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PROSES IDENTIFIKASI, ISOLASI, PEMURNIAN, PEWARNAAN GRAM, DAN PENGUJIAN BIOKIMIA BAKTERI AKUATIK DARI BERBAGAI SUMBER

IDENTIFICATION, ISOLATION, PURIFICATION, GRAM STAINING, AND BIOCHEMICHAL TESTING PROCESSES OF AQUATIC BACTERIA FROM DIFERENT SOURCES

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ABSTRAK

Bakteri akuatik memainkan peran penting dalam ekosistem dan memiliki aplikasi signifikan dalam bioteknologi dan akuakultur. Penelitian ini bertujuan untuk mengidentifikasi, mengisolasi, memurnikan, melakukan pewarnaan Gram, dan melakukan pengujian biokimia pada bakteri akuatik yang bersumber dari berbagai lingkungan. Metode ini melibatkan pemurnian isolat dan mengidentifikasi morfologi koloni makroskopis dan mikroskopis menggunakan pewarnaan Gram dan uji biokimia untuk menentukan keberadaan bakteri Gram-negatif dan Gram-positif di lingkungan akuatik. Hasil penelitian ini mengidentifikasi *Listeria* sp., *Staphylococcus* sp., dan *Enterobacter* sp. dari air tawar; *Vibrio cholerae* dan *Vibrio parahaemolyticus* dari air payau; dan *Bacillus* sp., *Corynebacterium* sp., dan *Vibrio* sp. dari air laut. Temuan ini memberikan wawasan tentang keragaman dan distribusi bakteri akuatik di berbagai sumber air.

ABSTRACT

Aquatic bacteria play a crucial role in ecosystems and have significant applications in biotechnology and aquaculture. This study aims to identify, isolate, purify, perform Gram staining, and conduct biochemical testing on aquatic bacteria sourced from various environments. The method involves purifying isolates and identifying macroscopic and microscopic colony morphology using Gram staining and biochemical tests to determine the presence of both Gram-negative and Gram-positive bacteria in aquatic environments. The results of this study identified Listeria sp., Staphylococcus sp., and Enterobacter sp. from freshwater; Vibrio cholerae and Vibrio parahaemolyticus from brackish water; and Bacillus sp., Corynebacterium sp., and Vibrio sp. from marine water. These findings provide insights into the diversity and distribution of aquatic bacteria across different water sources.

Keywords: Aquatic, Aquaculture, Bacteria, Microbiology

INTRODUCTION

Microorganisms are entities that cannot be seen directly, existing as microscopic organisms of various kinds that live either independently or in colonies. The identification and characterization of these bacteria are fundamental for understanding their roles in natural processes and their potential applications in industries such as biotechnology and aquaculture. The process of studying aquatic bacteria involves several steps, including isolation, purification, Gram staining, and biochemical testing. Isolation and purification are critical for obtaining pure bacterial cultures that can be accurately studied and characterized. Gram staining, a differential staining technique, helps in classifying bacteria into Gram-positive and Gram-negative groups based on their cell wall structure. Biochemical testing further elucidates the metabolic and enzymatic activities of the bacteria, providing deeper insights into their functional capabilities. There are two methods for observing these microorganisms: the hanging drop method and the simple native drop method, both of which are done without fixation or special fluids (Suroiyah et al., 2018). According to Woo et al. (2022), the isolation of bacteria is a process of extracting bacteria from a medium or from their original environment, and then cultivating them in an artificial medium to obtain a pure culture. Bacteria are single-celled organisms without a nucleus, living in water or soil. Gram staining is used to differentiate between two groups of bacteria: Gram-negative and Gram-positive, which are classified based on their cell wall structure. Gram-positive bacteria have simpler cell walls, while Gram-negative bacteria have more complex ones. Additionally, there are two types of stains: positive and negative. Positive stains contain basic dyes, while negative stains contain acidic dyes. Methylene blue is commonly used for simple and positive staining (Saputra et al., 2022). In this study, we aimed to identify bacteria present in different aquatic environments. Aquatic environments harbor a diverse range of bacterial species that are essential for maintaining ecological balance and contributing to various biogeochemical cycles.

MATERIALS AND METHODS

2.1. Collection and transportation of samples – This research was conducted at the Integrated Laboratory 1, FIKKIA, Universitas Airlangga Banyuwangi. The equipment used in this experiment includes an inoculating loop, Bunsen burner, test tubes, test tube rack, micropipette, incubator, petri dishes, Drigalski spatula, media refrigerator, bacterial isolate refrigerator, beaker glass, glass slides, microscope, basin, autoclave, oven, Erlenmeyer flasks, Durham tubes, hot plate, magnetic stirrer, analytical balance, spatula, volumetric pipette, and bulb. The materials used in this experiment include TSA+NaCl media, labels, markers, sample water, plastic wrap, alcohol, 95% ethanol, pure bacterial cultures, distilled water, crystal violet solution, potassium iodine (Lugol), safranin, oxidase strips, SIM media, OF media, TSIA media, SCA media, MRVP media, sugar test media, paraffin, aluminum foil, Kovac's reagent, methyl red, and Voges-Proskauer.

2.2. Sample processing and enrichment of bacteria - Aseptic measures were undertaken during the sampling procedure to prevent contamination of the samples. 8 sample waters were collected for microbiological test. These specimens were taken in a sterile chopping board and then minced properly and grinded together. Ten (10) gm of samples were homogenized with 90 milliliters(ml) of freshly prepared 0.1% peptone water and 0.1 ml of homogenized sample was inoculated according to standard methods on to selective media such as: Thiosulfate citrate bile salt sucrose (TCBS) agar (for *Vibrio* spp.), Tryptic Soy Agar (TSA) for enrichment of bacterial isolates and finally incubated at 37ºC for 24 hours (Ed-har et al., 2017).

2.3. Isolation of bacterial - Samples were stored in bottles and sealed. Before streaking the inoculating loop on the media, the bottle containing the water sample was shaken to evenly distribute the bacteria. Bacterial isolation was performed using two methods: spread and streak. The spread method used a Drigalski spatula, with 100 microliters of the water sample taken using a micropipette. The water sample was spread on the surface of the media and leveled with the Drigalski spatula, which was heated over a Bunsen burner beforehand to ensure aseptic technique. Petri dishes were covered with plastic wrap to minimize contamination. For the streak method, a loop was used. The loop was first sterilized by heating, then dipped into the water sample. The moistened loop was then streaked onto the surface of the media in a four-quadrant pattern. This procedure was carried out near a Bunsen burner to maintain aseptic conditions. Petri dishes were covered with plastic wrap to minimize contamination. After wrapping the petri dishes in plastic wrap, they were incubated at 37°C for 24-48 hours. Following incubation, the morphological characteristics of the colonies were identified to estimate the bacteria that grew on the media.

2.4 Culture and Purification of bacterial - The purification of bacterial isolates involves several stages, including preparing and sterilizing equipment and materials, creating a four-quadrant pattern on petri dishes containing TSA media to facilitate the streaking process, heating the inoculating loop to sterilize it, streaking the sterile loop on TSA media with bacterial growth to pick up bacterial colonies, and then streaking the loop containing the bacterial isolate onto the prepared TSA media using the four-quadrant streaking technique. The petri dishes are then sealed and wrapped with plastic wrap to prevent or reduce the risk of contamination. The TSA media can then be incubated in an incubator at a temperature of 35-37ºC for 24 hours, with the petri dishes inverted.

2.5 Gram staining of bacterial

The procedure for Gram staining begins with cleaning the glass slide using alcohol. The slide is then sterilized over a Bunsen burner until dry. Next, a loopful of bacteria is inoculated onto the slide, spread evenly, and a drop of distilled water is added. The sample is then heat-fixed over the flame of the Bunsen burner. Once dry, 2-3 drops of crystal violet solution are applied and left for 1 minute before rinsing with distilled water. After this, 2-3 drops of Lugol's iodine (potassium iodide) are added, left for 1 minute, and then rinsed with distilled water. Following this, 95% ethanol is applied for 30 seconds and rinsed with distilled water. The final solution is safranin, with 2-3 drops added and left for 2 minutes before rinsing with distilled water. The slide is then air-dried and observed under a microscope.

2.6 Biochemical test of bacterial

Suspected bacterial colonies obtained from different culture plates were isolated and then streaked on TSA and TSA + NaCl agar, followed by overnight incubation at 37°C. The pure isolates were characterized by bacterial cell morphology, hydrogen sulfide (H2S) and gas production, motility test, indole production, TSIA test, oxidase test, catalase test, Methyl Red (MR) test, Voges-Proskauer (VP) test, and glucose test. Gram staining techniques were performed to identify Gram-positive and Gram-negative bacteria. Biochemical tests were conducted to identify the pathogens following Bergey's Manual of Determinative Bacteriology (Cowan, 1974).

Media preparation for the MR-VP test involved weighing 1.7 grams of MR-VP media using an analytical balance. The media was then homogenized with 100 ml of distilled water using a hot plate and magnetic stirrer. After homogenization, the media was autoclaved for 1 hour at 121°C. The sterilized media was dispensed into test tubes with a volume of 2 ml and stored in a sterile refrigerator. A loopful of bacteria was inoculated into the media and incubated for one day. Methyl Red and Voges-Proskauer reagents were added to the media, and any color changes were observed. For SCA media preparation, 1.214 grams of SCA media was weighed using an analytical balance. The media was homogenized with 100 ml of distilled water using a hot plate and magnetic stirrer. The media was then autoclaved for 1 hour at 121°C, dispensed into test tubes with a volume of 5 ml, and stored in a sterile refrigerator. A sterilized inoculating needle was used to transfer a loopful of bacteria into the SCA media, which was then incubated for one day. Any color changes in the media were observed (Leboffe et al., 2012).

The preparation of sugar test media involved weighing 2.102 grams each of sucrose, lactose, maltose, and dextrose media using an analytical balance. The media was homogenized with 100 ml of distilled water using a hot plate and magnetic stirrer. The homogenized media was dispensed into 20 test tubes, with a Durham tube placed inside each test tube to collect any gas produced during incubation. The media was autoclaved for 1 hour at 121°C and stored in a sterile refrigerator. A loopful of bacteria was inoculated into each of the sucrose, maltose, lactose, and dextrose media, followed by one day of incubation. Any color changes in the media were then observed.

RESULT AND DISCUSSION

The isolated bacterial aquatic was founded from different source are presented in Table 1. Out of 8 water samples and among the total isolation of 8 bacterial strains are *Listeria* sp., *Staphylococcus* sp. and *Enterobacter* sp. from freshwater, *Vibrio chlorella* and *Vibrio parahaemolyticus* from brackish water, and *Bacillus* sp., *Corynobacterium* sp., and *Vibrio* sp from marine water. The identification of the pure bacterial isolates was performed by biochemical parameters included alkaline reaction, acidic reaction, H2S (hydrogen sulfide production) gas production, motility test, indole production, urea hydrolysis, catalase test, oxidase test, Methyl-Red (MR) test, Voges-Praskaure (VP) test are presented in Table 2. According to Woo et al. (2022), bacterial isolation is a process of taking bacteria from their original medium or environment and growing them in an artificial medium to obtain pure cultures. The purpose of bacterial isolation is to identify, characterize, and further study the types of bacteria present in the samples (Marzan et al., 2017).

Based on identification with Cowan and Steel, the results of biochemical tests and the biochemical characteristics produced by the bacteria indicate that the bacteria are *Listeria* sp., *Staphylococcus* sp., and *Enterobacter* sp. from freshwater; *Vibrio cholerae* and *Vibrio parahaemolyticus* from brackish water; and *Bacillus* sp., *Corynebacterium* sp., and *Vibrio* sp. from marine water in Table 2.

The findings of this study underscore the diverse and complex nature of aquatic bacterial communities across various aquatic environments. The identification of both Gram-negative and Gram-positive bacteria from freshwater, brackish, and marine water sources highlights the adaptability and ecological significance of these microorganisms. In freshwater environments, the isolation of *Listeria* sp*., Staphylococcus* sp., and *Enterobacter* sp. suggests the presence of bacteria that are not only pathogenic but also capable of thriving in nutrient-rich environments. *Listeria* sp. and *Staphylococcus* sp., known for their roles in foodborne illnesses and as opportunistic pathogens, respectively, indicate potential health risks associated with freshwater sources, particularly in aquaculture settings (Farber & Peterkin, 1991; Chambers & DeLeo, 2009). *Enterobacter* sp., a member of the Enterobacteriaceae family, is commonly found in water and soil and may play a role in nitrogen cycling within these ecosystems (Podschun & Ullmann, 1998).

Based on the water sample from the river, bacterial colonies grew with morphologies such as milky white to yellowish color, irregular round colonies, smooth surface, and elevated surface. The bacteria that grew, based on colony morphology is *Staphylococcus* sp. the bacteria obtained had cream-is supported by research conducted by Ma'fullah and Meishanti (2021), which states that they obtained several bacterial samples, one of which was *Staphylococcus* from the river. Additionally, research by Djunaidy and Putri (2020) states that *Staphylococcus* colonies appear white on TYC (Trypticase Yeast Cysteine) medium. Based on these references, it is presumed that the bacteria on the TSA medium are *Staphylococcus.* Gram staining is the most important and widely used differential staining technique to classify bacteria into two groups, Gram-positive and Gram-negative (Asfiyah et al., 2024). Gram staining is used to observe bacterial Gram characteristics and morphology. Gram staining was discovered by Hans Christian Gram in 1882 to identify bacteria or organisms causing pneumonia (Budiyanto et al., 2021). Gram-positive bacteria (+) retain crystal violet dye, making the bacterial cells appear blue or dark purple. Gram-negative bacteria (-) lose crystal violet when washed with alcohol and appear red because they absorb safranin stain (Asfiya et al., 2024).

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According to Asfiya *et al.* (2024), the ability of bacteria to absorb dye depends on their cell wall structure or peptidoglycan. Gram-positive bacteria have thicker cell walls than Gram-negative bacteria. This structure allows Gram-positive bacteria to retain crystal violet during the alcohol wash. Gram-negative bacteria have thinner cell walls. After washing with alcohol, the lipopolysaccharide layer in Gram-negative bacteria increases permeability, allowing the previous dye to be more easily removed from the cell. Consequently, during the safranin staining process, Gram-negative bacteria accept safranin and appear red. Based on Gram staining results, the identified bacteria were purple (Gram +), coccus-shaped or round, and clustered like grapes. The identified bacteria are *Staphylococcus* sp. According to Ora (2024), *Staphylococcus* sp. has characteristics of being coccus-shaped, clustered like grapes, non-flagellated, non-sporulating, non-capsulated, and Gram-positive. The advantages of

Gram staining include serving as a preliminary guide for identification, easy to perform to determine colony shape and arrangement. The disadvantages of Gram staining are that it can only determine the shape, color of the bacteria, and colonies, requiring further tests to confirm the identified bacterial species, expensive safranin dye, and safranin waste can impact health, causing irritation to the mouth, throat, respiratory system, and stomach (Asfiya et al., 2024).

Based on biochemical test results, the oxidase test showed positive results indicated by the oxidase strip turning blue, as the tested bacteria have the oxidase enzyme for aerobic respiration. The catalase test showed positive results indicated by the presence of foam or air bubbles, as the bacteria have the catalase enzyme that catalyzes the decomposition of H2O2 into water and oxygen. The SIM test showed positive motility indicated by the bacteria spreading and the medium turning black due to H2S production, indicating that the bacteria have flagella for movement (Reimena et al., 2017). The indole test showed negative results indicated by the absence of red color after adding Kovac's reagent, as the bacterial isolate could not break down the amino acid tryptophan to form indole (Mohamed et al., 2020).

The O/F test showed fermentative results indicated by the O/F medium with paraffin turning yellow while the O/F medium without paraffin did not change color, indicating that the bacteria can break down carbohydrates without oxygen (fermentation) and are anaerobic. The TSIA test showed a red color indicating an alkaline medium, black precipitate indicating H2S formation, and gaps in the medium indicating gas production. This indicates that the bacteria cannot ferment glucose, lactose, and sucrose as the medium did not change color (Reimena et al., 2017). The SCA test showed negative results indicated by the medium not turning blue, as the bacterial isolate lacks citrate permease or other necessary enzymes, thus not using citrate medium as a carbon source (Mohamed et al., 2020). The MR-VP test showed positive MR results indicated by red color and negative VP results indicated by no color change. The red color in MR indicates a pH drop by acids produced from sugar fermentation, while VP negative means the end product of bacterial fermentation is not Acetyl Methyl Carbinol (Acetoin). The sugar tests (sucrose, maltose, lactose, and dextrose) showed that only the dextrose medium turned yellow, while the others (sucrose, maltose, lactose) did not change color, indicating that the bacterial isolate produces acid from dextrose fermentation but cannot ferment sucrose, maltose, and lactose. Additionally, gas bubbles in the Durham tube of the dextrose and maltose media indicate gas production by the bacteria (Reimena et al., 2017).

The presence of *Vibrio cholerae* and *Vibrio parahaemolyticus* in brackish water highlights the unique challenges posed by transitional aquatic environments. Both species are well-known pathogens associated with seafood-related illnesses, particularly in areas where aquaculture practices are prevalent (Lipp et al., 2002; Su & Liu, 2007). Their detection in brackish water suggests that these environments may serve as reservoirs for pathogenic *Vibrio* species, posing a potential risk to public health and aquaculture industries.

Marine water samples revealed a broader diversity of bacteria, including *Bacillus* sp., *Corynebacterium* sp., and *Vibrio* sp. The detection of *Bacillus* sp., a genus known for its diverse metabolic capabilities and potential as a probiotic in aquaculture, points to its possible role in promoting the health and growth of marine organisms (Cutting, 2011). *Corynebacterium* sp., although less studied in marine environments, may contribute to the breakdown of organic matter and nutrient cycling (Bernard, 2012). The presence of *Vibrio* sp. in marine water, similar to brackish water, underscores the importance of monitoring *Vibrio* populations in marine aquaculture to mitigate risks associated with *Vibrio*-related diseases (Thompson et al., 2004).

CONCLUSION

Overall, the study provides valuable insights into the distribution of aquatic bacteria and their potential implications for aquatic ecosystems and aquaculture. The identification of pathogenic species, alongside beneficial ones, highlights the need for ongoing monitoring and management strategies to ensure the health and sustainability of aquatic environments. Future research should focus on understanding the functional roles of these bacteria and exploring their interactions with aquatic organisms to further elucidate their ecological and biotechnological significance.

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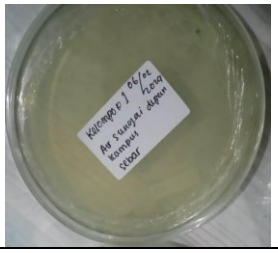
ATTACHMENT

Table 1. Isolation and Colony Morphology of Bacterial from Different Aquatic Environment

Source	Bacterial Identification	Morphology and Colony Description
Freshwater	*Listeria sp.*	Creamy yellow colonies on TSA medium
Freshwater	*Staphylococcus sp.*	Milky white to yellowish colonies, irregularly round, smooth surface, and elevated on TSA medium
Freshwater	*Enterobacter sp.*	Round colonies, creamy white, convex elevation with flat edges on TSA medium
Brackish water	*Vibrio cholerae*	Yellow colonies on selective TCBS medium
Brackish water	*Vibrio parahaemolyticus*	Greenish-yellow colonies on selective TCBS medium
Marine water	*Bacillus sp.*	Cream-colored colonies, round, uneven edges on TSA medium
Marine water	*Corynebacterium sp.*	Pure isolate colonies (cream-colored) on TSA medium
Marine water	*Vibrio sp.*	White to yellowish colonies on TSA + NaCl medium

Table 2. Colony and Morphology Bacterial Aquatic

Source	Bacterial Identification	Figure Colony
Freshwater	*Listeria* sp.	
Freshwater	*Staphylococcus* sp.	

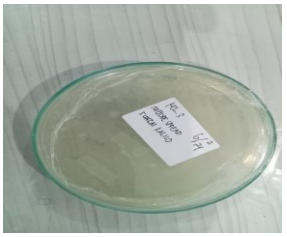
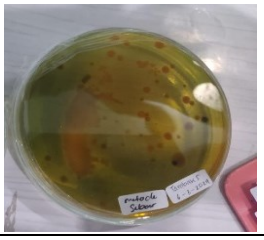
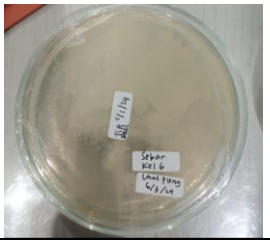
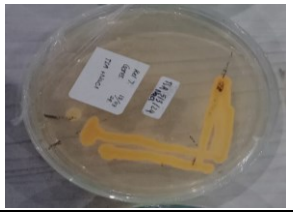
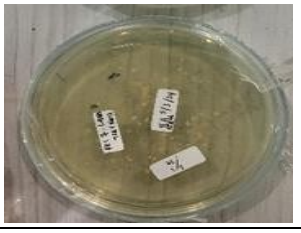
Freshwater	*Enterobacter* sp.	
Brackish water	*Vibrio cholerae*	
Brackish water	*Vibrio parahaemolyticus*	
Marine water	*Bacillus* sp.	
Marine water	*Corynobacterium* sp.	
Marine Water	*Vibrio* sp.	

Table 3. Results of biochemical tests of the isolated bacterial species from different aquatic environment

Aquatic Water Sample	Source	Bacterial isolates	Gram reaction	Oxidase	Catalase	Motility	OF	TSIA	Indole	SCA	MR	VP	Laktosa	Maltosa	Dextros	Maltosa	Sucrose
Freshwater	River	*Listeria* sp.	Gram positive	-	+	+	F	+	+	-	+	-	+	+	+	+	-
Freshwater	River	*Staphylococcus* sp.	Gram positive	+	+	+	F	-	-	-	+	-	-	-	+	-	-
Freshwater	River	*Enterobacter* sp.	Gram negative	-	+	+	F	+	-	-	+	-	+	+	+	+	-
Brackish water	Shrimp pond	*Vibrio cholerae*	Gram negative	-	+	-	F	-	-	+	+	+	-	+	+	+	+
Brackish water	Shrimp pond	*Vibrio parahaemolyticus*	Gram negative	-	+	+	F	-	+	+	+	-	-	-	+	-	-
Marine water	Mangrove forests	*Bacillus* sp.	Gram positive	+	+	-	O	-	-	+	+	+	-	+	+	+	+
Marine water	Estuaries	*Corynobacterium* sp.	Gram positive	-	+	-	F	+	+	+	+	-	+	+	+	+	+
Marine Water	Coastal area	*Vibrio* sp.	Gram negative	-	+	-	F	+	-	-	+	-	-	-	-	-	-

(+) =Positive; (-) =Negative reaction, O/F = Oxsidatif/ Fermentatif, TSIA = Triple Sugar Iron agar, SCA = Simmons Citrate Agar; MR= Methyl Red, VP= Voges Proskaure white colony characteristics., the bacteria are *Staphylococcus*. This assumpti