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Isolation and identification of Cr-resistant bacteria from the rhizosphere of *Tagetes* sp. and their ability to reduce Cr(VI)

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Abstract. *Meitiniarti VI, Putri EK, Runtu AE, Nugroho RA, Kasmiyati S. 2022. Isolation and identification of Cr-resistant bacteria from the rhizosphere of* Tagetes *sp. and their ability to reduce Cr(VI). Biodiversitas 23: 4117-4123.* Bioremediation involving the interaction of plants with microorganisms is a technique for improving the environment polluted with chromium hexavalent [Cr(VI)]. Marigolds (*Tagetes* sp.) are known to be a hyperaccumulator plant for Cr(VI) and there are microbes in its rhizosphere that enable the plant tolerant to heavy metals. The aims of this study were to isolate and identify indigenous rhizobacteria resistant to Cr(VI) from *Tagetes* sp. and to test the ability of these bacterial isolates to reduce Cr(VI). Three isolates of Cr(VI) resistant bacteria were obtained in this study, namely *Micrococcus luteus* RT-9, *Stenotrophomonas maltophilia* RT-12, and *Brevundimonas* sp. RT-23. *M. luteus* RT-9 and *S. maltophilia* RT-12 which were resistant to Cr(VI) 100 mg L^{-1} and their mixture were tested for their ability to reduce Cr(VI) in a liquid medium. *Microbacterium* sp. SpR3 obtained by the previous study was used as a reference. The results showed that the isolate of *S. maltophilia* RT-12 had a higher ability (2.2 mg L^{-1} h^{-1}) than *M. luteus* RT-9 (0.9 mg L^{-1} h^{-1}). *S. maltophilia* RT-12 had the ability to reduce Cr(VI) similar to *Microbacterium* sp. SpR3. The mixed inoculation of *M. luteus* RT-9, *S. maltophilia* RT-12, and *Microbacterium* sp. SpR3 did not show a difference in the reduction ability of Cr (VI) compared to pure cultures.

Keywords: Bioremediation, chromium hexavalent, *Micrococcus luteus* RT-9, mixed culture, *Stenotrophomonas maltophilia* RT-12

INTRODUCTION

Chromium is one of the heavy metals found on earth in increasing amounts due to human activities (Oliveira 2012; Ali et al. 2020; Seleiman et al. 2020), which oxidation levels ranging from chromium bivalent [Cr(II)] to chromium hexavalent [Cr(VI)] forms. Cr(VI) is widely found in industrial wastes, such as textile dyeing, metal plating, and leather tanning (Tchounwou et al. 2012). The excess level of chromium in the soil and water can reduce soil quality and fertility, water quality, and affect other organisms (Hidayat 2015; Shahid et al. 2020; Seleiman et al. 2021). The Cr(VI) form is one of the most significant sources of environmental pollution and is well known for its toxic, carcinogenic, and mutagenic effects on humans and other living organisms (Fatmawati et al. 2008; Seidel and Corwin 2013).

The simple, inexpensive, and eco-friendly solution that can be performed over a wide range of experimental conditions for the detoxification and elimination of chromium pollutants is bioremediation (Sharma 2019; Hossan et al. 2020; Kalsooma et al. 2021). There are various bioremediation techniques, one of which is utilizing plant and microbial interactions (Salem et al. 2018). According to Yan et al. (2020), plant-associated microbes could be used to improve plant performance for phytoremediation. This plant-microbe interaction can be improved by selecting microbial species capable of reducing metals, applying optimal conditions, and selecting plant species (Al-Battashi et al. 2016; Akhter et al. 2017; Alnaimat et al. 2017; Hassan et al. 2017).

In the application of bacteria for bioremediation of Cr(VI)-polluted soil, it is necessary to increase the quantity and diversity of Cr(VI)-resistant rhizobacteria (Akhter et al. 2020). Isolation of Cr(VI)-resistant bacteria from plant rhizosphere growing in polluted soils is a commonly used method for obtaining various bacterial species (Priadie 2012; Seneviratne et al. 2017). *Bacillus cereus* and *Microbacterium* sp. SpR3 are examples of Cr(VI)-resistant bacteria isolated from the rhizosphere of plants grown in soils polluted by wastewater from the textile and leather tanning industry (Meitiniarti et al. 2012, 2014; Akhter et al. 2020).

Among the plant species that are widely used as remediation agents, Marigolds (*Tagetes* sp.) is reported to be a hyperaccumulator plant for Cr(VI). This plant is able to live in polluted lands, such as soils polluted with wastes from the textile industry, leather tanning industry, or vehicle/machine repair workshops (Akhter et al. 2017). The phytoremediation ability of marigolds is also aided by the association with metals-reducing rhizobacteria (Akhter et al. 2020). This plant accumulates up to 94% of Cr(VI) in 35 days when associated with rhizobacteria (Chitraprabha and Sathyavathi 2018).

In *ex-situ* bioremediation practice, the use of mixed inoculum (consisting of various isolates) in the bioremediation process is often more promising and provides greater efficiency than single cultures (Hsu et al.

2010; Jakovljevic and Vrvic 2016; Polasko et al. 2019; Mahmood et al. 2022). In mixed culture condition, the presence of an isolate can support the existence and ability of other isolates (Vernans et al. 2019). To obtain bacterial isolates that have the potential to reduce Cr(VI), in this study bacteria were isolated from the rhizosphere of *Tagetes* sp. grown in Cr(VI) polluted soil from chromium containing leachate at the Ngronggo landfill and batik wastewater. The objectives of this study were to isolate and identify Cr(VI)-resistant rhizobacteria from the rhizosphere of *Tagetes* sp. and to test the ability of these bacterial isolates to reduce Cr(VI) from the environment, both in single and mixed cultures.

MATERIALS AND METHODS

Samples preparation and *Microbacterium* sp. culture

Tagetes sp. 3-4 weeks old and planting media were obtained from agricultural and nursery shops in the Kopeng area, Semarang Regency (-7.40342, 110.41854). The wastewater was collected from Ngronggo landfill, Semarang Regency (-7.37663, 110.48570), and batik industrial wastewater in the Solo area, Central Java (-7.57131, 110.79162).

One kilogram of sifted planting media (∅ 2 mm) was sterilized using an autoclave at 121°C for 60 minutes. Sterile planting media were put into new polybags and watered with 100 mL wastewater that had been diluted 50% or undiluted (contained Cr(VI) of 0.35 and 0.7 mg L^{-1}, respectively). The planting medium was then left overnight.

Tagetes sp. were transferred to planting media that have been polluted with wastewater. Plants were maintained for 14 days and watered every 2 days with 100 mL of tap water. The rhizosphere soils of these plants will be used as a source of isolates of Cr(VI)-resistant bacteria.

Isolates of *Microbacterium* sp. SpR3 used as a reference in the reduction of Cr(VI) was obtained from the SWCU Microbiology Laboratory. Bacterial isolates were kept in Luria Bertani (LB) agar medium containing 50 mg L^{-1} Cr(VI) and subcultured every 2 weeks.

Isolation and purification of Cr (VI)-resistant bacteria from rhizosphere of *Tagetes* sp.

One gram of rhizosphere soil of *Tagetes* sp. which has been polluted with wastewater was taken and diluted with 0.9% physiological saline solution until 10^{-4}. Isolation of rhizobacteria was carried out by enriched culture technique. One hundred µl of each dilution were spread on LB (Merck) agar plate medium containing Cr(VI) with concentrations of 25, 50, 75, and 100 mg L^{-1} and incubated in an incubator at 37°C. Each cell that forms a separate colony was selected based on its morphological characteristics and streaked on sterile LB agar plates for purification. The selected pure cultures were identified molecularly using 16S rRNA and their ability to reduce Cr(VI) was determined.

Molecular identification of Cr(VI)-resistant bacteria isolates

Three isolates of Cr(VI)-resistant bacteria that were able to survive at Cr(VI) concentrations of 75 and 100 mg L^{-1} were selected. The bacterial isolates on 48-hour-old slanted LB agar were extracted their genomic DNA using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005). The 16S rRNA gene was amplified using PCR and universal primers of 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5′-TACCTTGTTACGACTT-3') (Nugroho et al. 2020). All reactions were carried out in a 25 uL reaction mixture consisting of 12.5 µL of MyTaq HS Red Mix, 2x (Bioline, BIO-25046), 1 µL of each primer (10 µM), 1 µL of BSA (Biolabs, 10 mg mL^{-1}), 1 µL of DNA genome, and 8.5 µL ddH2O (MP Biomedicals). The amplification was done using a thermal cycler (Eppendorf Nexus GSX1). PCR was carried out with conditions of initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation step at 94°C for 30 s, primer annealing at 54°C for 30 s, and elongation at 72°C for 30 s (Nugroho et al. 2020). The reaction was completed with an extension step at 72°C for 10 min. PCR products (~1400 bp) were sent to 1st BASE Laboratories for sequencing with primers 27F and 1492R. To identify these strains of Cr-resistant bacteria, the nucleotide sequences were aligned and compared with the available standard sequences in the GenBank database (https://www.ncbi.nlm.nih.gov/) using BLAST. The nucleotide sequences of 16S rRNA from Cr(VI)-resistant bacteria isolates obtained in this study have been deposited in the GenBank database under Accession numbers listed in Table 2.

A phylogenetic tree was constructed using Mega software (Kumar et al. 2018) with sequences from closely related strains retrieved from GenBank. The sequences were aligned by ClustalW to reconstruct a phylogenetic tree using the Neighbor-joining tree methods and Kimura 2-parameter model (Tamura et al. 2021). Bootstrap values (more than 75%) based on 1000 replications were listed at nodes.

Determination of the ability to reduce Cr(VI) by bacterial isolates in single and mixed culture

Each bacterial isolate was inoculated into 50 mL liquid Luria Bertani (LB) medium containing 100 mg L^{-1} Cr(VI) (Nugroho et al. 2015) and incubated at room temperature on a shaker with 120 rpm. The optical density of culture was measured spectrophotometric at λ 600 nm every 24 hours. After the growth reached the logarithmic phase (OD value equal to 1), the cultures were harvested by centrifugation at 5,000 rpm for 10 minutes. Cell pellets were resuspended with sterile physiological saline and adjusted so that the absorbance of the cell suspensions of all cultures was the same. The cell suspension was ready for use in determining the growth rate and Cr(VI) reduction rate.

The growth rate of Cr(VI) resistant bacteria and their reduction ability were studied in 100 mL (working volume) liquid LB medium containing 100 mg L^{-1} Cr(VI). Bacterial cultures were inoculated at 10% of the working volume.

The inocula consisted of two inocula chosen because of their ability to survive at 100 mg L^{-1} Cr(VI), the mixture of these two inocula (1:1, v/v), and the mixture of these two inocula with *Microbacterium* sp. (1:1:1, v/v). All were done in 3 replicates. The cultures were incubated in a 100 rpm shaker at room temperature. Sampling was carried out every 2 hours (until the 8th hour) and then every 24 hours until the 72 hours.

The parameters observed were the optical density (OD) of cells and concentration of Cr(VI). OD of the cell was determined using a spectrophotometer (λ 600 nm) (Kalsooma et al. 2021) and the obtained OD values were converted into biomass (dry weight). The specific growth rate was determined as long as the culture was in the logarithmic phase (the first 8 hours). The concentration of Cr(VI) was determined spectrophotometrically using diphenyl carbazide method (Wiryawan et al. 2018). The rate of Cr(VI) reduction was calculated for the first 48 hours according to Franco et al. (2018).

RESULTS AND DISCUSSION

Isolation and characterization of Cr-resistant bacteria from rhizosphere of *Tagetes* sp.

Isolation of Cr(VI)-resistant bacteria from the rhizosphere of *Tagetes* sp. resulted in nine pure isolates. These nine bacterial isolates were Cr(VI) resistant at various concentrations (50-100 mg L^{-1}). The characteristics of the nine isolates are shown in Table 1. Three isolates that were able to grow on a medium with Cr(VI) 75 and 100 mg L^{-1} were selected to be identified molecularly using 16S rRNA. Two isolates, namely RT-9 and RT-12 were resistant to Cr(VI) 100 mg L^{-1}, and RT-23 was resistant to Cr(VI) 75 mg L^{-1}. This high Cr(VI) resistance ability indicated the ability to reduce Cr(VI) in the environment. Chitraprabha and Sathyavathi (2018) also reported that microbes associated with *Tagetes erecta* could accumulate and reduce Cr(VI).

Molecular and phylogenic analysis of Cr(VI)-resistant bacteria

The identities of the three isolates were analyzed based on the relationship between the 16S rRNA sequences using the BLAST method against the NCBI database. The results showed that isolates RT-9, RT-12, and RT-23 had almost 100% similarity to *Micrococcus luteus*, *Stenotrophomonas maltophilia*, and *Brevundimonas* sp., respectively (Table 2).

The phylogeny tree (Figure 1) below shows the relationship between the three isolates (RT-9, RT-12, and RT-23) with several bacterial species known to have resistance to chromium, such as *Micrococcus* sp. (Abamhekhelu et al. 2019), *S. maltophilia* (Mukherjee and Roy 2016; Raman et al. 2018), *Brevundimonas* sp. (Soto et al. 2021) and *E. coli* (Mohamed et al. 2020). Some researchers reported that *M. luteus*, *S. maltophilia*, and *Brevundimonas* sp. were known to have high resistance to heavy metals, including Cr(VI) (Marzan et al. 2017; Singh and Hiranmai 2021). According to Abamhekhelu et al. (2019), *Micrococcus* sp. has a better growth rate than *Staphylococcus aureus* and *Pseudomonas aeruginosa* on NB medium containing $K_2Cr_2O_7$. *M. luteus* is known to be a potential bacterium in Cr(VI) remediation (Abidin et al. 2019; Katyal and Kaur 2018). *Stenotrophomonas* sp. was found to be tolerant to Cr(VI) in the range of 10-500 mg L^{-1} and showed 100% reduction ability at a concentration of 10-70 mg L^{-1}. The ability to reduce chromium is due to the gene encoding the enzyme chromium reductase (*ChrR*) which plays a role in chromium reduction (Baldiris et al. 2018). Other studies have shown that *Brevundimonas* were found in places polluted with Cr(VI), such as metal mining areas (Romo et al. 2019; He et al. 2016).

Table 1. The characteristics of the nine isolates of Cr-resistant bacteria from the rhizosphere of *Tagetes* sp.

Colony code	Colony shape	Colony color	Cell shape	Gram test	KOH string	Catalase	Oxidase	Glucose ferm.	Cr(VI) resistant
RT-1	Irregular curled	Brownies white	Coccus	+	-	+	+	+	50 mg L^{-1}
RT-2	Circular entire	Yellowish	Diplo coccus	-	+	+	+	-	50 mg L^{-1}
RT-8	Circular entire	Red	Coccus	-	+	+	+	+	50 mg L^{-1}
RT-9	Circular entire	Yellow	Coccus tetrad	+	-	+	+	-	100 mg L^{-1}
RT-12	Circular undulate	Creamy	Rod	-	+	+	+	-	100 mg L^{-1}
RT-14	Irregular curled	Light brown	Irregular	+	-	+	+	-	50 mg L^{-1}
RT-15	Circular entire	Yellow	Coccus tetrad	+	-	+	-	+	50 mg L^{-1}
RT-23	Circular entire	Brown	Rod	-	+	+	+	-	75 mg L^{-1}
RT-35	Circular entire	Orange	Diplococcus	+	-	+	+	-	50 mg L^{-1}

Note: +: positive results or can produce enzymes, -: negative results or cannot produce enzymes, Glucose ferm.: Glucose fermentation

Table 2. Isolation code, molecular characteristics, and the value of the closeness of the species

Isolation code	Accession number	Sequence length (bp)	Species that are related	Related score
RT-9	OL687559	1391	*Micrococcus luteus*	100%
RT-12	OL687560	1423	*Stenotrophomonas maltophilia*	99%
RT-23	OL687561	1331	*Brevundimonas* sp.	100%

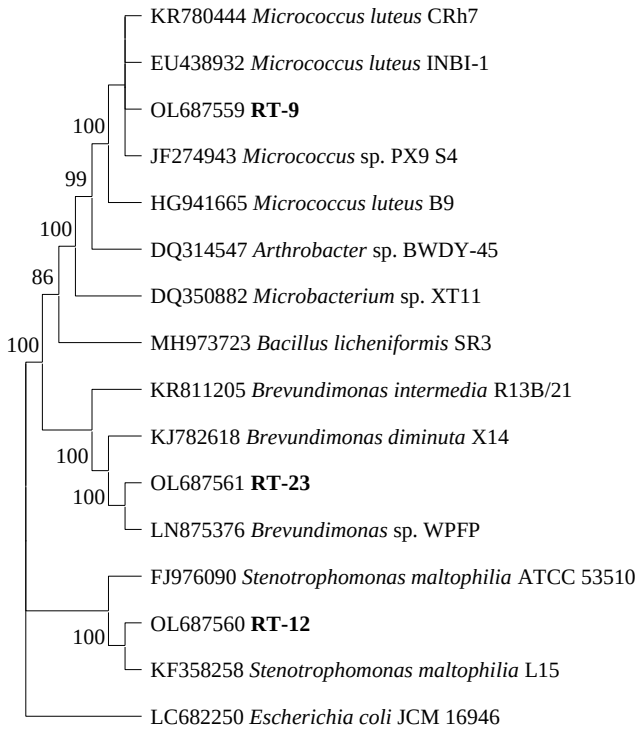

Figure 1. The relationship between RT-9, RT-12, and RT-23 isolates with several bacterial species known to have resistance to chromium. Sequences from this study are in bold

The growth of two isolates of Cr-resistant bacteria and their ability to reduce Cr(VI)

Two isolates RT-9 and RT-12 were further tested for their ability to grow and reduce Cr(VI) in the LB medium. The growth rate of RT-9 and RT-12 as a single culture, or in the form of a mixed culture of RT-9 + RT-12 (1:1, v/v) and a mixture of RT-9 + RT-12 + *Microbacterium* sp. SpR3 (1:1, v/v) could be seen in Figure 2. The mixed culture of RT-9 + RT-12 + *Microbacterium* sp. showed the fastest cell growth rate, while the RT-9 isolate showed the lowest cell growth rate compared to other isolates. This was confirmed by the specific growth rates of these three isolates (Table 3). This table showed that the specific growth rate of RT-9 isolates in the medium containing Cr(VI) was the lowest compared to RT-12 and *Microbacterium* sp. SpR3. According to Nugroho et al. (2015), the growth ability of Cr(VI)-resistant bacteria in a medium containing Cr(VI) is related to the ability to reduce Cr(VI). Aljerf and AlMasri (2018) stated that there are many factors other than the growth rate which affect the Cr(VI) reduction ability, including the availability of nutrients, environmental factors (pH, temperature, cofactors, inhibitory compounds), and interactions with other organisms.

The ability of a single and mixed culture of RT-9, RT-12, *Microbacterium* sp. SpR3 to reduce Cr(VI) and their Cr(VI) reduction rate could be seen in Figure 3 and Table 4. The pure culture of RT-9 needed a longer time than other isolates to reduce Cr(VI) concentration in the medium. These results indicate that the ability of bacteria to grow on media containing Cr(VI) affects the ability of bacteria to reduce Cr(VI) as reported by Nugroho et al. (2015). The fastest Cr(VI) reduction rate by pure culture was achieved by RT-12, *Microbacterium* sp. SpR3, and RT-9, respectively (Table 4). The Cr(VI) reduction rate of isolate RT-12 is similar to the Cr(VI) reduction rate of *Microbacterium* sp. SpR3 in soil (Innation et al. 2020). Based on the Cr(VI) reduction rate, the single culture of RT-12 showed the fastest Cr(VI) reduction rate (2.216 mg L^{-1} $hour^{-1}$). However, the Cr(VI) reduction rate of RT-12 was lower than *Desulfovibrio vulgaris* (Table 4). According to Franco et al. (2018), the growth of microorganisms will be stimulated if there are stoichiometrically balanced electron donors and acceptors. If the growth of microorganisms is fast, the reduction of Cr(VI) will take place efficiently.

Table 3. The specific growth rate of RT-9, RT-12, and *Microbacterium* sp. SpR3 in the LB medium containing Cr(VI)

Isolate	Specific growth rates (h^{-1})
RT-9	0.0205
RT-12	0.0908
Microbacterium sp. SpR3	0.0850

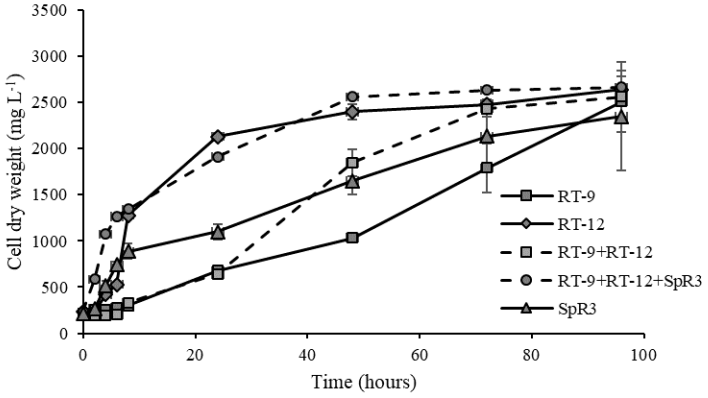

Figure 2. Cell growth of pure cultures RT-9, RT-12, *Microbacterium* sp. SpR3, mixed culture of RT-9+RT-12, and mixed culture of RT-9+RT-12+*Microbacterium* sp. SpR3

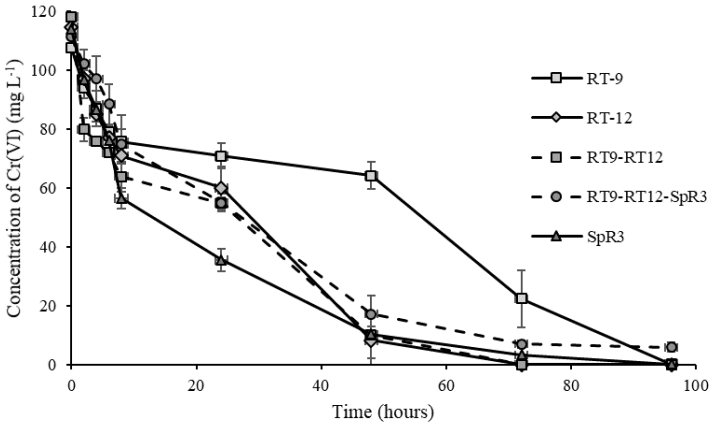

Figure 3. The concentration of Cr(VI) in LB medium inoculated with a single culture of RT-9, RT-12, *Microbacterium* sp. SpR3, mixed culture RT-9+RT-12, and RT-9+RT-12+*Microbacterium* sp. SpR3 during 96 hours of incubation

Table 4. The reduction rate of Cr(VI) by single and mixed cultures of RT-9, RT-12, *Microbacterium* sp SpR3, and several other bacteria for comparison

Inoculum	Cr-reduction rate (mg L^{-1} h^{-1})	Culture conditions	References
Single culture			
- RT-9	0.905	LB medium	*This study*
- RT-12	2.216	LB medium	*This study*
- *Microbacterium* sp. SpR3	2.157	LB medium	*This study*
- *Bacillus* sp. JDM-2-1	1.67	Acetate minimal medium	Zahoor and Rehman (2009)
- *Staphylococcus capitis*	1.21	Acetate minimal medium	Zahoor and Rehman (2009)
Desulfovibrio vulgaris at			
30°C	7.73	LS4D medium, electron-acceptor	Franco et al. 2018
20°C	2.25	limited (EAL)	
Mixed culture			
- RT-9+RT-12	2.251	LB medium	*This study*
- RT-9+RT-12+*Microbacterium* sp. SpR3	1.964	LB medium	*This study*

Table 4 also showed that the Cr(VI) reduction rate of the mixed culture of RT-9+RT-12 was higher than the mixed culture of RT-9+RT-12+*Microbacterium* sp. SpR3. This may be related to the growth of these two isolates in mixed cultures. It is possible that during the experiment the mixed culture of RT-9+RT-12 was dominated by RT-12 which has a higher growth rate than RT-9, so the Cr(VI) reduction rate in this mixed culture was similar to the Cr(VI) reduction rate of RT-12. Meanwhile, in the mixed culture of RT-9+RT-12+*Microbacterium* sp. SpR3, the

possibility of antagonism between RT-12 and *Microbacterium* sp. SpR3 may occur, resulting in a lower rate of Cr(VI) reduction. In mixed cultures, antagonism (Nugroho et al. 2020) or synergism (Dutta et al. 2021) effects can occur, so the use of mixed cultures needs further study before being applied in the environmental bioremediation process.

It can be concluded that three isolates of Cr(VI) resistant bacteria were obtained from the rhizosphere of *Tagetes* sp. These three isolates are *M. luteus* RT-9, *S. maltophilia* RT-12 and *Brevundimonas* sp. RT-23. The isolate *S. maltophilia* RT-12 was able to grow and reduced Cr(VI) better than the *M. luteus* RT-9. The mixed culture of *M. luteus* RT-9+*S. maltophilia* RT-12 and *M. luteus* RT-9+*S. maltophilia* RT-12+*Microbacterium* sp. SpR3 showed no difference in reducing Cr(VI) compared to the single culture of RT-12 and *Microbacterium* sp. SpR3. Further study on the reduction of Cr(VI) by the mixtures of *M. luteus* RT-9, *S. maltophilia* RT-12, and *Microbacterium* sp. SpR3 is needed. There can be a dominance of one isolate, as well as antagonism or synergism between types of bacteria in mixed cultures.

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