Brief Report

BIO Integration

Screening of antibacterial activities of *Bacillus* spp. isolated from the Parangkusumo coastal sand dunes, Indonesia

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Abstract

Background: The emergence of multidrug-resistant bacteria because of poor understanding of the issue and the misuse of antibiotics has become global health concern. Therefore, the discovery of novel antibacterial drugs is urgently needed. New antibacterial compounds may be found in the *Bacillus* species, which are abundant in sand dune ecosystems. Herein, we examined samples from the Parangkusumo coastal sand dunes in Indonesia.
Methods: Samples were collected from three areas in the sand dunes (the area closest to the sea, the core area of sand dunes, and the area farthest from the sea). The samples were inoculated on Luria Bertani agar. Morphological and molecular identification was performed on the basis of 16S rRNA. The samples' antimicrobial activity was evaluated with the disc diffusion method and compared with that of opportunistic pathogenic bacteria.
Results: Five species of *Bacillus* were successfully isolated from the Parangkusumo coastal sand dunes. To our knowledge, this is the first report of the isolation of *Bacillus aryabhattai* in Indonesia. All samples showed antimicrobial activity against pathogenic bacteria. *B. velezensis* and *B. subtilis* showed antibacterial activity against Gram-positive bacteria, whereas *B. aryabhattai* and *B. megaterium* showed antibacterial activity against Gram-negative bacteria, and *B. spizizenii* showed antibacterial activity toward Gram-positive and Gram-negative bacteria.
Conclusion: Five *Bacillus* species were successfully isolated from the Parangkusumo coastal sand dunes, Indonesia, and all samples showed antimicrobial activity toward opportunistic pathogenic bacteria. The crude antimicrobial compounds from *B. megaterium*, *B. aryabhattai*, *B. subtilis*, and *B. spizizenii* showed the highest growth-inhibition activity against *E. coli*, *P. aeruginosa*, *B. cereus*, and *S. aureus*, respectively.

Statement of Significance

This research is the first attempt to isolate and screen the antibacterial activity of bacterial species from the Parangkusumo sand dunes, Indonesia, one of the few tropical coastal sand dunes. The notable discoveries in this research included the first isolation of *B. aryabhattai* in Indonesia and the determination of the potential of this species to produce crude antimicrobial compounds (CACs) that inhibit the growth of pathogenic *Pseudomonas aeruginosa*.

Keywords

Antibacterial activity, *Bacillus*, Bioprospecting, Coastal sand dunes, Infectious disease.

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Introduction

The emergence of multidrug-resistant bacteria because of poor understanding of the issue and the misuse of antibiotics has become a global health concern [1, 2]. Every year, millions of cases of multidrug-resistant bacterial infections occur, causing tens of thousands of deaths and economic losses [3, 4]. Thus, the discovery of novel antibacterial drugs is urgently needed [5].

Studies aiming to discover new antibacterial compounds have shifted toward underexplored ecosystems, particularly marine and extreme environments [6]. One such ecosystem is the Parangkusumo coastal sand dunes in Yogyakarta, Indonesia, which are the only sand dunes in tropical Southeast Asia [7]. Coastal sand dunes are defined as

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mounds and narrow strips of sand with distinct boundaries determined by the sea and landward limits of sand transport [8]. The ecosystem is characterized by high salinity, low moisture, and low organic-matter content, and is hostile toward life forms including microorganisms [8–10]. One abundant genus in the coastal sand dune ecosystem is *Bacillus* [11, 12].

The *Bacillus* genus consists of more than 300 species of Gram-positive, rod-shaped, spore-forming bacteria that produce numerous antibacterial compounds [13], including bacteriocins, polyketides, and surfactins [14, 15].

Here, we conducted the first investigation of the antibacterial activity of bacterial isolates from Indonesian coastal sand dunes. The antibacterial activity of five *Bacillus* spp. isolated from the sand dunes were tested against four infectious-disease-causing bacteria.

Materials and Methods

Isolation and morphological characterization of the bacterial isolates

Soil samples were aseptically collected from three areas (the area closest to the sea, the core area of sand dunes, and the area farthest from the sea) of the Parangkusumo coastal sand dunes, at a depth of approximately 10 cm. Ten-gram soil samples from each sampling area were suspended in 90 ml sterile saline solution (0.85% NaCl) in 250-ml conical flasks and shaken on an orbital shaker at 180 rpm to obtain a homogenized soil suspension. One milliliter of each suspension was spread onto Luria-Bertani (LB) agar (10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride, and 15 g/L bacto agar) plates and incubated at 37°C for 24 h. Single colonies with various morphological characteristics, including shape, color, elevation, and margin, were identified from the plates. Five different dominant colonies were chosen and characterized through Gram's staining.

Molecular identification of bacterial isolates

Genomic DNA of the five isolates was extracted and purified with a Wizard® Genomic DNA Purification Kit (Promega, USA). Amplification of the target region of the 16S rRNA 27F gene was performed with 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) primers [16]. Purification of the PCR products was performed through DNA Clean & Concentrator™-5 (Zymo Research, USA) cleanup. The purified PCR products were sequenced by First Base (Singapore).

The deduced sequences were compared for 16S ribosomal RNA sequence homology against the NCBI database with Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The results of BLAST analysis were also confirmed with 16S-based ID from EZBioCloud (https://www.ezbiocloud.net/) [17].

Test bacteria

The test bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 2785, *Staphylococcus aureus* isolated from patient samples, and *Bacillus cereus* isolated from nature) used in this study were obtained from the Microbiology Laboratory, Department of Biology, Universitas Airlangga. The test bacteria were activated by inoculation of one colony from a stock plate into LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L sodium chloride) and incubation at 37°C for 24 h.

Crude antimicrobial compound production

One colony each of five bacterial isolates was inoculated into 3 ml LB medium and incubated in a rotary shaker (150 rpm) at 37°C for 24 h. Then each 1-ml overnight culture was inoculated into 100 ml LB medium and grown at 37°C (150 rpm) for 24–48 h. Supernatants containing crude antimicrobial compounds were harvested by centrifugation (16,000×g, for 1 min at 4°C). After centrifugation, supernatants were transferred into sterile conical tubes.

Evaluation of antibacterial activity with the disc diffusion method

The Kirby-Bauer method was used to study the antibacterial activity of the CACs from the five isolates against four test bacteria (*E. coli*, *P. aeruginosa*, *S. aureus*, and *B. cereus*). Before the antibacterial assays, one colony of each test bacterium was inoculated into 3-ml Mueller Hinton (MH) medium (Himedia, India) and incubated in a rotary shaker (150 rpm) at 37°C for 24 h. Subsequently, 30 µl of each overnight culture of test bacteria was inoculated into MH medium and cultured in a rotary shaker (150 rpm) at 37°C until an $OD_{600\ nm}$ equal to standard 0.5 McFarland solution ($1{\times}10^8$ CFU/ml) was reached [18].

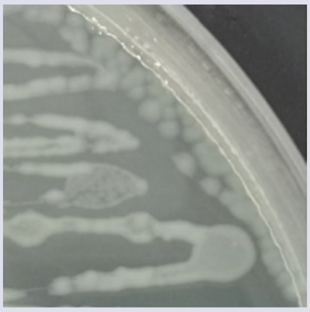
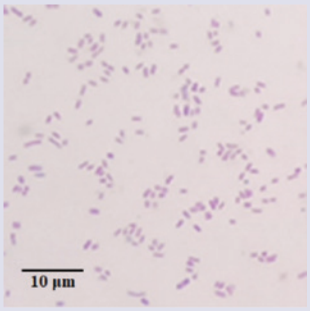
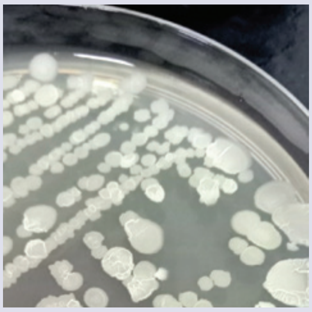
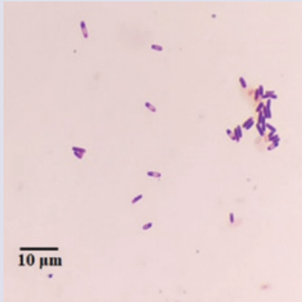
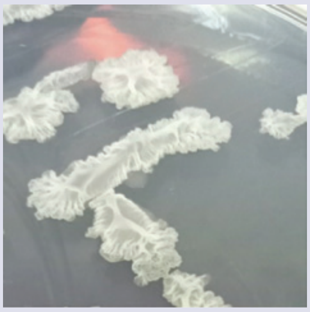
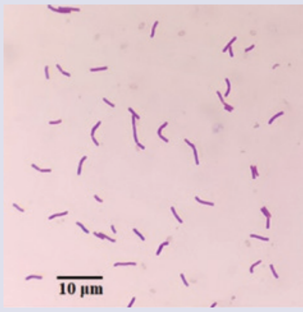
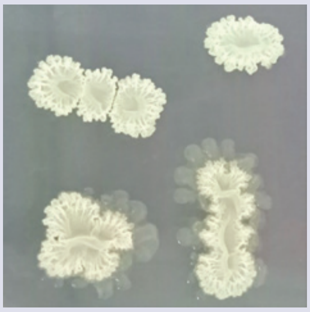
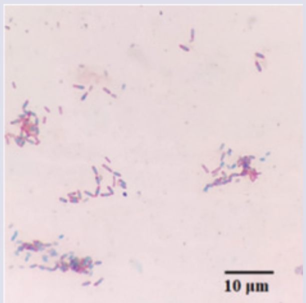
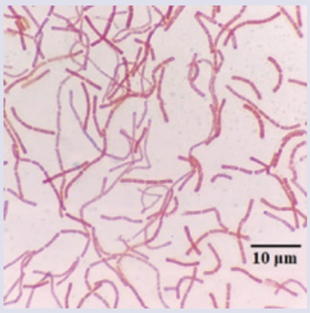
Each test bacterium was then inoculated onto an MH agar plate with a cotton swab. Sterile paper discs were infused with 20 µl of CACs from each of five bacterial isolates, or a positive control (40 mg/ml gentamicin) or negative control (LB medium), and placed onto the inoculated MH agar plates. The MH agar plates were incubated at 37°C for 24 h. After incubation, the diameter of the inhibition zone was measured with digital calipers.

Results and discussion

Identification of bacterial isolates

Macroscopic, microscopic, and molecular identification was performed for five isolated bacteria from the Parangkusumo coastal sand dunes (**Table 1**). All isolates were rod-shaped,

Table 1 Colony and Microscopic Characterization Results of the Isolates

Isolate Code	Colony Characterization		Microscopic Characterization	
PSD 1.2	White and opaque colonies with small size, circular shape, raised elevation, entire margin, smooth glistening surface, and moist consistency		Rod-shaped, Gram-positive	
PSD 2.1	White and opaque colonies with moderate size, circular shape, raised elevation, entire margin, smooth glistening surface, and viscid consistency		Rod-shaped, Gram-positive	
PSD 2.2	White and opaque colonies with moderate size, circular shape, flat elevation, entire margin, wrinkled surface, and viscid consistency		Rod-shaped, Gram-positive	
PSD 3.1	White and opaque colonies with moderate size, circular shape, raised elevation, entire margin, smooth glistening surface, and viscid consistency		Rod-shaped, Gram-positive	
PSD 40.1	White and opaque colonies with moderate size, circular shape, raised elevation, entire margin, smooth glistening surface, and viscid consistency		Rod-shaped, Gram-positive	

Gram-positive bacteria of the genus *Bacillus*. As reported previously, *Bacillus* is a dominant genus in sand dune environments that produces antimicrobial compounds [19]. Interestingly, one isolate was identified as *B. aryabhattai*. To our knowledge, this is the first report of the isolation of *B. aryabhattai* in Indonesia. This species was previously isolated from the upper atmosphere in India, and soil in South Korea and Spain [20–22].

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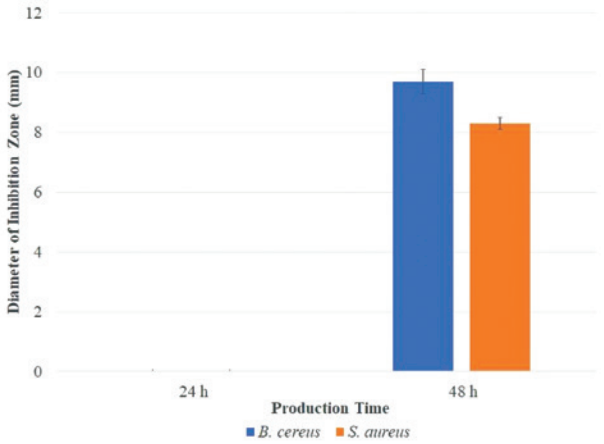

Figure 1 Antibacterial activity of CACs produced from 24- and 48-h culture of the isolate PSD 1.2

Table 2 Molecular Identification Results of the Isolates

Isolate Code	Closest Species	Identity (%)
PSD 1.2	*Bacillus velezensis*	98.13
PSD 2.1	*Bacillus aryabhattai*	99.72
PSD 2.2	*Bacillus spizizenii*	99.45
PSD 3.1	*Bacillus subtilis*	99.86
PSD 40.1	*Bacillus megaterium*	99.79

Antimicrobial activity of crude antimicrobial compounds produced by the Bacillus isolates

The antibacterial activity of CACs produced from 24- and 48-h culture of isolate PSD 1.2 was evaluated (**Figure 1**). The inhibition zone was observed only when CACs from 48-h culture were used. Previous reports have used an incubation time of 40–48 h for producing CACs from *B. velezensis* [23, 24]. Therefore, 48-h incubation was used for production of CACs from other *Bacillus* isolates.

The disc diffusion assay results indicated that all five *Bacillus* isolates showed antimicrobial activity toward some of the tested bacteria (**Table 2**). However, compared with that of gentamicin, the wide-spectrum-antibiotic positive control, the *Bacillus* spp. CACs had weak antibacterial activity (**Table 3**).

Isolates 1.2. (*B. velezensis*) and 3.1. (*B. subtilis*) showed growth inhibition against only Gram-positive test bacteria. These results were consistent with previous reports in which the supernatants of *B. velezensis* and *B. subtilis* showed antibacterial activity against Gram-positive bacteria [25–27]. However, *B. velezensis* has also been reported to inhibit Gram-negative bacteria [23]. *B. subtilis* has been reported to produce antimicrobial compounds, such as gageostatin linear lipopeptides and difficidin macrolides, that inhibit the growth of Gram-negative bacteria [15]. The production conditions used in this study might have favored the production of antimicrobial compounds from *B. velezensis*, such as antimicrobial peptides [27], surfactin lipopeptide [28], and macrolactin [29], and from *B. subtilis*, such as bacilysin, surfactins, and subtilosin [30], which effectively inhibit the growth of Gram-positive bacteria.

Isolates 2.1 (*B. aryabhattai*) and 40.1 (*B. megaterium*) showed inhibition against the growth of only Gram-negative test bacteria. In previous studies, *B. aryabhattai* and *B. megaterium* have been reported to show antibacterial activity against Gram-negative and Gram-positive bacteria [31, 32] by producing antimicrobial compounds such as megacins and tyrocidines [33], respectively. Moreover, isolate 2.2 was the only isolate showing antibacterial activity toward Gram-negative and Gram-positive bacteria. This result was consistent with findings from previous reports on the antimicrobial activity of *Bacillus spizizenii* [34, 35].

Microbial bioprospecting is the investigation of microbial biodiversity of an ecosystem to search new resources of commercial value which involves multidisciplinary approaches such as microbiology, molecular biology, pharmacy, medicine, and even social studies [36, 37]. Exploring unexplored or less explored ecosystems, such as coastal sand dunes, may increase the possibility of identifying microorganisms producing biologically active metabolites [38, 39]. Five *Bacillus* spp. isolated from the Parangkusumo sand dunes were found to be potential anti-bacterial compounds producers, despite the weak antibacterial activities of their CACs observed in the current study. In further studies, the antibacterial activities may be improved by optimizing production parameters such as the composition of the medium, incubation temperature, and time. More detailed and accurate evaluations of antibacterial activity, such as the determination of minimum inhibitory concentration and minimum bactericidal concentration of extracted compounds must also be performed.

Table 3 Antimicrobial Activity of *Bacillus* Species Toward Test Bacteria

***Bacillus* Species (code)**	**Inhibition Zone (mm)**			
	E. coli	***P. aeruginosa***	***B. cereus***	***S. aureus***
Bacillus velezensis (PSD 1.2)	NI	NI	9.7 ± 0.4*	8.3 ± 0.2*
Bacillus aryabhattai (PSD 2.1)	NI	9.3 ± 2.2*	NI	NI
Bacillus spizizenii (PSD 2.2)	NI	8.9 ± 2.9*	NI	8.7 ± 0.1*
Bacillus subtilis (PSD 3.1)	NI	NI	10.9 ± 2.2*	8.4 ± 0.3*
Bacillus megaterium (PSD 40.1)	9.8 ± 0.8*	7.2 ± 0.3*	NI	NI
Gentamicin (positive control)	31.2 ± 1.0*	28.4 ± 1.4*	27.9 ± 1.2*	26.7 ± 2.2*
LB medium (negative control)	NI	NI	NI	NI

NI: no inhibition.
*Values are the means ± standard deviations of duplicate measurements.

Furthermore, the mass production of single antibacterial compounds from those isolates are of great interest. One of the compounds is surfactins which not only showed broad spectrum antibacterial activities, but also antiviral, antifungal, and antitumoral activities [29, 40, 41]. Surfactins also utilized in food, pharmaceutical, and cosmetic industries as surfactant and emulsifier [42], as well as in petrochemical industries for enhancing oil recovery and bioremediation purposes [43, 44]. In order to produce single antibacterial compound of interest, such as surfactins, the optimization of fermentation conditions using the *Bacillus* isolates, as well as extraction and characterization methods of the compound need to be conducted.

Conclusions

Five CAC-producing *Bacillus* isolates were successfully isolated from the soil of the Parangkusumo sand dunes, Indonesia. The highest inhibitory activities against *E. coli*, *P. aeruginosa*, *B. cereus*, and *S. aureus* were shown by the CACs from *B. megaterium* PSD 40.1, *B. aryabhattai* PSD 2.1, *B. subtilis* PSD 3.1, and *B. spizizenii* PSD 2.2, respectively. This report of the first screening of the antimicrobial activity of bacterial isolates from Indonesian coastal sand dunes is expected to encourage further exploration of beneficial microorganisms from this ecosystem.

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