Identification of the ultisol land indigenous bacteria from Banyumas Regency based on the characteristics of morphology, physiology and biochemistry

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Abstract. This study aims to identify SR2 and TG4 isolates based on the characteristics of morphology, physiology, and biochemistry. Morphological characters are known through gram and endospore staining tests and microscopy observations. Physiological and biochemical characters are known based on a series of tests such as motility, catalase, sugar (glucose, mannitol, sucrose, lactose) fermentation ability, hydrolysis of starch, urease, methyl-red (MR), voges-prostekuer (VP), simons citrate, H2S production, oxidase, paraffin, and indole. The results showed that SR2 isolates were rod-shaped, gram-positive bacteria. TG4 isolates include rod-shaped, gram-negative bacteria. SR2 and TG4 are endospores-forming bacteria. The results of physiological and biochemical tests showed SR2 isolates were motile, had catalase activity, can ferment sugars (glucose, sucrose, and mannitol), unable to ferment lactose, hydrolyze starch, cannot hydrolyze urea, positive results for MR and indole test, but negative for VP test, simmons citrate, H2S production, and oxidase. TG4 isolates were motile, showed positive results for catalase test, sugar fermentation (glucose, sucrose, and mannitol), starch hydrolysis, MR test, but negative results for lactose fermentation, urease, VP, simmons citrate, H2S production, oxidase, and indole. SR2 and TG4 isolates include facultative aerobic / anaerobic bacteria. SR2 Isolates are probably Bacillus sp. and TG4 are Serratia liquefacieus.

1. Introduction
Two dominant isolates were selected, namely SR2 and TG4 successfully selected from 26 isolates obtained from ultisol soil samples in four locations in Banyumas Regency (Srowot, Karangrao, Tanggeran, and Pagaralang Village), Central Java Province. The selection of isolates is based on the best growth curve dan generation time, and the ability of the isolate to grow on the media containing buprofezin synthetic pesticides active ingredients [1]. The existence of synthetic pesticide residues has been known to suppress the growth and activity of soil bacteria [2]. Synthetic pesticide residues can reduce soil fertility because it disrupts the activity of enzymes produced by soil bacteria such as urease, protease, phosphatase [3], amylase and cellulase [4].

SR2 and TG4 isolates need to be identified based on morphological, physiological and biochemical characteristics. The identification of bacterial isolates to the level of the genus or species referring to
Bergey's Manual of Determinative Bacteriology. This study aims to identify SR2 and TG4 isolates based on morphological, physiological, and biochemical characteristics.

2. Materials and methods

2.1 Morphological tests

2.1.1 Gram staining. A heat-fixed smear of bacterial cells was prepared. Purple crystal was added to the sample until they are submerged and left for one minute. Samples were washed with distilled water (C'aya Qmia) and added by lugol (Merck) until submerged and allowed to stand for one minute. After that, the samples were washed with distilled water followed by 90% alcohol (Brataco) and dried with tissue paper. On the sample, a few drops of safranin (Merck) were added and then allowed to stand for 30-45 seconds. The sample were washed with distilled water and dried. The sample was observed by light microscope with 1000x magnification.

2.1.2 Schaeffer-fulton endospores staining. Smear of bacterial cells were prepared above the object glass. Malachite Green (Merck) dyes was added to the sample until submerged. The samples were fixed by heating for 10 minutes (every 1 minute added 1 drop of malachite green). The samples then cooled and washed with flowing distilled water. Safranin counter stain was added to the mixture and incubated for 1 minute. The samples were washed with flowing distilled water and air dried. The sample were added immerse oil (Merck) and observed by light microscope with 1000x magnification.

2.2 Physiological and biochemical testing

2.2.1 Motility test. Nutrient Agar (NA, Merck KGaA) in a test tube (Iwaki) was sterilized by autoclaving (Equitron) 121°C, 15 minutes. After the media solidified, pure bacterial culture was inserted into the NA media as deep as ¾ the media portion. The media was incubated 18-24 hours at 35-37°C in an oven/incubator (Memmert UF55). Bacterial growth was observed. If the growth is in a straight line, the test is stated to be negative (non-motile), whereas if it widens / spreads outside the inoculation line, the test results were positive (motile).

2.2.2 Catalase test. Pure bacterial cultures were inoculated into sterile Nutrient Broth (NB, Merck KGaA) in a test tube and incubated 24-48 hours at 37°C. A few drops of H2O2 (Mediss) 3% were added into the media. Positive test results were indicated by the formation of air bubbles.

2.2.3 Sugar fermentation test. Sterile NB media contains 0.5% sugar and a few drops of Brom Thymol Blue (BTB) indicator (Merck) were placed in a sterile test tube in which the durham tube is upside down. The media was autoclaved at 12°C, 15 minutes. After cold, the media was inoculated with a pure bacterial culture and incubated 24-48 hours at 37°C. Positive results were indicated by changes in the color of the media from blue to yellow and gas formation in the durham tube [2, 5]. Sugar fermentation tests can also use Triple Sugar Iron agar (TSIA) (Merck KGaA). Pure bacterial culture was inoculated using ose into 3/4 parts of the media and also scratched on the surface. The media was incubated 24-48 hours at 37°C. The results of a positive glucose fermentation test were indicated by the color of the TSIA media inside which turns yellow. The results of the sucrose and lactose fermentation tests were positive when there was a change in the color to yellow on the surface of the TSIA media.

2.2.4 Starch hydrolisis test. Sterile NA media containing 0.5% starch (Merck) in petri dishes (Anumbra) were inoculated with pure bacterial culture. The media was incubated at 37°C, 48 hours. Lugol (iodine in 95% alcohol and potassium iodide / KI) (Merck) was added to the medium and left
for 30 seconds. Positive test results were indicated by the presence of clear zones around bacterial colonies, while the purple color indicates the starch hydrolysis process did not occur [5].

2.2.5 Urease test. Urea broth (Merck KGaA) as a result of sterilization by a syringe filter (Allpure) was placed in a sterile test tube. Pure bacterial cultures were inoculated into the media and incubated at 37°C, 24-48 hours. Positive test results were indicated by a change in media color from orange to pink [5].

2.2.6 Methy-red (MR) test. Sterile MR-VP broth (HIMEDIA) was prepared in a test tube. Pure bacterial cultures were inoculated into the media and incubated 24-48 hours at 37°C. Five drops of 1% MR (Merck KGaA) indicator were added to the sample, shaken, and allowed to stand for 5 minutes. Positive test results were indicated by changes in media color to red [5].

2.2.7 Voges-prostekuer (VP) test. Sterile MR-VP broth in a test tube was inoculated with pure bacterial culture and incubated 24-48 hours at 37°C. Ten drops of 5% alpha naphthol (Merck) and 10 drops of 40% KOH (Merck) were added into the sample. The sample was shaken every 3-4 minutes. The observations were carried out 15 minutes after the addition of alpha naphthol and KOH. Positive test results were indicated by changes in the color of the media to pink [5].

2.2.8 Simmons citrate test. This test used autoclaved Simmons citrate media (HIMEDIA). Pure bacterial cultures were inoculated into the media. Media that already contains bacteria was then incubated 18-24 hours at 35°C. The test results show positive if there was a change in the color of the media from green to blue [5].

2.2.9 H2S production test. This test used TSIA media that has been sterilized by autoclaving. A pure bacterial culture was inserted into 3/4 parts of the media and also scratched onto the surface of the media. Media containing bacteria were incubated 18-24 hours at 35-37°C. Positive test results were indicated by black deposits on the media [5].

2.2.10 Oxidase test. Pure bacterial culture in the NA medium was applied to oxidase disch (HIMEDIA) using a sterile toothpick. Positive test results were indicated by changes in culture color to dark purple before 10 seconds. Negative test results are shown by the absence of color changes in the sample after 10 seconds or there is a color change after 10 seconds [6].

2.2.11 Paraffin test. Five drops of liquid paraffin (Lansida) were added to pure bacterial culture in NB media containing 0.5% mannitol. Cultures without paraffin also prepared. The sample was incubated 24-48 hours, at 37°C. If mannitol fermentation occurred in paraffin-containing media and did not contain paraffin, the bacteria were classified as aerobic/anaerobic facultative. If mannitol fermentation did not occur in paraffin-containing media, while mannitol fermentation occurs in media without paraffin, bacteria were aerobic obligates. If mannitol fermentation occurs in paraffin-containing media, mannitol fermentation did not occur in media without paraffin, the bacteria were anaerobic obligates.

2.2.12 Indole test. Sterile NA media containing 0.5% L-tryptophan (Merck KGaA) was inoculated with pure bacterial culture. The media was incubated 24-48 hours, at 37°C. Five drops of Kovac’s reagent (Merck KGaA) was added into the culture. Positive test results were indicated by the formation of red on the surface of the media.
3. Result and discussions

3.1 Morphological observations

Morphological observations based on gram staining showed that SR2 isolates included gram-positive bacteria, while TG4 isolates included gram-negative bacteria (Figure 1). Gram staining can distinguish the two groups of bacteria based on differential staining with crystal violet-iodine and safranin counterstain complexes. Gram-positive bacteria were purple, while gram-negative bacteria were pink [7].

![Figure 1](image1.png)

**Figure 1.** Gram staining results. SR2 isolates were purple (gram positive) (a) and TG4 were pink (gram negative) (b).

The results of endosporic observation showed isolates SR2 and TG4 were endospores-forming bacteria. Endospores are green when malachite green is applied to a heat-fixed smear of bacterial cells. The stain penetrates the endospore. When the safranin (red) is then applied, it stains the remainder of the cells red or pink [8] (Figure 2)

![Figure 2](image2.png)

**Figure 2.** The results of endosporic observation of SR2 (a) and TG4 (b) isolates. Both isolates were endospore-forming bacteria seen from the green color appeared after malacite green staining.
3.2 Physiological and biochemical characteristics

The physiological and biochemical test results of isolates SR2 and TG4 were presented in Table 1.

| Physiological and Biochemical Test       | Isolate   |
|------------------------------------------|-----------|
|                                          | SR2       | TG4       |
| Motility Test                            | +         | +         |
| Catalase Test                            | +         | +         |
| Glucose Fermentation Test                | Acid, without gas | Acid, without gas |
| Sucrose Fermentation Test                | Acid, without gas | Acid, without gas |
| Mannitol Fermentation Test               | Acid, without gas | Acid, without gas |
| Lactose Fermentation Test                | -         | -         |
| Starch Hydrolisis Test                   | +         | +         |
| Urease Test                              | -         | -         |
| MR Test                                  | +         | +         |
| VP Test                                  | -         | -         |
| Simmons Citrate Test                     | -         | -         |
| H₂S Production Test                      | -         | -         |
| Oxidase Test                             | -         | -         |
| Paraffin Test                            | Facultative Aerobes/Anaerobes | Facultative Aerobes/Anaerobes |
| Indole Test                              | +         | -         |

Remarks: + positive test results, - negative test results

3.2.1 Motility test. This test was to distinguish motile and non-motile bacteria. Bacteria that have motile characteristics will move beyond the inoculation line, while non-motile bacteria will grow only on the path of inoculation. Both isolates SR2 and TG4 were motile (Figure 3).

![Figure 3. Motility test result of SR2 (left) and TG4 (right) isolates. Motile bacteria were characterized by bacterial growth out of the inoculation line.](image_url)

3.2.2 Sugar fermentation test. This test was used to determine the ability of bacteria to ferment sugar to produce organic acids. Organic acids cause the pH of the media to become acidic so that the BTB indicator changes color from blue to yellow. Sometimes a gas was produced which is marked by the
presence of gas bubbles in the durham tube. In this study, isolates SR2 and TG4 were able to ferment glucose, sucrose, and mannitol. However, the sugar fermentation process that occurs did not produce gas (no gas was formed on the durham tube) (Figure 4).

![Figure 4](image)

**Figure 4.** Test results of sugar fermentation. NB media contains glucose 0.5% (a), sucrose 0.5% (b), and mannitol 0.5% (c). SR2 and TG4 isolates were able to ferment glucose, sucrose, and mannitol.

3.2.3 Starch hydrolysis test. This test was used to determine the ability of bacteria to hydrolyze starch (complex carbohydrates) into simple carbohydrate compounds. This ability was associated with the activity of amylase enzymes produced by bacteria. Isolates SR2 and TG4 showed the ability to hydrolyze starch (Figure 5).

![Figure 5](image)

**Figure 5.** Starch hydrolysis test results. Isolates SR2 and GT4 showed the existence of clear zones around isolates which indicate that starch has been hydrolyzed into simple compounds that did not form a purple complex with iodine.

3.2.4 MR test. Tests are used to determine the ability of bacteria to ferment glucose to produce mixed acids. Methyl red was used as an indicator. The indicator would be red when the media pH was 4.4.
(acid) and yellow when the media pH is 6.2. The isolates SR2 and TG4 showed positive results for the MR test (Figure 6).

![MR test results. Isolate SR2 (left), TG4 (middle), and control (right). SR2 and TG isolates were red which means positive results if compared with control.](image)

3.2.5 Paraffin test. Liquid paraffin that added to the bacterial culture medium will cover the upper surface of the media. If the presence of paraffin disrupts bacterial metabolism, the bacteria belong to the obligate aerob group. Conversely, if the presence or absence of paraffin in the media did not disrupts the process of bacterial metabolism, the bacteria was facultative aerobes/anaerobes. Bacteria belong to the obligate anaerob group, if the absence of paraffin in the media actually disrupts the process of bacterial metabolism. The results of the paraffin test showed that isolates SR2 and TG4 were grouped into facultative aerobic / anaerobic bacteria (Figure 7).

![Isolates SR2 and TG4 in NB media containing 0.5% mannitol. Bacterial metabolism was not disrupted by paraffin (a) or the absence of paraffin (b). Paraffin was marked by black arrow.](image)
3.2.6 *Indole test.* The indol test was used to determine the presence or absence of tryptophanase enzyme activity. This enzyme plays a role in catalyzing the conversion of tryptophan to indole. Indole compounds are precursors of the indole acetic acid (IAA) hormone. Indole compounds would form a red complex with Kovac’s reagent. Indole test results showed positive for SR2 isolates and negative for TG4 isolate (images not presented).

3.3 *Bacterial identification*

Based on Bergey’s Manual of Determinative Bacteriology [9], isolat SR2 probably *Bacillus* sp. and TG4 are *Serratia liquefacieus*. Genus *bacillus* were characterized by rod-shaped bacteria, gram-positive, capable of producing endospores, motile, generally capable of fermenting carbohydrates by producing acids and not producing gas, having catalase activity, facultative aerobes/anaerobes, indole produced, and commonly found in soil. Genus *serratia* were characterized by rod-shaped bacteria, gram-negative, produces acetic from glucose fermentation, not capable for fermenting lactose, indole not produced, urease negative, motile, H 2S production negative, facultative aerobes/anaerobes, found in soil and water [9].

4. Conclusions

SR2 isolates were gram-positive bacteria, TG4 isolates include gram-negative bacteria. Both isolates include endospora-forming bacteria in the form of rods. The results of physiology and biochemistry test showed that isolates SR2 and TG4 have similar characteristics, such as being motile, having catalase activity, capable of fermenting sugar (glucose, sucrose, and mannitol), unable to ferment lactose, can hydrolyze starch, have no enzyme activity of urease, showed positive results on MR test, but negative on VP test, simmons citrate, H 2S production, and oxidase. SR2 and TG4 isolates are grouped into facultative aerobic / anaerobic bacteria. The physiological and biochemical characters of isolates SR2 and TG4 differed only in the indol test: SR2 isolates showed positive, while TG4 isolates were negative. SR2 probably Isolate *Bacillus* sp. and TG4 were *Serratia liquefacieus*.

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