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PRELIMINARY STUDIES ON ISOLATION AND SCREENING OF ANTIBIOTIC PRODUCING SYMBIOTIC BACTERIA FROM STARFISH (*Protoreaster nodosus*) COLLECTED FROM COASTAL AREA TAKALAR REGENCY, INDONESIA

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KEYWORDS

Protoreaster nodosus

Symbiotic bacteria

Antibacterial activity

Disc diffusion assay

ABSTRACT

Marine organisms are well known for the availability of bioactive compounds which have various biological activities including antibacterial activity. Likewise, their symbiotic bacteria can also produce compounds that have similar activities. The purpose of this study was to isolate and screen the symbiotic bacteria from starfish (*Protoreaster nodosus*) collected from coastal area Takalar Regency, South Sulawesi, Indonesia. Isolation was carried out by the pour plate method using nutrient agar medium dissolved in sterile seawater. The isolated symbiotic bacteria were purified by using the quadrant method. The pure isolate was culture through submerged fermentation using nutrient broth media enriched with 1% yeast extract and sterile seawater for 7 days. The selected symbiont bacterial isolates were tested for their antibacterial activity against gram-positive and gram-negative bacteria using disc diffusion assays. The results of fermentation were separated from the biomasses and tested for antibacterial activity against *Staphylococcus aureus* (*S. aureus*, ATCC 25923), *Bacillus subtilis* (*B. subtilis*, ATCC 6633), *Salmonella typhi* (*S. typhi*, NCTC 786), and *Escherichia coli* (*E. coli*, ATCC 25923). The results of study revealed that four symbiotic bacteria (SB 1T, SB 2T, SB 3T, and SB 4T) were successfully isolated. All the SB isolates have good antibacterial activity against all tested bacterial strains with an average diameter of inhibition zone larger than 11 mm. Among all isolates, isolate SB 4T showed a remarkable size of zones growth inhibition (> 15 mm) against all tested bacterial strains. Thus, the symbiotic bacteria isolated from *P. nodosus* in this study have a promising broad-spectrum antibacterial activity.

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1 Introduction

Various marine organisms, including algae, sponges, and starfish have various bioactive compounds that can be used to produce medicinal natural products (Habbu et al., 2016; Blunt et al., 2018). Some starfish species such as *Certonardoa semiregularis, Stellaster equestris, Astropecten indicus, Protoreaster linckii,* and *P. nonudus* are well known for their antimicrobial activity (Chamundeeswari et al., 2012; Prabhu & Bragadeeswaran, 2013; Mohammed Husain, 2019). Several secondary metabolites such as steroids, steroidal glycosides, anthraquinones, alkaloids, phospholipids, peptides, and fatty acids were found in starfish. Among these, bioactive compounds such as polyhydroxylated sterols and their derivatives possess antibacterial activity against several strains of pathogen bacteria (Dong et al., 2011; Thao et al., 2015; Cockroft et al., 2019; Ivanchina et al., 2019).

Previous studies have shown that various symbiotic microorganisms were associated with marine invertebrates and produced secondary metabolites with antimicrobial properties (Kamke et al., 2010; Thomas et al., 2010; Sartini et al., 2014; Ismail et al., 2016). These microorganisms include bacteria, actinomycetes, and fungi. Santhi et al. (2017) reported the presence of symbiotic *Bacillus sp.* with the sponge. Similarly, Nakagawa et al. (2017) reported the presence of symbiotic bacteria in coelomic fluid isolated from two species of starfish i.e., *Patiria pectinifera* and *Asterias amurensis* which were identified as Helicobacter-related taxon (Nakagawa et al., 2017). However, to the best of our knowledge, no studies have been carried out on the isolation and screening of antibacterial-producing symbiotic bacteria from starfish particularly *P. nodosus* species.

Moreover, the differences in the environment where the starfish live can also affect the microbial symbiont strain found in the starfish. Similarly, Jackson et al. (2018) reported that anatomical regions of sea star, microbial community, and the difference in geographical location affect the sea star microbiome and in particular may reduce the bacterial cells (by 2-log reduction) in the coelomic fluid of sea stars. The combination of environmental factors such as temperature, salinity, and pH are believed to be the driving factors that affect the microbial community associated with sea stars (Jackson et al., 2018).

Therefore, the current study was carried out to investigate the symbiotic bacteria isolated from the starfish (*P. nodosus*) collected from coastal area Takalar Regency, South Sulawesi, Indonesia. Further, the screening of antibacterial activity of the isolated symbiotic bacteria against several gram-positive and gram-negative bacteria was also performed.

2 Materials and Methods

2.1 Materials

Starfish (*P. nodosus*) was collected from the coastal area Takalar Regency, South Sulawesi, Indonesia (Figure 1). Nutrient agar, nutrient broth, yeast extract, and Muller Hinton agar were purchased from Merck (Germany). Seawater (collected from the coastal area Takalar Regency, South Sulawesi, Indonesia) were sterilized using an autoclave. Bacterial test strain *Staphylococcus aureus* (*S. aureus*, ATCC 25923), *Bacillus subtilis* (*B. subtilis*, ATCC 6633), *Salmonella typhi* (*S. typhi*, NCTC 786), and *Escherichia coli* (*E. coli*, ATCC 25923) were obtained from the culture collection of Bacteriology Unit at Department of Microbiology, Faculty of Pharmacy Hasanuddin University, Indonesia. All other reagents and solvents used in this study were of the analytical grade.

2.2 Isolation of symbiotic bacteria

In the current study, for the isolation of symbiotic bacteria, the pour plate method was used. Briefly, samples of starfish (*P. nodosus*) were cut into pieces and weighed 25 g, this amount was then crushed with a blender and 250 mL volume was prepared by adding sterile seawater. This was followed by the serial dilution of the mixture up to 10^{-5} dilution factor. From each dilution, 100 µl was taken out by a micropipette and mixed into nutrient agar media which has been prepared in a petri dish by pour plate method. Furthermore, the culture was incubated for 24–48 hours at 37^{o}C. The purification of symbiotic bacteria isolates was performed through the quadrant method until pure isolates were obtained.

2.3 Screening of antibacterial chemical producing symbiotic bacteria

2.3.1 Production of bioactive compounds through submerged fermentation

Each pure symbiotic bacterial isolate was inoculated into the sterile nutrient broth and incubated for 24 hours at 37^{o}C. Culture starter (10 %v/v) was poured into Erlenmeyer flask containing nutrient broth enriched with 1% yeast extract, followed by incubation for 7 days at 37^{o}C under constant shaking (200 rpm). After 7 days of incubation, the fermented liquid (supernatant) was separated from the biomasses (sediment). This was followed by the extraction (3 times) of this fermented liquid with ethyl acetate solvent (1:1 v/v) in a separating funnel for 90 minutes. The extract was obtained by solvent evaporation and then stored in a desiccator for further use.

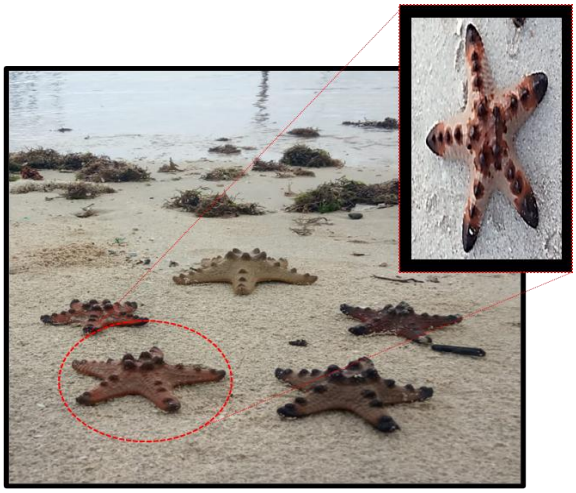

Figure 1 Starfish (*P. nodosus*) collected from coastal area Takalar Regency, South Sulawesi, Indonesia

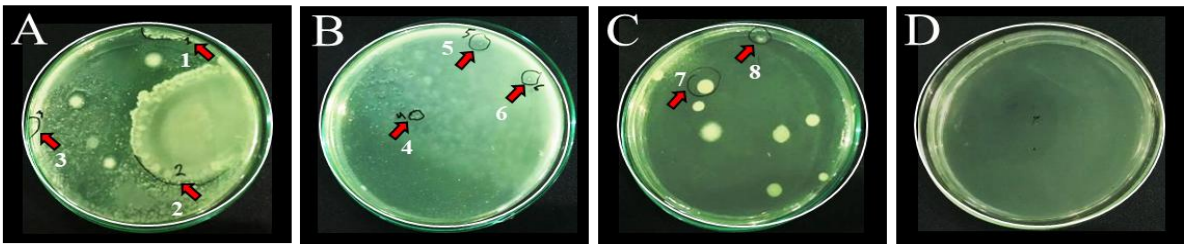

Figure 2 The growth of symbiotic bacteria isolated from starfish (*Protoreaster nodosus*) on nutrient agar media dissolved in sterile seawater after 2 days incubation at 37^0C

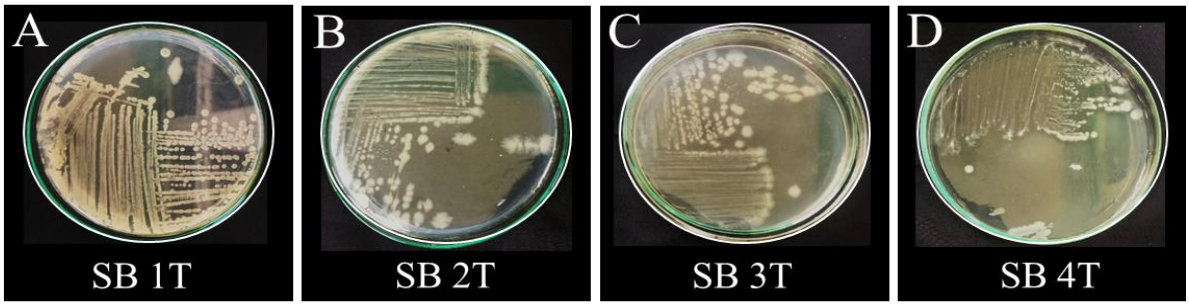

Figure 3 Four selected symbiotic bacteria isolates from starfish (*Protoreaster nodosus*) in nutrient agar media

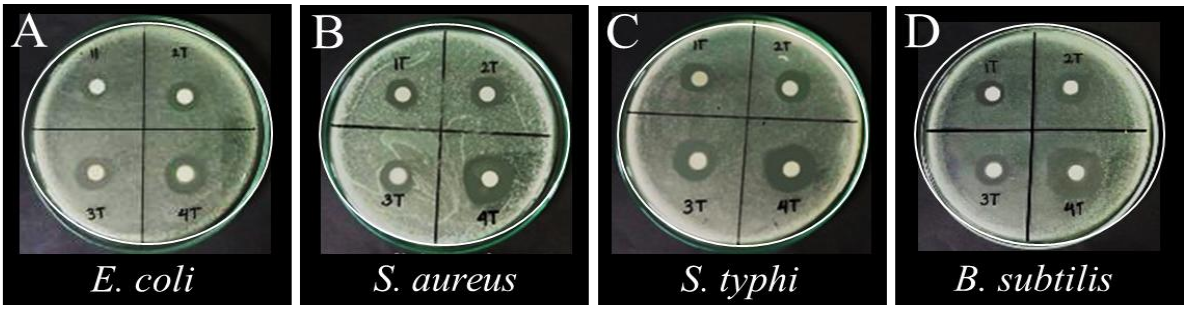

Figure 4 Antibacterial activity of symbiotic bacteria isolates after fermentation using disc diffusion assay

Table 1 Characteristic of the symbiotic bacteria isolates

Isolates	Macroscopic observation			
	Colony shape	Edge of colony	Elevation	Colour
SB 1T	Irregular	Undulate	Convex	White
SB 2T	Irregular	Undulate	Raised (height is visible, but flat over the entire surface	White
BS 3T	Round	Flat	Convex	White
BS 4T	Irregular	Flat	Convex	White

Table 2 Diameter of inhibition zone of symbiotic bacteria isolates after fermentation 2 days using disc diffusion assay

Isolates	Diameter of inhibition zone (mm ± SD)			
	E. coli	*S. aureus*	*S. typhosa*	*B. subtilis*
SB 1T	11.13 ± 0.02	11.72 ± 0.30	11.70 ± 0.08	11.28 ± 0.01
SB 2T	11.78 ± 0.04	11.78 ±0.12	11.16 ± 0.05	11.28 ± 0.06
BS 3T	12.71 ±0.20	11.27 ± 0.01	12.13 ± 0.03	12.08 ± 0.01
BS 4T	15.52 ± 0.28	17.36 ±0.36	18.46 ± 0.38	18.53 ± 0.23

Values are expressed as mean averages ± SD of three different batches of symbiotic bacteria

2.3.2. *In vitro* antibacterial assay using a disc diffusion assay

Antibacterial activity was evaluated using disc diffusion assay as described by Sartini et al. (2014) with slight modification. Briefly, 20 µL supernatant was pipetted on the paper disc (6-mm diameter). Each disc was then placed on to Muller Hinton agar (pH 7.0 ± 0.2) containing 1 mL of 10^8 cell/ml bacterial suspensions (*S. aureus* ATCC 25923, *B. subtilis* ATCC 6633, *S. typhi*NCTC 786, and *E. coli* ATCC 25923), this was followed by the 24 hours incubation at 37°C. The result of antibacterial activity is expressed as the diameter of the inhibition zone (DIZ).

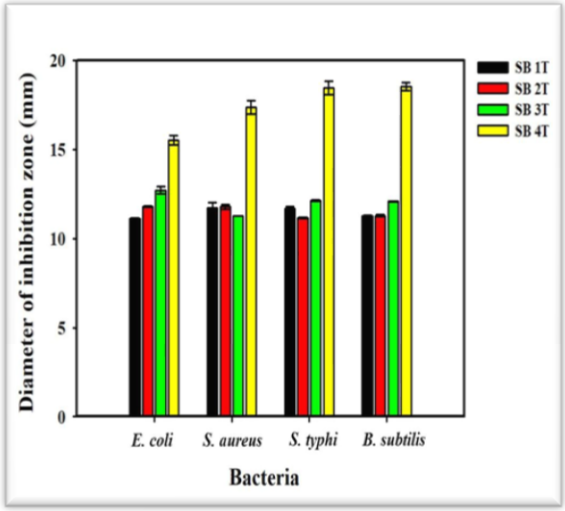

Figure 5 Diameter of inhibition zone of symbiotic bacteria isolates against *E. coli*, *S. aureus*, *S. typhi*, and *B. subtilis*

3 Results and Discussion

3.1 Isolation of symbiotic bacteria

The isolation of symbiotic bacteria from the starfish (*P. nodosus*) collected from coastal area Takalar Regency, South Sulawesi, Indonesia was carried out by using the pour plate method in nutrient broth media through 10^{-1} to 10^{-5} dilutions. After 2 days of incubation, the growths of bacteria in various dilutions (10^{-1} to 10^{-5}) were macroscopically observed. Based on the macroscopic differences in the shape of the colonies, the purification was performed using the quadrant method. The shape of bacterial colonies was shown in Figure 2. The results of this study revealed that 8 colonies can be continued for purification. These results confirmed the presence of microorganisms associated with starfish (*P. nodosus*) and the symbiotic bacteria were successfully isolated from the host. These results establishing the data related to the symbiotic bacteria that were isolated from starfish collected from coastal area Takalar Regency, South Sulawesi, Indonesia. Although various previous studies have been already reported the presence of several symbiotic bacteria from starfish species but this is the first study that evaluated the effect of the environment and geographical location of the Takalar Regency, South Sulawesi,

Indonesia on the occurrence of the symbiotic microorganism associated with starfish. As reported by Jackson et al. (2018), the combination of environmental (temperature, salinity, and pH) and host factors (mucosal layers, diet, exposure to coelomocytes, and secondary metabolites) affect the occurrence and variety of symbiotic bacteria of starfish (Jackson et al., 2018).

Further, figure 3 showed that the selected symbiotic bacterial isolates were free from the contamination after inoculation in nutrient agar media. These isolates namely SB 1T, SB 2T, SB 3T, and SB 4T have different shapes, edge of colonies, elevation, and colour (Table 1).

The shape of the colony varied from irregular shape to round shape. The edge of the colony was undulate and flat/even. The elevation of the colony was recognized as convex or raised and the colour of the isolate was observed to have a white colour. These 4 isolates were then further fermented to produce secondary metabolites that possess antibacterial activity.

3.2. Screening of antibacterial-producing symbiotic bacteria

Previous studies have been reported the isolation of symbiotic microbes from marine biota, especially from sponges, macroalga, and holothurian. These symbiotic microbes are well known to produce secondary metabolites that possess several biological activities including antimicrobial activity (Ismail et al., 2016; Kuo et al., 2019; Pringgenies et al., 2019). However, information's regarding the symbiotic bacteria from starfish that possess antibacterial activities are still lacking.

In the current study, four isolated isolates viz., SB 1T, SB 2T, SB 3T, and SB 4T were selected for evaluating their antibacterial potential against the selected gram-positive (*S. aureus* ATCC 25923 and *B. subtilis* ATCC 6633) and gram-negative (*E. coli* ATCC 25923 and *S. typhi*NCTC 786) bacteria by using zone of inhibition assays. These results are incorporated in table 2 and figure 4 & 5. The results of the study revealed that the average DIZ of SB 1T, SB 2T, SB 3T, and SB 4T against the tested bacterial strains are 11.45 mm, 11.49 mm, 12.05, and 17.47, respectively. All the SB isolates have good antibacterial activity against all tested bacterial strains and among these, isolate SB 4T showed a remarkable size of zones growth inhibition (> 15 mm). Thus, the symbiotic bacteria isolated from *P. nodosus* in this study have a promising broad-spectrum antibacterial activity for therapeutic purposes.

Conclusion

Four symbiotic bacteria i.e., SB 1T, SB 2T, SB 3T, and SB 4T were successfully isolated from the starfish collected from coastal area Takalar Regency, South Sulawesi, Indonesia. All the SB isolates have good antibacterial activity against the broad spectrum gram-positive and gram-negative bacterial strains and in particular, the isolate SB 4T showed a remarkable size of zones growth inhibition. However, in this study molecular identification of these isolates has not yet been conducted. Future studies are required for the characterization of these isolates through molecular identification.

Acknowledgements

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Conflict of Interest

Authors declare that they have no conflict of interest.

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