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Bioprospecting of bacterial symbionts of sponge *Spongia officinalis* from Savu Sea, Indonesia for antibacterial potential against multidrug-resistant bacteria

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Abstract. *Prastiyanto ME, Kartika AI, Darmawati S, Radjasa OK. 2022. Bioprospecting of bacterial symbionts of sponge* Spongia officinalis *from Savu Sea, East Nusa Tenggara, Indonesia for antibacterial potential against multidrug-resistant bacteria. Biodiversitas 23: 1118-1124.* Marine sponge *Spongia* sp. has been reported to have potential as an antibacterial agent. However, less information on the potential of bacterial symbionts of *Spongia* sp. as an antibacterial agent has been documented. The present investigation involves isolating bacterial symbionts of sponge *Spongia officinalis* and the characterization of antibacterial potential against multidrug-resistant bacteria isolated from clinical specimens. *Spongia officinalis* was collected from Savu Sea, East Nusa Tenggara, Indonesia and its symbionts were isolated with Zobell marine agar media. The overlay method was used to screen the antibacterial activity against selected six MDR bacteria. Antibacterial activity was determined by measuring the diameter of the inhibition zone. Identification of active bacterial symbionts was carried out based on the 16S rRNA gene sequencing. The results revealed that four out of 10 symbionts showed antibacterial activity against MDR bacteria with an inhibition index ranging from 4.8 to 12.6 mm. Prastiyanto-1A and Prastiyanto-1E isolates demonstrated antibacterial activity against ESβL- *Escherichia coli* and ESβL + CRE- *Klebsiella pneumoniae* subsp *pneumoniae*, Pratiyanto-2A isolate showed antibacterial activity against MRSA, while Prastiyanto-4A isolates showed antibacterial activity against CRPA. The selected four isolates were identified as *Bacillus subtilis*, *Bacillus mojavensis* and *Bacillus simplex* using 16S rRNA gene sequencing and BLASTn analysis. These results provide information about the potential of bacterial symbionts of *S. officinalis* as natural antibacterial sources against MDR bacteria.

Keyword: Antibacterial, bacterial symbionts, multidrug-resistant, sponge, *Spongia officinalis*

INTRODUCTION

Infectious disease attributed to multidrug resistance (MDR) bacteria has become a health problem worldwide, including Indonesia. The use of antibiotics without following the guidelines is one of the causes of bacterial resistance to antibiotics (Bologa et al. 2013; Kon 2015). Patients infected with MDR bacteria have a high risk due to difficult treatment and the need for more treatment sources compared to the patients suffering from infections not related to MDR (Word Health Organization 2018). The Center for Diseases Control and Prevention (CDC) identifies the top MDR bacteria based on their threats. Several pathogenic bacteria such as methicillin-resistant, ESβL-producing Enterobacteriaceae, carbapenemase-resistant and Vancomycin-resistant are considered MDR bacteria that cause serious dangers (CDC 2019).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most common resistant bacterium and is the main cause of nosocomial infections worldwide, including in Indonesia. The prevalence rate of MRSA in hospitals in several Asian countries such as Korea, Japan, South, Taiwan, and China is 70-80% (Song et al. 2011). The carriage MRSA rate is 4.3% (64 of 1,502) among surgery patients discharged from Indonesian hospitals (Mayer et al. 2010). Meanwhile, the resistance of Enterococci bacteria to the vancomycin group has also become a serious problem. The emergence of the vancomycin-resistant *Enterococci* (VRE)- *Enterococcus faecalis* strains has caused great difficulties in antibiotic therapy (Adhikari 2010).

β-lactam is the most commonly used antibiotic to fight against infection caused Gram-negative bacteria, so many Gram-negative bacteria are resistant to β-lactam antibiotics. ESβL-producing Enterobacteriaceae, especially *Escherichia coli* and *Klebsiella pneumoniae* subsp *pneumoniae* have increased dramatically over the past few years (Kim et al. 2002; Bayraktar et al. 2019). Nearly 30% of ESβL-producing *K. pneumoniae* were identified from the total positive cultures in clinical specimens of patients at a hospital in South Sulawesi, Indonesia (Waworuntu et al. 2021). The resistance of Gram-negative bacteria to the Carbapenem class antibiotics has entered the critical list (WHO 2017). A natural antibacterial agent must be taken from a biological source.

Natural anti-MDR bacterial agents can be obtained from fruits (Prastiyanto et al. 2020d), seeds (Prastiyanto et al. 2020a; Prastiyanto 2021), Latex (Prastiyanto et al. 2020c) lactic acid bacteria (Lestari et al. 2019), mushroom (Prastiyanto et al. 2020b, 2016) and bacterial isolates from marine organisms (Al-dhabi et al. 2020). In recent years, studies of marine bioactive compounds have yielded many drug candidates (Webster and Taylor 2012). The bioactive potential of marine sources is effectively fights pathogens that infect humans (Blunt et al. 2017). Marine organisms including sponges, corals, Cnidaria, Arthropods, Echinodermata, and Tunicates have attracted the attention of many scientists over the past few decades because of the beneficial bioactive compounds producers (Radjasa et al. 2013, 2011; Nalini et al. 2018). Sponges are one of the most potential marine organisms with the potential for bioactive compounds. The bioactive compounds have been used as the sources of drugs such as anti-tumor, anti-cancer, anti-inflammatory, cytotoxic, anti-malarial, antifouling, immunosuppressive, antiviral, antifungal, and antibacterial (Mayer et al. 2010; Anjum et al. 2016).

Many studies have reported that sponges occupy the highest position of marine life, which shows potential as antibacterial agents. A novel alkaloid isoaaptamine isolated from the sponge *Aaptos aaptos* inhibits sortase A (SrtA), an enzyme that plays a key role in the retention and virulence of cell wall proteins in *S. aureus* (Jang et al. 2007). The *Dysidea granulose* (marine sponge) produces three polybrominated diphenyl ethers. They possessed broad-spectrum activity against methicillin-sensitive *S. aureus* (MSSA) and MRSA (Sun et al. 2015). However, obtaining bioactive compounds from marine sources requires a lot of materials. This will damage the marine ecosystem if the sponge is exploited continuously.

Spongia sp. has been reported to have bioactive compounds in the form of merosesquiterpenes, which show antibacterial activity against *S. aureus* (Nguyen et al. 2017). Several studies have recounted that many bioactive compounds from marine life are similar to the bioactive compounds of microorganisms associated with these marine biotas (König et al. 2006). The present investigation deals with isolating bacterial symbionts of sponge *S. officinalis* and the characterization of antibacterial potential against multidrug-resistant bacteria.

MATERIALS AND METHODS

The collection of sponge samples

Sponge samples were collected approximately from a depth of 1.5 m from Savu Sea, Kupang, East Nusa Tenggara, Indonesia in 8 November 2019 at 10°08'22.0"S 123°37'39.2"E (Figure 1). The obtained samples were then put into sterile bags underwater, stored in a cooler (4°C), and brought to the laboratory. The identification and classification of sponges were carried out in the Diponegoro University Fisheries and Marine Laboratory, Semarang, Indonesia.

Figure 1. Map of the study areas in the Savu Sea, Kupang, East Nusa Tenggara, Indonesia. A-D, Sampling sites and E, *Spongia officinalis* collected from the sampling site

Isolation of bacterial symbionts of *Spongia officinalis*

The sponges were processed under aseptic conditions. One gram of *S. officinalis* was rinsed with sterile seawater three times, crushed, and added with 9 mL of sterile seawater. The sponge sample was then diluted with 10^{-4}, 100 μL of which was taken and spread on Zobell marine agar (Marine agar 2216) Himedia® media, and then incubated at 35 ± 2°C for one week. Colonies were selected based on morphological differences. Colonies with different morphologies were transferred to the same media to obtain a pure culture.

Bacterial preparation

Multidrug resistance bacteria were isolated from patients of the hospital Dr. Kariadi, Semarang City, Indonesia (Table 1). All isolates were identified and susceptibility patterns were obtained using Vitek®MS (bioM´erieux). The MDR bacteria were sub-cultured on 5% sheep blood agar plate overnight (24 h) at 35±2°C. The MDR bacterial colonies were homogenized and adjusted to 0.5 McFarland standards (5×10^{8} CFU/mL) using McFarland Densitometer.

Screening for antibacterial activities against MDR bacteria

Screening to determine the antibacterial activity against MDR bacteria was carried out using the overlay method (Radjasa et al. 2013). The pure culture was inoculated ± 1 cm2 on Zobell marine agar medium in triplicate. After the bacteria grew, depending on the growth rate of the bacteria, which was commonly 1-7 days, the surface of the media was covered with Muller Hilton soft agar (0.3% (w/v) Muller Hilton broth, 1% (w/v) NaCl and 0.7% (w/v) agar containing 1% (v/v) MDR bacteria (ESβL- *E. coli*, ESβL+CRE- *K. pneumoniae* subsp *pneumoniae*, CRPA, MRSA, and VRE- *E. faecalis*.

All the plates were then incubated aerobically at 35 ± 2°C for 24 hours. Antibacterial activity of the isolates was determined by measuring the diameter of the inhibition zone in mm around the bacterial isolates. The levels of antibacterial activity were categorized as follows: no antibacterial activity (-), 0-1 mm (+), 1-3 mm (++), 3-7 mm (+++) and 7-15 mm (++++) (Asagabaldan et al. 2019). The inhibition area was measured to confirm the antibacterial activity (Apsari et al. 2019).

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$$\text{Inhibition index (II)} = \frac{\text{Diameter inhibition area (mm)} - \text{diameter colony (mm)}}{\text{Diameter colony (mm)}}$$

Table 1. The organisms for *in vitro* antibacterial screening

Species	Source	Antibiotic resistance pattern
ESβL- Escherichia coli	Urine	Ampicillin, Cefazolin, Ceftazidime, Ceftriaxone, Cefepime, Aztreonam, Ciprofloxacin, Nitrofurantoin Sulfamethoxazole
ESβL + CR- Klebsiella pneumoniae subsp pneumoniae	Urine	Ampicillin, Sulbactam, Tazobactam, Cefazolin, Ceftazidime, Ceftriaxone, Cefepime, Aztreonam, Ertapenem, Meropenem, Ciprofloxacine, Sulfamethoxazole
CRPA	Sputum	Ampicillin, Sulbactam, Tazobactam, Cefazolin, Ceftazidime, Ceftriaxone, Cefepime, Aztreonam, Meropenem, Amikacin Gentamicin, Ciprofloxacin, Tigecycline, Nitrofurantoin, Sulfamethoxazole
MDRO-Acinetobacter baumanii	Urine	Ampicillin, Sulbactam, Tazobactam, Cefazolin, Ceftazidime, Ceftriaxone, Cefepime, Aztreonam, Meropenem, Amikacin, Gentamicin, Ciprofloxacine, Sulfamethoxazole
MRSA	Wound	Benzylpenicillin, Oxacillin, Gentamicin, Ciprofloxacin, Levofloxacin, Moxifloxacin, Nitrofurantoin, Sulfamethoxazole
VRE Enterococcus faecalis	Urine	Gentamicin, Streptomycin, Ciprofloxacin, Levofloxacin, Erythromycin, Dalfopristin, Vancomycin, Tetracycline

Note: ESβL: extended-spectrum beta-lactamase, CR: Carbapenem-resistant, CRPA: Carbapenem-resistant *Pseudomonas aeruginosa*, MDRO: Multidrug-Resistant Organisms, MRSA: Methicillin-resistant *Staphylococcus aureus*, VRE: Vancomycin-resistant Enterococci.

Molecular identification of active bacterial symbionts of *Spongia officinalis*

DNA was extracted from bacterial cells (up to 1 x 10^9) using Presto^{TM} Mini g DNA Bacteria Kit (Geneaid) according to the appropriate protocols in the manufacturer's instructions, with a final elution volume of 50 μL. Extracted DNA was stored at 4°C until required for PCR. The concentration of bacterial DNA used was 50 ng/μL. The volume of bacterial DNA was 2 μL and mix with 16S rRNA gene primer. This step using 2 μL of 16S rRNA gene primer 27F '5'-AGAGTTGATCMTGGCTCAG-3' and 2 μL of 16S rRNA gene primer 1492R '5'-CGGTTACCTTGTTACGACTT-3'. The final concentration of 10 μM primer was 10 μM. Formulation mixing is nuclease free water 6.5 μL, master mix (Promega) 12.5 μL, primer and DNA template. The amplification conditions of both PCRs were as follows. The heat started to activate the Taq polymerase enzyme at a temperature of 95°C for 4 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, primer annealing at 57°C for 30 seconds, extension at 72°C for 2 minutes, an extra extension for 72°C at 10 minutes, and cooling down at 4°C for 10 minutes on a Biometra Thermal cycler. PCR products were separated on a 2% agarose gel and DNA bands were visualized with Flourovue. Four microliters of FluoroVue were added to a mixture of 1 g agarose and 100 ml TAE. PCR product sequencing was carried out by Genetica Science Tangerang to analyze 16S rRNA gene sequences, then the tracking results through the Basic Local Alignment Search Tool (BLAST) database program at the National Center for Biotechnology Information NCBI), National Institute for Health, USA (www.ncbi.nlm.nih.gov)

Phylogenetic analysis

MEGA X software was used for phylogenetic analysis. The results of 16S rRNA gene sequencing were aligned using ClustalW. The phylogenetic trees were determined by the neighbor-joining method with Tamura-Nei model and completed with Non-parametric bootstrapping analysis (1000 datasets) from 16S rRNA gene sequences showing the phylogenetic relationships of closely related strains database available at NCBI GenBank.

RESULTS AND DISCUSSION

Bacterial symbionts of *Spongia officinalis*

The isolation of bacterial symbiont of *S. officinalis* resulted in 10 isolates (Figure 2). The outcomes showed different characters of bacterial isolates. The five out of 10 isolates were rod-shaped, Gram-positive, and endospore-forming. Four isolates were rod-shaped, Gram-positive, non-endospore-forming, while one isolate showed Gram-negative coccus (Table 2).

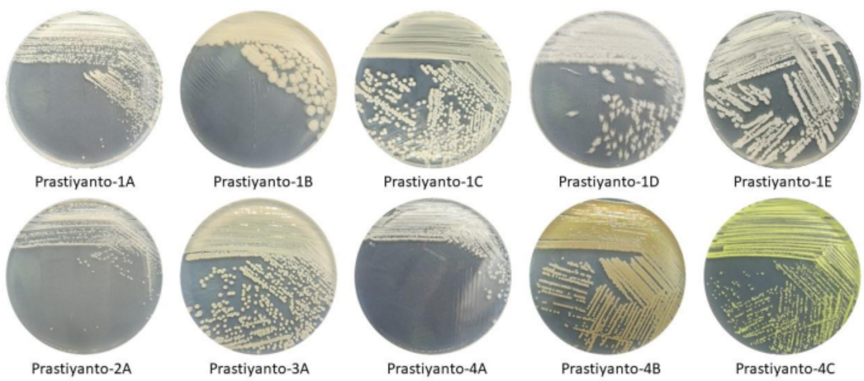


Figure 2. Macroscopic morphology of bacteria isolated from *Spongia officinalis* on Zobell marine agar

Table 2. Morphology and Gram staining of bacteria isolated from *Spongia officinalis*

Isolate	Morphology colony			Gram staining
	Form	Margin	Elevation	
Prastiyanto-1A	Circular	Curled	Convex	Rod-shaped, Gram-positive, endospore-forming
Prastiyanto-1B	Irregular	Undulate	Convex	Rod-shaped, Gram-negative, endospore-forming
Prastiyanto-1C	Circular	Entire	Convex	Coccus Gram-negative
Prastiyanto-1D	Irregular	Erose	Convex	Rod-shaped, Gram-positive, endospore-forming
Prastiyanto-1E	Circular	Curled	Convex	Rod-shaped, Gram-positive, endospore-forming
Prastiyanto-2A	Circular	Undulate	Convex	Rod-shaped, Gram-positive, endospore-forming
Prastiyanto-3A	Irregular	Undulate	Convex	Rod-shaped, Gram-positive, non- endospore-forming
Prastiyanto-4A	Circular	Undulate	Convex	Rod-shaped, Gram-positive, non- endospore-forming
Prastiyanto-4B	Circular	Entire	Convex	Rod-shaped, Gram-positive, non- endospore-forming
Prastiyanto-4C	Circular	Entire	Convex	Rod-shaped, Gram-positive, non- endospore-forming

Antibacterial activities against MDR bacteria

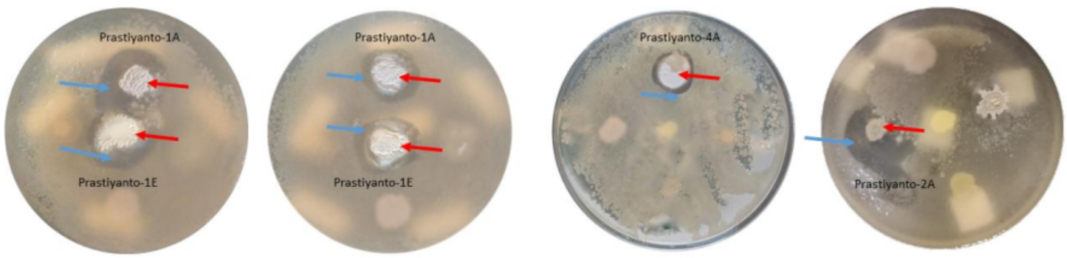
Antibacterial activity against MDR bacteria of bacterial of *S. officinalis* is indicated by the presence of an inhibition zone (Figure 3). The inhibition zone is a qualitative way to determine the ability of an antimicrobial agent to inhibit the growth of microorganisms, particularly the MDR bacteria. The results of this study revealed that four (40%) of 10 isolates showed antibacterial activity against MDR bacteria. Prastiyanto-1A and Prastiyanto-1E isolates demonstrated antibacterial activity against ESβL- *E. coli* and ESβL+CRE- *K. pneumoniae* subsp *pneumoniae*, Pratiyanto-2A isolate showed antibacterial activity against MRSA, while Prastiyanto-4A isolate proved antibacterial activity against CRPA.

Identification of bacteria symbionts of *Spongia officinalis*

Identification of the active bacterial symbionts *S. officinalis* that showed antibacterial activity against MDR was performed based on the 16S rRNA gene (Figure 4). The results showed that four isolates having antibacterial activity against MRD bacteria belonged to the members of the *Bacillus* genus. Prastiyanto-1A isolate demonstrated a close relationship with *Bacillus subtilis* SWI4a. Prastiyanto-1E isolate with *B. subtilis* PBBBS1, Prastiyanto-4A isolate with *Bacillus mojavensis* ifo 15718 and Prastiyanto-2A isolate was closely related to *Bacillus simplex* K1-6.

Phylogenetic analyses

Phylogenetic analysis showed that all strains related to the genera validly described species originating from marine habitats. Prastiyanto-1A isolate demonstrated a close relationship with *B. subtilis* SWI4a. *B. subtilis* SWI4a was a bacterium isolated from seaweed *Anthophycus longifolius* and has antibacterial activity (Chakraborty et al. 2014).



A B C D

Figure 3. Zones of inhibition of bacteria isolated from *Spongia officinalis* against MDR bacteria: A. ESβL- *Escherichia coli*, B. ESβL + CRE- *Klebsiella pneumoniae* subsp *pneumonia*, C. MRSA, and D. CRPA. → **:** Zone of inhibition, → : colony of bacteria isolated from *Spongia officinalis*

Table 3. Antibacterial activities of bacteria isolated from *Spongia officinalis* against multidrug-resistant bacteria

Isolate	ESβL- *Escherichia coli*		ESβL + CRE- *Klebsiella pneumoniae* subsp *pneumoniae*		CRPA		MDRO- *Acinetobacter baumanii*		MRSA		VRE- *Enterococcus faecalis*	
	Levels of activity	II (mm)	Levels of activity	II (mm)	Levels of activity	II (mm)	Levels of activity	II (mm)	Levels of activity	II (mm)	Levels of activity	II (mm)
Prastiyanto-1A	+++	6.4	++++	7.7	-	-	-	-	-	-	-	-
Prastiyanto-1B	-	-	-	-	-	-	-	-	-	-	-	-
Prastiyanto-1C	-	-	-	-	-	-	-	-	-	-	-	-
Prastiyanto-1D	-	-	-	-	-	-	-	-	-	-	-	-
Prastiyanto-1E	+++	6.1	++++	9.2	-	-	-	-	-	-	-	-
Prastiyanto-2A	-	-	-	-	-	-	-	-	++++	12.6	-	-
Prastiyanto-3A	-	-	-	-	-	-	-	-	-	-	-	-
Prastiyanto-4A	-	-	-	-	+++	4.8	-	-	-	-	-	-
Prastiyanto-4B	-	-	-	-	-	-	-	-	-	-	-	-
Prastiyanto-4C	-	-	-	-	-	-	-	-	-	-	-	-

Note: - :denotes no effect

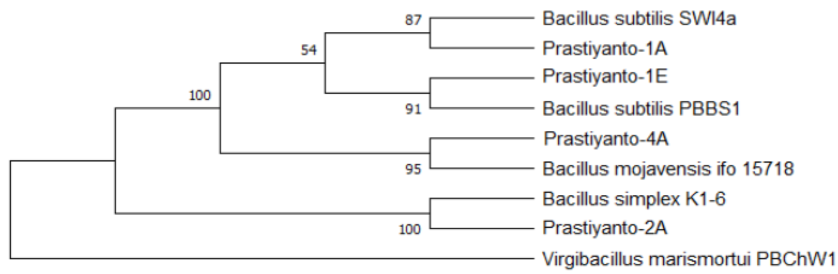


Figure 4. Neighbor-joining tree constructed with Tamura-Nei model from 16S rRNA gene sequences showing the phylogenetic relationships of strains from this study to closely related species

The total of 10 strains was selected for isolation of pure cultures according to morphology colony and Gram staining (Table 2). Forty percent (4 isolates) of the total isolates showed inhibitory activities against MDR bacteria (Table 3). Trianto et al. (2019) reported that 324 bacterial isolates associated with 55 sponges (isolates and Sponges ratio of 5.1-10.5) were produced. According to Webster and Thomas (2016), the diversity level of bacteria associated with sponges greatly varies among sponge species. Characterization of bacterial isolates associated with *S. officinalis* was carried out macroscopically (colony morphology) and microscopic (Gram staining).

The results of this study proved that Prastiyanto-1A, Prastiyanto-1E, Pratiyanto-2A and Prastiyanto-4A isolates associated with *S. officinalis* indicated antibacterial activity against MDR bacteria with an inhibition index ranging from 4.8 to 12.6 mm. These findings are consistent with the results of previous studies regarding the antibacterial activity of *S. officinalis* extract. *Spongia officinalis* extract with methanol-toluene solvent was able to inhibit the growth of *S. epidermidis* (5-12 mm), *Streptococcus lactis* (5-12 mm), and *B. subtilis* (2-5 mm) (McCaffrey and Endean 1985).

Relevant studies reported that extracts of *S. officinalis* could inhibit *S. aureus* and *P. aeruginosa* (Gonaález et al. 2012). Moreover, it was also reported that microorganisms associated with marine biota are the producers of bioactive compounds (König et al. 2006). Davidson and Haygood (1999) confirmed that the producer of the bryostatin compound from *Bugula neritina* was the microbial symbionts of *Candidatus Endobugula sertula*. The antibacterial activity against MDR bacteria belonged to the members of the *Bacillus* genus. Previous studies on bacteria associated with *Spongia* have shown similar results (Odekina et al. 2020). According to Mondol et al. (2013) *Bacillus* isolated from the sea produces secondary metabolites various antimicrobial activities (Mondol et al. 2013).

Phylogenetic analysis showed that Prastiyanto-1E isolate *B. subtilis* PBBS1, a bacterium isolated from the marine sediments of the Burmanallah coast (Cherian et al. 2019). Prastiyanto-4A isolate was very much linked to *B. mojavensis* ifo 15718, which was distilled from the sea and had antimicrobial potential (Ma et al. 2012) while Prastiyanto-2A isolate was associated with *B. simplex* K1-6 isolated from a coastal city, Izmir, Turkey (Cherian et al. 2019). The bioactive compounds of all isolates that have the potential to act as antibacterial agents against MDR bacteria in this study have not been investigated. However, several studies have reported the antibacterial potential of the *Bacillus* genus members. *Surfactin* produced by *B. subtilis* C9 exhibits antibacterial and antiviral activities (Kim et al. 1998). MacrolactinW extracted from ethyl acetate fraction from the fermentation of *Bacillus* sp. 09ID194 isolated from the sea showed strong antibacterial activity against *Escherichia coli*, *P. aeruginosa*, and *Staphylococcus aureus* (Mondol et al. 2011).

In conclusion, the bacterial symbionts of *S. officinalis* potential as antibacterial agents against MDR bacteria belong to *Bacillus*. These results provide information about the potential of bacteria associated with *S. officinalis* as natural antibacterial sources against MDR bacteria.

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