

Isolation and molecular characterization of potassium-solubilizing bacteria from limestone mountain of Bahorok, Langkat District, Indonesia

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Abstract. Bangun IH, Hanum H, Sabrina T. 2023. Isolation and molecular characterization of potassium-solubilizing bacteria from limestone mountain of Bahorok, Langkat District, Indonesia. *Biodiversitas* 24: 4175-4184. In agricultural practices, ensuring an adequate supply of potassium to plants is crucial for optimal growth and productivity. However, the exchangeable K is tightly bound to soil minerals such as mica, feldspar, and clay minerals, making it unavailable for plant uptake. K-solubilizing bacteria K can dissolve potassium from the mineral layer and be available to plants. The Previous study has found 11 bacterial isolates capable of solubilizing K in Aleksandrov solid media. This study aimed to select the best K-solubilizing bacteria for solubilizing K in soil and to perform molecular identification of these bacteria. A novel finding from this study is that specific KSBs enhance the levels of exchangeable K in the soil through various mechanisms, as evidenced by increased exchangeable Ca, Mg, and soil pH. Additionally, the research identified two newly discovered bacterial species capable of potassium solubilization: *Paraburkholderia phymatum* and *Burkholderia paludis*. Furthermore, the study suggests the existence of an unknown mechanism for K solubilization, indicated by the observed increase in soil pH during the process.

Keywords: Aleksandrov, biofertilizer, cation, feldspar, ion K

INTRODUCTION

Potassium (K) is an essential macronutrient that plants require in significant amounts (Salam et al. 2023). Two forms of K are found in the soil i.e. exchangeable and non-exchangeable (Eguchi et al. 2023). Exchangeable K is readily available to plants for immediate uptake, and it is held on to the surface of soil particles and can be easily exchanged with other cations (Brady et al. 2008; Dubus et al. 2023). Soil pH, cation exchangeable capacity, and soil texture significantly determine the amount of exchangeable K in the soil (Kome et al. 2019). The non-exchangeable K is tightly bound to soil minerals such as mica, feldspar, and clay minerals, making it unavailable for plant uptake. Its release into the soil solution is slow and limited due to its strong bonding, which restricts its contribution to plant nutrition (Dubus et al. 2023). Plants absorb K ions present in soil solutions (Çalışkan dan Çalışkan 2019) but very few. Most K, or > 90%, is non-exchangeable (Wakeel 2013). Fixation of K occurs rapidly, but the release of K through the exchangeable is prolonged due to the powerful binding of clay minerals (Shakeri 2018).

The usual method to address K deficiency in agricultural soil is using inorganic K fertilizers. The use of inorganic fertilizers is often seen as a quick solution, but it also presents considerable challenges, including environmental pollution, ecological damage, and increased production expenses (Purba et al. 2020). However, using K

fertilizers such as potassium chloride, potassium nitrate, monopotassium phosphate, and sulfate potash can result in various issues such as reduced soil fertility, disruption of biochemical energy flow, formation of complex compounds, and higher levels of salinity and soil acidity (Olaniyan et al. 2022). Another issue is that K can be trapped in clay minerals soil while competing with ammonium (Hernández et al. 2021). Vermiculite and micaceous are types of clay minerals that function as a reservoir for K, as they have a solid attraction for K ions and can retain them effectively (Dubus et al. 2023). Therefore, an alternative solution is necessary to overcome the issue of K bonding in the soil's sediment and clay minerals. One effective option is using K-solubilizing bacteria (KSB) to increase the soil's K level. These bacteria have the capability to solubilize K ions from both sedimentary and igneous minerals. By releasing organic acids like citric acid, malic acid, and acrylate acid into the soil, the KSB dissolves the K bond and makes it available for plant uptake (Meena et al. 2014). These organic acids then react with sediment and clay minerals containing K, accelerating K's weathering and release from the sediment and clay minerals. The chemical reaction between organic acids and clay minerals produces complex organic compounds of K soluble in water and quickly absorbed by plant roots (Kome et al. 2019).

The selection process is needed to obtain KSB that can weather K bound to soil and clay minerals by comparing their ability through bench-scale studies and pilot-scale

processes (Sattar et al. 2019; Sharma et al. 2022). This process resulted in KSB that effectively increased soil available K for plants, aiding in plant K uptake (Zhao et al. 2022). KSB in soil include *Bacillus* sp. (Zhou et al. 2022), *Bacillus mucilaginous* (Xiao et al. 2012), *Escherichia vulneris*, *Bacillus cereus* (Wang et al. 2022), *Bacillus cepacia*, *Serratia marcescens* (Angraini et al. 2016), *Pseudomonas* sp. (Sarikhani et al. 2018), and members of *Enterobacteriaceae*, *Pseudomonadaceae*, *Bacillaceae*, *Micrococcaceae* (Sharma et al. 2022), that can dissolve K in Aleksandrov media. Previous research conducted by Bangun et al. (2020) has found that 11 KSB isolates were isolated from soil samples around Bahorok Langkat limestone area that were capable of solubilizing K in Aleksandrov media. The mechanism of K solubilization was characterized by a decrease in pH in the growth medium, indicating the potential mechanism of mobilizing K from crystal-bound forms through the production of organic acids (Ghosh et al. 2023). Recent literature review analysis indicates that the number of published articles related to K solubilization mechanisms is scattered and fewer compared to the articles on K-solubilizing microbes, suggesting that the mechanisms of K solubilization by K-solubilizing microbes still require further exploration (Soumare et al. 2023). Therefore, this study aimed to select the best K-solubilizing bacteria for solubilizing K in soil and to perform molecular identification of these selected bacterial isolates.

MATERIALS AND METHODS

Study area

The research was conducted at the Soil Laboratory of Universitas Muhammadiyah Sumatera Utara at Kapt. Mukhtar Basri Street. No. 3 in Medan, Indonesia, with the 16S rRNA identification performed at the Biodiversity Biotechnology Indonesia Laboratory at Jl. Cilubang Nagrak No. 62, Bogor, Indonesia. The soil sample was collected from Besitang, Langkat, North Sumatra, Indonesia (98°14'65".21" N; 4°04'86".84" E; 17 m elevation). The research was conducted from March to August 2020.

Materials

This study consisted of three interconnected stages, which began with the selection of 11 KSB (Table 1) from a

previous study by Bangun et al. (2020). Eleven selected KSBs were subjected to bench-scale studies (dissolving K in solid media in the laboratory) using the dissolution index method. In the following stage, pilot-scale processes were conducted to test the KSBs' ability to dissolve K in controlled soil. Finally, the selected KSBs were molecularly identified using 16S rRNA. The materials utilized in this research include Aleksandrov media (Aleksandrov et al. 1967) (Feldspar (KAlSi_3O_8), CaPO_4 , glucose, yeast extract, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, FeCl_3 , CaCO_3 , distilled water, NaCl), nutrient broth media (peptone, beef extract, NaCl, yeast extract) physiological solution of NaCl 0.85%, KCl, isolate KSB, and soil sample.

Bench-scale studies dissolution index of K by KSB

The current study employed 11 (Table 1) types of KSB, which were selected from a previous study by Bangun et al. (2020) and tested for their dissolution index on Aleksandrov solid media in a laboratory setting using a non-factorial, completely randomized design (CRD). These KSB were: KSB.PA1, KSB.PA2, KSB.PA3, KSB.TA1, KSB.PB1, KSB.TB1, KSB.TB2, KSB.TB3, KSB.TB4, KSB.TB5, and KSB.TB6 from (Tabel 1). The experiment was conducted with three replications, resulting in 33 experimental units. The research began with the purification of 11 KSBs on Aleksandrov Agar media (pH 6±0.5). A single pure KSB from each of the 11 KSB was spot inoculated in the middle of a petri dish containing Aleksandrov medium (pH 6±0.5) and incubated for 7 days at a temperature of 27°C±2°C. The determination of the dissolution index of K followed the method described by (Angraini et al. 2016), where the clear zone formed around the colony was observed and measured with calipers, and the K dissolution index was calculated daily for 7 days to determine the bacteria's ability to dissolve K using the following equation:

$$DI = \frac{SZ - DC}{DC} \quad [1]$$

Where:

- DI : Dissolution index
SZ : Diameter of the colony and clear zone
DC : Diameter of the colony

Table 1. Source, origin, location, plant species, and isolate code of K solubilizing bacteria (KSB) used in this study

| Sources sample | Location | | Tree species | Isolate code |
|---|---------------------------|------------------|---------------------------|--------------|
| | Coordinate | Altitude (m asl) | | |
| The highest spot of the mount (soil depth 1-5 cm) | N: 3°28.11'; E: 98°08.53' | 338 | <i>Cassia javanica</i> | KSB.PA.1 |
| The highest spot of the mount (soil depth 1-5 cm) | N: 3°28.11'; E: 98°08.53' | 338 | <i>C. javanica</i> | KSB.PA.2 |
| The highest spot of the mount (soil depth 1-5 cm) | N: 3°28.05'; E: 98°08.51' | 364 | <i>Prunus avium</i> | KSB.PA.3 |
| The highest spot of the mount (soil depth 1-5 cm) | N: 3°28.05'; E: 98°08.51' | 364 | <i>P. avium</i> | KSB.PB.1 |
| The middle spot of the mount (soil depth 1-5 cm) | E: 3°28.01'; N: 98°08.53' | 281 | <i>Piper aduncum</i> | KSB.TA.1 |
| The middle spot of the mount (soil depth 5-10 cm) | E: 3°28.01'; N: 98°08.53' | 281 | <i>P. aduncum</i> | KSB.TB.1 |
| The middle spot of the mount (soil depth 5-10 cm) | E: 3°28.01'; N: 98°08.53' | 281 | <i>P. aduncum</i> | KSB.TB.2 |
| The middle spot of the mount (soil depth 5-10 cm) | E: 3°28.01'; N: 98°08.53' | 281 | <i>P. aduncum</i> | KSB.TB.3 |
| The middle spot of the mount (soil depth 5-10 cm) | E: 3°28.03'; N: 98°08.59' | 267 | <i>Hevea brasiliensis</i> | KSB.TB.4 |
| The middle spot of the mount (soil depth 5-10 cm) | E: 3°28.03'; N: 98°08.59' | 267 | <i>H. brasiliensis</i> | KSB.TB.5 |
| The middle spot of the mount (soil depth 5-10 cm) | E: 3°28.03'; N: 98°08.59' | 267 | <i>H. brasiliensis</i> | KSB.TB.6 |

Table 2. The details of the experimental treatment

| Bacterial isolates | Dosage KSB | Bacterial density |
|--------------------|-------------------------|---|
| Kontrol | no any KSB | - |
| KSB.PA1 | 10 mL pot ⁻¹ | 26 x 10 ⁸ CFU mL ⁻¹ |
| KSB.PA2 | 10 mL pot ⁻¹ | 39 x 10 ⁸ CFU mL ⁻¹ |
| KSB.PA3 | 10 mL pot ⁻¹ | 37 x 10 ⁸ CFU mL ⁻¹ |
| KSB.TA1 | 10 mL pot ⁻¹ | 40 x 10 ⁸ CFU mL ⁻¹ |
| KSB.PB1 | 10 mL pot ⁻¹ | 34 x 10 ⁸ CFU mL ⁻¹ |
| KSB.TB1 | 10 mL pot ⁻¹ | 37 x 10 ⁸ CFU mL ⁻¹ |
| KSB.TB2 | 10 mL pot ⁻¹ | 42 x 10 ⁸ CFU mL ⁻¹ |
| KSB.TB3 | 10 mL pot ⁻¹ | 47 x 10 ⁸ CFU mL ⁻¹ |
| KSB.TB4 | 10 mL pot ⁻¹ | 41 x 10 ⁸ CFU mL ⁻¹ |
| KSB.TB5 | 10 mL pot ⁻¹ | 35 x 10 ⁸ CFU mL ⁻¹ |
| KSB.TB6 | 10 mL pot ⁻¹ | 62 x 10 ⁸ CFU mL ⁻¹ |

Pilot-scale processes by KSB improving K-exchangeable in soil

The preparation of KSB isolates was carried out by culturing 11 KSBs in separate Erlenmeyer flasks containing 250 mL of sterile NB media (pH 6±2) and incubating them for 24 hours at a temperature of 27°C±2°C (population KSB 10⁸). Before conducting the field application, the total population of KSB was determined using the total plate count (TPC) method. The potential test was conducted on clay mineral soil from Besitang, Langkat, North Sumatra, Indonesia (98°14'65".21" N; 4°04'86".84" E; 17 m elevation). A complete randomized design (CRD) without factorial arrangement was replicated three times with 12 different treatments (Table 2). 18 kg of soil was finely grounded and placed in 36 pots (500-g soil pot⁻¹). Initial soil analysis was not performed because this study used control soil (without KSB). The natural state of KSB was assumed to be the same in each soil sample used. The 500-gram pots of soil were arranged in a room at 27°C±2°C and left for 3 days. Daily watering with distilled water was done until the field capacity was reached (around 20% to 35% of the soil's weight). Then, inoculation of KSB was carried out at a rate of 10 mL pot⁻¹ (10⁸ CFU mL⁻¹), and the pots were incubated in the laboratory for 1 month at a temperature of 27±5°C. Daily watering was done on all treatment pots until the field capacity was reached.

Chemical properties such as exchangeable K, Ca, Mg, and Na were evaluated in soil samples using a leaching technique. This involved soaking 10 g of dried soil in 100 mL of 1 M ammonium acetate solution with a pH buffer of 7 for 5-6 hours. The resulting leachates were collected and diluted to 100 mL with 1 N NH₄OAc solution before being analyzed using the Atomic absorption spectroscopy (AAS) method to measure the K-exchangeable, Ca-exchangeable, Mg-exchangeable, and Na-exchangeable. The H₂O (1:5) electrometry method measured the pH level. At the same time, the total K content was determined by digesting a representative soil sample with concentrated HNO₃ and then diluting and analyzing it with AAS using a series of K standards for calibration and quality control. To ensure consistent bacterial population density among isolates (Table 2), isolates with higher densities are diluted with sterilized water in a proportionate percentage (water

volume based on the density percentage of the lowest-density bacteria, KSB.PA1), thoroughly mixed, and the total volume of the solution is adjusted with other isolates to achieve a uniform density.

Identification 16S rRNA

The identification process involved the PCR-16S rRNA sequencing method. DNA isolation and PCR amplification were conducted simultaneously using a direct PCR Kit (KOD FX Neo, Toyobo) following the kit's instructions. The Universal Primer consisting of Primer F: 16F27/Sequence: AGA GTT TGA TCM TGC CTC AG and Primer R: 16R 1492/Sequence: TAC GGY TAC CTT GTT ACG ACT T was used, and the PCR machine used was the Eppendorf personal master cycler. The PCR product underwent a purification process, and the nitrogen base sequence was read by PT. Genetika Science. The sequencing results were edited using the MEGA 11 program. The edited sequence data were further analyzed in Basic Local Alignment Search Tool (BLAST) with genomic data registered at NCBI/National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST>) to determine the taxon/species with the closest molecular homology/similarity.

Evolutionary analysis

The Maximum Likelihood method and Tamura-Nei model (Tamura dan Nei 1993) were used to infer the evolutionary history. The tree with the highest log likelihood (-8971.27) is displayed, and the percentage of trees in which the associated taxa clustered together is indicated next to the branches. The initial tree(s) for the heuristic search were generated automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with the highest log likelihood value. This analysis involved 14 nucleotide sequences, and the final dataset contained 1541 positions. The evolutionary analyses were performed in MEGA11 (Tamura et al. 2021).

Data analysis

The data obtained from this study were analyzed for homogeneity and statistically analyzed using ANOVA. Any data significantly influencing the outcomes were further examined using Dunnett's Post Hoc test (significance level - 5%) (Koenig et al. 2008). Software like Microsoft Excel 2016 and GraphPad Prism (Version 9.5.1) was used while evolutionary analyses were conducted in MEGA11.

RESULTS AND DISCUSSION

Dissolution index of K by KSB

The dissolution index on Alexandrov media was used to test KSB isolates to determine their ability to solubilize K. The dissolution index on this media measures how well KSB can form solubilization zones on the solid medium (Figure 1).

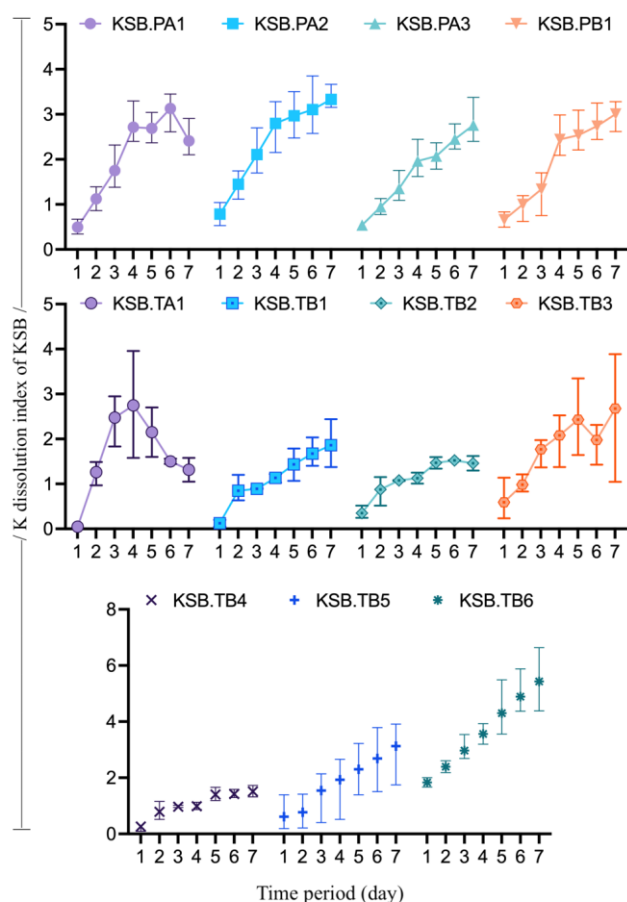


Figure 1. K dissolution index of K-solubilizing bacteria (KSB) on Alexandrov solid media during 7 days of observation. Standard deviation was used to represent the error bars in the data

The larger the solubilization zone, the better the KSB solubilizes K from the insoluble source. The results showed that the solubilization index of all KSB during the 7-day observation did not differ significantly (Figure 1). The increased dissolution index varied depending on the type of bacteria. However, it can be seen that from the first day to the seventh day, all bacteria showed an increasing trend. Out of the 11 observed bacterial isolates, it was found that KSB.TB6 and KSB.PA2 isolates consistently recorded the highest dissolution index compared to the other isolates. Furthermore, based on the conducted data analysis, it is evident that the K solubilization index of KSB does not consistently exhibit a parallel increase with bacterial growth across all isolates. For instance, in isolate KSB.TA1 and KSB.PA1, the solubilization index decreased on day 4 (KSB.TA1) and day 6 (KSB.PA1), and on the following days, it continued to decrease, even though the colony growth of KSB increased. A similar occurrence was noted in the case of KSB.TB3 and KSB.TB1 isolates where the K solubilization index by KSB was reduced on day 3 (KSB.TB1) and day 6 (KSB.TB3). However, unlike KSB.TA1 and KSB.PA1, both KSB.TB3 and KSB.TB1 showed a subsequent increase in the solubilization index in the following days.

Potential test of KSB improving K-exchangeable in soil

Inoculation of KSB has little impact on exchangeable K, Ca, Mg, and Na in the soil. Nevertheless, some KSB's enhanced the amount of exchangeable K in clay minerals following a 30-day incubation period (Figure 1). The increase in exchangeable K in clay minerals soil by different KSB during 30 days of incubation is as follows: KSB.PA1 (9.2%), KSB.PA3 (7.3%), KSB.TA1 (10.9%), KSB.PB1 (9.8%), KSB.TB1 (13.2%), KSB.TB2 (17.85%), KSB.TB3 (8%), KSB.TB4 (6.1%), KSB.TB5 (9.8%) and KSB.TB6 (9.8%), except for the KSB.PA2 bacteria, which exhibited a decrease of 1.4% compared to the control (Figure 2A). From the selection process, ten KSBs effectively released the total K in the soil through dissolution into exchangeable K in clay minerals during 30 days of incubation, with a coefficient of variation (CV) value of 10.47% (Figure 2A). The survival of bacteria varies depending on the supporting conditions such as pH, temperature, salinity, nutrient availability, etc. During the 30-day incubation period, no significant effect was observed on the exchangeable Ca in KSB inoculated soils (Figure 2B). However, the application of KSB was found to increase Ca-exchangeable levels in KSB.PA3 by 5.75%, KSB.TA1 by 12.19%, and KSB.PA1 by 9.21% compared to the control without KSB (CV: 18.24%). On the other hand, other isolates decreased Ca-exchangeable levels ranging from 3.74% to 40.07%. The implementation of all KSB treatments did not result in a significant impact on the measurement of exchangeable Mg in the soil (Figure 2C).

KSB inoculation can potentially increase the exchangeable Mg level in the soil, with percentage increments observed for KSB.PA1 (3.2%), KSB.PA3 (4.1%), KSB.TA1 (1.7%), KSB.PB1 (5.7%), KSB.TB1 (2%), KSB.TB2 (0.8%), KSB.TB4 (2.4%), KSB.TB5 (1.1%), and KSB.TB6 (1.2%) compared to the absence of KSB inoculation (CV: 3.79%). Nonetheless, some KSBs were found to lower the Mg-exchangeable level, namely the KSP.PA2 isolate with a reduction of 37.55% and KSB.TB3 with a decrease of 1.5%. Meanwhile, regarding the exchangeable Na level in the soil observation, using KSB can increase the exchangeable Na level in the soil (Figure 2D). However, the difference is insignificant as compared to the control group without KSB inoculation. Each isolate exhibited a different percentage of increase with KSB.TA1 showed the highest increase at 54.41%, followed by KSB.TB2 at 50.26%, and KSB.TB6 at 42.29%. Other isolates, such as KSB.PA1, KSP.PA2, KSB.PA3, KSB.PB1, KSB.TB1, KSB.TB3, KSB.TB4, and KSB.TB5 also shows an increase in the exchangeable Na level with varying percentages (CV: 46.22%).

The effect of the KSB-mediated increase in exchangeable K on the total soil K stock and pH are presented in Figure 4. There are various impacts of increasing exchangeable K in the soil after inoculation of various types of KSB, such as increasing exchangeable Ca, Mg, and Na. Still, total K did not show any significant effect after 30 days of incubation. However, in general, the total amount of K in the soil increased, and the best isolate was KSB.PA3, with an increase of 18.69%, followed by KSP.PA2 with an increase of 15.96%, and KSB.TB3 with

an increase of 13.04%, compared to without KSB inoculation. However, some isolates showed varying patterns of increase in the KSB.TA1, where the total K decreased by 8.6% compared to without KSB inoculation (CV: 12.64%). It should be noted that an increase in the total amount of K in the soil does not always indicate the presence of exchangeable K, as the state of K in the soil can vary. Meanwhile, in soil pH observation after inoculation KSB, although there was no significant effect, pH increased by 0.22% - 4.85%, except for the KSB.TB3 bacterial isolate, which decreased by 0.45%. The highest increase in pH was found in KSB.PA1 inoculated soil (4.85%), followed by KSB.PB1 (3.59%), and KSP.PA2 (3.31%) inoculated soils.

Molecular identification bacteria

Molecular identification was conducted on three selected isolates, including KSB.TB5, KSB.PB1 and

KSB.TB6. The results of 16S rRNA amplification were obtained using primers 63f and 1387r. The 16S rRNA gene amplification electrophoresis results for the three isolates are shown in (Figure 5).

Based on a BLAST search against the NCBI database, isolate KSB.PB1 showed a 98.95% homology and 100% query cover with *Paraburkholderia phymatum* strain STM6022 16S ribosomal RNA gene, partial sequence. Isolate KSB.TB5 showed a 100% homology and 100% query cover with *Burkholderia paludis* strain JRBHU-2 16S ribosomal RNA gene, partial sequence. Isolate KSB.TB6 showed a 100% homology and 100% query cover with *Burkholderia cepacia* strain yy-2 16S ribosomal RNA gene, partial sequence, based on the results of a BLAST search against the NCBI database. The phylogenetic analysis of the three bacteria that have been molecularly identified and sequenced for the 16S rRNA gene can be seen in Figure 6.

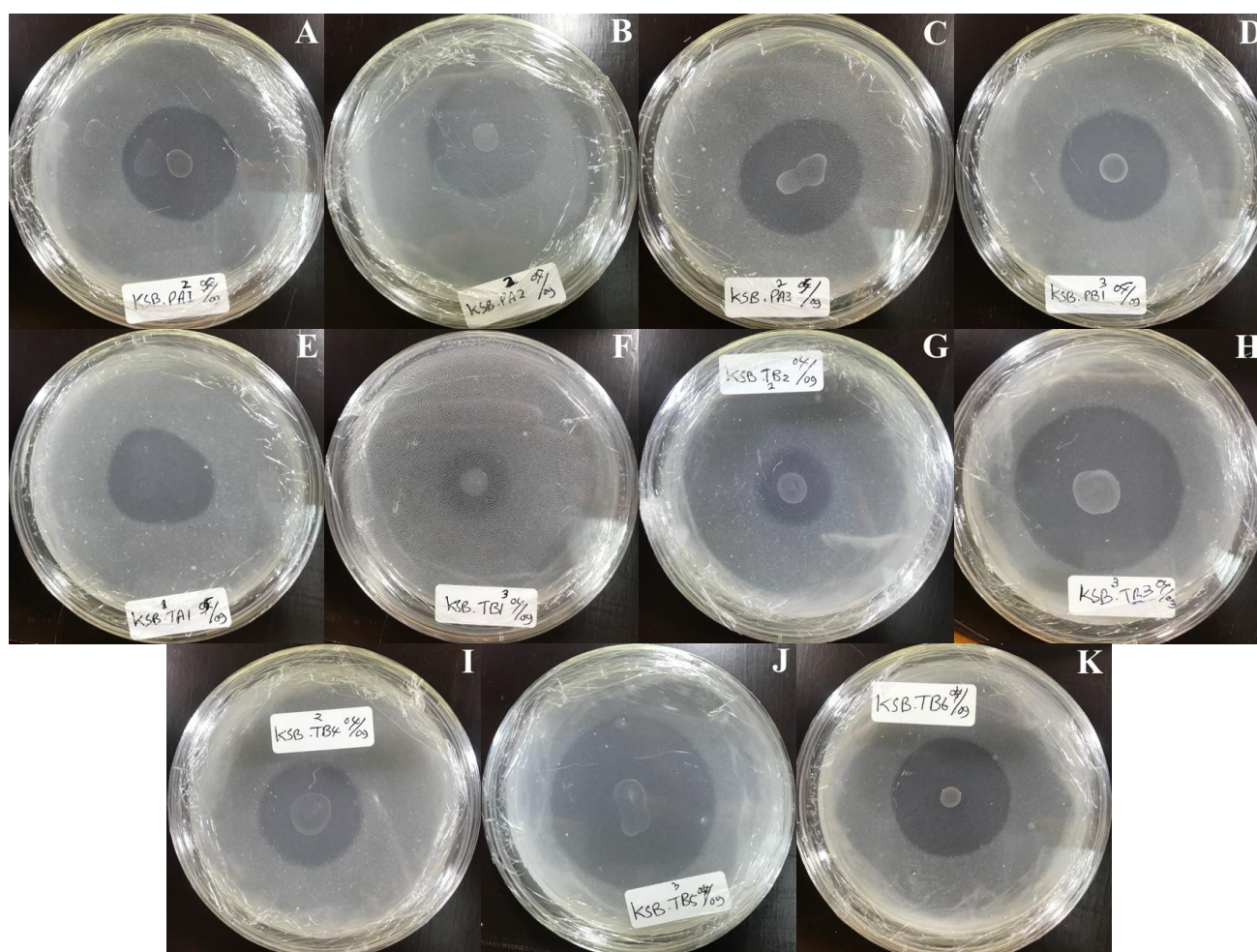


Figure 2. Dissolution index of 11 K solubilizing bacteria on Alexandrov media mineral source from feldspar (KAlSi_3O_8) after being cultured for 7 days. Note: A. KSB.PA1, B. KSB.PA2, C. KSB.PA3, D. KSB.PB1, E. KSB.TA1, F. KSB.TB1, G. KSB.TB2, H. KSB.TB3, I. KSB.TB4, J. KSB.TB5, K. KSB.TB6

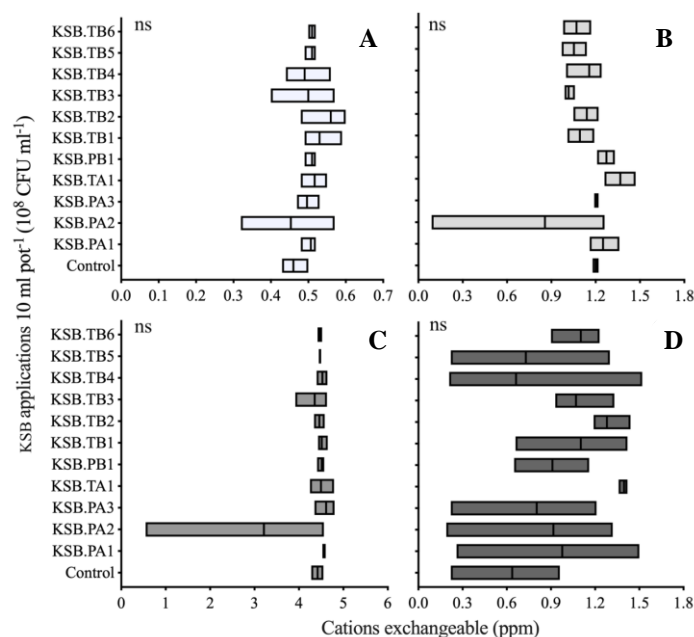


Figure 1. Cations exchangeable in clay minerals, such as: A. K-exchangeable, B. Ca-exchangeable, C. Mg-exchangeable, and D. Na-exchangeable, in a room after 30 days of incubation (27°C±2°C). Note: (1) Standard deviation was used to represent the separated low-high bars in the data. (2) "ns" indicates data that is not significantly different according to ANOVA

Molecular phylogenetic analysis

The evolutionary history was inferred using the Maximum Likelihood method and Tamura-Nei model (Tamura dan Nei 1993). The percentage of trees in which associated taxa clustered together is indicated next to the branches. This phylogenetic analysis involved 14 nucleotide sequences, and the final dataset contained 1541 positions (Figure 6).

The nucleotide gene relationship analysis showed that bacterial strain KSB.PB1 had a 77% similarity with *Paraburkholderia phymatum* STM6022. Furthermore, the nucleotide gene relationship analysis revealed that bacterial strain KSB.TB5 had a 64% similarity with *Burkholderia paludis* strain JRBHU-2. In the nucleotide gene relationship phylogenetic tree (Figure 5), bacterial strain KSB.TB6 showed a 95% similarity with *Burkholderia cepacia* strain yy-2. The evolution of these organisms is a complex process that involves various factors such as mutation, gene transfer, natural selection, and environmental factors. Based on the similarity percentages obtained from the nucleotide gene relationship analysis, it is difficult to draw definitive conclusions about the evolutionary history of these organisms. However, it can be inferred that they share a common ancestor at some point in the past. They have undergone divergent evolution since then, leading to the differences observed in their nucleotide sequences. The isolates' sequences were later submitted to NCBI, and accession numbers were acquired (Table 3).

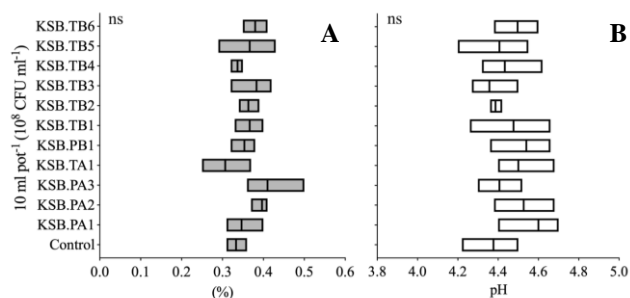


Figure 2. Total K (A) and pH (B) in the soil after 30 days of incubation (27°C±2°C). Note: (1) Standard deviation was used to represent the separated low-high bars in the data. (2) "ns" indicates data that is not significantly different according to ANOVA

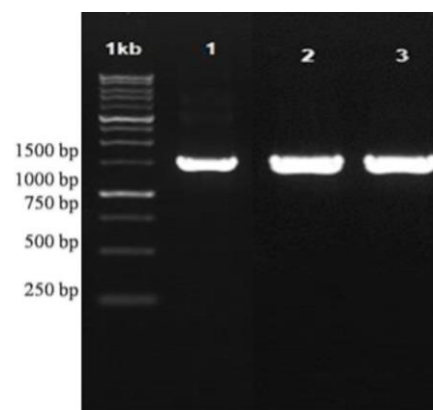


Figure 5. Amplification of 16S rRNA gene of isolating K solubilizing strains from the limestone mountain of Bahorok Langkat. Note: Lane 1 = KSB.PB1, lane 2 = KSB.TB5, and lane 3 = KSB.TB6. The sample volume loaded per lane = 1 uL, and the DNA ladder loaded per lane = 0.1 uL

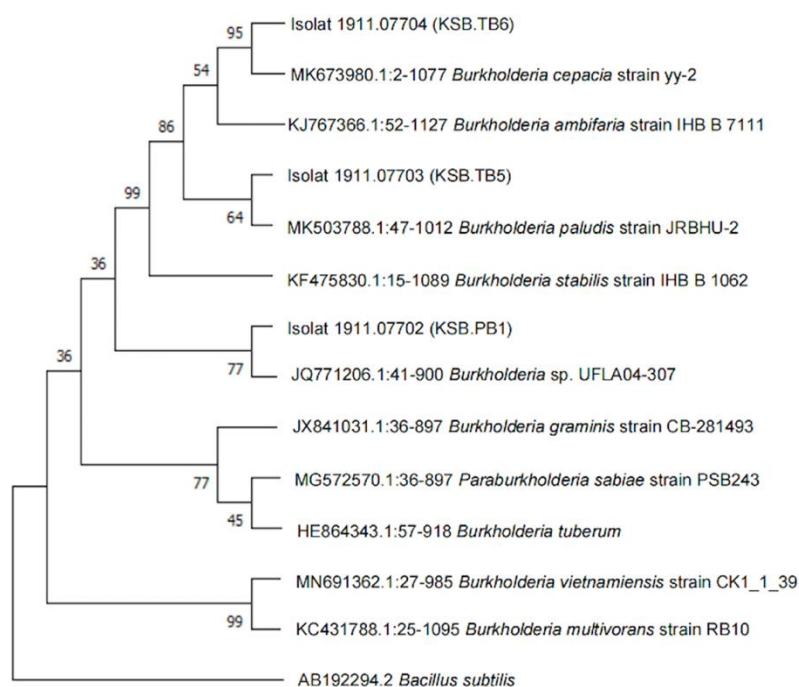


Figure 6. Phylogenetic relationship by and identifications of bacterial strains isolate based on 16S rRNA sequence through Maximum Likelihood method using 1541 bootstrap replicates

Table 3. Molecular analysis of three KSB isolates was performed using their 16S rDNA sequences, and the results were submitted to the NCBI GenBank database. The closest species and corresponding similarity percentages for each isolate were documented using the BLAST tool on NCBI

| KSB isolates | Isolates denoted in NCBI | Species similarity (%) | GenBank accession number |
|--------------|---|------------------------|--------------------------|
| KSB.TB5 | <i>Burkholderia paludis</i> strain JRBHU-2 | 100% | OR342151 |
| KSB.TB6 | <i>Burkholderia cepacia</i> strain yy-2 | 100% | OR342153 |
| KSB.PB1 | <i>Paraburkholderia phymatum</i> strain STM6022 | 98.95% | OR342197 |

Discussions

Generally, all selected bacteria found in the limestone mountain of Bahorok Langkat could dissolved K (Bangun et al. 2020). The selection process was divided into two stages: Bench-scale studies, a selection process that tests K dissolution in the laboratory on solid Aleksandrov media, and Pilot-scale processes, which test K solubilization on soil media. The study showed that 11 bacteria from Bahorok Langkat Limestone Mountain in Bench-scale studies could dissolve K in 7 days of observation, but each KSB isolate had different abilities. The research conducted by (Setiawati dan Mutmainnah 2016) showed that the KSB could solubilize K from insoluble K-bearing minerals sources such as feldspar and formed solubilization zones on the solid medium. Microbes involved in K solubilization can be classified based on the dissolution index as low ($DI < 2$), moderate ($2 < DI < 4$), and high ($DI > 4$) (Marra et al. 2011). The top-performing isolates among the 11 bacteria tested using a completely random design on solid Aleksandrov media for K solubilizations were found to be KSB.TB6, KSB.PA2, and KSB.PB1. The mechanism of K solubilization is also attributed to either media acidification

or the chelation of cations that usually bind to K (Etesami et al. 2017).

In addition, KSB isolates that were able to solubilize K in the laboratory using the dissolution index observation method may experience a decrease in their dissolution index due to faster colony growth than the formation of clear zones. The KSB.TB2 bacterial isolate exhibited an inconsistent increase and the lowest dissolution index at all observation times. The weakness of the dissolution index method in observing K solubilization by KSB may not accurately reflect the actual solubilization potential of the bacteria. This is because the dissolution index is based on the formation of clear zones around bacterial colonies on solid media, which can be influenced by factors such as colony growth rate, medium composition, and the size of the inoculum. Therefore, the dissolution index may not always be a reliable indicator of the ability of KSB to solubilize K under different conditions. Furthermore, the inconsistency between the solubilization index and bacterial growth was observed in some isolates, such as the KSB.TB2 bacterial isolate highlights the limitations of relying solely on the dissolution index to evaluate K

solubilization by KSB. In order to determine the best KSB, the selection process is carried out at the Pilot-scale processes stage by applying it to the soil. The study found that after 30 days of incubation, KSB application increased K-exchangeable levels in most isolates, except for KSB.PA2 bacteria experienced a decrease compared to the control group. However, some isolates have high deviation diversity values.

The ability of bacteria to dissolve K-exchangeable depends on supporting conditions such as pH, temperature, salinity, nutrient availability, etc (Roszak dan Colwell 1987; Serrazanetti et al. 2009). Based on the diversity values, certain isolates, when subjected to various environmental conditions, exhibit the ability to enhance the exchangeable K levels in the soil. The most effective isolates for increasing K-exchangeable are KSB.PB1, KSB.TB5, and KSB.TB6 (Figure 3A). In contrast, the presence of certain KSBs has a different impact on the exchangeable Ca levels in the soil. Generally, applying KSBs leads to a decrease in Ca-exchangeable levels. However, exceptions exist, as the isolates KSB.PA3, KSB.TA1, and KSB.PA1 shows an increase compared to the control group without KSB (Figure 3B). Regarding Mg exchangeable levels, the application of KSBs results in a slight increase in most cases, albeit with low percentages. However, two isolates, KSP.PA2, and KSB.TB, show a decrease in Mg-exchangeable levels by 37.55% (Figure 3C). The mechanism of K solubilization has been previously reported to involve the production of organic acids and acidification (Etesami et al. 2017), which can cause a decrease in the Ca-exchangeable and Mg-exchangeable levels in the soil. The soil acidification caused by several factors can decrease the Ca-exchangeable level in the soil (Johnson et al. 2008; Bedison et al. 2010).

The application of KSB increased the Na-exchangeable level in the soil, although with a high coefficient of variation of percentages (Figure 3D). The increase in Na-exchangeable level varied from 4% (KSB.TB4) up to 54.41% (KSB.TA1). Currently, no study has examined the impact of KSB on Na levels. Thus there is no point of reference available for comparison purposes. In general, the application of KSB contributes to the release of exchangeable K in the soil through several mechanisms. Specifically, in this study, the total K amount in the soil increased after the inoculation of KSB, although the K-exchangeable level also increased (Figure 4A). For example, using isolates KSB.PA1, KSB.PA3, KSB.PB1, KSB.TB1, KSB.TB2, KSB.TB3, KSB.TB4, KSB.TB5, and KSB.TB6 increased total K, and K-exchangeable also increased. However, there were instances where an increase in total K resulted in a decrease in K-exchangeable, such as in the KSP.PA2 treatment, K-exchangeable decreased by 1.4% while total K increased by 15.96%. Conversely, using KSB.TA1 bacteria increased the K-exchangeable level by 10.96%, while total K decreased by 8.6%. The use of KSB.TB6 showed the best ability to increase K-exchangeable by 9.8% and total K by 12.28%. However, it is important to note that an increase in

the total amount of K in the soil does not always indicate an increase in K-exchangeable.

The simultaneous application of KSBs leads to an increase in exchangeable K levels and soil pH. The rise in exchangeable Ca and Mg percentages also indicates an increase in pH. According to some studies, the mechanism of K dissolution in the absence of K can be dissolved due to the production of various types of organic acids accompanied by acid exchange reactions or pH reduction (Liu et al. 2016). This increase in pH is very interesting because this study is thought to be caused by the mechanism of K dissolution into K-exchangeable, which has a different mechanism from several previous studies. In contrast to the study by Uroz et al. (2009), K-solubilizing bacteria lower the pH and cause the bacteria to release protons and organic acids. Some isolates also produce malic and syringic acids, which help dissolve K in the soil (Soumare et al. 2022). Therefore, it is evident that the mechanism of K dissolution can vary depending on the bacterial isolates and soil conditions. The mechanism by which KSB increase K-exchangeable in the soil is not fully understood. However, some studies have shown that the main mechanism of K solubilization is producing organic acids and rhizosphere acidification (Soumare et al. 2022). Furthermore, KSB can enhance the soil's cation exchange capacity and increase plant nutrient availability. The improvement in exchangeable K can also affect the availability of other exchangeable cations, such as calcium, magnesium, and sodium. In some previous studies, increased K-exchangeable levels in the soil can decreased the exchangeable calcium and magnesium. However, in this study, the increase in K-exchangeable also increased exchangeable Ca and Mg. The effect on Na-exchangeable was previously not understood, but new findings in this study suggest that KSB can increase Na-exchangeable levels even at the highly varying coefficient of variation. In this study, an interesting aspect was found. The increase in pH in this study is considered due to the mechanism of K dissolution into exchangeable K, which has a different mechanism from several previous studies.

A homology value of 98% or less indicates a different species or can be considered a new species (Patel et al. 2004). However, bacteria can be classified into new species in some cases, although the 16S rDNA homology is above 98%. It happens if the homology value of DNA-DNA hybridization is low or less than 70% (Patel et al. 2004). Studies suggest that *P. phymatum* is a Gram-negative, non-sporulating bacteria with a straight rod shape (Vandamme et al. 2002). This bacterium is a prominent model organism used in the research on beta-rhizobia-legume symbiosis (Moulin et al. 2001). *P. phymatum* is not a common bacterium in potassium solubilization. Instead, it stands out for its ability to establish N₂-fixing symbiosis with various legume species, including *Phaseolus vulgaris* (Lardi et al. 2017). The formation of rhizobia-legume nitrogen-fixing symbiosis is a highly complex and regulated process that involves the exchange of specific signals between bacteria and plants. Among these signals, bacterial lipochitooligosaccharides (Nod factors), surface polysaccharides, and flavonoids secreted by plant roots

play crucial roles (Downie 2010). *B. paludis* has not been extensively studied in relation to K solubilization in Alexandrov media or soil. *B. paludis* is a Gram-negative, facultatively anaerobic, rod-shaped bacterium with a width of about 0.6-0.8 µm and a length of 1.6-2.1 µm. The bacterial colonies are round, regular, transparent, moist, and have a 1.0-3.0 mm diameter. *B. paludis* can grow at 15-40°C (optimum 30°C) and pH 4.0-10.0 (optimum pH 7.0) on NB and produce enzymes such as phosphatase, esterase, esterase lipase, and others (Ong et al. 2016). *B. paludis* was recently isolated and could produce a type of siderophore with antimicrobial properties such as pyochelin (Ong et al. 2016). *B. cepacia*, discovered by Yabuuchi et al. (1992) and Palleroni and Holmes (1981), is a bacteria that can dissolve K. Other studies have also been conducted, showing that *B. cepacia* can dissolve K in the soil (Zhang dan Kong 2014; Pratama dan Anas 2016; Saha et al. 2016). *B. cepacia* is a phosphate-solubilizing bacteria efficiently used as biological fertilizer in agricultural soils (Gupta et al. 2012). *B. cepacia*, which solubilizes phosphate, can also produce indole acetic acid under in vitro conditions (Singh et al. 2013).

The conclusion demonstrates that all KSBs were able to solubilize K in both Aleksandrov media and soil, although their abilities varied. A novel finding from this study is that specific KSBs enhance the levels of exchangeable K in the soil through various mechanisms, as evidenced by increased exchangeable Ca, Mg, and soil pH. Additionally, the research identified two newly discovered bacterial species capable of potassium solubilization: *P. phymatum* and *B. paludis*. Furthermore, the study suggests the existence of an unknown mechanism for K solubilization, indicated by the observed increase in soil pH during the process. Further exploration of the K solubilization mechanism will provide valuable insights into addressing soil acidification, a common occurrence during the K solubilization process through acidolysis.

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