revealed through 16S rRNA sequencing analysis. The assembled sequence yielded the final contigs with a length of 1484 bp which showed 99.58 % similarity with *Peterkaempfera griseoplana* as determined using EzBioCloud database (Figure 6). The identification of 16S rRNA gene sequencing revealed that K22S-63 is belong to the genus and family *Peterkaempfera* and *Streptomycetaceae*, respectively.

Peterkaempfera griseoplana is a gram-positive bacteria species belongs to the newly genus *Peterkaempfera* (Madhaiyan et al. 2022). This species was previously classified under the genera *Streptomyces* (Backus et al. 1957) and *Streptadichiphilus* (Nouioui et al. 2019) based on genetic and phylogenomic information. *Peterkaempfera* gen. nov. is a novel genus revealed by Madhaiyan et al. (2022), which is classified in *Streptomycetes* group characterised by the production of branched mycelium and aerial hyphae. The study by Boeck et al. (1971) discover that, *Streptomyces griseoplana* can produce the bioactive com-

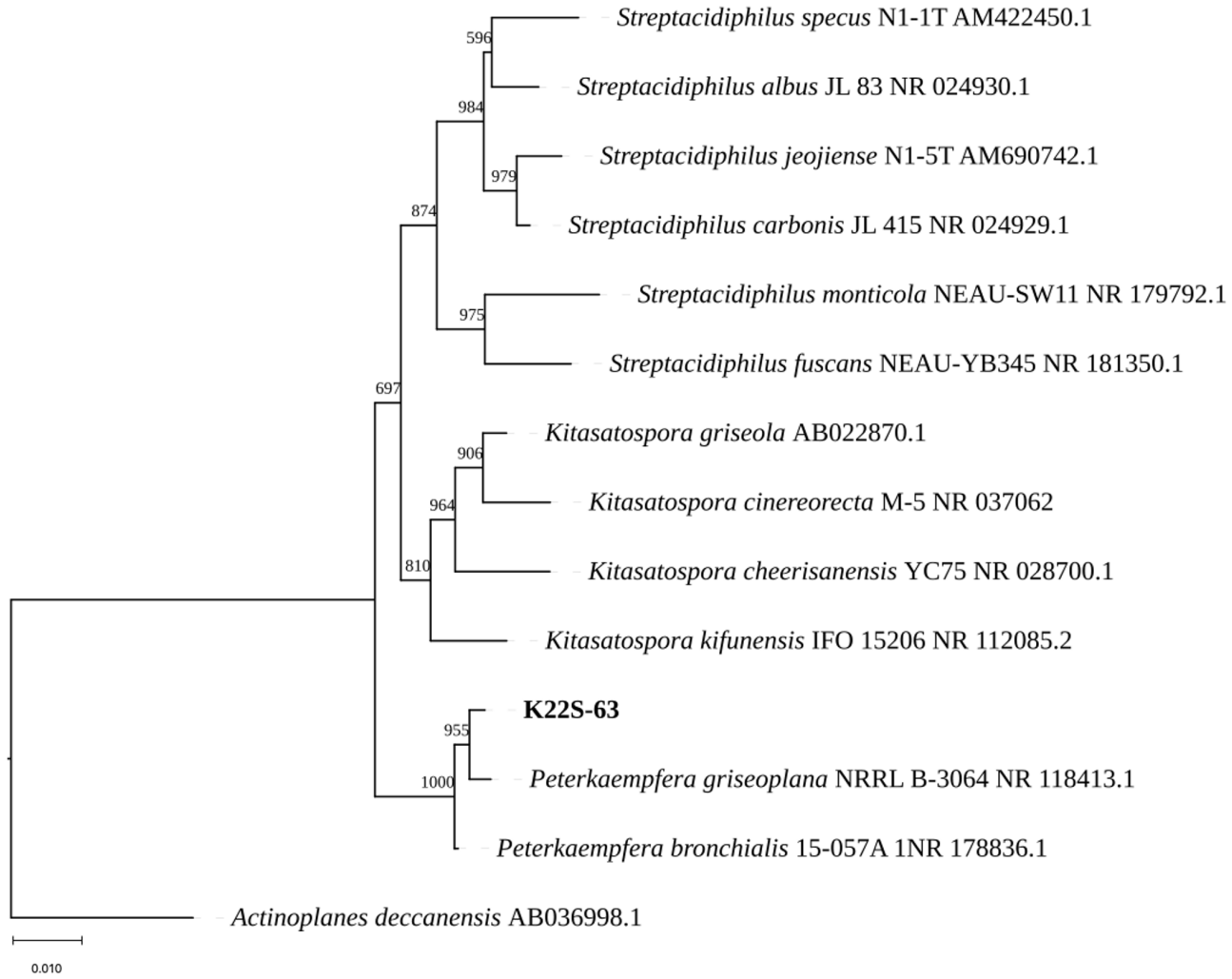

Figure 6. Phylogenetic tree of K22S-63 isolates based on 16S rRNA gene sequencing using Neighbor-Joining method with bootstrap analysis and Kimura-2-Model Parameter.

pound anticapsin, which is well known for the ability to produce a wide range of secondary metabolites, including antibiotics, antifungals and immunosuppressants (Caulier et al. 2019). Mohamed et al. (2020), reported that S1SHA1 strain had the antimicrobial activity against *Bacillus subtilis* NRRL B 543, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922 and *Candida albicans* MTCC183. Moreover, this strain is identified as *Streptomyces griseoplanus* based on 16S rRNA gene sequence, morphological, physiological and biochemical characteristic. Nouioui et al. (2018), stated that *S. griseoplanus* can act as a biocontrol agent against plant disease, as well as antifungal compound production including *diketopiperazine* dan surfactant. Extracellular extract of SAI-25, which identified as *S. griseoplanus*, have the potency as biosurfactant due to a broad range of insecticidal activity against *lepidopteran armigera*, *Spodoptera litura* (Fabricius), dan *Chilo partellus* (Swinhoe) in laboratory condition (Vijayabharathi et al. 2014).

CONCLUSIONS

This study unveiled the potential of actinobacteria isolated from the unspoiled protected forest in Indonesia. Out of 96 actinobacteria isolates, K22S-63 possess the highest phosphate solubilisation and *Indole-3-Acetic Acid* (IAA) production activities, with the values of 41.51 and 43.80 µg mL^{-1}, respectively. The detection of PGPR-encoding genes using PCR amplification was validates the potential of K22S-63 as biofertiliser candidate. Additionally, the presence of *PKS* and *NRPS* genes suggest the antimicrobial potency of K22S-

63 actinobacteria. This finding is particularly noteworthy given the limited research and exploration of the genus *Peterkaempfera*, which currently comprises only two recognised species. Further investigations including identification of agroactive compounds, genome mining and bioprospecting of K22S-63 for the PGPR traits, are remains essential to explore the potential of this isolate, which may extend beyond agriculture into broader field such as environmental sustainability and clinical biotechnology.

AUTHOR CONTRIBUTION

All authors are contributed equally to this work and reviewed the manuscript. I.S.H. conducted all research activities and wrote the manuscript. T.K.D. collected soil samples and provided reagents. S.W. designed the research framework and supervised the methodology. S.R. facilitated the provision of equipment and materials, and isolated the actinobacteria from soil sample. E.R. provided full supervision and mentorship throughout the research and manuscript process.

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CONFLICT OF INTEREST

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