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Study of potassium solubilizing bacteria from limestone mining area in Palimanan, Cirebon Quarry

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ABSTRACT

Aims: Potassium is an essential macronutrient for the growth and development of plants. Most of potassium in the soil presented in mineral forms or non-exchangeable forms which are not available for plants. The microbial activity facilitated to release of mineral forms or non-exchangeable potassium to the exchangeable or water-soluble. This study was aimed to isolate, select, and characterize of the selected potassium solubilizing bacteria from limestone mining area in Palimanan, Cirebon Quarry.
Methodology and results: Isolation and selection of bacteria was done based on potassium dissolving index in Aleksandrov media containing feldspar, non-exchangeable potassium. Thirty seven isolates of potassium solubilizing bacteria were obtained in this study. Three isolates showed higher dissolution index, namely KQC.4B.1, KQC.5A.4, and KQC.5C.5. All of isolates were Gram negative bacteria, short-rod formed, and able to dissolve potassium concentration on 10^{th} and 20^{th} days. The three isolates showed 99.9% physiologically similar with *Burkholderia cepacia*. Futhermore by using 16S rRNA gene identification, isolate KQC.5C.5 closely related with *B. cepacia* with 99% identity. The application of isolate KQC.5C.5 on soil showed that the isolate was able to release the solution K formed after 10^{th} day incubation.
Conclusion, significance and impact of study: Potassium solubilizing bacteria (*B. cepacia*) could use as a biological fertilizer for providing potassium which is available to plants grown on reclamation area of limestone quarry.

Keywords: biological fertilizer, *Burkholderia cepacia*, dissolution index, feldspar

INTRODUCTION

Fertilizer is one of critical production factor in agriculture sector in Indonesia, but farmers are preferably using inorganic (chemical) fertilizer. The excessive use of inorganic fertilizers can lead to decrease in the quality of soil and environmental pollution (Lynn *et al.*, 2013). Biological fertilizer application is considered to restrict inorganic fertilizers usage, thereby reducing environmental pollution, reducing farming costs, and maximizing yields.

The potassium solubilizing bacteria is a rhizospheric bacteria which solubilises the insoluble potassium to soluble forms for plant growth and yield. The use of potassium solubilizing bacteria as a biological fertilizer was suggested as a solution to improve plant nutrition. Potassium play a role in enzyme activation, maintaining cell turgor, transportation of sugars and starches, played a role in improving crop quality, increasing resistance against pests and diseases, and helping crops on stress conditions (Meena *et al*., 2014).

The concentration of dissolved potassium in soil around 1-2% and more than 90% of that potassium are in insoluble rocks form and silicate minerals (mica, muscovite, feldspar, microline, orthoklas), largely it unavailable for plant uptake (Parmar and Sindhu, 2013). Some of potassium solubilizing bacteria that capable in dissolving potassium in the soil such as *Bacillus* sp., *Paenibacillus* sp., *B. mucilaginosus* and *B. edaphicus* (Muralikannan, 1996; Sheng, 2005; Sugumaran and Janarthanam, 2007; Liu *et al.*, 2012). Soil microbes play an essential role in potassium cycle, such as *Pseudomonas* and *Bacillus* which known as potassium solubilizing bacteria in silicate form (Murali *et al.*, 2005).

The limestone quarry in Palimanan Cirebon is one of open limestone quarry in Indonesia. Praptisih *et al.* (2012) described that in Mount Kromong area such as Palimanan Quarry, various fossils which allegedly contain potassium were founded. Palimanan Quarry also has a high biodiversity such as plants and animals, and in this area is also suspected to have variety of beneficial soil bacteria such as potassium solubilizing bacteria. Mubarik *et al.* (2014) have successfully isolated the potassium

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solubilizing bacteria using Pikovskaya medium around limestone quarry Palimanan (buffer zone), but did not success to obtain potassium solubilizing bacteria. Based on that, this research was conducted to obtain potassium solubilizing bacteria, and then to select and characterize its ability. In the future, the potassium solubilizing bacteria can be used as a biological fertilizer to support the growth of plants which planted on reclamation area of limestone quarry.

MATERIALS AND METHODS

Materials

Soil samples were taken from limestone quarry at PT Indocement Tbk, Palimanan, Cirebon and Darmaga, Bogor, Indonesia.

Soil sampling

Soil samples were obtained from three different areas with 13 sampling spots, namely the active quarry land area (5 sampling spots), limestone mined land area (3 sampling spots), and reclamation area (5 sampling spots). The sample in reclamation area was obtained around plants roots and 0-15 cm depth from ground level.

Isolation of potassium solubilizing bacteria

Three grams of soil samples were diluted into 27 mL physiological saline solution (NaCl 0.85%), then agitated on a shaker for 1 h at 120 rpm. Serial dilutions was prepared from 10^{-1} to 10^{-4}. One milliliter of 10^{-2}, 10^{-3}, and 10^{-4} suspension respectively spread on Aleksandrov medium (5 g glucose, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.006 g $FeCl_3$,0.1 g $CaCO_3$, 2 g Ca_3PO_4, 3 g feldspar (as potassium source) and 20 g bacto agar in 1 L distillated water (aquadest) and incubated in 28 °C for 3-7 days (Prajapati and Modi, 2012). Colony of potassium solubilizing bacteria was surrounded by a clear zone. Each colony then purified using quadrant streak method and stored as a stock in nutrient slant agar.

Screening of potassium solubilizing bacteria for potassium solubilization

The single colony of potassium solubilizing bacteria on Aleksandrov agar incubated for 7 days at room temperature. The clear zone formed around the colony was observed and measured with a ruler or calipers then calculate each potassium dissolution index (DI) to determine the ability of bacteria to dissolve potassium by using the following equation:

$$DI = \frac{\text{clear zone diameter (mm)} - \text{colony diameter (mm)}}{\text{colony diameter (mm)}}$$

Growth curve of selected isolates

A loop of selected isolates which incubated for 24 h was inoculated into 50 mL Nutrient Broth (NB) medium and incubated in rotary incubator until the cells reached 10^8 bacterial cells/mL. One milliliter bacterial culture (10^8 cells/mL) was inoculated into 100 mL NB medium and incubated in shaker incubator at 100 rpm in 37 °C. Cell density measurements performed every 3 h using a spectrophotometer with 620 nm wavelength for 24-48 h.

Hypersensitivity test on tobacco leaves

Hypersensitivity test was done by injecting the selected bacterial isolate (10^8 cells/mL) on the lower surface of tobacco leaves *Nicotiana tabacum* L. If there was discoloration or change in color to yellow on the in injected area after 24-48 h, it showed necrosis symptoms, and the bacteria were suspected as plant pathogen. On this test, distilled water and *Xanthomonas oryzae* pv. *oryzae* were used as a negative control and positive control, respectively.

Quantitative test of potassium solubilization by bacteria

The quantitative test of potassium solubilization was measured based on Parmar and Sindhu (2013) method. A loop of selected isolate was inoculated into Erlenmeyer which contain 50 mL Aleksandrov liquid medium and incubated for 48 h until the bacterial cell achieved 10^8 cells/mL. As much as of 0.5 mL of bacterial culture was inoculated into 50 mL Aleksandrov liquid medium then incubated in shaker incubator at 100 rpm, 28 °C for 25 days. Every 5 days, 1 mL of culture samples was taken from the culture and used on serial dilutions up to 10^{-7} thereafter plated on Aleksandrov medium to determine the number of cells/mL. The potassium solubilization quantitative test was done by centrifuged 1.5 mL bacterial suspension at 10,000 rpm by using Eppendorf miniSpin with F45-12-11 rotor for 10 min. One milliliter supernatant suspended with distilled water up to 5 mL total volume and mixed thoroughly. Afterward the suspension was measured by atomic absorption spectrophotometer (AA-7000 AAS with water flame-C_2H_2) at 765.5 nm wavelength.

Identification of bacterial isolates

The selected bacterial isolates were identified based on morphology of colony (color, form, elevation, margin), Gram staining, biochemical characteristic (using Analytical Profile Index (API) 20 NE kit from BioMérieux, Inc. Durham, USA), and molecular identification.

Molecular identification

DNA Extraction

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A pure single bacteria colony that has been quadrant streaked out on Nutrient Agar (NA) medium incubated at 37 °C for 24 h. The grown bacteria then retrieved using a sterile toothpick. Bacterial cells then centrifuged at 10,000 rpm for 1 min. DNA extraction was done following the procedures of PrestoTM gDNA Bacteria Mini Kit (Geneaid). The extracted DNA concentration and purity were measured using NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

16S rRNA gene amplification, DNA sequencing and phylogenetic analysis

The 16S rRNA gene was amplified using Polymerase Chain Reaction (PCR) machine. Fifty microliters of PCR Mix was created with composition: 25 µL GoTaq Green Master Mix 2 (Promega, Madison, WI, USA), 0.5 µL (100 pmol) for each primer: 63f (5'-CAGGCCTAACACATGCAAGTC-3') and 1387r (5'-GGGCGGWGTGTACAAGGC-3 ') (Marchesi *et al*., 1998), 2 µL (64.4 ng/µL) of DNA templates and 22 µL nuclease free water. The PCR conditions used, namely pre-denaturation (94 °C, 4 min), denaturation (94 °C, 30 sec), annealing (55 °C, 30 sec), elongation (72 °C, 1 min), and post-elongation (72 °C, 7 min). The PCR process was done for 30 cycles. Finally the temperature was declined to 4 °C for 10 min to stop PCR reaction. PCR products were subjected to gel electrophoresis using 1% agarose gel at 70 V for 45 min and visualized under UV light. PCR products were sequenced by sending it to sequencing service company (First Base Malaysia). The sequence results were compared with GenBank sequences using BLASTN program then phylogenetic trees were constructed using MEGA 5.05 program (MegaSoftware, Inc, Arizona, USA).

Application of selected isolate in soil

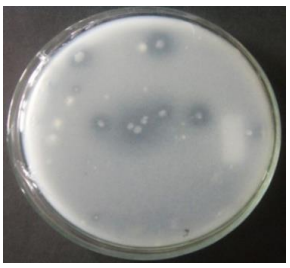
The potassium availability and water content of soils derived from Cirebon and Darmaga were analyzed. The soil that contains less potassium was used for further experiment. Approximately 100 g soil (sterilized in autoclave) with 6 repetitions used into 2 treatments which are (1) control (without isolate) and (2) isolate KQC.5C.5 (10^9 cells/mL and 10%) incubated for 10 days at 28 °C. The potassium availability analysis, potassium total analysis, and moisture content were done in day 0 and 10.

RESULTS

Isolation of potassium solubilizing bacteria

A total of 37 potassium solubilizing bacteria isolates were obtained from limestone quarry area soil (13 samples) of PT Indocement Tbk, Palimanan Cirebon using Aleksandrov medium. The potassium solubilizing bacteria isolates were characterized by a clear zone that formed around the colony (Figure 1).

Screening of potassium solubilizing bacteria for potassium solubilization



A total of 37 potassium solubilizing purified isolates were tested on solubilized potassium qualitatively

Figure 1: The growth of bacterial colony in the media potassium Aleksandrov characterized by the clear zone around the colony.

Three isolates were selected based on the highest of dissolution index (DI), namely KQC.4B.1, KQC.5A.4, and KQC.5C.5 (Table 1).

Growth curve of selected isolates

The growth curve is required to determine the bacterial growth phase. All selected isolates indicated similarity growth phase pattern, even though the number of bacterial cells were not the same on each phase (Figure 2).

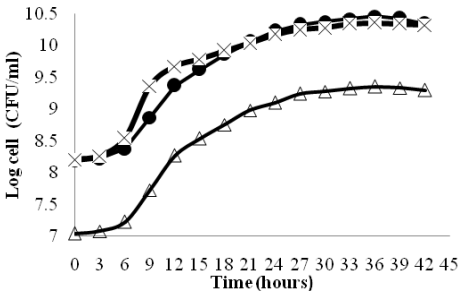


Figure 2: The growth curve of 3 selected isolates on Nutrient Broth medium at 37 °C. **X,** KQC.4B.1; ●, KQC.5A.4, ∆ KQC.5C.5.

Hypersensitivity test on tobacco leaves

The hypersensitivity test of KQC.4B.1, KQC.5A.4 and KQC.5C.5 isolates were negative, because each did not indicate necrosis symptoms on tobacco leaves after injection for 24-48 h.

Hypersensitivity test on tobacco leaves

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Quantitative test of potassium solubilization by bacteria

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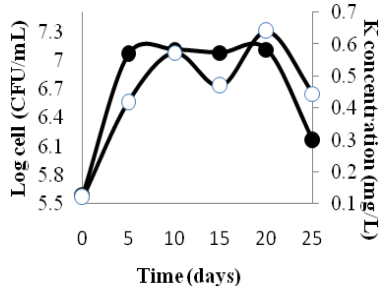
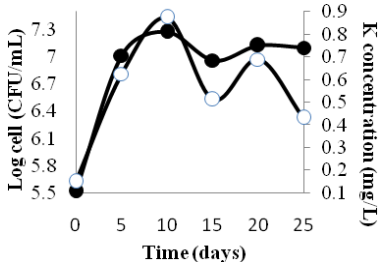
The potassium solubilizing quantitative estimation value by selected isolates showed different results. On 20 days

Table 1: Potassium solubilizing bacteria isolated from limestone quarry at Cirebon, Indonesia.

Area sample	Bacterial isolates code	K dissolution index (mm)	Area sample	Bacterial isolates code	K dissolution index (mm)
Limestone mined land	KQC.1B.1	0.22	Reclamation area 2	KQC.5B.5	0.23
Active quarry land	KQC.4A.1	0.705	Reclamation area 2	KQC.5B.6	0.63
Active quarry land	KQC.4A.2	0.63	Reclamation area 2	KQC.5B.7	0.65
Active quarry land	KQC.4B.1	2.2	Reclamation area 2	KQC.5B.8	not clear
Active quarry land	KQC.4B.2	0.645	Reclamation area 2	KQC.5B.9	0.625
Active quarry land	KQC.4B.3	0.58	Reclamation area 2	KQC.5B.10	0.995
Reclamation area 1	KQC.5A.1	1.05	Reclamation area 3	KQC.5C.1	0.525
Reclamation area 1	KQC.5A.2	0.51	Reclamation area 3	KQC.5C.2	1
Reclamation area 1	KQC.5A.3	0.38	Reclamation area 3	KQC.5C.3	not clear
Reclamation area 1	KQC.5A.4	3.125	Reclamation area 3	KQC.5C.4	0.585
Reclamation area 1	KQC.5A.5	0.8	Reclamation area 3	KQC.5C.5	3.125
Reclamation area 1	KQC.5A.6	0.27	Reclamation area 4	KQC.7A.1	not clear
Reclamation area 1	KQC.5A.7	0.43	Reclamation area 4	KQC.7A.2	not clear
Reclamation area 1	KQC.5A.8	0.47	Reclamation area 4	KQC.7A.3	0.57
Reclamation area 2	KQC.5B.1	not clear	Reclamation area 4	KQC.7A.4	0.57
Reclamation area 2	KQC.5B.2	0.355	Reclamation area 4	KQC.7A.5	not clear
Reclamation area 2	KQC.5B.3	0.305	Reclamation area 4	KQC.7A.6	0.54
Reclamation area 2	KQC.5B.4	0.35	Reclamation area 4	KQC.7A.7	0.235
			Reclamation area 5	KQC.7B.1	0.31



(a)



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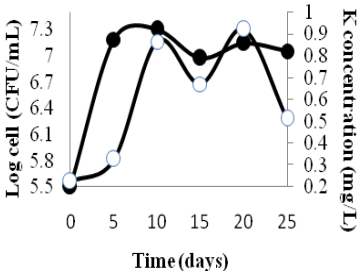

(c)

Figure 3: The ability of bacterial isolates to solubilize potassium, a, KQC.4B.1; b, KQC.5A.4; c, KQC.5C.5. ○, Concentration of potassium; ● Log cell (CFU/mL).

incubation KQC.4B.1 and KQC.5C.5 solubilized the highest potassium concentration with value 0.64 mg/L and 0.92 mg/L, respectively, while KQC.5A.4 was able to solubilize 0.87 mg/L potassium concentration on 10 days incubation (Figure 3). The three selected isolates had similar pattern on potassium solubilizing in media. An increase in the number of cells will be followed by an increase of potassium dissolution, and vice versa.

Morphological and physiological bacteria identification

The selected bacterial isolates are similar morphologically, such as a round shape with raised margin, white color, and smooth margin. All isolates are Gram negative and short rod shaped. Physiological identification was done using API 20 NE kit. Isolates KQC.5A.4, and KQC.5C.5 showed positive results on tests: gelatin, 4-nitrophenil-β-D-galactopyranoside, D-glucose (assimilation), D-arabinose, D-mannose, D-mannitol, N-acetyl glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malate acid, trisodium citrate, phenylacetic acid, and oxidase. KQC.4B.1 isolate has the same test result with isolates KQC.5A.4 and KQC.5C.5 but positive results were also found in D-glucose test (fermentation), L-arginine, urea, and aesculin ferric citrate. Identification result using Apiweb™ identification software with the

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database (V4.0) indicated that the three isolates had 99.9% similarity level with *Burkholderia cepacia*.

Molecular identification

Molecular identification was represented on isolate KQC.5C.5 because of the ability to dissolve the highest potassium as qualitatively and quantitatively. Results of 16S rRNA amplification using primers 63f and 1387r (Marchesi *et al*. 1998) produced around 1300 bp length band (Figure 4). Phylogenetic analysis using Neigbour Joining (NJ) method with 1000 bootstrap value. Based on BLASTN result, isolate KQC.5C.5 has 99% identity level with *Burkholderia cepacia* (Figure 5).

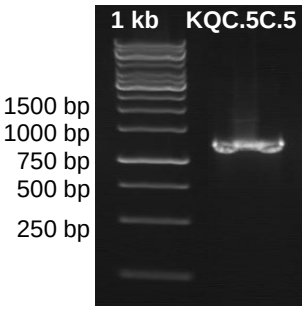

Figure 4: Electrophoresis result of the 16S rRNA gene amplification of isolate KQC.5C.5.

The application of selected isolate in soil treatments

Potassium availability in soil from Darmaga and Cirebon was 263.72 mg/kg and 438.32 mg/kg, respectively. The Darmaga soil is chosen for application with bacteria because it contained less soluble potassium. The results showed that value of K available and K total in bacterial treatments were increased after 10 days incubation, in contrast on control (without bacteria), the value of K available and K total was lower than the treatment with bacteria addition either in day 0 or day 10th (Table 2).

DISCUSSION

The potassium solubilizing bacteria was characterized by the ability of bacteria to forming a clear zone in Aleksandrov medium (Parmar and Sindhu, 2013) (Figure 1). Three isolates were selected based on the highest dissolution index (DI), namely KQC.4B.1, KQC.5A.4, and KQC.5C.5 (Table 1). The differences of dissolution index values in each isolates might be caused by the ability of each bacterium in producing organic acids such as oxalic acid and tartaric acid, and also the production of polysaccharides contributed on silicate minerals dissolution like feldspar and illite to release potassium (Sheng and He, 2006). Potassium bound in the form of silicate minerals such as muscovite, orthoclase, biotite, feldspar, illite, mica, vermiculite, smectite can be dissolved by organic acid producing bacteria which will be available for plants (Ullman *et al*., 1996).

Each of isolate KQC.4B.1, KQC.5A.4, and KQC.5C.5 achieved log phase at 6th to 30th h, initial stationary phase at 33rd to 36th h, and in 39th to 42nd h a slightly declining phase (Figure 2). The difference of time phase and amount of bacterial growth can be caused by several factors such as nutrients, oxygen, carbon dioxide, pH, and temperature (Moat *et al*. 2002). Three isolates were categorized as negative hypersensitive, because they did not cause necrosis symptoms on tobacco leaves after incubation for 24-48 h. According to Wahyudi *et al*. (2011), hypersensitivity reactions are rapid cell death and localized program. This reaction appears on infectedplants during introduction phase of pathogens, along with an attempt to inhibit the growth of pathogen.

Isolates KQC.4B.1 and KQC.5C.5 were able to solubilize highest potassium concentration with the highest value, i.e 0.64 mg/L and 0.92 mg/L, respectively on 20 days incubation, while KQC.5A.4 was able to solubilize 0.87 mg/L potassium concentration on 10 days incubation (Figure 3). The solubilization of potassium in soil caused by organic acids or polysaccharides which produced by bacteria. *Bacillus mucilaginosus* isolated from soil, rocks, and minerals could solubilize potassium in media containing mica as much as 4.29 mg/L (Sugumaran and Janarthanam, 2007). According to Parmar and Sindhu (2013) bacteria strains WPS73 and NNY43 were able to solubilize potassium at 41.0 and 48.0 mg/L. Bacteria strain WPS73 solubilized 49.0 mg/L potassium at 25 °C. This bacteria was able to solubilize potassium from soil in form of soluble rock and silicate minerals such as mica, illite, and orthoklas by produced and excreted organic acids that either directly released at potassium rocks or silicate ion which able to solubilize potassium thus plants can absorb it directly. Zhang and Kong (2014) described that there were 17 bacteria from *Klebsiella variicola*, *Enterobacter cloacae*, *E. asburiae*, *E. aerogenes*, *Pantoea agglomerans*, *Agrobacterium tumefaciens*, *Microbacterium foliorum*, *Myroides odoratimimus*, and *Burkholderia cepacia* bacterium which able to solubilize potassium ranged from 0.59 mg/L up to 4.4 mg/L. The bacteria were isolated from tobacco rhizosphere soil from various Province, namely Sichuan, Shandong, and Hubei. Potassium has four main roles for bacteria, to adjust turgor pressure and osmotic solutes, regulate internal pH, and activating intracellular enzyme (Epstein, 2003).

Three isolates showed 99.9% physiologically similar with *B. cepacia*. Furthermore by using 16S rRNA gene identification, isolate KQC.5C.5 closely related with *B. cepacia* with 99% identity (Figure 5). Various kinds of bacteria are *Pseudomonas*, *Burkholderia*, *Acidithiobacillus ferrooxidans*, *Bacillus mucilaginosus*, *B. edaphicus*, *B. circulans*, and *Paenibacillus* sp. has been reported to dissolve potassium in the soil (Lian *et al.*, 2002; Sheng, 2005; Lie *et al*., 2006; Liu *et al.*, 2012). Potassium solubilizing bacteria also found from rhizosphere at pine trees in the Guangxi province of China is *Burkholderia* genus (Lü and Huang, 2010). According to Calvaruso *et al*. (2006) inoculation of *Burkholderia glathei* PML1 (12) was

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significantly improved plant nutrition, and number of lateral roots and root hairs of pine plants mainly because of its effect on mineral weathering. Application of control (without isolate) and KQC.5C.5 isolate in sterile soil Darmaga increasing K total and K available in day 0 and 10th. Isolate KQC.5C.5 able to solubilize soil bounded potassium after incubated for 10 days. The value of K available and K total in all treatments were increased after incubated for 10 days, but for treatment without bacteria (control), the value of K available and K total was lower than the treatment with bacteria either in day 0 or day 10th (Table 2). This result showed that isolate KQC.5C.5 able to increase the K availability in soil, thus plants can used it. According to Cahyani (2009), soil sterilization treatment was significantly affected the soil nutrient condition. The P availability level was significantly increased in soil with autoclave sterilization treatment method. Autoclave and steam sterilization methods kill fungi, but bacteria and actinomycetes can survive with negligible amount less than 30 colonies/mL.

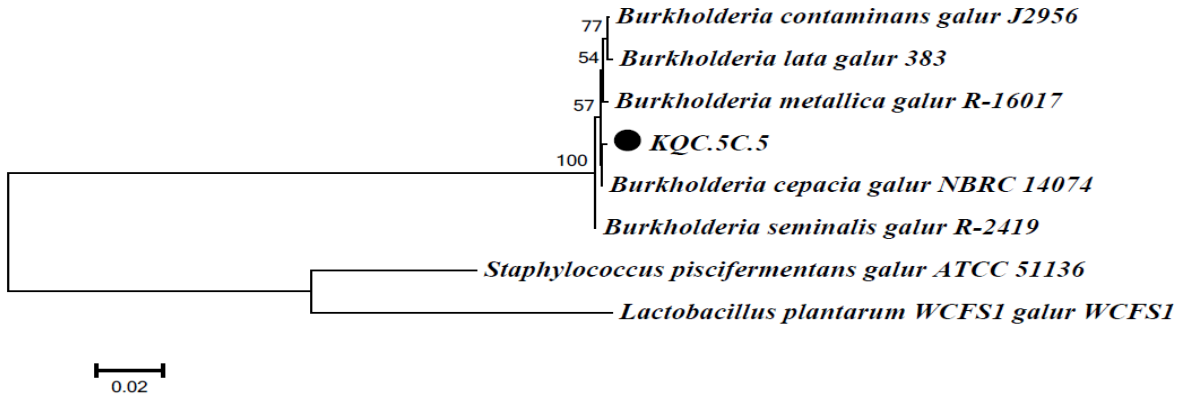

Figure 5: The phylogenetic tree construction of isolate KQC.5C.5.

Table 2: Potassium availability and potassium total from culture of KQC.5C.5 in Aleksandrov medium at 0 and 10th day incubation at room temperature.

Treatment	Day 0 Potassium (ppm)		Day 10th Potassium (ppm)	
	Available	Total	Available	Total
Control (without isolate)	57.68	182.65	121.1	210.44
Isolate KQC.5C.5	82.33	204.98	132.1	264.09

CONCLUSION

The selected bacterial isolates from 37 isolates based on the highest solubilizing index are KQC.4B.1, KQC.5A.4 and KQC.5C.5, respectively. Three isolates have similar characteristics which are Gram negative bacteria, short rod shaped, negative hypersensitive, able solubilizing highest potassium concentration in 10 and 20 days incubation. Based on 16S rRNA identification isolate KQC.5C.5 has 99% identity with *Burkholderia cepacia*. Isolate KQC.5C.5 also able to solubilize potassium in soil on 10 days incubation.

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