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Co-culturing potassium-solubilizing bacteria in a common liquid medium for the development of a consortium fertilizer formulation

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Abstract

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The co-culture technique is considered one of the approaches for producing microbial biomass for various microbial products. This study employed the method of co-culturing in a standard liquid medium (CLM) to promote the proliferation of potassium-solubilizing bacteria (KSB). KSB strains were screened for solubility index and compatibility on an agar plate, as well as growth rate in a liquid medium. The selected strains of KSB were co-cultured in Luria Bertani broth as CLM. The co-culture efficiency was evaluated based on the cell density of each partner and the total cell density in the broth. Two strains of *Enterobacter* sp. GTC5.13.1 and *Serratia* sp. GTC5.19.1 was screened from nine indigenous KSB strains. The ratio of starters and the initial inoculation size had a significant effect on the co-culture efficiency of these two KSB strains. The 4:1 ratio of starters of *Enterobacter* sp. GTC5.13.1 and *Serratia* sp. GTC5.19.1 and the initial inoculation size at 0.5 – 1% were suitable for co-culture. Under these conditions, the obtained co-culture exhibited total cell density and solubilization efficiency on mica, kaolin, and feldspar comparable to those of monoculture cultures. Preliminary results proved that the co-culture technique in CLM could be applied to produce biomass of *Enterobacter* sp. GTC5.13.1 and *Serratia* sp. GTC5.19.1 for the development of KSB fertilizer.

Keywords: the co-culture technique; common liquid medium; *Enterobacter*; potassium solubilizing bacteria; proliferation; *Serratia*
Abbreviations: KSB-potassium solubilizing bacteria; CLM-communal liquid medium; SI-solubility index; SE-solubilization efficiency

Introduction

The role of potassium-solubilizing bacteria (KSB) in plants has recently been a topic of interest to many researchers. Numerous publications about KSB are continuously published worldwide. Primarily, numerous publications have been published on isolation and screening to collect suitable KSB strains for developing microbial biofertilizers. Literature on KSB isolation and screening is constantly being published and is easily found via Google search, such as Sun et al. (2020); Muthuraja and Muthukumar (2021), and Wang et al. (2022).

KSB has been effective on a wide variety of crops, including wheat (Sheng and He, 2006), tea (Bagyalakshmi

et al., 2012), cucumber (Prajapati and Modi, 2016), maize (Goswami et al., 2019), and tomato (Pokluda et al., 2021), among others. KSB can be applied in hydroponic farming systems (Prajapati and Modi, 2016) and traditional soil farming systems, including those in pots (Sheng and He, 2006), artificial growing chambers (Singh et al., 2010), and in the field (Bagyalakshmi et al., 2012). The trials, in general, have shown that KSB increases the amount of available K in the soil and the amount of K absorbed by plants, reduces the need for chemical K fertilizer, and has a positive effect on many other biological parameters of crops.

To date, there is much evidence that applying microbial consortia can also help increase efficiency compared to applying individual strains. For example, the combined use of multiple strains of inorganic phosphate-solubilizing bacteria has been studied by Demissie et al. (2013) on Faba beans and Zolfaghari et al. (2021) on *Quercus brantii*. Potassium-solubilizing microorganisms can also be applied simultaneously, as reported by Muthuraja and Muthukumar (2022) for many bacterial and fungal strains, or by Gore and Navale (2017) for three KSB strains belonging to *Pseudomonas*. The combined application of KSB with microorganisms possessing other plant growth-promoting activities has also shown positive effects on plants, as discussed by Basak and Biswas (2010), Sheikhalipour et al. (2016), Biswas and Shivaprakash (2021), Dar et al. (2021), and Sherpa et al. (2021).

However, there are still many difficulties in applying a microbial consortium. The microbial consortium may not increase the effectiveness of the target activity as reported by Nugroho et al. (2021). Additionally, developing a microbial consortium formulation is more complex than developing a single-strain formulation (Roell et al., 2019). The complexity of bacterial consortium formulation encompasses not only the complexity of formulating each component strain but also the complexity of interactions between partners during consortium formation.

In the development of biofertilizer formulations containing microbial consortia, the co-culture technique is one alternative approach to creating a source of microbial biomass. Previously, the co-culture technique has been widely applied in research on ecology (Marchand and Collins, 2013), in the field of food (Stadie et al., 2013), and in the production of secondary metabolites (Ola et al., 2013) and pharmaceuticals (Fox et al., 2014). However, the co-culture technique remains limited in the development of consortium biofertilizer formulations.

This study was conducted to apply the technique of co-culture in liquid in a communal liquid medium (CLM) to produce indigenous KSB biomass for the development of biofertilizer formulations. The co-culture efficiency was evaluated based on two criteria, including cell density and solubilization efficiency of the resulting biomass.

Materials and Methods

Microorganisms

KSB strains included *Enterobacter* sp. GTC5.13.1, *Enterobacter* sp. GTC5.13.3, *Enterobacter* sp. GTC5.13.4, *Enterobacter* sp. GTC5.13.5, *Enterobacter* sp. GTC5.13.6, *Burkholderia* sp. GTC5.14.1, *Serratia* sp. GTC5.19.1, *Acinetobacter* sp. GTC5.21.1, and *Pseudomonas* sp. GTC5.8.4. These KSB strains had previously been isolated, screened, and identified by our own research team. KSB strains were cultured and maintained on slant Luria-Bertani agar.

Media and uses

Luria-Bertani Agar was used for preservation culture on slants, compatibility assessment, and characterization of colony morphology. Media, including MacConkey Agar, Eosin Methylene Blue Agar, and Simmons Citrate Agar, were used for the morphological determination of bacterial colonies. Luria Bertani broth was used for co-culturing. The ingredients of all the above media were presented in Atlas (2010). Aleksandrov Agar, modified (Amaresan et al., 2022), was used to evaluate potassium solubility.

Evaluation of potassium solubility on an agar plate

Two-day-old bacterial cultures on Luria-Bertani slants were inoculated with three-point spots on a plate of modified Aleksandrov Agar to determine potassium solubility (Amaresan et al., 2022). The plates were incubated at 30-33°C for 3-4 days. The experiment was repeated on three plates for each bacterial strain. The transparent, soluble ring around the bacterial growth zone was formed because the medium, supplemented with mica, an insoluble mineral bearing potassium, made the medium opaque. Diameter of the halo zone (D) and diameter of the bacterial growth zone (d) were measured in cm. Potassium solubility was assessed based on the solubility index (SI) (Equation 1).

$$SI = (D - d) / d \quad (1)$$

Evaluation of the compatibility of KSB strains

A KSB loop from a two-day-old Luria-Bertani slant culture was inoculated into an Erlenmeyer containing 50mL of Luria-Bertani broth. The Erlenmeyer was shaken at 200rpm at 30-33°C for two days. The culture was diluted with Luria-Bertani broth to prepare a suspension with an $OD_{600\ nm}$ of 0.5. Suspensions of each KSB strain were similarly prepared. Then, 1mL of the suspension of the first KSB strain

was spread on a Luria-Bertani agar plate. Wells were created by aseptic manipulation on the plate immediately after being inoculated with the first strain of KSB. Ten mL of suspensions of each remaining KSB strain were then placed in separate wells on the plate to evaluate the compatibility of the first KSB strain with these remaining KSB strains. Each KSB strain was evaluated for compatibility according to this procedure. Plates were incubated at 30-33°C for 2 days, and the experiment was performed in triplicate. The presence of a growth inhibitory zone between each pair of KSB strains was observed and measured to assess their compatibility (Long et al., 2013).

Determination of growth curves of KSB strains

A KSB loop from a two-day-old Luria-Bertani slant culture was inoculated into an Erlenmeyer containing 50mL of Luria-Bertani broth. Erlenmeyer was shaken at 200rpm at 30-33°C for 2 days. The culture was diluted with Luria Bertani broth to prepare a suspension with an $OD_{600\ nm}$ of 0.5. Suspensions of each KSB strain were similarly prepared. Then, 1mL of this bacterial suspension was inoculated into an Erlenmeyer containing 50mL of fresh Luria-Bertani broth, and the culture conditions were similar. Each KSB strain was cultured individually. The culture was harvested every two hours and diluted fivefold with Luria-Bertani broth prior to measuring the $OD_{600\ nm}$ for evaluation of growth. Luria Bertani broth was used to calibrate the instrument. The growth curves of each KSB strain were figured based on the change in $OD_{600\ nm}$ over time. Simultaneously, the cell density of the cultures obtained at the stationary phase was also determined using standard plate counting techniques on Luria-Bertani agar.

Selection of the medium to differentiate the colony morphology of KSB strains

The KSB strains were streaked on four different media, including Luria-Bertani Agar, MacConkey Agar, Eosin-Methylene Blue, and Simmons Citrate Agar. The plates were incubated at 30-33°C for 2 days to observe the morphological characteristics of the colonies. The medium that clearly distinguished the most KSB strains was used to count the cell density of the KSB strains present in the culture after co-culturing using the standard plate counting technique.

KSBs co-culturing trials

The compatible strains with similar growth phases and cell densities were co-cultured in a standard liquid medium. The procedure included the steps summarized as follows: (1) preparation of two-day-old Luria Bertani slant culture of each KSB strain; (2) a loop of slant culture was inoculated into Luria Bertani broth and was shaken at 200rpm at 30-33°C until the bacteria entered the stationary phase, each KSB strain was prepared separately; (3) the obtained cultures from each KSB strain were adjusted with Luria Bertani broth to prepare the bacterial suspension with $OD_{600\ nm}$ of 0.5; (4) the suspensions were mixed and one mL of the mixture was inoculated into an Erlenmeyer containing 200mL of the fresh medium for co-culture, then these new Erlenmeyers were shaken at 200rpm at 30-33°C until the bacteria enter the stationary phase. The controls were KSB strains individually inoculated at a volume equal to the total volume of all strains in co-culture. The monocultures and co-cultures after cultivation were measured for cell density using the standard plate-counting technique, which utilized the selected agar medium to differentiate colonies between co-cultured strains based on colony morphology.

The K-solubility of the resulting co-culture and monocultures was also examined, as described by Sun et al. (2019). The cultures were diluted 1000-fold with sterile distilled water. Ten µL of this prepared suspension was inoculated at one point on Aleksandrov Agar, three spots per dish. Then, the Petri dish was incubated at 30-33°C for four days. The free-cell filtrate of the suspension was also examined using a similar procedure to the control, to eliminate the effects of K-solubilizing factors present in the suspension. The diameters of the halo zone on the test dish (D_1) and on the control plate (D_2), and the bacterial growth zone on the test dish (d) were measured in cm. The solubilization efficiency (SE) of the bacterial cells in the cultures was calculated using Equation 2.

$$SE = (((D_1 - D_2) - d) / d) \times 100\ (\%) \quad (2)$$

Data analysis

All experiments were conducted in a completely randomized design, and the results represent the average of multiple repetitions. Comparisons of means were made using SPSS v. 20.1 (IBM, New York, USA) with one-way analysis of variance (ANOVA) and the Duncan test at a significance level of $P < 0.05$.

Results and Discussion

Preliminary screening of KSB strains

Nine strains of KSB were retested for their ability to dissolve mica (an insoluble form of K-bearing inorganic compound). Unfortunately, *Burkholderia* sp. GTC5.14.1 strain lost the activity (Table 1). The remaining eight strains all exhibited solubilization activity with statistically different SIs. All nine retested bacterial strains were KSBs in our previous studies; however, the strain *Burkholderia* sp. GTC5.14.1

ceased to show activity in solubilizing mica. The degeneration had been previously reported for various activities in many microorganisms. The cause of degeneration could be due to inappropriate storage media of the bacteria (Angshumanjana et al., 2016). This activity of bacterial strains could also be affected by cold storage conditions. Kandil and El Soda (2015) showed the intracellular enzymatic activity of 26 strains of *Lactobacillus* spp. Significantly decreased after six months of frozen storage. Abdelsamei et al. (2015) noted that the antibacterial activity of *L. acidophilus* decreased after 90 days of storage at 4°C. This was thought to be due to an osmotic imbalance, causing physiological and biochemical changes, as well as cold lesions in the bacteria. In addition, repeated subcultures could alter genes and affect microbial availability activities. Neha et al. (2019) reported that two bacterial strains isolated from *Meretrix meretrix* decreased activity against *Staphylococcus aureus* after several repeated subcultures.

Table 1. K solubility of KSB strains on Aleksandrov Agar

KSB strain	SI
Pseudomonas sp. GTC5.8.4	3.60^{a}
Enterobacter sp. GTC5.13.1	3.36^{a}
Enterobacter sp. GTC5.13.3	2.47^{bc}
Enterobacter sp. GTC5.13.4	2.34^{c}
Enterobacter sp. GTC5.13.5	1.21^{d}
Enterobacter sp. GTC5.13.6	1.73^{cd}
Burkholderia sp. GTC5.14.1	–
Serratia sp. GTC5.19.1	2.92^{ab}
Acinetobacter sp. GTC5.21.1	3.30^{a}

– : No halo zone.
In the same column, means followed by the same letter(s) indicate insignificant differences (Duncan test, $P < 0.05$).
Source: Authors' own elaboration

To date, screening for KSB strains has been dependent on the author. With the exact calculation of SI, for example, Mursyida et al. (2015) selected two potential isolates out of 12 KSB isolates, and the SIs of these two isolates were 1.29 and 3.73, respectively. Meanwhile, Parmar et al. (2016) selected five isolates out of 25 isolates with the lowest selected SI of 2.18 (this data was recalculated according to the SI calculation method in this study). This was possible because K solubility depended on the KSB strain, the form of K-bearing mineral, and the liquid or solid culture conditions, as discussed by Pérez-Pérez et al. (2021) for eight strains of KSB. According to Pérez-Pérez et al. (2021), among the KSB strains, the strain had a higher solubility of feldspar powder but possibly a lower solubility of muscovite. Alternatively, the strain had a higher solubility in solid media but could have a lower solubility in liquid medium for feldspar powder. However, the changed order in the eight KSB strains occurred only between those with approximately the same K solubility. Previously, Rajawat et al. (2016) also showed that KSB strains with high SI on agar would have high K solubility in liquid medium; however, the order of K solubility of these strains on agar and liquid media was not compared. With the results of this experiment, the four strains of KSB, including *Pseudomonas* sp. GTC5.8.4, and *Enterobacter* sp. GTC5.13.1, *Serratia* sp. GTC5.19.1, and *Acinetobacter* sp. GTC5.21.1 was selected for co-culture studies.

Compatibility of the selected strains of KSB

One of the basic requirements was that bacterial strains must not antagonize each other to be co-cultured in CLM (Jiang et al., 2017; Che and Men, 2019) or formulated in a consortium (Gore and Navale, 2017; Kaur et al., 2022). Gore and Navale (2017) established a consortium of K-soluble bacterial and fungal strains based on this requirement for effective testing on banana. The *Pseudomonas aeruginosa* PA14 strain and the *E. aerogenes* strain were previously co-cultured in bioelectrochemical systems to enhance electrochemical activity by Venkataraman et al. (2011). These two bacterial strains had a mutualistic interaction. Meanwhile, to date, there have been no reports on the compatibility between strains of the genera *Pseudomonas*, *Serratia*, and *Acinetobacter* and between strains of the genera *Enterobacter*, *Serratia*, and *Acinetobacter*. In this study, all four strains of *Pseudomonas* sp. GTC5.8.4, *Enterobacter* sp. GTC5.13.1, *Serratia* sp. GTC5.19.1, and *Acinetobacter* sp. GTC5.21.1 did not show antagonism to each other on

Table 2. Compatibility of the selected KSB strains

KSB strain	GTC5.8.4	GTC5.13.1	GTC5.19.1	GTC5.21.1
GTC5.8.4		+	+	+
GTC5.13.1	+		+	+
GTC5.19.1	+	+		+
GTC5.21.1	+	+	+	

+: Compatibility.
GTC5.8.4: *Pseudomonas* sp. GTC5.8.4, GTC5.13.1: *Enterobacter* sp. GTC5.13.1, GTC5.19.1: *Serratia* sp. GTC5.19.1, and GTC5.21.1: *Acinetobacter* sp. GTC5.21.1.
Source: Authors' own elaboration

Luria-Bertani Agar according to the random arrangement of pairs of strains (Table 2). Inhibition rings were not formed around all agar wells. Therefore, these strains could all be studied in co-culture in a standard liquid medium.

Growth curves of the selected KSB strains

The shape of the growth curves of all four selected KSB strains was nearly identical in Luria-Bertani broth (Figure 1). The lag phase of *Pseudomonas* sp. GTC5.8.4 prolonged for four hours, while that of the three other strains lasted only two hours. All four strains exhibited an exponential phase that lasted until the 18th hour of cultivation, after which they entered the stationary phase. The cell density in the cultures at the stationary phase was statistically different at the level of significance $P < 0.05$ (Table 3). The cell density of *Enterobacter* sp. GTC5.13.1 and *Serratia* sp. GTC5.19.1 was similar to and higher than the two other selected KSB. This proved that *Enterobacter* sp. GTC5.13.1 and *Serratia* sp. GTC5.19.1 had a similar and the highest growth rate, followed by *Pseudomonas* sp. GTC5.8.4, and the lowest was *Acinetobacter* sp. GTC5.21.1. The initial population ratio and growth rate were parameters that should be considered in the screening of strains for co-culture (Goers et al., 2014; Diender et al., 2021; Kapoore et al., 2021). *Enterobacter* sp. GTC5.13.1 and *Serratia* sp. GTC5.19.1 were two suitable strains for co-culturing due to their similar growth curves and growth rates.

Table 3. The cell density in the selected KSB cultures harvested at the stationary phase

KSB strain	The cell density (CFU/ml of culture)
Pseudomonas sp. GTC5.8.4	7.7×10^{9b}
Enterobacter sp. GTC5.13.1	8.7×10^{9a}
Serratia sp. GTC5.19.1	8.5×10^{9a}
Acinetobacter sp. GTC5.21.1	1.4×10^{9c}

In the same column, means followed by the same letter(s) indicate insignificant differences (Duncan test, $P < 0.05$).

Source: Authors' own elaboration

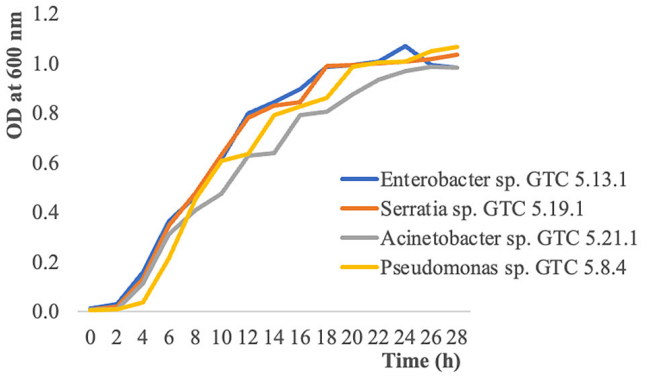

Fig. 1. Growth curves of the selected KSB in Luria Bertani broth

Source: Authors' own elaboration

Selecting the agar medium to differentiate the KSB strains

Enterobacter sp. GTC5.13.1 and *Serratia* sp. GTC5.19.1 could not be distinguished based on colony morphology on Luria Bertani Agar (Figure 2A) and Simmons Citrate Agar (Figure 2B). The colony characteristics of both KSB strains were round, opaque, white, with a raised elevation, an entire margin, and a smooth surface. Colonies of *Enterobacter* sp. GTC5.13.1 were round in configuration, with irregular margins, raised elevations, smooth surfaces, and dark pink, while colonies of *Serratia* sp. GTC5.19.1 were smaller, convex elevations, round configuration, entire margin, smooth surface, and varied color from pale pink to deep purple on Eosin Methylene Blue medium (Figure 2C). The two selected KSB strains were clearly differentiated on MacConkey Agar (Figure 2D). The characteristics of colony morphology of these two strains of KSB on MacConkey Agar are shown in Table 4.

Nutrient Agar, Luria-Bertani Agar, Peptone Meat Extract Agar, and many other Nutrient-Rich media have been commonly used to determine the density of bacterial cells in monocultures by the standard plate counting technique (Atlas, 2010). However, these media were usually only able to determine the total number of cells, but not the cell density of individual strains in multi-strain samples and co-cultures, because it was difficult to distinguish them based on colony morphology. Therefore, in this study, two selected strains of *Enterobacter* sp. GTC5.13.1 and *Serratia* sp. GTC5.19.1 was examined for colony morphology on four different media. MacConkey Agar was selected as the medium to determine the cell density of each strain in co-cultures (Figure 3). MacConkey Agar containing selective ingredients could reduce the number of cells counted on the plate compared

Table 4. Colony characteristics of two selected strains on MacConKey Agar after 2 days of incubation

KSB strain	Colony characteristics
Enterobacter sp. GTC5.13.1	Large, round configuration, entire margin, umbonate elevation, light pink, smooth surface, sticky, moist-looking
Serratia sp. GTC5.19.1	Medium, L-form configuration, umbonate elevation, pale pink, smooth surface, sticky, moist-looking

Source: Authors' own elaboration

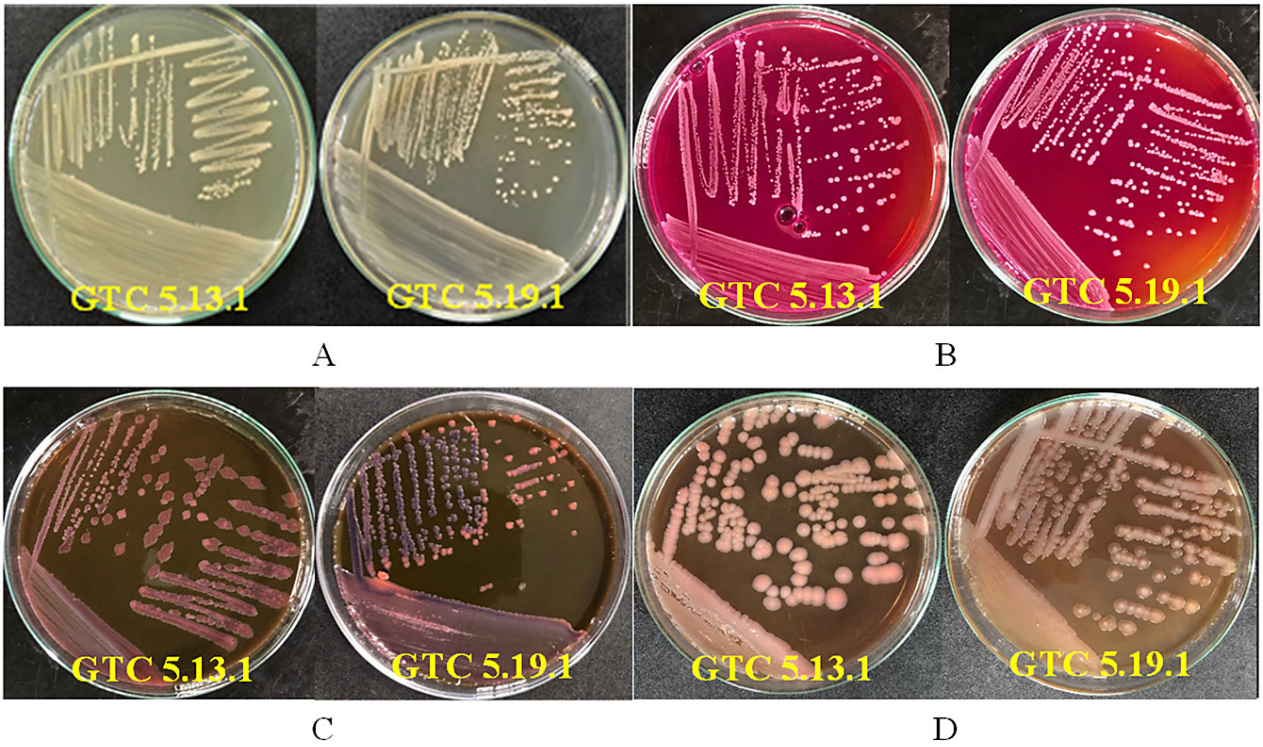

Fig. 2. Colony morphology of two selected KSB after two days of incubation on Luria-Bertani Agar (A), Simmons Citrate Agar (B), Eosin Methylene Blue Agar (C), and MacConKey Agar (D)
Source: Authors' own elaboration

to the actual number in the sample. In this study, the growth kinetics rather than the exact cell density of *Enterobacter* sp. GTC5.13.1 and *Serratia* sp. GTC5.19.1 was taken into account in order to select suitable conditions for cultivation. The exact cell density of each strain, as well as the exact total number in the samples, would be of interest in a future study employing a different approach.

Effect of the initial inoculation ratio of the KSB strains on proliferation efficiency in co-cultivation

The final cell density of *Enterobacter* sp. GTC5.13.1 and *Serratia* sp. GTC5.19.1 were the highest in the monocultures (the initial inoculation ratio between *Enterobacter* sp. GTC5.13.1 and *Serratia* sp. GTC5.19.1 was 1:0 and 0:1, respectively) (Table 5). Any variation of this initial inoculation ratio significantly changed the final cell density of each partner and the total cell density in the resulting co-culture. The final cell densities of each KSB in the co-cultures were significantly lower than those in the monocultures in all cases of the surveyed initial inoculation ratio. When this ratio increased, the cell density of *Enterobacter* sp. GTC5.13.1 increased while this ratio decreased, the density of *Serratia* sp.

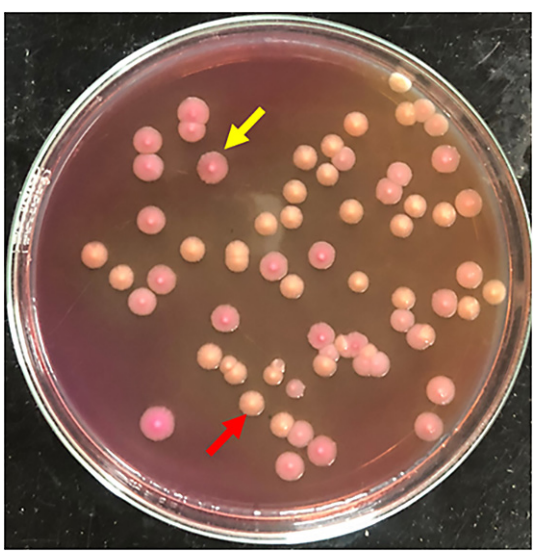

Fig. 3. MacConkey Agar was used to determine the cell density of each strain in co-cultures. *Enterobacter* sp. GTC5.13.1 (yellow arrow) and *Serratia* sp. GTC5.19.1 (red arrow).
Source: Authors' own elaboration

Table 5. The effect of ratio of partners in the initial inoculum on the growth of the selected KSB strains co-cultured in Luria Bertani broth

Ratio of starters		Cell density (CFU/ml of culture)		
GTC5.13.1	GTC5.19.1	Total	GTC5.13.1	GTC5.19.1
1	4	8.6×10^{9a}	1.5×10^{9e}	7.1×10^{9b}
1	3	7.6×10^{9bc}	1.8×10^{9e}	5.8×10^{9c}
1	2	6.8×10^{9cd}	1.9×10^{9de}	4.9×10^{9d}
1	1	4.7×10^{9e}	1.5×10^{9e}	3.2×10^{9f}
2	1	6.2×10^{9d}	2.2×10^{9d}	4.0×10^{9e}
3	1	6.8×10^{9cd}	2.8×10^{9c}	4.0×10^{9e}
4	1	8.1×10^{9ab}	4.0×10^{9b}	4.1×10^{9e}
1	0	–	8.7×10^{9a}	–
0	1	–	–	8.5×10^{9a}

In the same column, means followed by the same letter(s) indicate insignificant differences (Duncan test, $P < 0.05$).
GTC5.13.1: *Enterobacter* sp. GTC5.13.1, GTC5.19.1: *Serratia* sp. GTC5.19.1.
Source: Authors' own elaboration

GTC5.19.1 increased. The obtained co-culture had the lowest total cell density at the 1:1 initial ratio. Meanwhile, the total final cell density in the co-cultures was closer to the cell density in monoculture when this ratio increased or decreased more.

Although the two selected strains of KSB did not exhibit antagonism towards each other on Luria Bertani Agar (Table 2) and had a similar growth rate in Luria Bertani broth (Table 3), the cell density of each strain in the co-cultures was lower than that in monocultures (Table 5). Perhaps, there was competition or sharing for nutrients between these two strains of KSB in co-culture (Jiang et al., 2017). *Enterobacter* sp. GTC5.13.1 was probably less competitive for nutrients. Therefore, the final cell density of *Enterobacter* sp. GTC5.13.1 was all lower than that of *Serratia* sp. GTC5.19.1 in the co-cultures from most of the surveyed initial inoculation ratios. Additionally, the components in the culture medium were likely more suitable for the growth of *Serratia* sp. GTC 5.19.1. Gao et al. (2021) previously reported that the final densities of *Pseudomonas putida* KT2440 were always higher than those of *Escherichia coli* K-12 in co-cultures in all three treatments with the initial inoculation ratios of 1000:1, 1:1, and 1:1000. The reason was that the carbon source was more suitable for *P. putida* KT2440. Gore and Navale (2017) co-cultured three KSB strains of *Pseudomonas* sp. in five different liquid media with an equal starter ratio between strains to select the appropriate medium for liquid formulation; however, the cell density after culture was not detailed. In this study, the total final densities at 1:4 and 4:1 ratios were all roughly equal to the cell densities in the monocultures. They were significantly higher than counts at other ratios. There could be positive or negative effects on the growth and metabolic patterns of each co-cultured partner, as discussed by Chandra et al. (2012), Yan and Dong (2018), and Gao et al. (2021). Therefore, Kapoore et al. (2021) also suggested that the initial inoculation ratio should be established in co-culture systems. In this experiment, a 4:1 initial inoculum ratio was more suitable for the co-culture of *Enterobacter* sp. GTC5.13.1 and *Serratia* sp. GTC5.19.1.

Effect of the initial inoculum size of two selected KSB strains on proliferation efficiency in co-cultivation

In the co-culture technique, in addition to the ratio of populations of partners that needs to be prioritized to establish, many other parameters, such as nutrient source, initial mixed inoculum size, time of adding each variety, pH, salinity, etc., also affect the growth as well as the metabolic pattern of microorganisms (Kapoore et al., 2021). The initial inoculum size is a crucial parameter that affects the growth rate and, consequently, the fermentation time. A small initial inoculum size requires a longer time to achieve fermentation, whereas a large initial inoculum size leads to a rapid increase in biomass and can reduce cultivation time. However, an inoculum size that is too large may not result in an increase in the target product. The yield of the target product can decrease when the inoculum size is too large because the microorganisms use a significant amount of nutrients to grow and maintain a high cell density. The optimal initial inoculum size varies with each co-culture system. For example, an initial inoculum size of 5% was suitable for co-culturing *Bacillus cereus* CCM2010 and *B. thuringiensis* Bt407 (with a ratio of 1:1 v/v) to obtain maximum ferulic acid (Samad and Zainol, 2017). The optimal condition for caproic acid production by co-culturing *Clostridium kluyveri* H068 and *Methanogen* 166 was 10% initial inoculum size (ratio between the two strains was 2:1 v/v) (Yan and Dong, 2018).

In this experiment, increasing the initial inoculum size from 0.5% to 1% resulted in a higher final cell density for both co-cultured KSB strains (Table 6). However, the increase was statistically insignificant. The cell density of both strains was significantly reduced when the initial inoculation size was higher than 1%. The appropriate initial inoculum size was 0.5–1% of a mixture of two strains of *Enterobacter* sp. GTC5.13.1 and *Serratia* sp. GTC5.19.1 in the 4:1 ratio.

Solubilization efficiency of the resulting co-culture and monocultures

Cells of the two KSB strains in the monocultures and the resulting co-culture, which was co-cultivated at an initial inoculum size of 0.5%, were evaluated for their ability to solubilize K in mica, kaolin, and feldspar separately added to modified Aleksandrov Agar. The cell-free filtrate of the KSB suspension from all three cultures did not express a halo zone on the agar plate. This could be that neither of the

Table 6. The effect of the initial inoculum size on the growth of the selected KSB strains co-cultured in Luria Bertani broth

The initial inoculum size (%)*	Cell density (CFU/ml)		
	Total	GTC5.13.1	GTC5.19.1
0.5	6.7×10^{9ab}	3.9×10^{9ab}	2.9×10^{9ab}
1.0	7.1×10^{9a}	4.0×10^{9a}	3.1×10^{9a}
1.5	6.3×10^{9bc}	3.5×10^{9bc}	2.8×10^{9b}
2.0	6.0×10^{9c}	3.2×10^{9c}	2.8×10^{9b}
2.5	6.1×10^{9c}	3.3×10^{9c}	2.8×10^{9b}

In the same column, means followed by the same letter(s) indicate insignificant differences (Duncan test, $P < 0.05$).
GTC5.13.1: *Enterobacter* sp. GTC5.13.1, GTC5.19.1: *Serratia* sp. GTC5.19.1.
*: The ratio of *Enterobacter* sp. GTC5.13.1 and *Serratia* sp. GTC5.19.1 in the initial inoculum was 4:1 (v/v).
Source: Authors' own elaboration

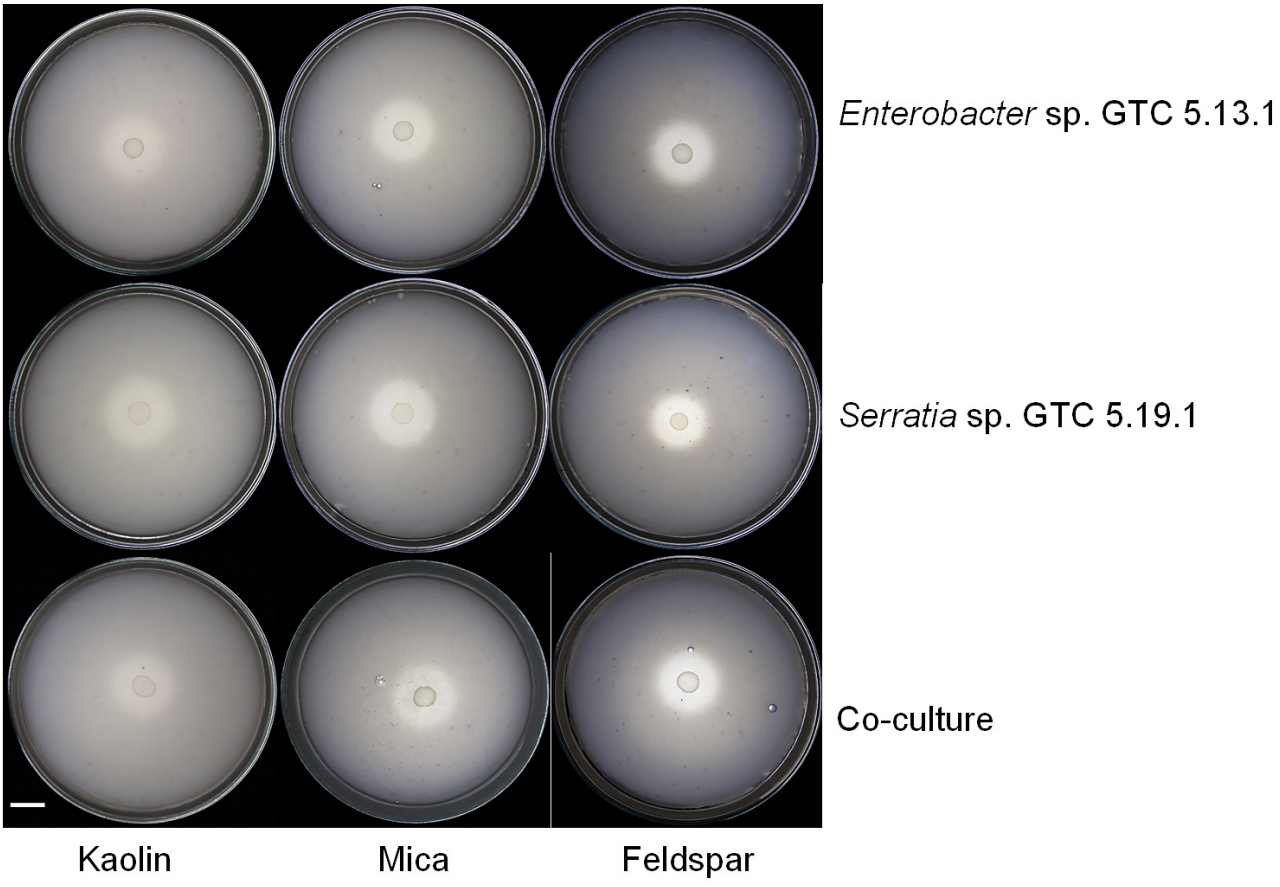

Fig. 4. The halo zones of the monocultures and the co-culture on modified Aleksandrov Agar with three different K sources
Source: Authors' own elaboration

two KSB strains released K-solubilizing compounds into the broth. Therefore, there was no halo zone on the agar plate inoculated with the cell-free filtrates. Meanwhile, halo zones appeared on plates inoculated with KSB suspensions (Figure 4). This proved that KSB cells still retained the target activity. However, the quantitative data on solubilization efficiency were not significantly different ($P < 0.05$) between the monocultures and the co-culture for all three investigated K sources (Table 7).

Table 7. The solubilization efficiency of the monocultures and the co-culture

Cultures	SE (%)		
K sources	Mica	Kaolin	Feldspar
Enterobacter sp. GTC5.13.1	620	390	740
Serratia sp. GTC5.19.1	490	380	660
The co-culture	500	430	650

In the same column, no insignificant differences at $P < 0.05$.
Source: Authors' own elaboration

The cells of these two strains still retained the target activity, which is one of the important requirements for developing microbial fertilizer formulations. There had been no previous reports directly evaluating the ability to retain K-solubility activity on agar plates after proliferation in both monoculture and co-culture. Meanwhile, numerous reports have been conducted to assess the effectiveness of K solubilization in KSB monoculture or co-culture, as measured by the content of soluble K in soil or biochemical parameters in plants. Gore and Navale (2017) obtained a co-culture containing three KSB strains of *Pseudomonas* sp., and the liquid formulation from this broth gave a positive effect on bananas. However, there was no comparison between this co-culture and monocultures of each *Pseudomonas* sp. strain in Gore and Navale (2017). In this study, the cultures were able to dissolve K in three different mineral sources, including mica, kaolin, and feldspar. This result increased the application potential of these cultures. A formulation from the co-culture was expected to have a higher application potential than the two monocultures due to the advantages of the consortium, such as the mutual interactions between strains, as well as its ability to function under various field conditions. However, additional research, such as formulation and field evaluation, is still needed.

Conclusions

Enterobacter sp. GTC5.13.1 and *Serratia* sp. GTC5.19.1 was screened from nine indigenous KSB strains and was suitable for co-culture. These two KSB strains had SI on agar plates of 3.36 and 2.92, respectively, and showed compatibility together, with similar growth rates in Luria-Bertani broth. MacConKey Agar was suitable for counting the cell densities of each strain in the co-culture based on the differences in colony morphology. At the co-culture conditions, including a 4:1 ratio of starters and an initial inoculum size of 0.5 – 1% in Luria-Bertani broth, the resulting co-culture had a total cell density comparable to that of the monocultures. After co-culture proliferation, the cells of *Enterobacter* sp. GTC5.13.1 and *Serratia* sp. GTC5.19.1 could solubilize K on mica, kaolin, and feldspar on an agar plate. The technique of co-culturing in CLM could obviously be applied to two strains of *Enterobacter* sp. GTC5.13.1 and *Serratia* sp. GTC5.19.1. Furthermore, studies were needed to optimize co-culture conditions for obtaining biomass sources to develop consortium formulations from these two indigenous KSB strains.

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