HAYATI
Journal of Biosciences

Vol. 32 No. 1, January 2025 55-69
DOI:10.4308/hjb.32.1.55-69
ISSN: 1978-3019
EISSN: 2086-4094

Research Article

# Characterization of Antibacterial Compounds from Marine Sponge-associated *Streptomyces* spp. against Some Pathogenic Bacteria

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ARTICLE INFO

*Article history:*
Received July 1, 2024
Received in revised form August 23, 2024
Accepted September 4, 2024

*KEYWORDS:*
antibacterial,
antibiotic resistance,
LC-MS/MS,
modified molasses medium,
pathogenic bacteria,
*Streptomyces*



## ABSTRACT

The increasing trend of antibiotic resistance among pathogenic bacteria is a worldwide problem. *Streptomyces* produce a number of bioactive compounds such as antibacterial. This study aimed to investigate the effect of different media and incubation time in increasing the antibacterial activity of marine sponge-associated *Streptomyces* spp. and characterize antibacterial compounds of marine sponge-associated *Streptomyces* spp. against pathogenic bacteria. Among the three tested media and some days of incubation times, *Streptomyces* spp. produce more antibacterial activity when grown using modified molasses medium at 15 days incubation. The ethyl acetate extracts of Dbi28t exhibited a significant inhibitory zone against Extended Spectrum β-Lactamase (ESBL)-producing *Escherichia coli, Providencia rettgeri* then followed by *Proteus mirabilis* and *Pseudomonas putida* and the results were higher than some commercial antibiotics. This study has identified nine antibacterial compounds in Dbi28t using Liquid Chromatography-tandem Mass Spectrometric (LC-MS/MS) analysis, with the most abundance belonging to pumilacidin A, then followed by pumilacidin B, surfactin B, surfactin A, phenazostatin B, chalcomycin B, neopyrrolomycin C, saquayamycin A and saphenamycin. This work provides the first report from a *Streptomyces* sp. Dbi28t produced pumilacidin, surfactin and other bioactive compounds with the modified molasses medium for optimization of characterization of its antibacterial compounds.

## 1. Introduction

Treatment of pathogenic bacterial infections has confirmed difficult over time, along with the appearance of antibiotic-resistant abilities in various pathogenic bacteria (Biernbaum and Kudva 2022). Extended Spectrum β-Lactamase (ESBL)-producing *Escherichia coli* are a major cause of resistance to β-lactam antibiotics listed as critical priority pathogens (WHO 2015; Nweze *et al.* 2020; Ibrahim *et al.* 2023). In addition, *Proteus mirabilis* and *Pseudomonas putida* are known as opportunistic bacteria and designated as class 2 pathogens. *Providencia rettgeri*, are also the most common clinical isolates cause infectious diseases (Dunbar *et al.* 2020). These various bacteria cause several types of infectious diseases such as gastroenteritis, diarrhea, bacteremia, and urinary tract infections (UTIs). Interestingly some of these pathogenic bacteria have shown were multidrug resistant (Mazzariol *et al.* 2017; Tan *et al.* 2019; Chamon *et al.* 2020; Yuan *et al.* 2020). As a result, some approaches have been investigated to control the pathogenesis of these pathogens, in addition to great interest in the exploration of natural antibiotic sources, owing to their potential therapeutic efficacy (Bhakyashree and Kannabiran 2020). In this regard, the pharmaceutical industries in the world are attracted to producing antibiotics from *Streptomyces* due to their ability to synthesize the antibiotics (Amin *et al.* 2017).

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*Streptomyces*, Gram-positive bacteria, well-known in antibiotic producing. Almost 2/3 (80%) of all commercial antibiotics used today are isolated from *Streptomyces* (Barka *et al.* 2016; Krishnan 2023). *Streptomyces* are continuously being explored for the discovery of new antibacterial compounds with better efficacy and lower side effects (Nicault *et al.* 2020) including from marine environments. Among them, our previous studies have shown that marine sponge-associated *Streptomyces* spp. have potential for antibacterial against pathogenic bacteria including ESBL-producing *E. coli*, but the resulting antibacterial activity is not optimal (Efendi 2022). Therefore, optimization for growth particularly focusing on external growth factors is essential (Sandoval-Powers *et al.* 2021). Among these factors are the optimal culture conditions, including culture medium and incubation time required for *Streptomyces* spp. Consequently, its metabolism can be effectively regulated, so an improvement of the producing antibacterial compounds (Chandrakar and Gupta 2018).

Previous studies have well-documented that the International *Streptomyces* Project (ISP) is known as a common media to growth and produce antibacterial compounds in *Streptomyces* (Shirling and Gottlied 1966). However, the high cost of the medium components in ISP media prompted researchers to analyze waste products as substrates for *Streptomyces* spp. cultures such as molasses. The molasses medium has been previously utilized for producing antibacterial compounds such as magnamycin by *Streptomyces halstedii* (Abou-zeid *et al.* 1980). However, in this study employs a modified molasses medium with varying ingredients composition. Production and optimization of antibacterial compounds by marine sponge-associated *Streptomyces* spp. on the modified molasses medium have not been reported yet. Therefore, this study aimed to investigate the effect of different media and incubation time in increasing the antibacterial activity of marine sponge-associated *Streptomyces* spp. isolates, as well as characterize antibacterial compounds of these isolates against some pathogenic bacteria including ESBL-producing *E. coli, P. mirabilis, P. rettgeri* and *P. putida*.

## 2. Materials and Methods

### 2.1. Sample Information

Three *Streptomyces* spp. isolates associated with marine sponge of Pulau Panggang, Seribu Islands National Park, Indonesia (collection of Prof. Dr. Ir. Yulin Lestari) were used for this study (collection of Prof. Dr. Ir. Yulin Lestari). *Streptomyces* spp. isolates Dbi28t and Car21t have been identified based on the 16s rRNA in previous studies and have similarities with *Streptomyces* spp. The third isolate, Crc27t, has not been molecularly identified. However, its macroscopic and microscopic characterization is suspected to be *Streptomyces* spp. All isolates of *Streptomyces* spp. were from previous studies, with detailed descriptions provided in Table 1. Furthermore, four isolates of pathogenic bacteria were used for antibacterial assays. The pathogenic bacteria were ESBL-producing *E. coli* (collection of the Department of Parasitology, Faculty of Medicine, University of Indonesia (rectal swab from patient in hospital), *P. mirabilis* strain CIFRI.C4 RS39, *P. rettgeri* strain RS39, and *P. putida* strain NY5709 (collection of Prof. Dr. dr. Sri Budiarti).

### 2.2. Antibiotic Susceptibility Test for Pathogenic Bacteria

Antibiotic Experiments were conducted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2020) and European Committee on Antimicrobial Susceptibility Testing (EUCAST)

Table 1. *Streptomyces* spp. isolates data used in this study

| Isolate code | Result of 16s rRNA analysis | Accession number | Antibacterial potential | References |
|---|---|---|---|---|
| Car21t | *Streptomyces tendae* ATCC 19812<br>*Streptomyces violacerubidus* LMG 20319 | D63873<br>AJ81374 | *Xanthomonas oryzae* and ESBL-producing E. coli | Retnowati *et al.* 2019; Efendi 2022 |
| Dbi28t | *S. tendae* ATCC 19812<br>*Streptomyces rubrogriseus* LMG 20318 | D63873<br>AJ81373 | *X. oryzae* and ESBL-producing *E. coli* | Retnowati *et al.* 2019; Efendi 2022 |
| Crc27t | na[a] | na[a] | ESBL-producing *E. coli* | Retnowati *et al.* 2019; Efendi 2022 |

guidelines (Kittinger *et al.* 2016; EUCAST 2024). The test was carried out using 12 commercially available antibiotic discs (Oxoid, UK) (10 µg penicillin, 10 µg ampicillin, 30 µg aztreonam, 30 µg cefotaxime, 5 µg cefixime, 30 µg ceftriaxone, 10 µg meropenem, 10 µg streptomycin, 30 µg kanamycin, 5 µg ciprofloxacin, 30 µg chloramphenicol and 30 µg tetracycline). The bacterial suspension volume was adjusted to achieve approximately 2 × $10^8$ CFU/ml, and antibiotic susceptibility test are performed in triplicate, incubated at 37°C for 18-20 h. The diameter of inhibition zone was measured using Image J software (mm) (https://imagej.net/ij/). The zone of clearing was interpreted according to standard measurements were defined in accordance with the CLSI 2020 and EUCAST 2024 guidelines.

### 2.3. Marine Sponge-associated Streptomyces spp. Rejuvenation

A total of three marine sponge-associated *Streptomyces* spp. isolates were rejuvenated in an agar medium of ISP2, ISP4, and modified molasses agar medium (solid medium) w/v i.e., ISP2 (10 g/L of malt extract (Merck), 4 g/L of glucose (Merck), 4 g/L of yeast extract (Merck), 20 g/L pure agar) (Shirling and Gottlieb 1966; Marzoug *et al.* 2023), ISP4 (10 g/L of soluble starch (Merck), 1 g/L of $K_2HPO_4$ (Merck), 1 g/L of $MgSO_4 \cdot 7H_2O$, 1 g/L of NaCl (Merck), 2 g/L of $(NH_4)_2SO_4$ (Merck), 2 g/L of $CaCO_3$ (Merck), 0.001 g/L of $FeSO_4 \cdot 7H_2O$ (Merck), 0.001 g/L of $MnCl_2 \cdot 7H_2O$ (Merck), 0.001 g/L of $ZnSO_4 \cdot 7H_2O$, 20 g/L pure agar) (Shirling and Gottlieb 1966) and modified molasses medium (not publish, prepare for patented). The initial pH of the media was adjusted to 6.8-7.0. The plates were aerobically incubated at 27°C for 14 days. The daily observation of each plate revealed the development and appearance of *Streptomyces*, which were easily detected by their distinctive calcareous to leathery appearance. The ISP2, ISP4, and modified molasses plates were used to individually harvest colonies and subculture them. In addition, the morphology of single colony of *Streptomyces* spp. was observed using a stereomicroscope at 16× magnification (Leica EZ4 Jerman).

### 2.4. Selection of Suitable Medium and Time Optimization for Antibacterial Compounds Production

The selection of suitable medium for antibacterial compounds production was done by growing *Streptomyces* spp. isolates in three liquid media i.e., ISP2 (Shirling and Gottlieb 1966; Marzoug *et al.* 2023), ISP4 (Shirling and Gottlieb 1966), and modified molasses medium. The initial pH of the medium was adjusted to 6.8-7.0. Cultures were placed into a shaking incubator (100 rpm, 27°C) respectively 5, 10, 15, and 20 days for determine the optimum time used to produce large amounts of antibacterial compounds. At every end of incubation time, the broth cultures each isolate were centrifugated at 13,000 rpm, 27°C, 15 min. Furthermore, supernatant of each isolate was assayed the antibacterial activity against the pathogenic bacteria, using Kirby-Bauer discs diffusion method (Hudzicki 2009) in triplicate. The antibacterial test began by growing the pathogenic bacteria on 20 ml of Luria Bertani (LB) broth, 37°C. As many as 1% of pathogenic bacterial cultures that have achieved approximately 2 × $10^8$ CFU/ml ($OD_{625\ nm}$ = 0.8) are mixed with 100 ml of warm Mueller Hinton (MH) agar (Merck, Germany) (40-50°C). Paper disc (6 mm) is placed on solidified MH agar, then dripped 20 µL of *Streptomyces* spp. supernatant. Positive control used 5 µg ciprofloxacin and modified molasses, ISP4 and ISP2 broth media without culture of *Streptomyces* spp. served as the negative control. Incubation was carried out for 16-18 h, 37°C and the inhibitory zone was measured using Image J software.

### 2.5. Production and Extraction of Antibacterial Compounds

The production of antibacterial compounds by *Streptomyces* spp. was done by growing the isolates on the best medium culture and optimum time of isolates to inhibit some pathogenic bacteria (Chandrakar and Gupta (2018) with modifications. *Streptomyces* spp. isolates were aseptically inoculated into 1,000 ml Erlenmeyer flasks containing 500 ml of production medium and incubated in optimum condition at 27°C in a shaker incubator at 100 rpm. A total of 5 L of each *Streptomyces* spp. culture was collected for the separation of antibacterial compounds. The extraction of antibacterial compounds was performed based on the previous methods by Jose *et al.* (2013); Chandrakar and Gupta (2018); Elias *et al.* (2022) with modifications in extraction time for agitation and temperature for evaporation. Ethyl acetate as the solvent was added to each culture of marine sponge-associated *Streptomyces* spp. isolates in 1:1 (v/v) ratio. The mixture was allowed to stand overnight and agitated for 30 min, then kept stationary for 15 minutes. The bioactive compounds-containing organic solvent phase was then separated

from the aqueous phase in a separatory funnel, collected, and evaporated in a vacuum rotary evaporator at 40°C. Each isolate's crude extract (ethyl acetate extract) was diluted in dimethyl sulfoxide (DMSO) (Merck, Germany).

### 2.6. Antibacterial Assays of Streptomyces spp.'s Extract

Antibacterial assays of each the ethyl acetate extracts of *Streptomyces* spp. against pathogenic bacteria were done by Kirby-Bauer discs diffusion method same with previous method (Hudzicki 2009) in triplicate and was measured the inhibitory zone formed using Image J software (mm). The positive control was used 5 µg ciprofloxacin and 10% DMSO was used as a negative control.

### 2.7. Characterization of Antibacterial Compounds Using LC-MS/MS

The ethyl acetate extracts of *Streptomyces* spp. were analysed by LC-MS/MS analysis was performed on an Acquity Ultra-Performance Liquid Chromatography (UPLC) system coupled to a mass spectrometer QToF-MS detector (Xevo G2-S QTof, Waters, USA) using electrospray ionization. Eluent for chromatographic method consisted of solvent A (Water + 5 mM Ammonium Formic) and B (Acetonitrile + 0.05% Formic acid). The ethyl acetate extracts were injected into the LC with a 5 µL microsyringe and inserted four times into the UPLC (LC: ACQUITY UPLC® H-Class System, Waters, USA) column ACQUITY UPLC BEH (Ethylene Bridge Hybride) C18 (1.8 µm 2.1 × 50 mm; Waters, USA). The MS analysis was determined in positive ion mode (positive (+) ESI charge) with a mass range of 50-1,200 m/z. Masslynx 4.1 software, PubChem (https://pubchem.ncbi.nlm.nih.gov/), and ChemSpider (https://www.chemspider.com/) were used to process and analyze data files from the LC-MS/MS (Pratama *et al.* 2023 with less modification).

### 2.8. Statistical Analysis

Data were statistically evaluated using one-way analysis of variance (ANOVA) by SPSS software package (version 16.0), while the significant difference was verified using the Duncan test at $P<0.05$.

## 3. Results

### 3.1. Antibiotic Resistant Profile of the Pathogenic Bacteria

The findings of the antibacterial susceptibility testing showed that the pathogenic bacteria viz., ESBL-producing *E. coli, P. rettgeri, P. mirabilis* and *P. putida* were multidrugs resistant pathogens (resistant to at least three different classes of antibiotics). These pathogenic bacteria exhibited resistance to the three antibiotic classes including β-lactam, fluoroquinolones, and aminoglycosides. Extended Spectrum β-Lactamase (ESBL)-producing *E. coli* was only susceptible to two of the tested antibiotics (chloramphenicol and tetracycline). Similarly, *P. rettgeri* was also susceptible to two of the tested antibiotics (ceftriaxone and tetracycline) and intermediate to aztreonam and chloramphenicol. Furthermore, *P. mirabilis* was only susceptible to one of the tested antibiotics (tetracycline) and intermediate to cefixime and chloramphenicol. In contrast, *P. putida* was resistant to all the tested antibiotics (Table 2).

### 3.2. Selection of Suitable Medium and Time for Antibacterial Compounds Production

Optimum culture conditions are a key factor for the growth and antibacterial production of *Streptomyces* spp. In the present study, marine sponge-associated *Streptomyces* spp. produces better antibacterial activity when grown using a modified molasses medium, then followed by ISP4 and ISP2 medium after an incubation period of 15 d 27°C. In addition, all of *Streptomyces* spp. isolates produce better antibacterial activity against ESBL-producing *E. coli* and *P. rettgeri*, compared to *P. mirablis* and *P. putida* when grown using a modified molasses and ISP4 medium. The highest antibacterial activity against *P. mirablis* and *P. putida* was observed when grown using an ISP2 medium, but only was observed in Dbi28t isolates against *P. mirablis* as well as only observed in Car21t isolates against *P. putida*. However overall, the highest antibacterial activity was achieved using the modified molasses medium after an incubation period of 15 d 27°C (Table 3; Figure 1). Hence, a modified molasses medium and incubation

Table 2. Inhibition zone of antibiotics against pathogenic bacteria

| AB[a] | Antibiotics class | ESBL EC[b] | | PM[b] | | PR[b] | | PP[b] | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1[c] | 2[d] | 1[c] | 2[d] | 1[c] | 2[d] | 1[c] | 2[d] |
| AMP | β-Lactam | 0±0 | R | 0±0 | R | 0±0 | R | 0±0 | R |
| P | β-Lactam | 0±0 | R | 0±0 | R | 0±0 | R | 0±0 | R |
| ATM | β-Lactam | 8.24±0.20 | R | 17.04±0.15 | R | 19.21±0.18 | I | 0±0 | R |
| CTX | β-Lactam | 0±0 | R | 11.50±0.25 | R | 10.29±0.26 | R | 0±0 | R |
| CFM | β-Lactam | 0±0 | R | 16.50±0.50 | I | 15.84±0.63 | R | 9.94±0.66 | R |
| CRO | β-Lactam | 0±0 | R | 16.24±0.72 | R | 27.05±2.02 | S | 0±0 | R |
| MEM | β-Lactam | 13.32±0.60 | R | 11.05±0.70 | R | 17.61±0.72 | R | 0±0 | R |
| S | Aminoglycoside | 0±0 | R | 10.25±0.58 | R | 8.85±0.63 | R | 10.88±0.66 | R |
| K | Aminoglycoside | 12.84±0.33 | R | 11.47±0.47 | R | 12.15±0.83 | R | 14.16±0.93 | R |
| CIP | Fluoroquinolone | 13.99±0.82 | R | 14.30±1.84 | R | 16.02±2.19 | R | 15.39±1.18 | R |
| C | Phenicol | 24.38±0.56 | S | 17.43±1.03 | I | 14.50±1.07 | I | 9.98±1.42 | R |
| TE | Tetracycline | 22.28±1.61 | S | 15.29±0.92 | S | 16.70±0.79 | S | 13.29±0.91 | R |

[a]AB: Antibiotics; AMP: ampicillin; P: penicillin; ATM: aztreonam; CTX: cefotaxime; CFM: cefixime; CRO: ceftriaxone; MEM: meropenem; S: streptomycin; K: kanamycin; CIP: ciprofloxacin; C: chloramphenicol; TE: tetracycline. [b]ESBL EC: Extended Spectrum β-Lactamase (ESBL)-producing *Escherichia coli*; PM: *Proteus mirabilis*; PR: *Providencia rettgeri*; PP: *Pseudomonas putida*. [c]1: Inhibition zone (mm). [d]2: Interpretive criteria based on CLSI 2020 and EUCAST 2024 (R: Resistant, I: Intermediate, S: Susceptible)

Table 3. Effect of media on antibacterial compounds production by *Streptomyces* spp. isolates

| Isolate code | Media | Diameter of zone inhibition (mm) | | | |
|---|---|---|---|---|---|
| | | ESBL EC[a] | PM[a] | PR[a] | PP[b] |
| Car21t | Modified molasses | 11.25±0.59[a] | 8.08±0.13[b] | 10.56±0.06[a] | 9.48±0.49[b] |
| | ISP2 | 9.95±0.42[b] | 9.05±1.00[b] | 8.99±0.09[b] | 10.02±0.04[b] |
| | ISP4 | 11.09±0.16[a] | 10.74±0.65[a] | 10.01±0.98[ab] | 9.31±0.72[b] |
| Crc27t | Modified molasses | 10.38±0.47[a] | 10.08±0.07[b] | 13.39±0.53[a] | 9.12±0.21[a] |
| | ISP2 | 8.65±0.22[b] | 9.65±0.79[b] | 10.01±0.02[b] | 8.58±0.23[b] |
| | ISP4 | 10.05±0.09[a] | 9.52±0.56[b] | 11.45±1.27[b] | 7.91±0.15[c] |
| Dbi28t | Modified molasses | 14.06±0.14[a] | 9.80±0.50[b] | 16.20±0.24[a] | 9.82±0.27[a] |
| | ISP2 | 10.57±0.82[b] | 12.33±0.61[a] | 9.96±0.05[c] | 9.12±0.21[a] |
| | ISP4 | 9.69±0.53[b] | 10.30±0.46[b] | 12.88±0.22[b] | 9.00±0.82[a] |

[a]ESBL EC: Extended Spectrum β-Lactamase (ESBL)-producing *Escherichia coli*; PM: *Proteus mirabilis*; PR: *Providencia rettgeri*; PP: *Pseudomonas putida*

time of 15 days were further selected for the production of antibacterial compounds.

Morphological colony observations of three isolates of marine sponge-associated *Streptomyces* spp. in the best medium in this study, modified molasses medium exhibited good growth after a 15-day incubation period (Figure 2). The colonies on a modified molasses agar medium displayed characteristic feature such as substrate mycelium, aerial mycelium, and sporulation. Morphological characterization of *Streptomyces* spp. in modified molasses giving good sporulation that produces spores in a solid culture's medium. The result of this study indicated that a modified molasses medium promoting the sporulation of *Streptomyces* spp. This medium is composed of sugarcane molasses as a carbon source and nitrogen source, yeast extracts and urea as an additional nitrogen source, as well as phosphate, inorganic salts, and trace salt solution.

### 3.3. Antibacterial Activity of *Streptomyces* spp.

The current study demonstrated the first report to identify the antibacterial activity from marine sponge-associated *Streptomyces* spp. with modified molasses medium as the medium of antibacterial production. Although control antibiotics had a greater inhibition zone than the ethyl acetate extracts of *Streptomyces* spp. in most cases, the ethyl acetate extracts of *Streptomyces* sp. Dbi28t isolate showed a significant difference at $p<0.05$ for the greatest zone of inhibition than the antibiotic as a positive control against ESBL-producing *E. coli* and *P. rettgeri*. Overall, the ethyl acetate extract *Streptomyces* sp. Dbi28t has the best activity towards ESBL-producing *E. coli, P. rettgeri* then followed by *P. mirabilis* and *P. putida* at a concentration of 1,000 µg/ml. In general, the ethyl acetate extract *Streptomyces* sp. Dbi28t has better antibacterial activity against all pathogenic bacteria than Car21t and Crc27t. No

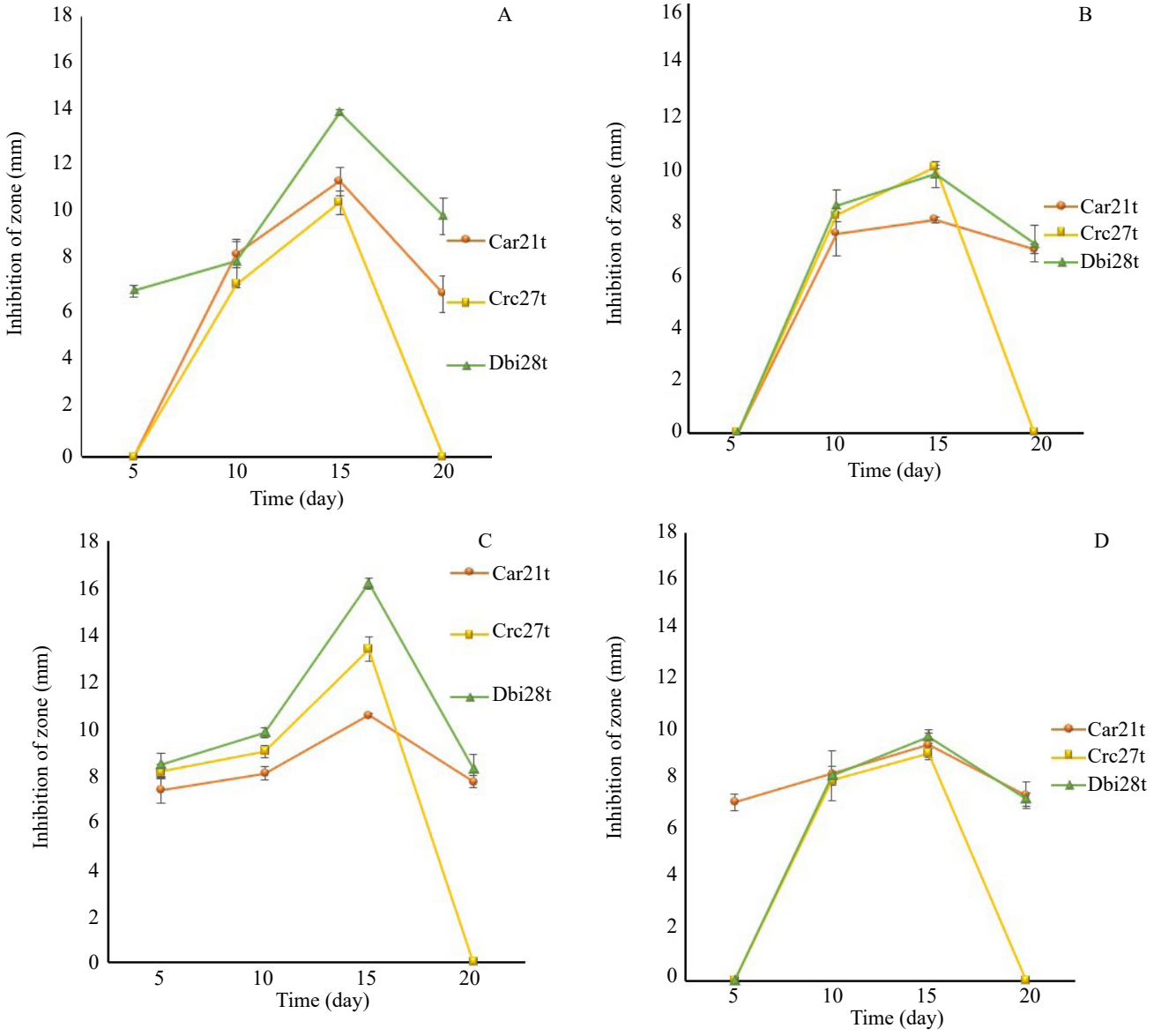


Figure 1. Time optimization of antibacterial compounds production by marine sponge-associated *Streptomyces* spp. at modified molasses medium and antibacterial activity against (A) ESBL-producing *E. coli*, (B) *P. mirabilis*, (C) *P. rettgeri*, and (D) *P. putida*

antibacterial effect was observed for the negative control (10% DMSO) (Table 4; Figure 3).

The result showed that the best antibacterial agent in this study, ethyl acetate extract *Streptomyces* sp. Dbi28t exhibited a higher inhibition zone against pathogenic bacteria compared with the inhibition zone by commercial antibiotics against pathogenic bacteria. Overall, the ethyl acetate extract Dbi28t exhibited better antibacterial activity than penicillin, ampicillin, cefotaxime, meropenem, and streptomycin in all the tested pathogenic bacteria. The present results indicated that marine sponge-associated *Streptomyces* sp. Dbi28t has antibacterial potential against pathogenic bacteria (Figure 4).

### 3.4. Characteristics of Antibacterial Compounds *Streptomyces* spp.

Characterization of antibacterial compounds of *Streptomyces* sp. Dbi28t using LC-MS/MS, found antibacterial compounds including pumilacidin A, then followed by pumilacidin B, surfactin B, surfactin A, phenazostatin B, chalcomycin B, neopyrrolomycin C, saquayamycin A and saphenamycin. Based on the result, the ethyl acetate extracts of *Streptomyces* sp. Dbi28t were revealed to have 9 major peaks. The ethyl acetate extracts of *Streptomyces* sp. Dbi28t were rich in lipopeptides, and other antibacterial compounds (Table 5; Figure 5).

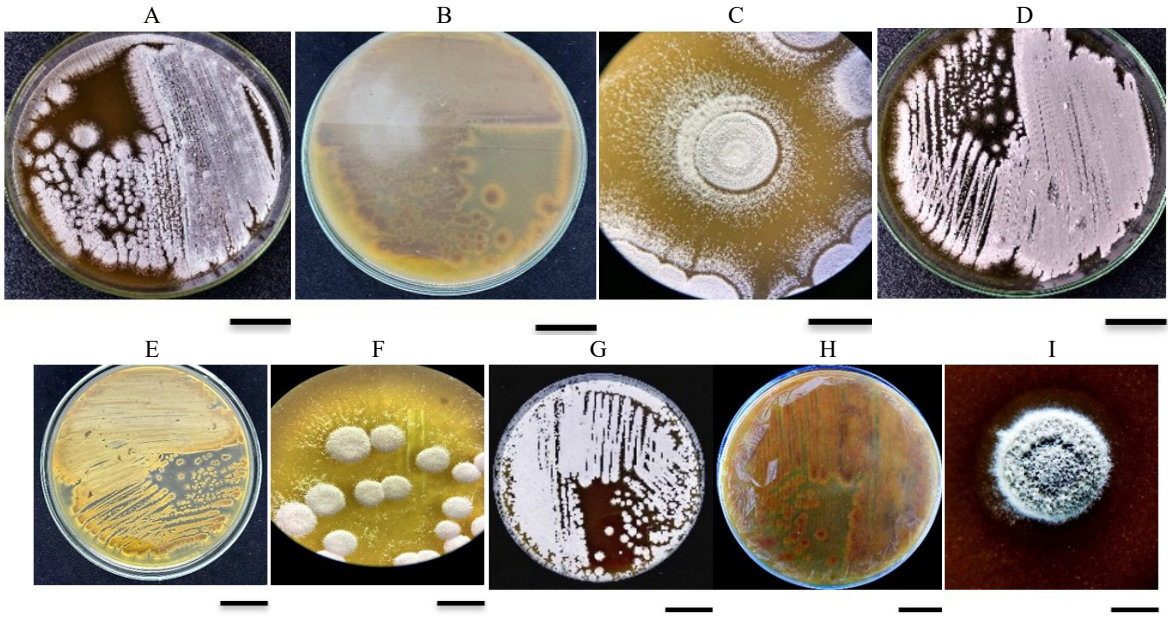


Figure 2. The morphological characteristics of *Streptomyces* spp. isolates in an agar medium of modified molasses medium at 15 days incubation 27°C, (A) (B) (C) Car21t, (D) (E) (F) Crc27t, (G) (H) (I) Dbi28t. Aerial and substrate mycelia *Streptomyces* spp. appearances on modified molasses medium (A) (D) (G) aerial mycelia, (B) (E) (H) substrate mycelia, (C) (F) (I) morphology of single colony of *Streptomyces* spp. isolates using a stereomicroscope at 16× magnification (Leica EZ4 Jerman). Bars represent 1 cm

Table 4. Antibacterial activity of the ethyl acetate extracts of *Streptomyces* spp.

| Extract code | Concentration (µg/ml) | Diameter of zone inhibition (mm) | | | |
|---|---|---|---|---|---|
| | | ESBL EC[a] | PM[a] | PR[a] | PP[b] |
| Dbi28t | 250 | 8.51±0.45$^{d}$ | 7.17±0.07$^{e}$ | 8.45±0.28$^{e}$ | 9.90±0.57$^{d}$ |
| | 500 | 9.68±0.55$^{c}$ | 8.55±0.22$^{bc}$ | 9.67±0.32$^{d}$ | 10.94±0.42$^{c}$ |
| | 750 | 14.54±0.74$^{b}$ | 9.04±0.02$^{b}$ | 13.09±0.15$^{c}$ | 11.64±0.13$^{b}$ |
| | 1,000 | 22.83±0.37$^{a}$ | 14.70±0.32$^{a}$ | 22.03±0.12$^{a}$ | 12.18±0.29$^{b}$ |
| | Ciprofloxacin 5 µg | 14.29±0.30$^{b}$ | 14.83±1.89$^{a}$ | 18.3±0.61$^{b}$ | 14.71±0.51$^{a}$ |
| | DMSO 10% | 0±0$^{e}$ | 0±0$^{d}$ | 0±0$^{f}$ | 0±0$^{e}$ |
| Dbi28t | 250 | 7.10±0.10$^{e}$ | 6.69±0.44$^{d}$ | 6.96±0.07$^{e}$ | 10.12±0.31$^{c}$ |
| | 500 | 8.65±0.52$^{d}$ | 7.59±0.30$^{d}$ | 7.78±0.15$^{d}$ | 11.13±0.58$^{bc}$ |
| | 750 | 9.68±0.21$^{c}$ | 9.45±0.36$^{c}$ | 8.57±0.49$^{c}$ | 11.26±0.46$^{b}$ |
| | 1,000 | 12.29±0.46$^{b}$ | 11.45±0.62$^{b}$ | 9.67±0.42$^{b}$ | 11.52±0.49$^{b}$ |
| | Ciprofloxacin 5 µg | 15.50±0.86$^{a}$ | 14.49±1.31$^{a}$ | 19.29±0.51$^{a}$ | 15.69±1.13$^{a}$ |
| | DMSO 10% | 0±0f | 0±0$^{e}$ | 0±0$^{f}$ | 0±0$^{d}$ |
| Crc27t | 250 | 6.52±0.22$^{c}$ | 6.31±0.16$^{d}$ | 7.36±0.40$^{d}$ | 9.85±0.24$^{d}$ |
| | 500 | 6.90±0.12$^{bc}$ | 6.80±0.13$^{cd}$ | 7.74±0.05$^{cd}$ | 10±0.00$^{d}$ |
| | 750 | 7±0.00$^{bc}$ | 7.24±0.48$^{bc}$ | 8.03±0.04$^{c}$ | 10.59±0.12$^{c}$ |
| | 1,000 | 7.46±0.32$^{b}$ | 7.67±0.27$^{b}$ | 8.97±0.06$^{b}$ | 11.91±0.04$^{b}$ |
| | Ciprofloxacin 5 µg | 13.64±0.64$^{a}$ | 14.57±0.48$^{a}$ | 17.60±0.52$^{a}$ | 15.45±0.69$^{a}$ |
| | DMSO 10% | 0±0$^{d}$ | 0±0$^{e}$ | 0±0$^{e}$ | 0±0$^{e}$ |

[a]ESBL EC: Extended Spectrum β-Lactamase (ESBL)-producing *Escherichia coli*; PM: *Proteus mirabilis*; PR: *Providencia rettgeri*; PP: *Pseudomonas putida*

The values listed above are the means and standard deviations in three replicates. Statistically significant differences were determined by one-way ANOVA followed by the Duncan test (p-values <0.05). Value with the different superscript letters in the same column is significantly different

## 4. Discussion

The family Enterobacteriaceae including *E. coli, P. mirabilis*, and *P. rettgeri*, as well as the family Pseudomonadaceae such as *Pseudomonas putida* are members of Gram-negative bacteria, which may have many resistance mechanisms to help them survive antibiotic treatment (Yang *et al.* 2020; Fanelli *et al.* 2021; Ibrahim *et al.* 2023). This resistance is partly attributed to the ESBL-producing Enterobacteriaceae, which are

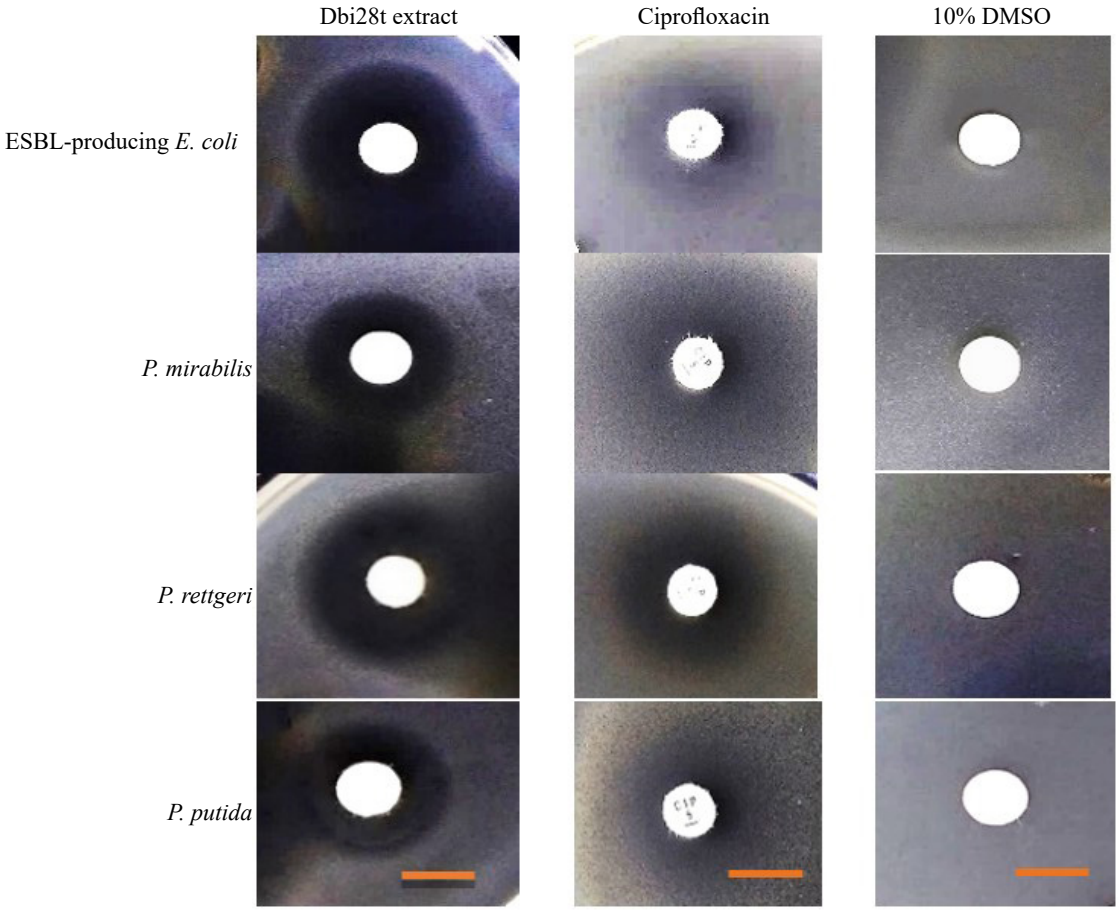


Figure 3. Representation of antibacterial activity via disc diffusion method. The ethyl acetate extracts of *Streptomyces* sp. Dbi28t applied on each concentration of 1,000 µg/ml. 10% DMSO and 5 µg ciprofloxacin used for negative and positive control, respectively. Bars represent 6 mm

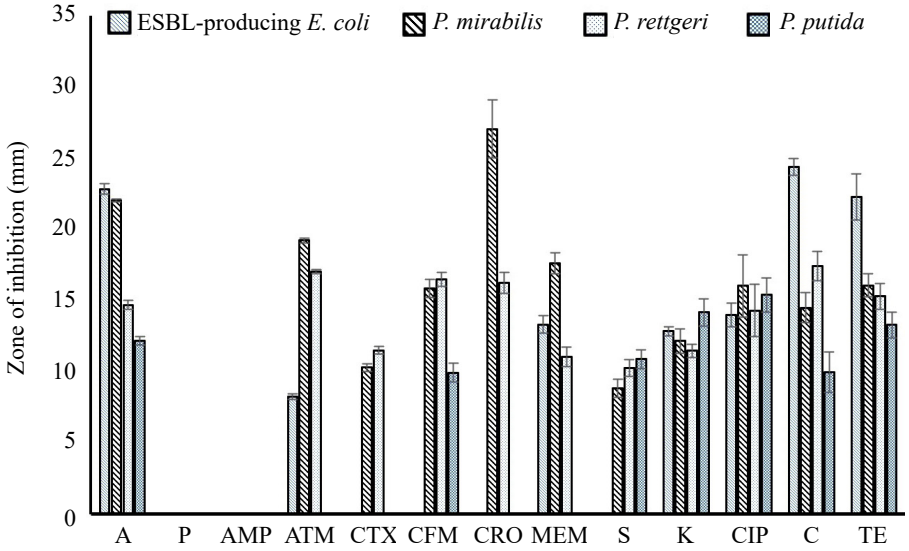


Figure 4. Antibacterial activity of the ethyl acetate extracts of *Streptomyces* sp. Dbi28t on concentration of 1,000 µg/ml against pathogenic bacteria compared with commercial antibiotics. A: ethyl acetate extracts of *Streptomyces* sp. Dbi28t on concentration of 1,000 µg/ml; P: penicillin; AMP: ampicillin; ATM: aztreonam; CTX: cefotaxime; CFM: cefixime; CRO: ceftriaxone; MEM: meropenem

Table 5. Major antibacterial compounds found in *Streptomyces* sp. Dbi28t

| Compounds | Group of compounds | RT (min) | Molecular weight (g/mol) | $MS^2$ fragmentation pattern (m/z) | Molecular formula | Biological activity | References |
|---|---|---|---|---|---|---|---|
| Neopyrrolomycin C | Pyrole alkaloid | 6.555 | 331.4 | 255.06<br>329.04<br>331.04<br>345.13<br>428.28<br>519.15<br>521.15 | $C_{10}H_4Cl_5NO$ | Antibacterial | Hopp *et al.* 2009 |
| Phenazostatin B | Aromatic | 8.439 | 530.38 | 531.15<br>532.16<br>532.16<br>534.15<br>535.15 | $C_{32}H_{26}N_4O_4$ | Antibacterial | Li *et al.* 2015 |
| Saphenamycin | Phenazines | 10.774 | 402.4 | 211.09<br>327.12<br>369.17<br>401.19<br>402.2<br>403.2 | $C_{23}H_{18}N_2O_5$ | Antibacterial | Kitahara *et al.* 1982 |
| Chalcomycin B | Lactone | 12.461 | 812.9 | 812.5<br>813.5<br>814.5<br>856.52<br>857.53<br>900.55<br>901.55<br>944.58<br>945.58 | $C_{41}H_{64}O_{16}$ | Antibacterial | Asolkar *et al.* 2002 |
| Saquayamycin A | Benzoquinone | 14.394 | 820.8 | 732.54<br>776.57<br>777.57<br>820.59<br>821.6<br>822.6 | $C_{43}H_{48}O_{16}$ | Antibacterial | Aouiche *et al.* 2014 |
| Surfactin A | Lipopeptide | 15.499 | 1008.3 | 1008.66<br>1009.66<br>1010.66 | $C_{51}H_{89}N_7O_{13}$ | Antibacterial | Lau and Rinehart 1994 |
| Surfactin B | Lipopeptide | 15.871 | 1022.3 | 1022.67<br>1023.67<br>1024.68<br>1025.68 | $C_{52}H_{91}N_7O_{13}$ | Antibacterial | Chen *et al.* 2019; Thapa *et al.* 2024 |
| Pumilacidin B | Lipopeptide | 16.286 | 1036.3 | 1036.19<br>1037.69<br>1038.69<br>1039.69 | $C_{53}H_{93}N_7O_{13}$ | Antibacterial | Chen *et al.* 2019 |
| Pumilacidin A | Lipopeptide | 16.679 | 1050.4 | 1036.69<br>1037.69<br>1038.7<br>1050.71<br>1051.71<br>1052.71<br>1053.72 | $C_{54}H_{95}N_7O_{13}$ | Antibacterial | Chen *et al.* 2019 |

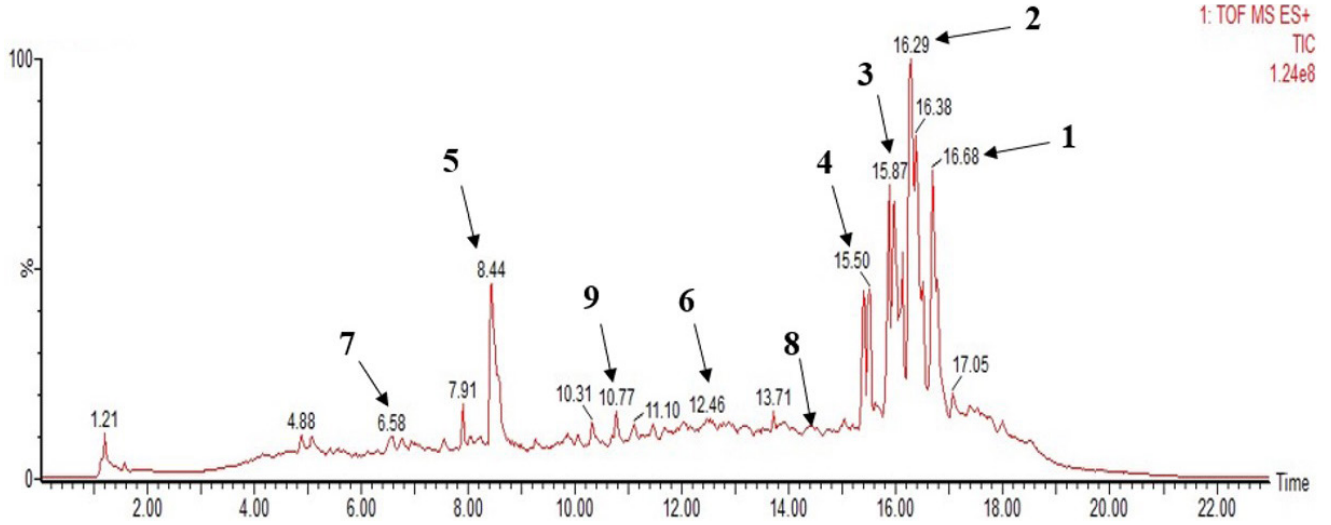


Figure 5. The LC-MS/MS chromatogram of *Streptomyces* sp. Dbi28t. The abundant of antibacterial compounds was pointed by the black arrows, with the most abundance belonging to pumilacidin A (1), then followed by pumilacidin B (2), surfactin B (3), surfactin A (4), phenazostatin B (5), chalcomycin B (6), neopyrrolomycin C (7), saquayamycin A (8) and saphenamycin (9)

generally resistant to penicillins and extended-spectrum cephalosporins and may also be cross-resistant to other antibiotic classes (such as aminoglycosides and fluoroquinolones) (Lee *et al.* 2018). Similarly, in this study, the findings of the antibiotic susceptibility testing showed that the pathogenic bacteria including ESBL-producing *E. coli, P. rettgeri, P. mirabilis*, and *P. putida* were multidrug-resistant pathogens. These pathogenic bacteria exhibited resistance to antibiotic classes including β-lactam, fluoroquinolones, and aminoglycosides (Table 2).

Many researchers have reported that resistance to β-lactam antibiotics is primarily due to the bacteria produced β-lactamases, which hydrolyze the β-lactam ring, thereby inactivating the drug e.g ESBL-producing *E. coli, P. mirabilis* (Lee *et al.* 2018; Li *et al.* 2023) and *Providencia* spp. (Yuan *et al.* 2020). Similarly, a previous study showed that revealed a high prevalence of ESBL and carbapenemases production among *P. mirabilis* clinical isolates (Shaaban *et al.* 2022). Furthermore, *P. putida* displayed resistance to several classes of antibiotics, and it also formed huge amounts of biofilm (Fanelli *et al.* 2021). In addition, Gram-negative bacteria have distinct cell walls a hydrophobic outer membrane bilayer, containing lipopolysaccharide (LPS) molecules, phospholipids, and outer membrane proteins (OMPs), which enhance their inherent and acquired resistance mechanisms. Although some antibiotics target the cell wall, most antibacterial chemicals cannot pass through it to reach their target, hence the cell wall functions as a barrier. This means that when creating new treatments, the ensuing resistance mechanisms must be considered (Impey *et al.* 2020).

Marine sponge-associated *Streptomyces* spp. is known to be an excellent producer of antibacterial with great commercial value (Li *et al.* 2023). Nevertheless, enhancing production and cutting costs remains a significant problem in antibacterial production. A various type of approach for increasing of antibacterial production continue to be carried out such as the supplemented of inexpensive materials in culture media like molasses. The present study, biotechnological approaches for producing antibacterial compounds in *Streptomyces* spp. involve the use of a modified molasses medium, where molasses can serve as a substrate to provide the stimulators for increasing the growth and production of antibacterial compounds that being used against pathogenic bacteria. This approach is advantageous for being more supportive of sustainability, economically due to a reduction in production cost and environmentally friendly due to molasses being agroindustrial wastes, so it is a cheap alternative to medium components in a modified molasses medium compared to ISP4 and ISP2 media are a costly media.

In this study, sugarcane molasses as by-products from the agro-industrial field were used as a production medium to produce antibacterial compounds, and the composition of the medium was modified from common molasses-based medium with supplementation of various nitrogen sources and phosphate, and various

inorganic minerals. The results of this study showed that modified molasses medium promotes the growth and higher antibacterial activities by marine sponge-associated *Streptomyces* spp. against pathogenic bacteria compared to ISP4 and ISP2 medium. At the present work during the selection of a suitable medium and optimization of the time incubation in the liquid culture media showed that the supernatant free-cell of marine sponge-associated *Streptomyces* spp. produces better antibacterial activity against pathogenic bacteria when cultured using a modified molasses medium at 15 days incubation. Similarly, in the previous work, the highest antimicrobial activity was obtained at 15 days incubation in *Streptomyces* spp. when grown using a molasses-soybean meal medium (Ulya 2009). According to Chandrakar and Gupta (2018); Marzoug *et al.* (2023), the main factors influencing the production of antibacterial compounds include carbon sources, nitrogen sources, phosphate availability, and mineral composition.

The present results indicated that a modified molasses medium is the best culture medium to produce higher antibacterial activity, composed of a carbon source (molasses), nitrogen sources (yeast extract and urea), phosphate, as well as various inorganic minerals. According to Rocha *et al.* (2020), molasses as substrate in the molasses-based medium is one of the cheapest sources of carbohydrates (glucose, fructose, and sucrose), water, nitrogen, and vitamins. Glucose and sucrose in molasses are the main carbon sources in growth and antibacterial compounds production (Al-Ansari *et al.* 2020). Previous studies have reported that glucose and sucrose in molasses are the best carbon sources for antibiotic production such as erythromycin by *Saccharopolyspora erythraea* (El-Enshasy *et al.* 2008). Most bacteria preferred using the simplest available carbohydrate, such as glucose rather than starch. In the ISP4 medium, starch was the only available carbohydrate. So, to sustain cell growth, the *Streptomyces* spp. would synthesize amylase to break down starch into glucose that would be ready for use (Solihin *et al.* 2021). The two main components of starch are the highly branched amylopectin molecule and the amylose molecule. Both amylose and amylopectin are polymers of glucose (Arijaje and Wang 2017). Hence the glucose in molasses will be directly used as a carbon source, while the starch in ISP4 must first be degraded into shorter-chain compounds by producing the amylase enzyme which aims to break down the starch to form glucose. Thus, explains why the modified molasses medium produces better antibacterial activity against pathogenic bacteria rather than the ISP4 medium for producing antibacterial compounds by marine sponge-associated *Streptomyces* spp.

The all isolates of marine sponge-associated *Streptomyces* spp. exhibited good antibacterial activity in a modified molasses medium. Hence, it was chosen for antibacterial compounds production, then extracted with ethyl acetate. The extraction of antibacterial compounds of marine sponge-associated *Streptomyces* spp. with ethyl acetate is in accordance with some previous reports. Ethyl acetate was found to be the best solvent to extract antibacterial compounds from *Streptomyces* spp. against pathogenic bacteria (Marzoug *et al.* 2023). Similarly, the *Streptomyces* sp. NLKPB 45 strain exhibited good activity against pathogenic bacteria such as *E. coli* when it was extracted using ethyl acetate solvents compared to other solvents (Kalyani *et al.* 2019).

The ethyl acetate extracts of *Streptomyces* sp. Dbi28t showed extensive inhibition to the growth of ESBL-producing *E. coli, P. rettgeri*, then followed by *P. mirabilis* and *P. putida* at a concentration of 1,000 µg/ml whereas the strength of inhibition is categorized as very strong and strong. The strength of inhibition is categorized according to Davis and Stout (1971): very strong (>20 mm), strong (10-20 mm), medium (5-10 mm), and weak (<5 mm). Similar findings have been reported in the ethyl acetate extracts of *Streptomyces* sp. Al-Dhabi-90 possessed good antibacterial activities against drug-resistant ESBL pathogens such as *E. coli* and *P. mirabilis* (Al-Dhabi *et al.* 2019). Similarly, a marine *Streptomyces* sp. PM49 which had an inhibitory effect against ESBL-producing *E. coli*. This strain was efficient in inhibiting the β-lactamase enzymes of these pathogens (Shanthi *et al.* 2015). Noteworthy, *Streptomyces* spp. can produce β-lactamase inhibitors to inhibit the β-lactamase enzymes of pathogenic bacteria (Marques *et al.* 2018). Moreover, a previous study has reported *Streptomyces* spp. exhibited strong antibacterial activity against pathogenic bacteria such as *P. putida* and *P. rettgeri* (Shetty *et al.* 2014; Wang *et al.* 2021).

The activities of antibacterial compounds have shown the factor responsible for those antibacterial activities that are produced (Thapa *et al.* 2024). Hence, to get a deep understanding of the antibacterial compound composition from *Streptomyces* sp. Dbi28t, LCMS/MS analysis has been carried out. Based on the result, the ethyl acetate extracts of Dbi28t were rich

in lipopeptides and other antibacterial compounds which are known as antibiotics from *Streptomyces* species as per the existing literature (Table 5; Figure 5). *Streptomyces* are one of the most important sources of lipopeptides, with the exploration of such lipopeptides greatly contributing to the development of modern antibiotics for therapeutic use. Arguably the most important lipopeptide from *Streptomyces* is daptomycin (Zhang *et al.* 2023). Other lipopeptides mainly pumilacidin A and B as well as surfactin A and B, are reported as antibacterial compounds which typically involve the breakdown of the cell membrane of pathogens, as well as are known as power lipopeptides produced by *Bacillus* spp. (Li *et al.* 2023; Zhang *et al.* 2023; Castaldi *et al.* 2024). However, these compounds turn out not only to be found in Bacillus but also in *Streptomyces* spp. with antibacterial activities that are mainly mediated by permeabilising cellular membranes (Brack *et al.* 2015; Zhang *et al.* 2023; Thapa *et al.* 2024). In addition, its antibacterial activities may be potent as novel non-β-lactam inhibitors especially those that do not have the β-lactam ring like β-lactamase inhibitory peptide (Alaybeyoglu *et al.* 2017). Furthermore, similar findings have been reported that surfactin B was identified from the ethyl acetate extracts of Streptomyces sp. PC1 using LC-MS/MS analysis and demonstrating potent antibacterial activities against pathogenic bacteria (Thapa *et al.* 2024). Moreover, surfactin was also found in the ethyl acetate extracts of marine sponge-associated *Streptomyces* sp. RM66 (Alkhalifah 2021).

Characterization of antibacterial compounds of marine sponge-associated *Streptomyces* sp. Dbi28t using LC-MS/MS analysis also showed the existence of other antibacterial compounds including phenazostatin B, chalcomycin B, neopyrrolomycin C, saquayamycin A, and saphenamycin. A previous study reported phenazostatin B was isolated from the *Streptomyces* genus and marine actinobacteria isolate *Pseudonocardia* sp. showed potent antibacterial against pathogenic bacteria such as *E. coli* (Maskey *et al.* 2003; Li *et al.* 2015). Furthermore, chalcomycin B was previously isolated from marine-derived Streptomyces sp. B7064 showed potent activity against *E. coli* (Asolkar *et al.* 2002). Similarly, marine-derived *Streptomyces* sp. HK-2006-1 produced chalcomycin B which has potent as an antibacterial (Wang *et al.* 2016).

The other antibacterial compound in this study is neopyrrolomycin C. Neopyrrolomycin C has been reported in a previous study and was obtained from the ethyl acetate extracts of a *Streptomyces* sp. AMRI-33844. These compounds displayed good antibacterial activity against pathogenic bacteria including ESBL-producing *E. coli*, the activity was 2 orders of magnitude greater than vancomycin or ciprofloxacin in many instances (Hopp *et al.* 2009). Another antibacterial compound found in marine sponge-associated *Streptomyces* sp. Dbi28t was saquayamycin A. These compounds are known for anticancer and antibiotics. Saquayamycin A was discovered in *Streptomyces* sp. PAL114, exhibiting potent activity against multidrug-resistant pathogens. Moreover, the characterization of antibacterial compounds of marine sponge-associated *Streptomyces* sp. Dbi28t also found saphenamycin. This result is in accordance with that of a previous study by Kitahara *et al.* (1982) that saphenamycin is a known antibiotic from the phenazines group that has been isolated from the cultured broth of *Streptomyces* MG314-hF8 which has antibacterial activity against pathogenic bacteria like *E. coli* K12 and *Proteus* species.

In conclusions, marine sponge-associated *Streptomyces* spp. produces better antibacterial activity against pathogenic bacteria when grown using modified molasses medium at 15 days incubation. The ethyl acetate extract of marine sponge-associated *Streptomyces* sp. Dbi28t exhibited a significant inhibitory zone against pathogenic bacteria including ESBL-producing *E. coli*, and *P. rettgeri*, then followed by *P. mirabilis* and *P. putida* whereas the strength of inhibition is categorized as very strong and strong, and the results of antibacterial activity against these pathogenic bacteria were higher than some commercial antibiotics. Characterization of antibacterial compounds of *Streptomyces* sp. Dbi28t using LC-MS/MS, found antibacterial compounds including pumilacidin A, pumilacidin B, sufactin B, surfactin A, phenazostatin B, chalcomycin B, neopyrrolomycin C, saquayamycin A, and saphenamycin. The antibacterial compounds identified in this study can be further validated through detection of encoding genes known to produce these compounds in *Streptomyces* spp. isolates. Understanding the action mechanisms of these antibacterial compounds is crucial for achieving

successful outcomes in combating Gram-negative pathogenic bacteria.

## Acknowledgements

The authors are thankful to Prof. Dr. Ir. Yulin Lestari for providing financial research support. This work also was supported by Microbiology Division, Department of Biology, Faculty of Sciences, IPB University for providing the necessary laboratory facilities. We also thank Mr. Azhar Darlan, Puslabfor Mabes Polri, for conducting the LC-MS/MS analysis.

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