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Isolation and Morphological Characterization of Tunicate-Associated Bacterial Colonies from the Shipwreck of Kumbang Karimunjawa, Jepara, Indonesia

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A B S T R A C T

Tunicates (Ascidia) are hosts for organisms associated with them which include bacteria, archaea, and fungi. Tunicates and their associated bacteria are able to produce bioactive compounds that have various functions, such as antibacterial, antiviral, antifungal, anticancer, antitumor, and cytotoxic compounds. The selection of appropriate media in the isolation of tunicate-associated bacteria increases the opportunity to obtain pure cultures that have the potential to produce bioactive compounds. In this study, a total of 6 tunicates were identified based on morphological characteristics. Isolation of associated bacteria was conducted by serial dilution method using Zobell and M1 media. Colonies that grew on the surface of the media were characterized based on colony morphology and purified by streak plate method. The isolation results 29 isolates that grew on Zobell 2216 media and 82 isolates on M1 media. Purification left 41% isolates from Zobell 2216 media and 84% isolates from M1 media. The decrease in the number of isolates is due to the unsuitable environmental conditions compared to their natural habitat or the inability of isolates to utilize media nutrients for a long time. M1 media is better than Zobell 2216 media in the percentage of colonies that grow during isolation and the obtaining of pure isolates.

Keywords: Tunicates, Bacterial Isolation, Marine agar

1. Introduction

The Shipwreck of Kumbang Karimunjawa is located at a depth of 2-13 meters, providing habitat for sessile organisms, including tunicates. Tunicates can be found in the form of colonies or solitary, filter feeders by filtering food through inhalant siphons and then removing it through excurrent siphons (Curran et al., 2015). Tunicates live attached to the seabed both in shallow water and in deep sea waters (Kurabayasi et al., 2003), together with corals, fish, and other avertebrates compose the coral reef ecosystem (Idris et al., 2019).

Tunicates host a wide variety of symbiont microorganisms due to their unique filtration and digestion systems (Song, et.al., 2019). Bacteria have the highest abundance and diversity compared to Archaea and Fungi that have been successfully isolated from tunicates (Bauermeister, et al., 2019). Tunicates and their associated bacteria are bioresources that are less explored in Indonesia (Ayuningrum et al., 2020), even though they are able to produce bioactive compounds that have various functions, such as antibacterial, antiviral, antifungal, anticancer, antitumor, and cytotoxic compounds (Ramesh et al., 2021). This is evidence that tunicates have the potential as a source of marine natural products (MPNs) that can be further explored as an object of search for new chemical compounds.

Extraction of chemical compounds from tunicates in large quantities will disrupt the ecological stability of coral reefs. Exploitation of tunicates as raw materials for the exploration of new chemical compounds can be avoided by discovering the chemical compounds produced by their associated bacteria. Not all bacteria associated with tunicates can be cultured on bacterial medium under laboratory conditions. Growth media contains nutrients that are used to support bacterial growth, so the selection and preparation of media for bacterial culture must be done carefully in order to increase success in laboratory culture (Madigan et al., 2015). Characterization of the colony morphology of each bacterial isolate code needs to be done to ease the purification of bacterial isolates and contrast them with contaminants that may appear when culturing in the laboratory.

2. Methods

2.1 Sample Collection

Tunicates samples were collected with diving equipment at a depth of 14 meters at the shipwreck of Kumbang Island Karimunjawa, Jepara, Indonesia. Samples were cut using a scalpel, placed in a plastic ziplock, labeled, and stored in a coolbox. This aims to maintain bacteria in the sample and sample degradation before bacterial isolation.

2.2 Preparation of Bacterial Growth Media

The media used for isolation, culture, and storage of pure isolates are Zobell 2216 and M1 media. Zobell 2216 media was made by mixing 40.25 g of Zobell broth powder and 15 g of agar into 1 L of seawater, while the media was made by mixing starch 10 g, yeast extract 4 g, peptone 2 g, and agar 15 g in 1 L of seawater, heated until homogeneous, then sterilized using an autoclave at 121℃ for 15 minutes. Sterilized media was poured during the liquid phase (warm temperature) into a petri dish, waited until it solidified and was ready for use.

2.3 Isolation and Purification of Tunicate Associated Bacteria

Bacterial isolation was performed using the dilution method and separation of bacterial colonies based on the morphology of colonies growing on the agar surface (Benson, 2001). Tunicate tissue as much as 1 g was diluted in stages. A total of 30 µl of 10^3 and 10^4 dilutions were spread on the surface of Zobell 2216 and M1 agar media that had been added with 25 mg/L nystatin, then incubated for 2 days at room temperature. The colonies of bacteria that grow were separated based on differences in colony morphology using the streak method on the same media for isolation.

3. Result and Discussions

The results of the investigation obtained 6 tunicates samples, labeled KJ2-02, KJ2-03, KJ2-11, KJ2-15, KJ2-18, and KJ2-25. All samples were aseptically rinsed using sterile seawater before bacterial isolation. Bacterial culture media based on their consistency are grouped into solid media (1.5-2.0% agar), semisolid (0.5% agar), and liquid/broth (no agar). Solid media is used for bacterial isolation and to differentiate the colony characteristics of isolates, semisolid media for determining bacterial movement, and liquid media for growing bacterial populations (Sukhorukov, 2021). Agar media (solid) limits the movement of bacterial cells, allowing bacteria that grow on the surface of the media to form macroscopic colonies. Bacterial colonies growing on the agar surface (Figure 1) were characterized based on differences in colony morphology including configuration, margin, elevation, and color. The total number of colonies that grew on the surface of the media was 111 colonies, including 29 colonies on Zobell 2216 media (Table 1) and 82 colonies on M1 media (Table 2).

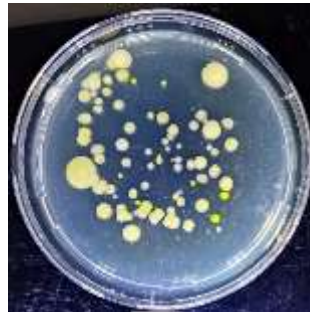

Fig. 1 - Bacterial colonies that grow on the agar surface

Table 1. Morphological Characteristics of Colonies on Zobell 2216 media

Code	Bacterial Colony Morphology			
	Configuration	Margin	Elevation	Colour
Sample KJ2-02				
KZ302-01	Concentric	Undulate	Flat	Cream
KZ302-02	L-form	Undulate	flat	orange
KZ302-03	Round with raised margin	Smooth	Convex	Cream
KZ302-04	Irregular	Smooth	Convex	Cream
KZ302-05	Round	Smooth	Convex	Orange

KZ302-06	Round	Concentric	Flat	White
KZ302-07	Round	Irregular	Flat	White
KZ402-01	Round with scalloped margin	Smooth	Convex	Cream
KZ402-02	Round	Smooth	Raised	Yellow
KZ402-03	Concentric	Smooth	Flat	White
Sample KJ2-11				
KZ311-01	Round	Wavy	Flat	Yellow
KZ311-02	Round	Smooth	Raised	White
KZ411-01	Round with raised margin	Smooth	Raised	Cream
KZ411-02	Round	Lobate	Flat	Cream
KZ411-03	Round	Lobate	Flat	Light cream
KZ411-04	Round	Irregular	Flat	Cream
Sample KJ2-15				
KZ315-01	Round	Lobate	Flat	Cream
KZ315-02	Round	Smooth	Raised	Cream
KZ315-03	Round with margin	Smooth	Flat	Cream
KZ415-01	Round	Smooth	Flat	Cream
Sample KJ2-18				
KZ318-01	Round	Smooth	Flat	Cream
KZ318-02	Round	Ciliate	Convex	Cream
KZ418-01	Round with margin	Smooth	Convex	Cream
Sample KJ2-25				
KZ325-01	Round	Smooth	Flat	Cream
KZ325-02	Round	Smooth	Convex	Cream
KZ325-03	Concentric	Smooth	Flat	Cream
KZ325-04	Round	Smooth	Convex	Cream
KZ425-01	Round	Smooth	Raised	Yellow
KZ425-02	Round	Smooth	Flat	White

Table 2. Morphological Characteristics of Colonies on M1 media

Code	**Bacterial Colony Morphology**			
	Configuration	**Margin**	**Elevation**	**Colour**
Sample KJ2-02				
KM302-01	Round	Smooth	Drop like	Cream
KM302-02	Round	Smooth	Convex	Yellow
KM302-03	Round	Smooth	Convex	White
KM302-04	Round	Smooth	Flat	White
KM402-01	Round with raised margin	Smooth	Flat	Orange
KM402-02	Wrinkled	Wavy	Flat	Yellow

KM402-03	Wrinkled	Irregular	Flat	White
KM402-04	Wrinkled	Wavy	Flat	White
KM402-05	Round	Smooth	Convex	Cream
KM402-06	Round with scalloped margin	Smooth	Flat	White
Sample KJ2-11				
KM311-01	Round	Smooth	Flat	Cream
KM311-02	Round	Smooth	Convex	Yellow
KM311-03	Round	Smooth	Umbonate	Yellow
KM311-04	Round	Smooth	Umbonate	White
KM311-05	L-form	Smooth	Raised	White
KM311-06	Round	Smooth	Raised	Yellow-Orange
KM311-07	Round	Wavy	Flat	Cream
KM311-08	Round	Smooth	Raised	Yellow
KM411-01	Concentric	Ciliate	Flat	Yellow
KM411-02	Round	Ciliate	Convex	Orange
KM411-03	Round	Smooth	Drop like	Yellow
KM411-04	Wrinkled	Smooth	Flat	White
KM411-05	Round	Wavy	Flat	White
KM411-06	Round	Smooth	Hilly	White
Sample KJ2-15				
KM315-01	Round	Smooth	Raised	Cream
KM315-02	Round	Smooth	Flat	Yellow
KM315-03	Round	Lobate	Flat	Yellow
KM315-04	Round	Smooth	Convex	White
KM315-05	Round with raised margin	Smooth	Flat	White
KM315-06	Round	Smooth	Flat	White
KM315-07	Round	Smooth	Flat	White-purple
KM315-08	Wrinkled	Smooth	Flat	White
KM315-09	Round	Smooth	Raised	Yellow
KM315-10	Round	Smooth	Convex	Cream
KM415-01	Round	Smooth	Raised	Yellow
KM415-02	Round	Wavy	Raised	Cream
KM415-03	Round	Smooth	Raised	White
KM415-04	Round	Smooth	Convex	White
KM415-05	Concentric	Wavy	Flat	White
KM415-06	Round	Smooth	Flat	Yellow
KM415-07	Round	Irregular	Convex	Yellow
KM415-08	Concentric	Smooth	Flat	Cream

KM415-09	Round with raised margin	Smooth	Flat	Yellow-cream
KM415-10	Concentric	Smooth	Flat	Yellow
Sample KJ2-18				
KM318-01	Round	Smooth	Flat	Yellow
KM318-02	Wrinkled	Lobate	Flat	White
KM318-03	Round	Irregular	Flat	Cream
KM318-04	Round	Smooth	Flat	Cream
KM318-05	Wrinkled	Wavy	Flat	Cream
KM318-06	Round	Round	Raised	White
KM418-01	L-form	Smooth	Flat	Cream
KM418-02	Round	Smooth	Flat	Yellow
KM418-03	Round	Smooth	Flat	Cream
KM418-04	Round	Smooth	Raised	Cream
KM418-05	Concentric	Wavy	Flat	Cream
KM418-06	Round	Smooth	Flat	White
KM418-07	Round	Wavy	Flat	White
KM418-08	Wrinkled	Irregular	Flat	White
KM418-09	Round	Wavy	Flat	Cream
KM418-10	Round with raised margin	Wavy	Flat	Cream
KM418-11	L-form	Smooth	Flat	Cream
KM418-12	Round with scalloped margin	Wavy	Flat	Cream
Sample KJ2-25				
KM325-01	Round	Smooth	Flat	Orange
KM325-02	Round	Smooth	Flat	Yellow
KM325-03	Round	Smooth	hilly	Cream
KM325-04	Round	Smooth	Flat	Cream-purple
KM325-05	Round with scalloped margin	Smooth	Raised	Cream
KM325-06	Round	Smooth	Flat	Cream
KM325-07	Round	Smooth	Convex	Orange-cream
KM325-08	Round	Smooth	Convex	Orange
KM425-01	Round with scalloped margin	Wavy	Convex	Orange-cream
KM425-02	Round	Smooth	Convex	Orange- yellow
KM425-03	Round	Smooth	Flat	Cream
KM425-04	Round	Wavy	Convex	Orange-cream
KM425-05	Round with raised margin	Smooth	Flat	Cream
KM425-06	Round	Smooth	Flat	Yellow
KM425-07	Wrinkled	Wavy	Flat	Cream
KM425-08	Round	Smooth	Flat	White-cream

KM425-09	Round	Smooth	Flat	Bright Yellow
KM425-10	Round	Smooth	Flat	Yellow
KM425-11	Round	Smooth	Convex	Yellow
KM425-12	Round	Smooth	Convex	Cream

Table 2 shows 10 colonies grew from sample KJ2-02, 6 colonies from KJ2-11, 4 colonies from KJ2-15, 3 colonies from KJ2-18, and 6 colonies from KJ2-25. Table 3 shows 10 colonies grew from sample KJ2-02, 14 colonies from KJ2-11, 20 colonies from KJ2-15, 18 colonies from KJ2-18, and 20 colonies from KJ2-25. The difference in the number of colonies that are able to grow from each sample with different growth media (Fig. 2) is affected by differences in the composition of the media. Small differences in the composition of a medium can produce very different growth characteristics of microorganisms (Atlas, 2010).

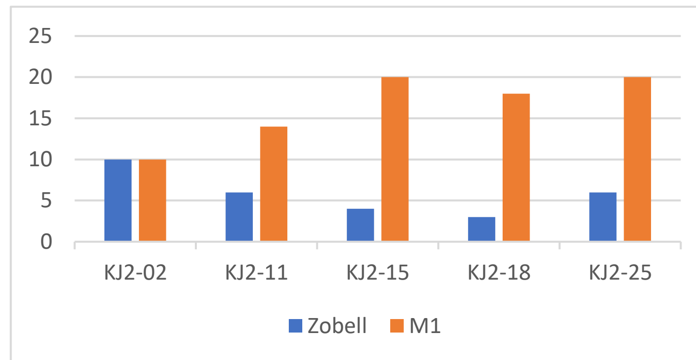

Fig. 2 - Comparison of colony counts on Zobell 2216 and M1 media

The use of various culture conditions including different culture media can increase the diversity of bacteria obtained (Zhao et al., 2020). Zobell 2216 medium is used to enumerate marine heterotrophic bacteria (Bonnet et al., 2019). NaCl in Zobell 2216E medium is used to select halophilic bacteria. Medium M1 in its preparation uses seawater as a solvent, so this medium is also selective of halophilic bacteria only (Petersen & McLaughlin, 2016). In addition, seawater from the waters around the island of Java contains micronutrients such as Na^+, K^+, Mg^{2+}, Ca^{2+}, Br^-, F^-, Cl^-, SO_4^{2-}, and PO_4^{2-} (Apriani et al., 2018) which support bacterial growth. The differences in the composition of M1 and Zobell 2216 media are that in M1 media there is starch. Most bacteria require organic carbon sources as a component of 50% of the dry weight of bacterial cells which can be obtained from amino acids, fatty acids, organic acids, sugars, and other organic components (Madigan et al., 2015). The prevalence of isolates on M1 media is due to the presence of starch as the main organic carbon source. Ayuningrum et al (2019) obtained similar results, i.e. tunicates association bacterial isolates that grew on M1 media were more numerous than on Zobell 2216 media.

One colony generally comes from one bacterial species, but the diversity of colonies in one petri allows contamination between bacterial colonies. Therefore, purification is necessary to obtain pure isolates. Purification is conducted by transferring bacterial colonies to new solid media according to those used in isolation. The process of transferring isolates to new media can reduce the viability of the next generation of isolates. The purification results of bacterial colonies that grew on the media left 81 isolates including 12 colonies on Zobell media (15% of total isolate) and 69 colonies on M1 media (85% of total isolate) (Fig. 3). The decrease in the number of isolates on each media can be caused by the next generation not being able to grow during the purification process. In addition, the bacteria are neither in the appropriate habitat nor able to utilize the media nutrients for a long period of time. An obstacle in bacterial culture is the difficulty in mimicking the specific environmental conditions that certain bacteria need to grow, as variations in the adjustment of environmental conditions (nutrients, pH, temperature, osmolarity, or many more) create a range of possibilities that cannot be fully resolved with sufficient time and effort (Stewart, 2012).

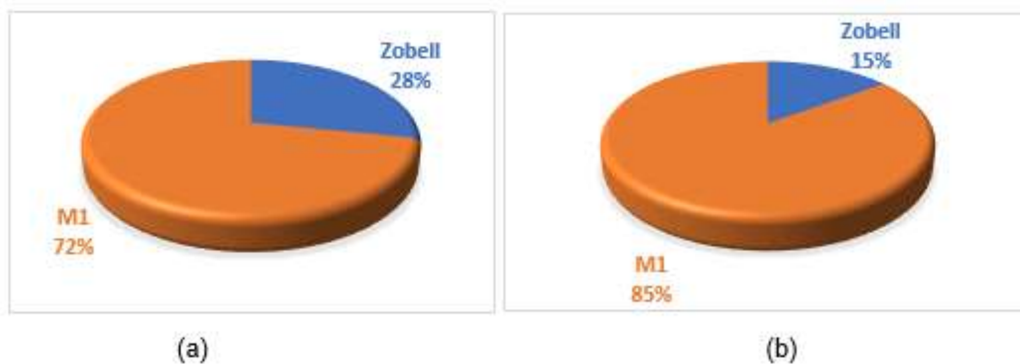

Fig. 3 - Isolation (a) and purification (b) results

Many species of marine bacteria have unknown growth characteristics and yet to be cultured (Bernard et al., 2000). Bacteria from sample KJ2-03 were not successfully isolated using Zobell 2216 and M1 media. This may be due to errors in tissue collection or a less than optimal grinding process. Unculturable bacteria may include known species with inappropriate culture conditions or entering conditions that cannot be cultured or unknown species that have never been cultured before due to the absence of suitable methods (Amann et al., 1995). Research by Rodrigues & Carvalho (2022) broke the dogma of less than 1% of bacteria growing in laboratory conditions, by culturing bacteria using various media using standard agar plate techniques. Bacterial isolation from sample KJ2-03 can be optimized using media other than Zobell and M1.

4. Conclusions

The selection of bacterial growth media affects the success of tunicates association bacteria isolation. The amount and composition of nutrients needed to support bacterial growth need to be adjusted to the characteristics of the bacteria to be isolated. M1 media gives better results in supporting bacterial growth compared to Zobell 2216 media. Colonies that grow on Zobell 2216 media show different characteristics from colonies on M1 media. This proves that bacterial isolation should use a variety of different media to increase the success of obtaining pure isolates.

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