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Isolation and Characterization of New Antibiotics from Indonesian Coastal Marine Bacteria

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Antibiotics are organic compounds produced by various microorganisms and have the ability to inhibit the growth or kill other microorganisms. However, the irrational application of antibiotics lead to resistance of microorganisms so that they become ineffective. The objectives of this study were to isolate and characterize new antibiotics from Indonesian coastal marine bacteria. In this study, a total of 141 isolates consisting of seven *Streptomyces* sp. isolates and 134 isolates of other marine bacteria, were obtained from Indonesian coastal regions. Based on antimicrobial activity assay, four *Streptomyces* sp. and five marine bacteria isolates showed antimicrobial activity towards *Bacillus cereus* and *Staphylococcus* aureus with the diameter of inhibition of 3-12 mm. Further, antimicrobial compounds were produced successfully extracted with six organic solvents, such as 1-butanol, dichloromethane, n-hexane, chloroform, and toluene. The best solvent to extract antimicrobial compounds from *Streptomyces* sp. isolates was 1-butanol, while the best solvent to extract antimicrobial compounds from other marine bacteria isolates could not be specified. Antimicrobial compounds were successfully separated by thin layer chromatography with mobile phase used were 1-butanol, acetic acid, and water at a ratio of 4:1:2 and retention values obtained at 0.50 and 0.63.

Key words: antibiotics, marine bacteria, *Streptomyces* sp., extraction, thin layer chromatography

Antibiotik merupakan senyawa organik yang dihasilkan oleh berbagai mikroorganisme dan mempunyai kemampuan untuk menghambat pertumbuhan maupun membunuh mikroorganisme lain. Namun, cara penggunaan antibiotik yang kurang tepat telah memicu terjadinya resistensi mikroorganisme, sehingga penggunaan antibiotik menjadi tidak efektif lagi. Tujuan dari studi ini ialah mengisolasi dan mengkarakterisasi antibiotik baru dari bakteri laut daerah pantai Indonesia. Dalam studi ini, sebanyak 141 isolat yang terdiri dari tujuh isolat *Streptomyces* sp. dan 134 isolat bakteri laut lainnya, berhasil diisolasi dari daerah pantai Indonesia. Berdasarkan uji aktivitas antimikroba, empat isolat *Streptomyces* sp. dan lima isolat bakteri laut lainnya menunjukkan aktivitas antimikroba terhadap *Bacillus cereus* dan *Staphylococcus aureus* dengan diameter zona penghambatan sekitar 3-12 mm. Selanjutnya, senyawa antimikroba yang dihasilkan berhasil diekstraksi dengan enam pelarut organik, yaitu 1-butanol, diklorometana, heksana, kloroform, dan toluena. Pelarut terbaik untuk mengekstraksi senyawa antimikroba dari isolat *Streptomyces* sp. adalah 1-butanol, sedangkan pelarut terbaik untuk mengekstraksi senyawa antimikroba dari isolat bakteri laut lainnya tidak spesifik. Senyawa antimikroba tersebut berhasil dipisahkan dengan metode kromatografi lapis tipis dengan fase gerak yang digunakan adalah 1-butanol, asam asetat, dan akuades dengan rasio 4:1:2 dan diperoleh nilai faktor retensi sebesar 0.50 dan 0.63.

Kata kunci :antibiotik, bakteri laut, *Streptomyces* sp., ekstraksi, kromatografi lapis tipis

Antibiotics are organic compounds produced by various microorganisms and have the ability to inhibit the growth or kill other microorganisms. However, the irrational application of antibiotics lead to resistance of microorganisms so that they become ineffective (Sunaryanto *et al.* 2009). In 2003, antibiotics used for treatment of diseases were 84% of hospitalized patients in Indonesia. Meanwhile, in 2012 the results of surveillance showed decline of inappropriate use of antibiotics, however extended-spectrum β-lactamase-producing *Escherichia coli* and methicillin-resistant *Staphylococcus aureus* increased. In addition, 81% of the hospitalized patients carried multiresistant *E. coli*, such as resistance to ampicillin (73%), trimethoprim (56%), chloramphenicol (43%), ciprofloxacin (22%), and gentamicin (18%) (Hadi *et al.* 2013). This triggers research to renew the mechanism of existing antibiotics and to seek and obtain new antibiotics from new resources, such as coastal region.

Almost 70% of antibiotics originated from *Actinomycetes*, mostly from the genera *Streptomyces*. *Actinomycetes* has the ability to synthesize secondary metabolites, such as enzymes and antibiotics. One of the countries with largest coastal region is Indonesia -

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an archipelago which has sea covering up two-thirds of its entire region. Moreover, sea has greater biodiversity than land so that the type of microorganisms obtained from the coastal region will also be more varied compared to soil microorganisms. However, the potential of marine *Actinomycetes* and other marine bacteria producing antimicrobial compounds has not been explored (Sunaryanto *et al.* 2009; Gokulkrishnan *et al.* 2011). The objectives of this study were to isolate and characterize new antibiotics from Indonesian coastal marine bacteria.

MATERIALS AND METHODS

Samples Collection. Samples were collected from 22 coastal regions in Java, Sumatera, Kalimantan, and Sulawesi. Sea sediments and water samples (approximately 80-100 cm below sea level) were collected and stored at 4 °C.

Bacterial Isolation. Sediment samples were heated at 65 °C for 60 min to suppress Gram-negative bacteria and to optimize the growth of marine *Streptomyces* sp. (Sunaryanto *et al*. 2009). One gram of dried sample were inoculated into 100 mL modified Starch Casein Broth and incubated at 28 °C with 120 rpm agitation for 7 d. After 7 d incubation, samples were diluted to 10^{-2} and spread to modified Starch Casein Agar (SCA) and Marine Agar (MA) (Difco). Both media were incubated at 28 °C for 7 d. Colonies with different morphologies were picked and cultivated in SCA for *Streptomyces* sp. and MA for other marine bacteria (Poorani *et al.* 2009).

Preliminary Screening of Antimicrobial Activity. Each isolate was inoculated into 10 mL Brain Heart Infusion (BHI) (Oxoid) and incubated at 28 °C with 120 rpm agitation for 6 days for *Streptomyces* sp. and 3 days for other marine bacteria. After incubation, the medium were centrifuged at 13 684 ×g (Thermo Scientific) for 15 min. The supernatant was collected and used in the antimicrobial activity assay. Test bacteria used for the antimicrobial activity assay were foodborne pathogens. The assay was done using agar well diffusion against *Bacillus cereus*, *Staphylococcus aureus* (ATCC 25923), *Salmonella typhi*, *Pseudomonas aeruginosa*, and Enteropathogenic *E. coli.* All of them were obtained from Atma Jaya Culture Collection. The clear zone around each well were interpreted as positive results of antimicrobial activity.

Extraction of Antimicrobial Compound. Pure cultures that have antimicrobial activity were inoculated into 60 mL BHI and incubated at 28 °C with 120 rpm agitation for 6 d for *Streptomyces* sp. and 3 days for other marine bacteria. After incubation, the medium were centrifuged at 5 857 ×g (Thermo Scientific) for 15 min. The supernatant was collected and transferred to a new tube. Extraction of antimicrobial compound was done with method of Augustine *et al*. (2005). The organic solvents used were 1-butanol, dichloromethane, chloroform, n-hexane, and toluene (Merck). The organic phase was collected, air-dried overnight, and dissolved in 1 mL Phosphate Buffered Saline (PBS). The crude extract are to be used in the next step.

Secondary Screening of Antimicrobial Activity. Crude extract from previous step was used in the antimicrobial activity assay. The antimicrobial activity assay was done using agar well diffusion against *Bacillus cereus*, *Staphylococcus aureus* (ATCC 25923), *Salmonella typhi*, *Pseudomonas aeruginosa*, and Enteropathogenic *E. coli.* Positive results of antimicrobial activity were determined by measuring the sizes of inhibitory zone around the wells.

Separation of Antimicrobial Compounds. About 2 µl of crude extract was spotted on silica 60 F_{254} (Merck). The plate was placed in TLC chamber which contains the mobile phase. Several mobile phase combination were used, such as 1-butanol:acetic acid:water (4:1:2) (Dharmaraj *et al*. 2010), ethanol:water:chloroform (4:4:2) (Augustine *et al.* 2005), methanol:dichloromethane (1:10) (Zheng *et al*. 2005a), and methanol:acetic acid:benzene (3:1:1) (Selvendran and Babu 2013). The plate was dried and visualized under ultraviolet (UV) light or sprayed with H_2SO_4. The retention factor (Rf) value for each spot were measured using standard formula.

$$\text{R}_\text{f}\text{ value} = \frac{\text{distance travelled by solute}}{\text{distance travelled by solvent.}}$$

RESULTS

Bacterial Isolation. A total of 141 isolates with different morphologies from 22 coastal regions were obtained from SCA and MA medium (Table 1). From 141 isolates, only seven isolates were *Actinomycetes* and all of them were isolated from Sindhu Sanur Beach, Bali.

Table 1 Isolates obtained from 22 coastal regions in Indonesia

Region	Sample Code	Origin of Location	Number of Isolates
Java	A1	Carita Beach	7
	A2	Pari Island (near fish cages)	7
	A3	Pari Island (near coral reef)	7
	A4	Indrayanti Private Beach	5
	A5	Indrayanti Public Beach	3
	A6	Alam Indah Beach	5
	A7	Pangandaran Beach	4
	A8	Burung Indah Island	3
	A9	Kongsi Island	3
	A10	Pasir Perawan Beach	6
	A11	Pasir Perawan Beach (near mangrove)	7
Sumatra	B1	Tanjung Pendam Beach	9
	B2	Pasir Padi Beach	7
	B3	Mabay Beach	5
	B4	Lengkuas Beach	4
Sulawesi	C1	Akarena Beach	7
Kalimantan	D1	Singkawang Beach	6
Bali-Nusa Tenggara	F1	Bali Beach	9
	F2	Kuta Beach	3
	F3	Sindhu Sanur Beach	23
	F4	Gili Island	3
	F5	Tuban Beach	8

Preliminary Screening of Antimicrobial Activity. From 141 isolates, only nine isolates showed antimicrobial activity. There were four *Streptomyces* sp. isolates and five isolates of other marine bacteria from Sindhu Sanur Beach, Bali. Four *Streptomyces* sp. isolates showed antimicrobial activity towards *B. cereus*, *S. aureus*, and *E. coli*, while five isolates of other marine bacteria showed antimicrobial activity only towards *B. cereus* and *S. aureus* (Table 2).

Table 2 Antimicrobial activity of nine isolates from Sindhu Sanur Beach

Isolate	Diameter of inhibition (mm)					
	B. cereus	*S. aureus*	*S. typhi*	*P. aeruginosa*	*E. coli*	EPEC
F3.1	7.5 ± 0.7	6.5 ± 0.7	-	-	-	-
F3.5	5.5 ± 0.7	4.0 ± 0.0	-	-	-	-
F3.6	2.5 ± 0.7	-	-	-	-	-
F3.7	5.0 ± 1.4	3.0 ± 0.0	-	-	-	-
F3.8	5.0 ± 0.0	-	-	-	-	-
F3.S1	5.5 ± 0.7	15.5 ± 0.7	-	-	4.5 ± 0.7	-
F3.S2	3.5 ± 0.7	14.5 ± 0.7	-	-	-	-
F3.S3	3.5 ± 0.7	16.0 ± 0.0	-	-	4.0 ± 0.0	-
F3.S6	-	9.5 ± 0.7	-	-	-	-

Results are mean ± standard deviation (n=2).

Table 3 Antimicrobial activity towards *B. cereus* after extraction

Isolate	Diameter of inhibition (mm)					
	Cell -free supernatant	Extracted by				
		1-butanol	Dichlorometh ane	n-hexane	Chloroform	Toluene
F3.1	2.0 ± 0.0	4.5 ± 0.7	5.5 ± 0.7	5.0 ± 0.0	4.5 ± 0.7	5.0 ± 0.0
F3.5	4.5 ± 0.7	10.0 ± 0.0	9.0 ± 0.0	12.0 ± 0.0	8.5 ± 0.7	8.5 ± 0.7
F3.6	2.5 ± 0.7	5.5 ± 0.7	6.5 ± 0.7	6.0 ± 1.4	3.5 ± 2.1	6.0 ± 1.4
F3.7	3.0 ± 0.0	5.5 ± 0. 7	7.5 ± 0.7	8.5 ± 0.7	4.5 ± 0.7	5.5 ± 0.7
F3.8	7.0 ± 0.0	12.0 ± 0.0	8.0 ± 0.0	9.0 ± 0.0	10.0 ± 0.0	8.5 ± 0.7

Results are mean ± standard deviation (n=2).

Secondary Screening of Antimicrobial Activity. Those antimicrobial compounds from nine isolates were extracted using various organic solvents and showed antimicrobial activity towards *B. cereus* and *S. aureus* (Table 3, Table 4). Almost all isolates depicted greater inhibition zones after extraction using organic solvents.

Separation of Antimicrobial Compounds. Antimicrobial compound from those isolates were separated by TLC with various mobile phase combination. The result showed different number and position of spots, and also different Rf value (Table 5).

The result of TLC with 1-butanol:acetic acid:water (4:1:2) as mobile phase, were two spots with different Rf values (Table 5). F3.1 was chosen as a representative isolate producing two antimicrobial compounds. Each spot from isolate F3.1 was dissolved by PBS and used for antimicrobial activity assay. The second spot with 0.63 Rf value can inhibit *B. cereus*, while the first spot with 0.50 Rf value cannot inhibit *B. cereus*. Diameter of zone inhibition was 3 mm (Fig 1).

Table 4 Antimicrobial activity towards *S. aureus* after extraction

Isolate	Diameter of inhibition (mm)					
	Cell -free supernatant	Extracted by				
		1-butanol	Dichloromethane	n-hexane	Chloroform	Toluene
F3.8	3.0 ± 0.0	4.0 ± 0.0	5.0 ± 0.0	6.0 ± 0.0	7.0 ± 0.0	6.0 ± 0.0
F3.S1	3.0 ± 0.0	9.0 ± 0.0	6.0 ± 0.0	-	5.5 ± 0.7	3.5 ± 0.7
F3.S2	2.0 ± 0.0	5.5 ± 0.7	4.0 ± 0.0	-	5.0 ± 0.0	5.0 ± 0.0
F3.S3	3.0 ± 0.0	5.5 ± 0.7	3.5 ± 0.7	2.5 ± 0.7	3.5 ± 0.7	3.0 ± 0.0
F3.S6	1.0 ± 0.0	3.0 ± 0.0	-	-	-	-

Results are mean ± standard deviation (n=2); - showed no inhibition zone.

Table 5 Rf value from various mobile phase combination

Isolate	Rf Value from Various Mobile Phase Combination			
	A	B	C	D
F3.1	0.50 ; 0.63	0.81	0.06	0.71
F3.5	0.50 ; 0.63	0.88	0.06	0.71
F3.6	0.63	0.88	0.06	0.71
F3.7	0.50	0.88	0.06	0.71
F3.8	0.63	0.88	0.06	0.75
F3.S1	0.50	0.88	0.04	0.75
F3.S2	0.50	0.88	0.05	0.75
F3.S3	0.50	0.88	0.06	0.75
F3.S6	0.50	0.88	0.08	0.73

(A) 1-butanol:acetic acid:water (4:1:2), (B) ethanol:water:chloroform (4:4:2), (C) methanol:dichloromethane (1:10), (D) methanol:acetic acid:benzene (3:1:1).

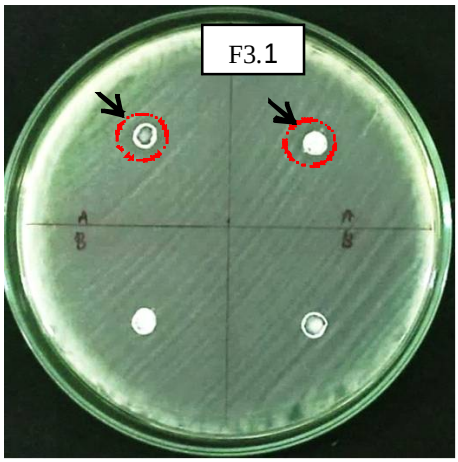

Fig 1 Antimicrobial activity by second spot with 0.63 Rf value from isolate F3.1. Bottom: first spot with 0.50 Rf value; Top: second spot with 0.63 Rf value.

DISCUSSION

The microorganisms living and growing in the marine environment are metabolically and physiologically more diverse compared to terrestrial microorganisms. However, the potential of marine microorganism has not been explored, especially marine microorganisms in Indonesian's marine environment. The most studied marine microorganisms are *Actinomycetes*, while other marine bacteria have not yet been extensively studied. From previous study, marine *Actinomycetes* with antimicrobial activity were successfully isolated from three beaches in Indonesia, such as Cirebon Desa Gebang Beach, Anyer Beach, and Kukup Gunung Kidul Yogyakarta Beach (Sunaryanto *et al.* 2009). In this study, the objective was to isolate *Actinomycetes* and other marine bacteria from different location in Indonesia possessing antimicrobial activity.

From 141 marine bacteria that were successfully isolated from 22 coastal regions in Indonesia, only seven isolates were *Actinomycetes*. Previous study also obtained similar results (Sunaryanto *et al.* 2009). The number of *Actinomycetes* that were successfully isolated from the marine environment tends to be less than the number of *Actinomycetes* that were isolated from soil and brackish water. It suggests soil structure, humidity, and pH in soil and brackish water are optimum condition for *Actinomycetes* growth.

Furthermore, only nine from 141 isolates (6%) showed antimicrobial activity in this study. Similar result were also obtained by a previous study reporting that only five from 77 isolates (6%), from sediment and sea water, have antimicrobial activity (Zheng *et al*. 2005b). Usually, bacteria are associated with marine invertebrates and seaweeds have greater activity than bacteria isolated from sediment and sea water. These bacteria could acquire the necessary nutrition from their hosts, while on the other hand, they could excrete products such as antibiotic and toxin to improve the chemical defense capability of the hosts.

The result of antimicrobial activity assay showed that the isolates were active against Gram-positive bacteria (*B. cereus* and *S. aureus*) compared to Gram-negative bacteria. It is caused by the structural differences between these microorganisms. Gram-negative bacteria having an outer polysaccharide membrane that makes the cell wall impermeable to lipophilic solutes, while Gram-positive bacteria having only a peptidoglycan layer which is not an effective permeability barrier (Khajure and Rathod 2011).

Throughout secondary screening, all isolates showed different activities compared to preliminary screening. Some isolates lost their activities, such as isolate F3.S1 and F3.S3 were only inhibited by *S. aureus* whereas previously they could inhibit *S. aureus, B. cereus,* and *E. coli* (Table 4). According to Khajure and Rathod (2011), during screening of antimicrobial compounds, marine bacteria often showed antimicrobial activity on agar but not in liquid medium. However, F3.8 showed improved activity depicting greater inhibition zone after extraction (Table 3, Table 4).

When compared with marine *Actinomycetes* isolates obtained by Sunaryanto *et al.* (2009), marine *Actinomycetes* in this study had a lower activity. Their isolates could inhibit *E. coli, S. aureus, P. aeruginosa*, and *Bacillus subtilis*. To get a better activity, isolates in this study should be grown in a richer medium and incubated for longer time.

Based on the results, the antimicrobial compounds can be extracted with 1-butanol, dichloromethane, n-hexane, chloroform, and toluene. These results differ from previous study reporting that antimicrobial compounds can only be extracted with n-hexane and petroleum ether, and cannot be extracted using other solvents such as n-butanol, ethyl acetate, chloroform, benzene, and xylene (Augustine *et al*. 2005). It showed that antimicrobial compounds should be extracted with different solvents depending on the suitability of antimicrobial compounds and solvents polarity.

The best solvent to extract antimicrobial compounds from *Streptomyces* sp. isolates was 1-butanol, however the best solvent to extract antimicrobial compounds from marine bacteria isolates cannot be specified. In this study, solvents with higher polarity yield better extraction of antimicrobial compounds. Similar result was also obtained by previous study that used a range of solvents like n-hexane, chloroform, ethyl acetate, benzene, n-butanol, and ethanol to extract the antimicrobial compounds from *Actinomycetes* (Rabah *et al.* 2007). They reported that n-hexane and chloroform were poor solvents, while n-butanol was a good solvent to extract the antimicrobial compounds. The reason is that n-butanol is more polar compared to n-hexane and chloroform.

In this study, inhibition zones produced from crude extract is greater than inhibition zones from cell free supernatant. Similar result were also obtained by previous study that inhibition zones after extraction with ethyl acetate is greater than before extraction (Jafarzade *et al*. 2013). Extraction of antimicrobial

compounds will increase the purity and therefore will result in a better zone of inhibition since the impurities contained in the cell free supernatant could interfere in the antimicrobial activity.

Separation using TLC with various mobile phase combinations showed different number and position of spots, and also Rf values. It depends on the suitability of antimicrobial compounds, stationary phase, and mobile phase polarity. The best mobile phase combination is 1-butanol, acetic acid, and water because it produced two spots that showed there are two compounds in one solution. The second spot with 0.63 Rf value can inhibit *B. cereus*, while the first spot with 0.50 Rf value cannot inhibit *B. cereus*. It showed that the second spot is an antimicrobial compound that has a high purity because it is still able to inhibit bacterial growth with low concentration (marked with a thin spot on TLC plate). However the first spot may not necessarily be an antimicrobial compound. The first spot may be an antimicrobial compound that loses its activity because both of these compounds are synergistic so that when they were separated it will reduce their activity. In addition, the first spot may have antimicrobial activity against other test bacteria (Mangunwardoyo *et al*. 2009).

Other mobile phase combination is ethanol, water, and chloroform with ratio 4:4:2. Polarity between antimicrobial compound and mobile phase is almost similar so that the compound was carried by the mobile phase. Other mobile phase combination is methanol and dichloromethane with ratio 1:10. Polarity between antimicrobial compound and mobile phase is not similar so that the compound is not carried by the mobile phase and not separated properly. These results differ from previous studies. It showed that the antimicrobial compounds and Rf value were different (Zheng *et al*. 2005a; Selvendran and Babu 2013).

Four *Streptomyces* sp. and five marine bacteria isolates from Sindhu Sanur Beach, Bali showed antimicrobial activities towards *B. cereus* and *S. aureus* with the diameter of inhibition about 3-12 mm. All of the solvents can be used to extract the antimicrobial compounds depending on the suitability of antimicrobial compounds and solvents polarity. The best solvent to extract the antimicrobial compound from *Streptomyces* sp. isolates was 1-butanol, while the best solvent to extract antimicrobial compound from marine bacteria isolates could not be specified. The best mobile phase combination on TLC for separating the antimicrobial compounds is 1-butanol, acetic acid, and water (4:1:2) with 0.50 and 0.63 Rf values.

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