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Isolation and Characterization of *Schmutzdecke* in Slow Sand Filter for Treating Domestic Wastewater

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ABSTRACT

This research aimed to discover the macroscopic, microscopic, and physiological characteristics and the genus of heterotrophic bacteria found in the *schmutzdecke* or biofilm layer in slow sand filters. The isolation of heterotrophic bacteria in the *schmutzdecke* applied the pour plate and quadrant streak method, while the characterization used macroscopic, microscopic, and physiological tests. Samples were taken from the *schmutzdecke* on top of the filter layer, and they were diluted 3, 4, and 5 times, then grown in Nutrient Agar media in order to isolate heterotrophic bacteria. The results of the research were analyzed using the identification manual books titled Bergey's Manual of Determinative Bacteriology, 9th edition and Manual for The Identification of Medical Bacteria, 3rd edition. These manuals show the names of the genus of bacteria in the *schmutzdecke* layer. On the basis of the identification results from macroscopic, microscopic, and physiological tests, there were 4 dominant genera out of 18 living isolates obtained from the *schmutzdecke* layer, namely *Kurthia gibsonii*, *Bacillus badius*, *Bacillus firmus,* and *Bacillus lentus*. The similarity percentage of these 4 isolates was 83%, 81%, 85%, and 77% respectively.

Keywords: heterotrophic bacteria, biofilm, *schmutzdecke*, slow sand filters.

INTRODUCTION

In Indonesia, domestic wastewater is the largest contaminant that pollutes water bodies (Wulandari et al., 2019). As population grows rapidly, clean water consumption also increases, which results in the surge of the amount of wastewater that can pollute the environment (Purwanti et al., 2018b). Wastewater comes from domestic activities, industries, and other public places (Ighalo et al., 2022; Igwegbe et al., 2022; Kurniawan et al., 2022c). It contains the materials that can endanger the life of humans and other living beings and disturbs environmental sustainability (Al-Ajalin et al., 2020). Greywater is the wastewater that is usually generated from the activities in the kitchen, baths, laundry, and from air conditioners (Samayamanthula et al., 2019). Greywater represents one of the best water reserves, because it contains fewer contaminants than sewage water. Besides greywater, households also generate human waste, which is known as blackwater. Therefore, greywater needs to be utilized as water reserve using a simple filtration device, namely slow sand filters (Fitriani et al., 2020).

Slow sand filter (SSF) is a simple filtration device known for drinking water treatment and it is widely used for the production of drinking water that is stable and able to remove particles (Urfer, 2017). The biological process of water treatment in slow sand filters occurs on the *schmutzdecke* layer (Zhao et al., 2019). Contaminant filtration

in slow sand filters occurs particularly on the collation or *schmutzdecke* layer, a biologically active layer consisting of algae, bacteria, diatoms, and zooplanktons (Abu Hasan et al., 2022; Joubert and Pillay, 2008; Kurniawan et al., 2022a).

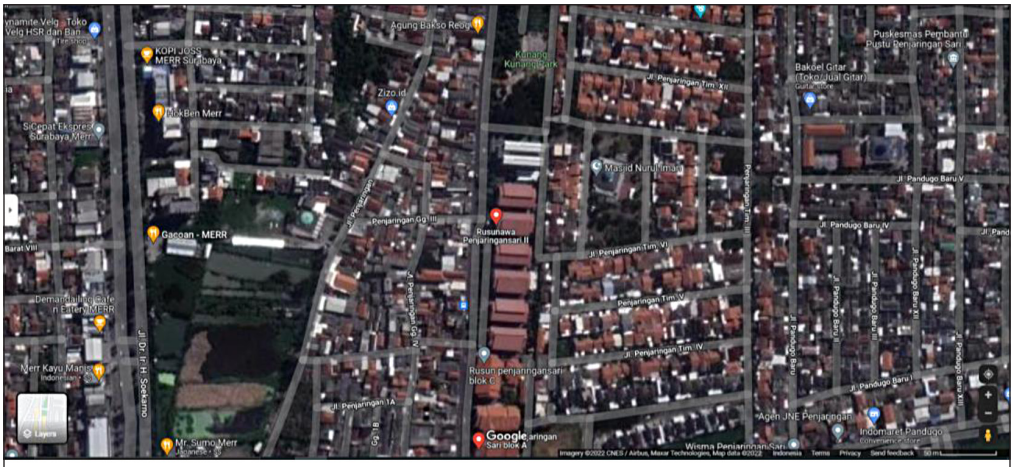
Slow sand filters have a complex ecosystem in the infiltration filter, since the species of microorganisms are not determined by the depth of the media but are distributed entirely. It is affected by several factors, including physical, chemical, and biological ones (Fitriani et al., 2020). Bacteria are one of the microorganisms found in the *schmutzdecke* layer. Bacteria are unique and unicellular prokaryotes that have no structure bounded by a membrane in their cytoplasm (Muhamad et al., 2021; Russell, 2007; Said et al., 2021). The identification process can be conducted using a conventional method. On the basis of the research by Imron et al. (2019), bacterial identification can be carried out quickly with a conventional method using an identification kit. Using a conventional method to identify bacteria enables the macroscopic and microscopic morphology to be seen, which can be used to reinforce the identification results described in Bergey's Manual of Determinative and Systematic Bacteriology, 1998 (Bergey and Holt, 1994) and Manual for The Identification of Medical Bacteria, 3rd edition (Cowan, 1974).

MATERIAL AND METHODS

Time and place of research

The samples for this research were taken from the domestic wastewater in the inlet of Domestic Wastewater Treatment Plants of Rusunawa Penjaringan Sari 2 Block C (-7° 19' 10.5" N, 112° 47' 2.2" S) (Figure 1a) and Rusunawa Keputih Block C (-7° 17' 52.68" N, 112° 48' 12.48" S) (Figure 1b). The identification of bacteria in the *schmutzdecke* layer took place in the Microbiology Laboratory, Integrated Laboratory, and Wet Laboratory of Faculty of Science and Technology of Universitas Airlangga. This research was

a)



b)

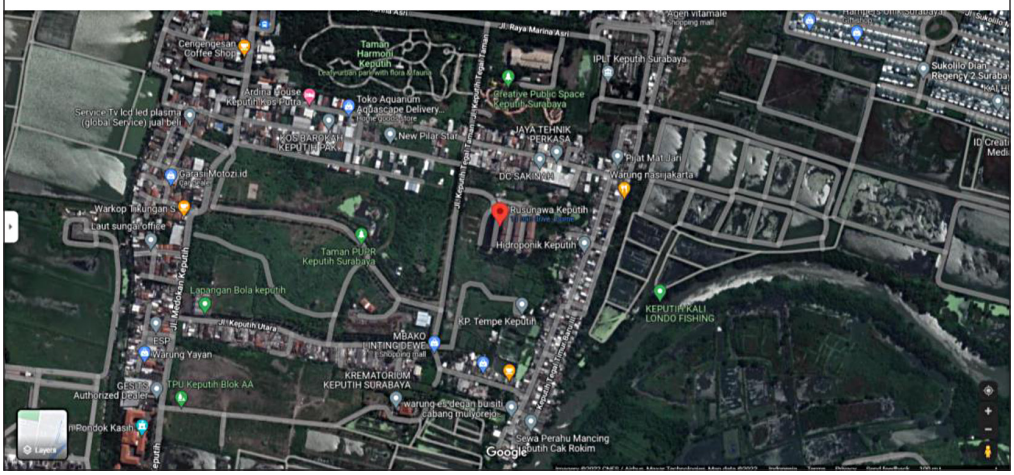


Figure 1. Location of wastewater sampling

conducted from December 2019 to March 2020. The locations of the wastewater and bacterial sampling can be seen in Figure 1.

Reactor setup

The main tools used in this research were waste reactors or slow sand filters and identification kits (Microbact, Oxoid). Other utilized tools included a microscope, object glass, cover glass, test tubes, Petri dishes, inoculation loops, a water bath, Erlenmeyer flasks, 250 mL bottles, micropipettes with a volume of 0.2–1000 µL and tip, pipettes, an analytical balance, vortex, Beaker glass, plastic wrap, aluminum foil, a microwave, a refrigerator, and an incubator. The details of the slow sand filter reactor design with a dimension of 32×13×110 are provided in Table 1. The slow sand filter reactor can be seen in Figure 2.

The main material that was used in this research was the *schmutzdecke* layer from slow sand filter waste reactors. Other utilized materials were Nutrient Agar (NA, Oxoid), griseofulvin, sterile physiological saline, Malachite green coloring, Gram-hucker's staining kit (violet crystal, iodine, alcohol, and safranin), H_2O_2, oxidation test strips, Microbact reagents (Nitrate A, Nitrate B, immersion oil, VPI, VPII, and TDA), and 70% ethanol.

Bacterial isolation

A total of 2 grams of the *schmutzdecke* sample were taken using a sterile spatula from 3 points in order to isolate the bacteria to discover the species of bacteria found in the *schmutzdecke* layer. The *schmutzdecke* layer was suspended by being placed into a bottle filled with physiological saline and then vortexing it. The dilution was conducted until the 10^5 dilutions. Isolates were obtained from the last three dilutions, namely 10^5, 10^4, 10^3. Then, 1 mL of the *schmutzdecke* suspension solution was poured onto a Petri dish using a micropipette. After that, Nutrient Agar and griseofulvin were added onto the Petri dish containing the suspension. The Petri dish was then closed and homogenized by rotating it with an 8-figure motion. Afterwards, the sides of the Petri dish were sealed with plastic wrap and the Petri dish was incubated in room temperature until the media solidified. The Petri dish was incubated for 24 hours. After the bacterial colonies grew, the process to observe the macroscopic characteristics of the colonies, the microscopic characteristics of the cells, and the physiological characteristics of the bacteria could be carried out (Purwanti et al., 2018a).

Table 1. Details of the slow sand filter reactor design

Detail	Size
Length of reactor	32 cm
Width of reactor	13 cm
Length of compartment I	16 cm
Length of compartment II	16 cm
Height of freeboard	10 cm
Height of buffer media	20 cm
Height of media compartment I	30 cm
Height of media compartment II	60 cm

Isolate purification

Isolates were purified by streaking each isolate, which has been identified as different macroscopically, on the NA media in different Petri dishes (Kurniawan et al., 2021). The isolates were streaked with the four-quadrant method. Then, the isolates were incubated for 24 hours and the pureness of the isolates was observed based on

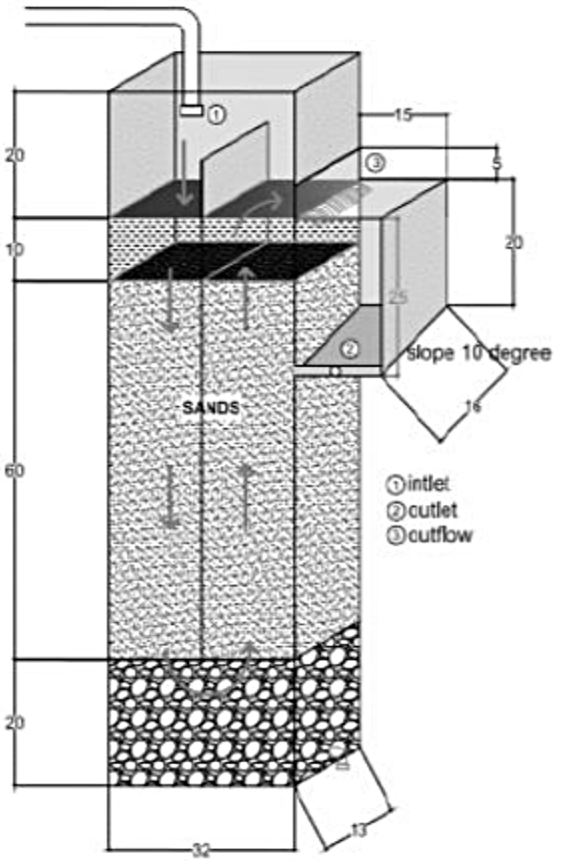


Figure 2. Slow sand filter reactor

the macroscopic and microscopic characteristics, which were conducted to discover whether the isolates are pure (Buhari et al., 2022). If the isolate was not pure, the isolate underwent another purification process until the isolate was pure. The isolate that was already pure was rejuvenated.

Isolate stocking

Isolates were stocked by taking one loop of each of the pure isolates. The isolates were then inoculated into different test tubes containing Nutrient Agar slants. After that, the isolates were incubated in an incubator in 37°C for 24 hours (Al-Ajalin et al., 2022; Imron et al., 2019). Each grown isolate was used for the microscopic and physiological identification tests.

Observation of the macroscopic characteristics of bacteria

The macroscopic characteristics of the bacterial colonies that were observed included the size, shape, elevation, edge, opacity, and pigmentation (Purwanti et al., 2018a).

Observation of the microscopic characteristics of bacteria

The microscopic characteristics of bacteria were observed using Gram staining. The first step of bacterial staining was to take the pure culture of bacteria from the test tube. Then, the bacteria taken from the test tube were smeared on the object glass that has 1 drop of distilled water on it. The cultured bacteria were smeared using a sterile inoculation loop. The sample in the object glass was then fixed by being passed through Bunsen burner flame until it dried (Al-Ajalin et al., 2022).

The bacteria on the object glass were then added with a drop of crystal violet solution and rested for one minute. After one minute, the object glass was rinsed with water and dried using a paper towel. The next step was to add a drop of Lugol's iodine solution to the object glass and let it rest for one minute, rinse it with water, and dry it with a paper towel. Lugol's iodine solution functions to bond the main color of the cell wall. The dried sample on the object glass was added with a drop of acetone alcohol solution, rested for 30 seconds, rinsed with water, and dried with a paper towel. Acetone alcohol functions as color remover. The final step was to add safranin solution. The sample that has been added with a drop of safranin was rested for one minute, then rinsed with water and dried. Safranin functions as a counterstain. The bacterial sample was then placed on a microscope to have its color and shape observed. If the bacteria are blue or purple, they are Gram-positive bacteria, whereas if the bacteria are red, they are Gram-negative bacteria (Ni'matuzahroh et al., 2020).

Identification through physiological test

A total of 3 loops from each of the bacterial isolates at 24 hours of age was taken using an inoculation loop and suspended into separate bottles filled with 10 mL of 0.8% sterile physiological saline. After that, 250 µL of the suspension was placed onto the wells of a Microbact kit 12A and 12B. The wells that have black circles were added with immersion oil if they tested positive for the oxidation test, while the wells with dotted circles were added with immersion oil if they tested negative for the oxidation test. Afterwards, the suspension on the kit was incubated for 24 hours. After the incubation, well 8 (indole) was added with 2 drops of nitrate A and nitrate B reagents and observed after 1 minute, well 10 (VP) was added with 1 drop of VPI and VPII reagents and observed after 30 minutes, while well 12 (TDA) was added with 1 drop of TDA reagent and observed immediately. Each well was observed based on the change of color, which means positive or negative. Besides using Microbact 12A and 12B kit, this research also involved an endospore staining test using Malachite green, catalase test using H_2O_2, motility test using SIM agar media, and endurance test in NaCl 5% solution.

Scanning electron microscope (SEM) analysis

SEM analysis was carried out on the control samples of clean sand times and shells as well as the samples from the filter media of three SSF reactors that have been grown with *schmutzdecke*. A total of 1–2 grams of samples were taken using a modified spatula aseptically from a certain point to a depth of ± 1 mm. The wet samples were dried naturally at room temperature for 24–48 hours and coated with gold (Au) using DC Sputter (Kurniawan et al., 2021). The samples that have become conductive were then observed using FE-SEM at 100x to 30,000x magnification, so several high-resolution SEM micrographs were obtained.

Data analysis

The data on the macroscopic, microscopic, and physiological characteristics from the biochemical identification tests were analyzed with Bergey's Manual of Determinative Bacteriology, 9th edition (Gerhardt et al., 1994) and Manual for The Identification of Medical Bacteria, 3rd edition (Cowan, 1974). The analysis results of the physiological characteristics show a percentage of species similarity, which is obtained through the Jaccard index (Real and Vargas, 1996). The following is the formula of the Jaccard index:

$$\text{Jaccard index} = \frac{a}{b} \times 100\% \quad (1)$$

where: a – the number of key characteristics of species that match the test results; b – the number of identification tests conducted.

RESULTS AND DISCUSSION

Macroscopic test results

On the basis of the macroscopic test results of 18 isolates found in different acclimatization periods, namely week 2 and week 3, the bacterial colonies in the *schmutzdecke* of slow sand filter reactors 1, 2, and 3 are as follows (Table 2 and Table 3).

Microscopic test results

On the basis of the results of the microscopic test of 18 isolates found in different acclimatization periods, namely week 2 and week 3, the shape, Gram stain, and endospore stain of bacteria in the *schmutzdecke* of slow sand filter reactors 1, 2, and 3 are as follows (Table 4, 5, and 6).

Physiological test results

On the basis of the results of the physiological test on 4 isolates found in different acclimatization periods, namely week 2 and week 3, the cell shape, Gram stain, and endospore stain of bacteria in the *schmutzdecke* layer of slow sand filter reactors 1, 2, and 3 are as follows (Table 7).

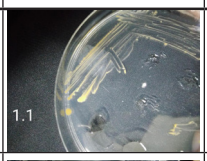
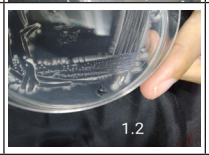
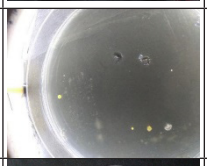
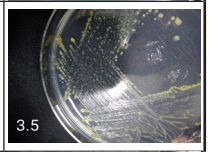
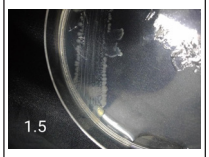
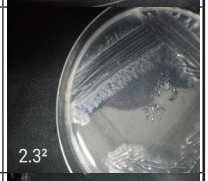
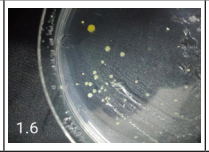
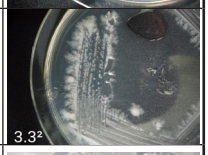
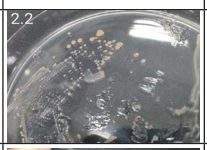
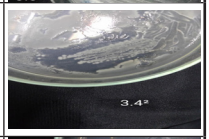
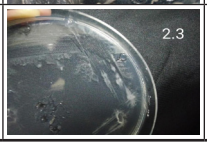
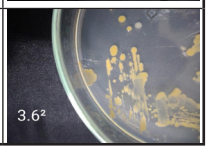
Identification of bacteria

On the basis of the identification results of macroscopic (Figure 3), microscopic, and physiological tests, the four isolates, namely isolates 1.6, 2.2, 3.4, and 3.6^2 were identified as *Kurthia gibsonii*, *Bacillus badius*, *Bacillus firmus,* and *Bacillus lentus* respectively. The similarities of these four isolates to the identified species were 83%, 81%, 85%, and 77% respectively. The percentage

Table 2. Morphological characteristics of bacterial colonies

No.	Isolate code	Characteristics					
		Shape	Edge	Elevation	Color	Size	Opacity
1.	1.1	Circular	Entire	Raised	Yellowish orange	Medium	Opaque
2.	1.2	Circular	Entire	Raised	White	Large	Opaque
3.	1.3	Circular	Entire	Raised	Clear	Small	Translucent
4.	1.4	Irregular	Undulate	Flat	White	Large	Opaque
5.	1.5	Irregular	Undulate	Flat	Clear	Medium	Translucent
6.	1.6	Circular	Entire	Raised	Yellow	Small	Opaque
7.	2.1	Circular	Entire	Flat	Yellow in the middle and white on the edge	Small	Translucent
8.	2.2	Circular	Entire	Raised	Pinkish orange	Small	Translucent
9.	2.3	Irregular	Filiform	Flat	White	Small	Translucent
10.	2.6	Circular	Entire	Raised	White	Small	Translucent
11.	3.4	Circular	Curled	Raised	White	Medium	Opaque
12.	3.5	Circular	Entire	Raised	Yellow	Medium	Opaque
13.	2.3^2	Irregular	Lobate	Raised	White	Medium	Opaque
14.	2.4^2	Circular	Curled	Flat	White	Medium	Translucent
15.	3.1^2	Circular	Entire	Flat	White	Large	Translucent
16.	3.3^2	Irregular	Lobate	Raised	White	Small	Translucent
17.	3.4^2	Circular	Entire	Raised	White	Medium	Opaque
18.	3.6^2	Circular	Entire	Raised	Orange	Medium	Translucent

Table 3. Macroscopic characteristics of bacteria

No.	Isolate code	Figure	No.	Isolate code	Figure
1.	1.1		10.	2.6	
2.	1.2		11.	3.4 (A)	
3	1.3		12.	3.5	
4.	1.4		13.	2.4^2	
5.	1.5		14.	2.3^2	
6.	1.6 (A)		15.	3.1^2	
7.	2.1		16.	3.3^2	
8.	2.2 (A)		17.	3.4^2	
9.	2.3		18.	3.6^2 (A)	

Note: A – abundant.

was calculated according to their macroscopic, microscopic, and physiological characteristics that have been matched with the information in the identification books.

The identification results from the macroscopic, microscopic, and physiological tests of the four dominant isolates show the similarity percentage as follows. Isolate 1.6 has 83% similarity to *Kurthia gibsonii* in terms of macroscopic, microscopic, and physiological characteristics. The macroscopic, microscopic, and physiological characteristics of isolate 2.2 have 81% similarity to those of *Bacillus badius*. The macroscopic, microscopic, and physiological characteristics of isolate 3.4 are 85% similar to those of *Bacillus firmus.* Finally, isolate 3.6^2 is 77% similar to *Bacillus lentus* in terms of macroscopic, microscopic, and physiological characteristics. The percentage was based on the analysis results using Jaccard index. The index

Table 4. Observation results of the shape and gram staining of bacteria

No	Isolate code	Staining result		Note
		Shape	Gram	
1.	1.1	Rod-shaped	+	1000x magnification
2.	1.2	Chain rod-shaped	+	1000x magnification
3.	1.3	Rod-shaped	+	1000x magnification
4.	1.4	Chain rod-shaped	+	1000x magnification
5.	1.5	Rod-shaped	+	1000x magnification
6.	1.6 (dominant)	Rod-shaped	+	1000x magnification
7.	2.1	Rod-shaped	+	1000x magnification
8.	2.2 (dominant)	Rod-shaped	+	1000x magnification
9.	2.3	Chain rod-shaped	+	1000x magnification
10.	2.6	Rod-shaped	+	1000x magnification
11.	3.4 (dominant)	Rod-shaped	+	1000x magnification
12.	3.5	Chain rod-shaped	+	1000x magnification
13.	2.3^2	Chain rod-shaped	+	1000x magnification
14.	2.4^2	Rod-shaped	+	1000x magnification
15.	3.1^2	Rod-shaped	+	1000x magnification
16.	3.3^2	Rod-shaped	+	1000x magnification
17.	3.4^2	Chain rod-shaped	+	1000x magnification
18.	3.6^2 (dominant)	Rod-shaped	+	1000x magnification

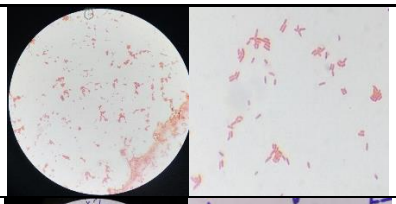
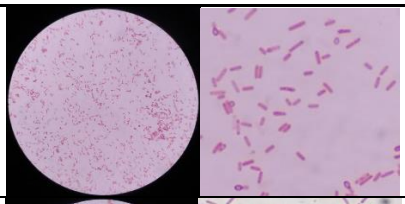
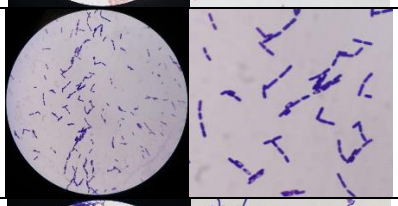
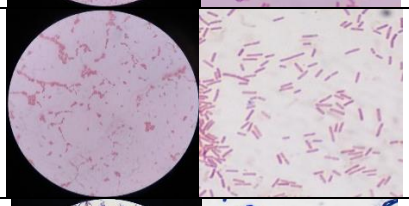
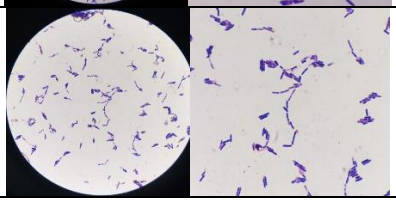
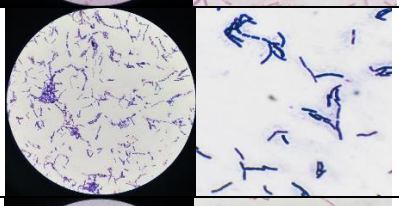
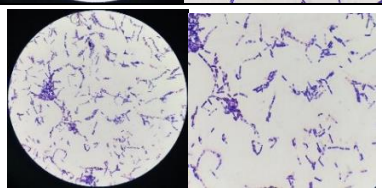
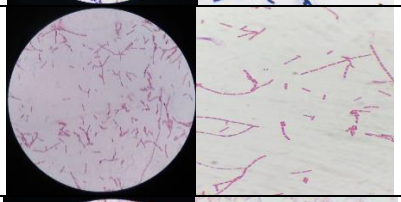
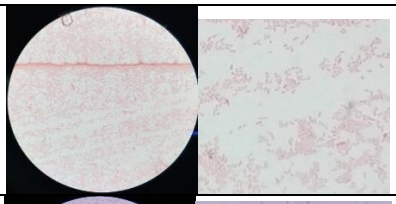
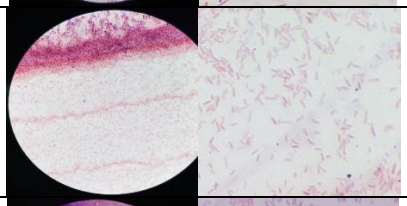
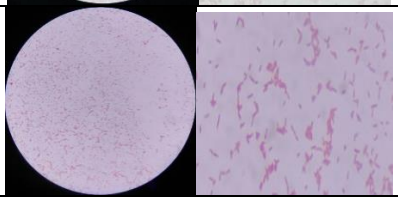
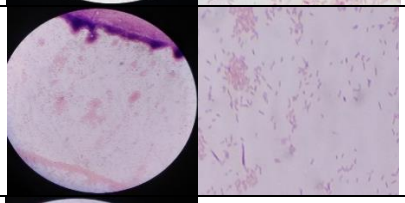
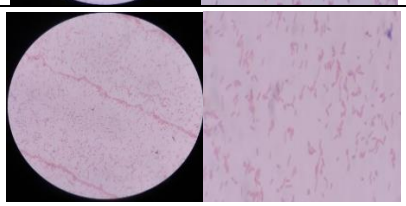
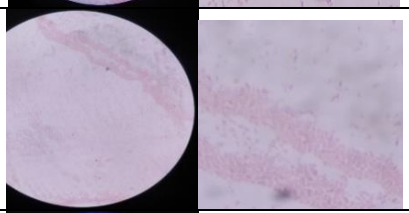
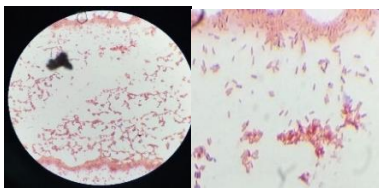
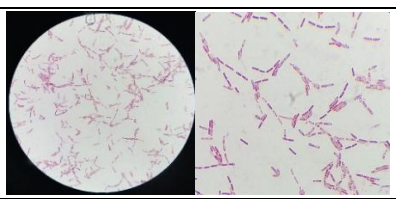
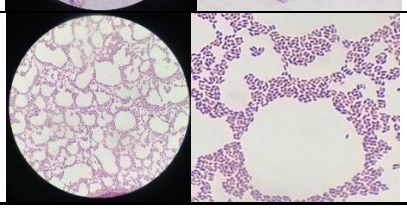
Table 5. Results of endospore observation

Isolate code	Test result	Note
1.6	-	1000x magnification
2.2	+	1000x magnification
3.4	+	1000x magnification
3.6^2	-	1000x magnification

analysis was carried out according to the calculation of macroscopic, microscopic, and physiological characteristics that were matched with the information in Bergey's Manual of Determinative Bacteriology, 9th edition and Manual for The Identification of Medical Bacteria, 3rd edition. *Kurthia gibsonii* has the same microscopic characteristics as isolate 1.6. Its cell is rod-shaped, its size is smaller than 3µ, is has no endospores, and it is Gram positive. Macroscopically, the bacteria on the Nutrient Agar media formed a colony with a circular shape, entire edge, raised elevation, yellow color, small size, and opaque opacity. Physiologically, isolate 1.6 can hydrolyze gelatin, generate catalase enzyme, hydrolyze amylum, and reduce nitrate. However, *Kurthia gibsonii* cannot hydrolyze amylum nor reduce nitrate. Besides, isolate 1.6 is motile and did not reduce H_2S, generate acetoin during VP test, nor ferment glucose, xylose, mannitol, rhamnose, and sucrose on Microbact 12A and 12B kit (Cowan, 1974). *Kurthia gibsonii* is also easily isolated from meat, fat, wastewater, etc. (Stackebrandt et al., 2006). According to Samayamanthula et al. (2019), greywater is the product of domestic waste from kitchen activities, baths, laundry, and air conditioners, so *Kurthia gibsonii* can be found in domestic wastewater.

The microscopic characteristics of isolate 2.2 include a non-chain rod-shaped cell, size smaller than 3µ, endospores that are central, oval-shaped, and non-swelling, and Gram-positive bacteria. *Bacillus badius* has the same microscopic characteristics as isolate 2.2, except in size and cell shape. *B. badius* is chain rod-shaped bacteria. Macroscopically, isolate 2.2 formed a colony in the Nutrient Agar media that is circular, curled-edged, raised, pinkish orange, small, and translucent. Physiologically, isolate 2.2 can generate oxidase enzyme and hydrolyze amylum, whereas *B. badius* cannot hydrolyze amylum. Isolate 2.2 cannot produce indole by hydrolyzing tryptophan, generate urease enzyme, nor reduce nitrate. Moreover, isolate 2.2 is motile, unable to generate β-galactosidase during the ONPG test, unable to generate acetoin during the VP test, unable to ferment glucose, raffinose, xylose, or salicin, and unable to reduce citrate as

Table 6. Microscopic characteristics of bacteria

No	Isolate code	Figure (1000x magnification)	No	Isolate code	Figure (1000x magnification)
1.	1.1		10.	2.6	
2.	1.2		11.	3.4 (A)	
3.	1.3		12.	3.5	
4.	1.4		13.	2.3[2]	
5.	1.5		14.	2.4[2]	
6.	1.6 (A)		15.	3.1[2]	
7.	2.1		16.	3.3[2]	
8.	2.2 (A)		17.	3.4[2]	
9.	2.3		18.	3.6[2] (A)	

Note: A = Abundant

Table 7. Results of physiological observation

Identification	Isolate code			
	1.6	2.2	3.4	3.6²
Gram	+	+	+	+
Cell structure	-	-	-	-
Motility	+	+	-	+
Length of cell > 3µ	-	-	-	-
Shape and position of endospores	-	Central and oval	Central and oval	-
Swelling cell due to endospores	-	-	-	-
Arginine	+	-	+	-
Adonitol	-	-	-	-
Arabinose	-	-	-	-
Lactose	-	-	-	-
Sucrose	-	-	-	-
Rhamnose	-	-	-	-
Sorbitol	-	-	-	-
Inositol	-	-	-	-
Malonate	-	-	-	-
Gelatine	+	+	-	+
TDA	-	-	-	-
Mannitol	-	-	-	-
H_2S	-	-	-	-
Ornithine	-	-	-	-
Lysine	-	-	-	-
Glucose	-	-	-	+
Raffinose	-	-	-	-
Salicin	-	-	-	-
Xylose	-	-	-	-
ONPG	+	-	-	-
Citrate	+	-	-	-
Urease	-	-	-	-
Indole	-	-	-	-
Nitrate	+	-	+	-
VP	-	-	-	-
Oxidase	-	+	+	+
Catalase	+	+	+	+
Amylum hydrolysis	+	+	+	+

Note: * Yellow is the characteristic used in and matched with the identification books.

the source of carbon that increases pH on Microbact 12A and 12B kit. *Bacillus badius* is widely distributed in the environment, with soil as its primary habitat and seawater as well as freshwater as its secondary habitats. Due to the lack of interest in this species of bacteria, little is known about the role of the bacteria in the environment besides producing antibiotics such as gramicidin and tyrocidine (Priest, 2014).

The microscopic characteristics of isolate 3.4 consist of a non-chain rod-shaped cell, size smaller than 3µ, Gram-positive type, and non-swelling, central, and oval-shaped endospores. Macroscopically, the isolate formed a colony on the Nutrient Agar medium that has a circular shape, entire edge, raised elevation, white color, medium size, and opaque opacity. Physiologically, isolate 3.4 cannot produce indole by hydrolyzing tryptophan, generate urease enzyme, nor reduce nitrate. It is not motile, but according to the identification books, *B. firmus* is motile. Furthermore, isolate 3.4 did not generate acetoin

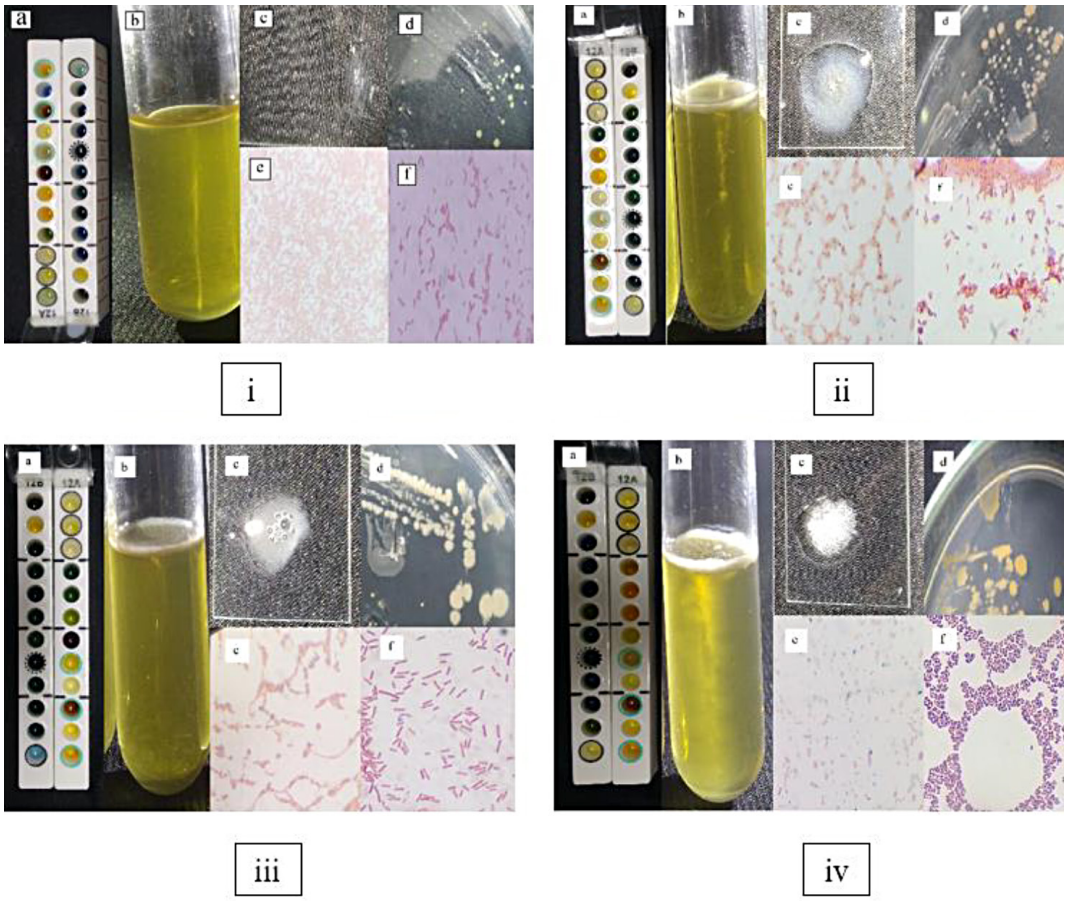


Figure 3. Physiological test results of isolates 1.6 (i), 2.2 (ii), 3.4 (iii), and 3.62 (iv); a) Microbact 12A and 12B test kit; b) motility test on SIM agar media; c) catalase test using H_2O_2 reagent; d) macroscopic shape of the colony; e) endospore staining test results; f) cell shape and Gram type of bacteria based on Gram staining results

during the VP test, ferment glucose, raffinose, xylose, or salicin, nor reduce citrate as the source of carbon that increases pH on Microbact 12A and 12B kit. Isolate 3.6^2 is a non-chain, rod-shaped, Gram-positive bacteria that is smaller than 3µ and has no endospores. *Bacillus lentus* has the same microscopic characteristics as isolate 3.6^2, except it has endospores. The endospores of *B. lentus* are oval-shaped, central, and non-swelling. Macroscopically, the isolate formed a colony in the Nutrient Agar media that is circular, entire-edged, raised in elevation, orange, medium-sized, and translucent. Physiologically, isolate 3.6^2 can generate oxidase enzyme, hydrolyze amylum, and ferment glucose. Isolate 3.6^2 cannot generate urease enzyme or β-galactosidase during the ONPG test, whereas *B. lentus* can produce urease enzyme and β-galactosidase during the ONPG test. Isolate 3.6^2 cannot generate acetoine during the VP test, produce indole by hydrolyzing tryptophan, ferment raffinose, xylose, or salicin, nor reduce citrate as the source of carbon that increases pH on Microbact 12A and 12B kit. *Bacillus lentus* and *Bacillus firmus* are halotolerant bacteria that are commonly found in seawater or estuary habitats. They are alkaliphilic bacteria that are spread in soil and able to survive under alkaline pH conditions (Priest, 2014). Greywater is wastewater consisting of waste from kitchen, laundry, and air conditioners (Samayamanthula et al., 2019), so it enables *Bacillus lentus* and *Bacillus firmus* to grow under such environmental conditions.

SEM analysis

On the basis of Figure 4, the surface morphology of the sand grain control sample is very rough and irregular (Kurniawan et al., 2022b). It has uneven edges and a lot of pores with the size of approximately 0.1 µm. It is different from the surface of the *Anadara granosa* shell grain control sample, which seems to have a structure that resembles layered rod-shaped sheets with sharp-edged flakes of a similar shape and a lot of empty cavities with a diameter larger than 0.1 µm (Moideen et al., 2016). The morphological

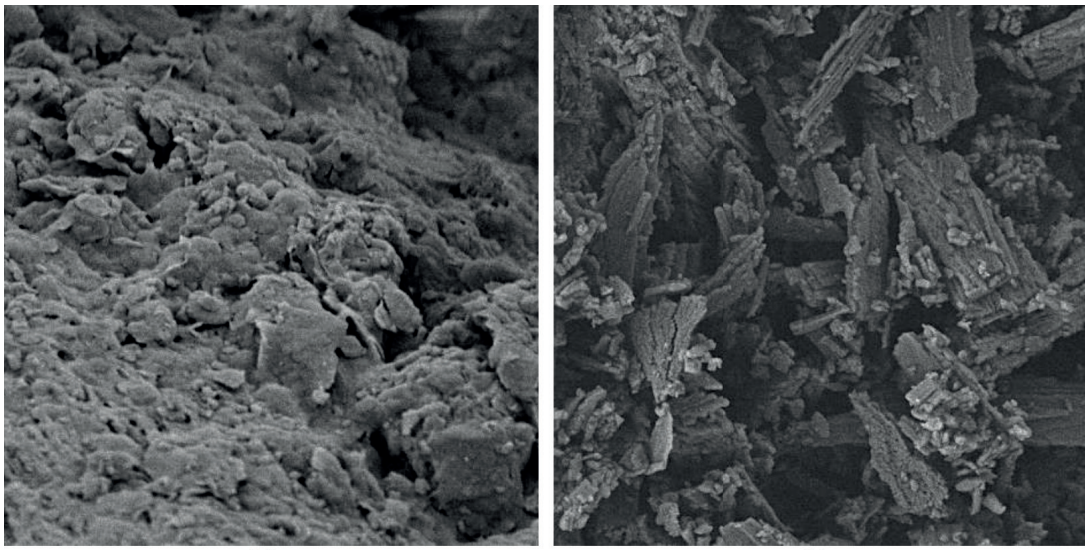


Figure 4. SEM micrographs of (a) sand and (b) shell control samples

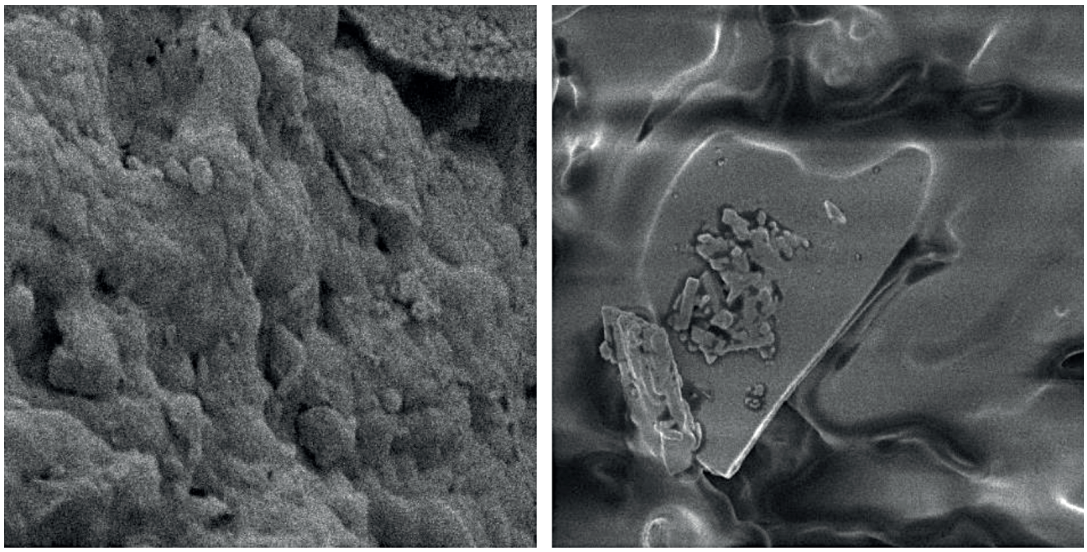


Figure 5. SEM micrographs of (a) sand and (b) shell samples after acclimatization

characteristics of *Anadara granosa* shell grains are formed by the accumulation of rod particles with single fiber, each of which has the size of 15–50 nm (Harahap et al., 2015).

Each of the sample media that has been covered by a *schmutzdecke* layer in each SSF reactor has a different morphological appearance (Figure 5). In general, the micropores of sand grains, which previously seemed clean and could be seen clearly, started to be covered by a slime-like layer (biofilm) and the accumulation of pollutant particles carried by greywater. The most significant change occurred to the *Anadara granosa* shell sample obtained from SSF 2 (Figure 5b), which had a shiny slime layer covering the entire media surface. It could be seen very clearly despite only using 5,000x magnification. The microenvironment created on the top part of the SSF reactor still lacks nutrients at the start of the acclimatization period (Joubert and Pillay, 2008). Over time, the media proliferation process and extracellular slime production by bacterial population become evident. The slime is the beginning of the *schmutzdecke* growth as the media where microbes, including heterotrophic bacteria, stick (Law et al., 2001).

CONCLUSIONS

On the basis of the identification results from macroscopic, microscopic, and physiological tests, the species of the four dominant isolates, namely isolates 1.6, 2.2, 3.4, and 3.6[2], are *Kurthia gibsonii*, *Bacillus badius*, *Bacillus firmus,* and *Bacillus lentus*, respectively. The four isolates have a similarity percentage of 83%, 81%, 85%, and 77%, respectively.

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