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Preliminary Identification of Bacteria Associated with Sea Cucumber (*Holothuria sp.*) Using Gram Staining Technique from Jepara, Central Java, Indonesia

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ABSTRACT:

Sea cucumbers (*Holothuria sp.*) are marine organisms that play important ecological and economic roles and are known to host various types of bacteria. This study aimed to perform a preliminary identification of bacteria associated with Holothuria sp. collected from the waters of Jepara, Central Java, Indonesia, using the Gram staining technique. The isolation method used was the pour plate technique, and purification was carried out using the streak method. Bacterial samples were isolated from the surface, digestive tract, flesh, and skin of the sea cucumbers, then cultured on nutrient media before Gram staining to determine their morphological characteristics and basic bacterial classification. The results indicated the presence of Gram-negative bacteria with coccoid morphological variations.

Keywords: Bacteria, Gram Technique, Jepara, Sea Cucumber

1. Introduction

Sea Cucumber (*Holothuria sp.*) is widely found throughout the Indo-Pacific and is abundant in many shallow coastal ecosystems. Species of the genus Holothuria are among the most common holothurians in these environments and play key ecological roles, such as sediment bioturbation, organic matter breakdown, and nutrient remineralization, all of which help maintain benthic ecosystem stability (Purcell et al., 2016; Robinson et al., 2018). Besides their ecological importance, sea cucumbers are valued for their nutritional and medicinal uses, making them a major fishery resource in many Asian regions, including Indonesia. Marine invertebrates like sea cucumbers host diverse microbial communities on their body surfaces, within their digestive tracts, and in their coelomic fluids (Enomoto et al., 2012). These microbes participate in various biological processes, including nutrient breakdown, improving digestion, boosting immune responses, and defending against environmental stresses (Amaro et al., 2009; Hess et al., 2011; Amaro et al., 2012). Sea cucumbers often ingest sediment rich in microbial biomass, making bacteria a key part of their diet and digestive ecology (Roberts et al., 2001; Gao et al., 2010). Previous studies have identified several dominant bacterial genera associated with sea cucumbers, especially Vibrio, Bacillus, and Acinetobacter, which may reflect environmental conditions and host-microbe interactions (Zhou et al., 2015; Zhang et al., 2021).

Recent research highlighted the importance of bacterial community structure in sea cucumber aquaculture systems. For example, studies in Apostichopus japonicus culture ponds demonstrated correlations between sediment, water, and gut bacterial assemblages, indicating that environmental microbial profiles influence host-associated communities (Zhou et al., 2022). Other investigations have characterized aerobic culturable bacteria from the sea cucumber gut and revealed their roles in metabolism, nutrient assimilation, and potential host health (Zhang et al., 2013). More recent work using whole genome sequencing and metabolomics has provided deeper insight into bacterial traits and functional capacities associated with sea cucumber-derived isolates (Luo et al., 2024). Reviews of culturable microorganisms from sea cucumbers further indicate that these bacteria may serve as important sources of bioactive compounds with biotechnological potential (Marine Drugs, 2021). Despite Indonesia’s high diversity of sea cucumbers, information on their associated bacterial communities remains limited. Only a few studies have examined microbial assemblages of Holothuria spp. from Indonesian waters, and most provide general observations without focusing on preliminary bacterial identification (Mulyani et al., 2021; Khotimah et al., 2023). Establishing baseline microbial data is important for understanding host health, detecting potential pathogens, and supporting future aquaculture development in regions where sea cucumbers are harvested or cultured.

Gram staining remains a rapid and widely applied technique to classify bacteria into Gram-positive and Gram-negative groups based on cell wall characteristics. Although Gram staining does not provide species-level identification, it offers essential information about bacterial morphology and structural groups and serves as a foundation for subsequent biochemical or molecular analyses (Madigan et al., 2017; Cappuccino & Welsh, 2020). For sea cucumbers, this basic categorization helps to understand dominant microbial groups and their potential ecological or pathological roles. This study aims to perform a preliminary identification of bacteria associated with Holothuria sp. collected from Jepara, Central Java, Indonesia, using the Gram staining technique. The results are expected to provide foundational data on the bacterial groups inhabiting the external and internal components of sea cucumbers and support future investigations on microbial diversity, environmental interactions, and health-related implications in local sea cucumber populations.

2. Material and methods

2.1. Location and Time of Research

This research was conducted in October 2025. Field sampling was carried out using an exploratory method. Sea cucumber samples were taken by diving, then stored in 70% alcohol before being isolated. Initial isolation was carried out directly that day. Gram staining and purification were carried out in the biotechnology laboratory, Faculty of Fisheries and Marine Sciences, Diponegoro University.

2.2. Preparation of Isolation Medium

Isolation of bacteria associated with Holothuria sp. was performed using general-purpose nutrient media. Nutrient Agar (NA) was prepared according to standard microbiological procedures. For each medium, the required amount of commercial powder NA was weighed based on the manufacturer's instructions. The powder was dissolved in distilled water and heated gently while stirring until fully homogenized.

The prepared medium solution was then distributed into Erlenmeyer flasks or culture bottles and sterilized using an autoclave at 121°C and 15 psi for 15 minutes. After autoclaving, the medium was allowed to cool to approximately 45–50°C. Under aseptic conditions inside a laminar flow cabinet, the molten medium was poured into sterile Petri dishes (approximately 15–20 mL per plate) and left to solidify at room temperature.

Once solidified, the plates were stored inverted at 4°C to prevent condensation and contamination. Prior to use, all plates were placed at room temperature for 15–30 minutes to minimize moisture accumulation on the agar surface. The prepared media were then ready for bacterial isolation from sea cucumber samples.

2.3. Isolation and Purification of Sea Cucumber-Associated Bacteria

Isolation of bacteria from sea cucumbers begins with the sea cucumber sectio. The bacteria taken were the skin, flesh, and intestines of sea cucumbers. Each part was ground with mortar and then diluted to 10^3. Isolation of bacteria from the dilution results was carried out using the pour plate method on Nutrient agar media. After isolation, wait 1 x 24 hours to see the results of the bacteria growing.

The isolation results were then purified in nutrient agar. Purification is carried out using the streak method by taking bacterial cultures using a loop needle. Then, to see the results of the purification, wait 1x24 hours.

2.4. Gram Staining of Sea Cucumber Associated Bacteria

A thin bacterial smear was first prepared by transferring a small amount of a single colony onto a clean glass slide and mixing it with a drop of sterile distilled water. The smear was allowed to air-dry and subsequently heat-fixed by passing the slide briefly over a flame to ensure cell adherence and inactivation. The smear was then flooded with crystal violet for one minute and gently rinsed with water, followed by the application of Gram's iodine for one minute to form the crystal violet–iodine complex within the cells. Decolorization was performed using 95% ethanol for approximately 10–20 seconds until the runoff became clear, after which the slide was immediately rinsed to prevent over-decolorization. The smear was counterstained with safranin for one minute and rinsed again before drying. Finally, the stained preparation was examined under a light microscope at 100× magnification using immersion oil to determine whether the isolates were Gram-positive (purple) or Gram-negative (pink/red).

3. Results

3.1. Isolation of Sea Cucumber Associated Bacteria

The research results showed that samples taken from the skin, flesh and intestines of the HA species were Holothuria atra and Holothuria scabra. Figure 1 shows the results of dilutions at 10^2 and 10^3 from intestine. Figure 2 isolation results from flesh and figure 3 is result from skin. These results show that bacteria grow in NA media in a spread manner.

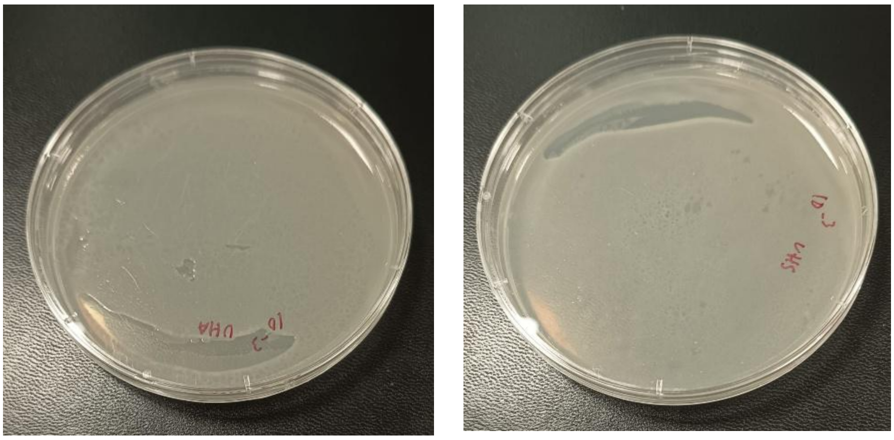

Figure 1. *Isolate UHA and UHS in dilution 10^3*

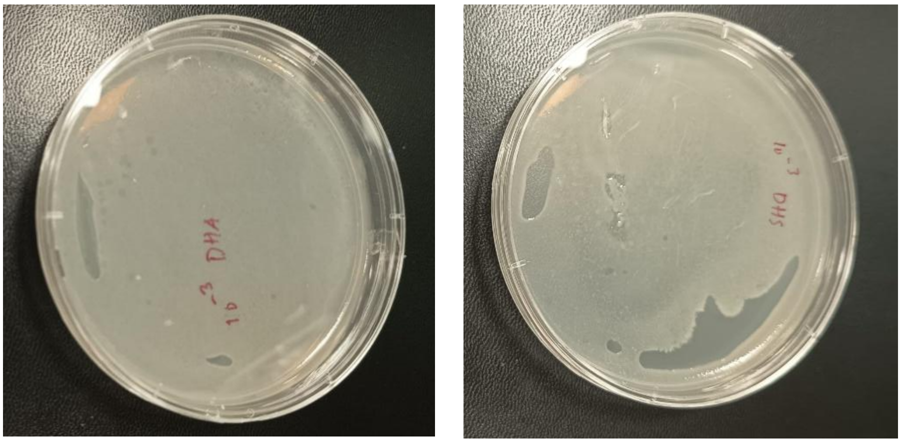

Figure 2.. *Isolate DHA and DHS in dilution 10^3*

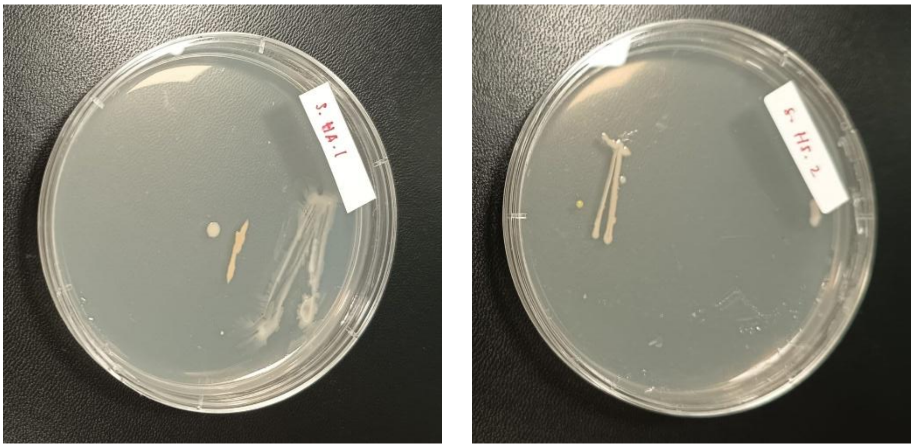

Figure 3.. *Isolate S.HA.1 and S.HS. 2*

3.2. Purification of Sea Cucumber Associated Bacteria

Purification of bacterial isolates is an essential step to ensure that each culture used for further characterization originates from a single bacterial colony. In this study, purification was performed using the streak plate technique after initial isolation by the pour plate method. The streaking process enables progressive dilution of bacterial cells across the agar surface, facilitating the separation of individual colonies with distinct growth patterns (Prescott et al., 2008). This technique is widely used in microbiology due to its effectiveness in obtaining pure cultures from mixed microbial populations, particularly those associated with marine organisms such as sea cucumbers (Ghanbari et al., 2015). Bacterial samples obtained from the surface, digestive tract, flesh, and skin of Holothuria sp. initially exhibited diverse colony morphologies, indicating a mixed bacterial community. Repeated streaking enabled the isolation of stable colonies with consistent characteristics in shape, color, texture, and margin. Ensuring colony uniformity is crucial, as pure cultures form the foundation of reliable biochemical, morphological, and molecular analyses (Madigan et al., 2017).

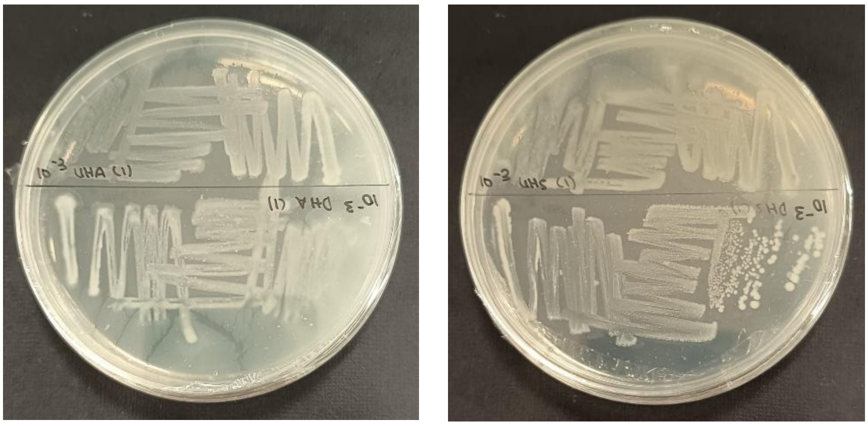

Figure 4. *Isolate UHS, UHA, DHA and DHS*

Figure 5.. *Isolate S.HS and S.HA*

3.3. Gram Staining of Sea Cucumber Associated Bacteria

This technique differentiates bacteria into Gram-positive and Gram-negative groups according to their ability to retain crystal violet dye during the staining process (Madigan et al., 2017). In the present study, Gram staining was performed on purified bacterial isolates obtained from various anatomical parts of Holothuria sp., including the skin, flesh, digestive tract, and body surface. The results revealed that the isolates consistently exhibited Gram-negative reactions, indicated by their pink coloration after counterstaining with safranin. This outcome suggests that the dominant bacteria associated with Holothuria sp. possess thin peptidoglycan layers and an outer membrane, characteristics typical of Gram-negative bacteria (Prescott et al., 2008). The Gram-negative coccoid morphology observed aligns with previous studies on marine invertebrates, where members of the *Vibrionaceae, Pseudoalteromonadaceae,* and other Gram-negative families commonly dominate the microbiota (Jami et al., 2014; Ghanbari et al., 2015).

The prevalence of Gram-negative cocci in sea cucumbers may be influenced by several ecological factors, including the nutrient-rich mucus layer on the organism's surface, the composition of surrounding seawater, and potential symbiotic or commensal interactions within the host's digestive tract. Such bacteria may play beneficial roles, including aiding nutrient absorption or contributing to host immune modulation (He et al., 2018).

Gram staining confirmed that Gram-negative coccoid bacteria represent the predominant morphological group associated with Holothuria sp. from Jepara waters. While these findings provide an important initial characterization, further molecular and biochemical analyses are necessary to determine the diversity, ecological roles, and potential functional significance of these Gram-negative bacterial communities in sea cucumbers.

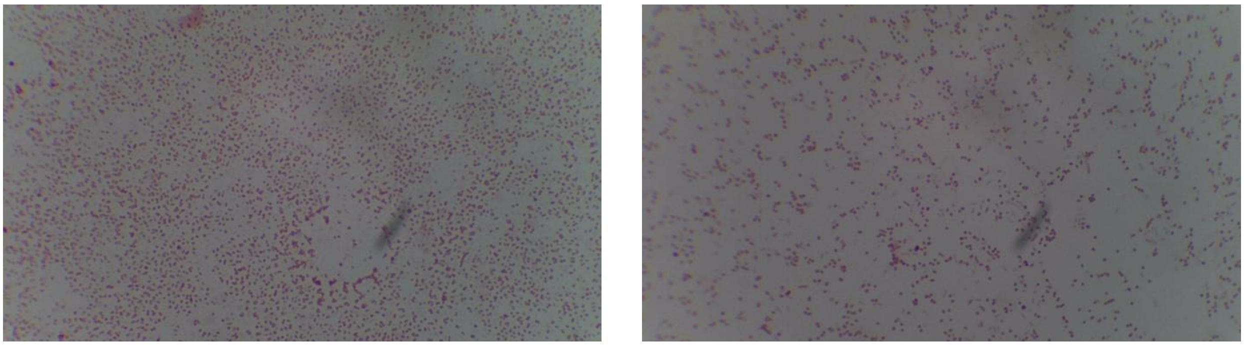

***Figure 6**. Isolate DHA and Isolate DHS*

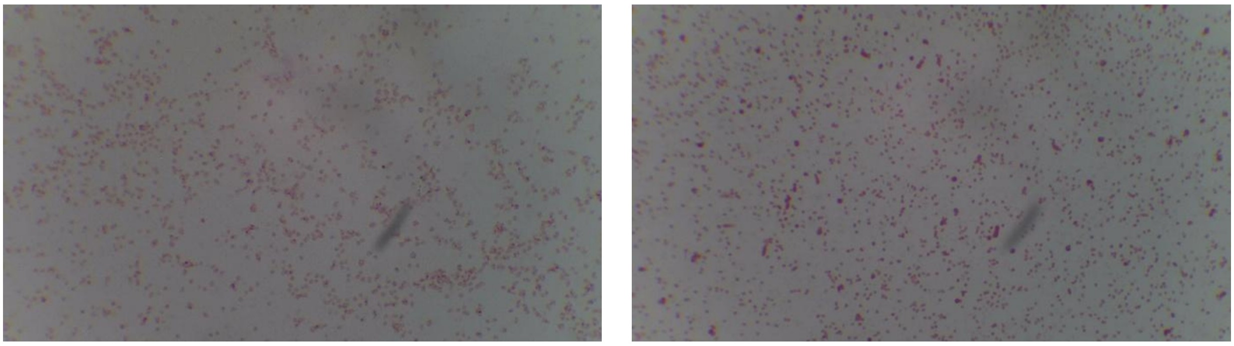

***Figure 7.**. Isolate UHA and Isolate UHS*

4. Conclusion

Based on the results of this study on bacteria associated with Holothuria sp. from the waters of Jepara, Central Java, it can be concluded that bacteria can be isolated from various parts of the sea cucumber, including the surface, skin, flesh, and digestive tract, using the pour plate method. Purification of the isolates through the streak plate technique. Gram staining revealed that the dominant isolates were Gram-negative cocci, indicating that the bacterial community associated with Holothuria sp. is predominantly composed of Gram-negative bacteria. This preliminary identification provides essential baseline information on the microbial diversity associated with Holothuria sp. and serves as a foundation for future studies using molecular approaches to determine bacterial species and their ecological or functional roles within the sea cucumber microbiome.

Conflict of interest

The authors declare that there are no conflicts of interest.

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