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ANTIBACTERIAL, CYTOTOXICITY, AND BIOSYNTHETIC GENE CLUSTER ANALYSIS OF CORAL-ASSOCIATED ACTINOBACTERIA FROM MENJANGAN BESAR ISLAND, KARIMUNJAWA NATIONAL PARK

Mada T. Sibero[1,2,✉], Evan H. Frederick[1,2], Kristina Lubis[1], Diah P. Wijayanti[1], D. Haryanti[1], Anggun P. Siswanto[3], Delianis Pringgenies[1], Retno Murwani[2] and Yasuhiro Igarashi[4]

[1]Department of Marine Science, Faculty of Fisheries and Marine Science, Universitas Diponegoro, Tembalang-50275, Central Java, Indonesia.
[2]Natural Product Laboratory, Integrated Laboratory for Research and Services, Universitas Diponegoro, Tembalang-50275, Central Java, Indonesia.
[3]Chemical Engineering, Vocational School, Universitas Diponegoro, Tembalang-50275, Central Java, Indonesia.
[4]Biotechnology Research Center, Department of Biotechnology, Toyama Prefectural University, Imizu-939-0398, Toyama, Japan.
✉Corresponding Author: madatriandala@hotmail.com

ABSTRACT

Coral-associated actinobacteria have been abundantly reported for their biological properties, particularly as antimicrobial and anticancer agents. This study was carried out to investigate the potential of coral-associated actinobacteria as antibacterial and cytotoxic agents and analyze the biosynthetic gene clusters (BGCs) of the most remarkable isolate. Coral *Porites cylindrica* was collected from Menjangan Besar Island, Karimunjawa National Park, Indonesia. Based on the antibacterial screening, three out of fifteen isolates demonstrated the potential as antibacterial agents, such as CSM 11, CSM 13, and CSM 18. They were identified as *Streptomyces rochei*, *S. xiamenensis*, and *S. albus*, respectively. There were no crude extracts that demonstrated antibacterial activity. *S. rochei* CSM 11 from A11 medium showed a strong cytotoxic activity with an IC_{50} value of 4.50 µg/mL. The antiSMASH analysis described that *S. rochei* contains 33 clusters with various bioactive compounds.

Keywords: Actinobacteria, Antibacterial, AntiSMASH, Coral, Cytotoxicity

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INTRODUCTION

Exploration of antimicrobial and cytotoxic compounds is still the center of attention as the number of multidrug-resistant (MDR) bacterial infections and cancer cases keep increasing. The infection of MDR bacteria will prolong the medication time due to the limited option of effective antibiotic treatment, retard the recovery, and increase the mortality ratio.[1] This phenomenon is triggered by the irrational use of antibiotics in many sectors, including in personal care products consisting of synthesized antibiotics such as retinoids and benzoyl peroxide.[2–4] The utilization of these products is reported to promote antibiotic resistance in skin opportunistic bacteria, leading to a severe acne condition.[5] Following this issue, the World Health Organization (WHO) stated that the number of cancer cases escalates every year.[6] The unhealthy lifestyle in society is the major factor that influences this escalation.[7] Hence, the exploration of novel antibacterial and anticancer compounds is needed to overcome these issues. As a tropical country, Indonesia preserves various untapped marine resources with outstanding potential in the biopharmaceutical industry.[8,9] Corals are noted to have abundant holobiont, providing a unique and elusive competition interaction among them which trigger the production of numerous natural products.[10,11] As one of the coral-associated microbes, actinobacteria become the rising star to obtain novel antibacterial and anticancer compounds. It was highlighted from prior studies that several genera of coral-associated actinobacteria such

as *Brevibacterium*, *Gordonia*, *Kocuria*, *Micrococcus*, *Micromonospora*, and *Streptomyces* exhibited antimicrobial and cytotoxic effects.[12,13] This ability comes from unique combinations of biosynthetic gene clusters (BGCs) located in their DNA that encode a particular biosynthetic pathway to produce secondary metabolites.[14,15] Discovering this fact, this study was performed to evaluate the potential of Indonesia's indigenous coral-associated actinobacteria as promising antibacterial and anticancer agents; to analyze the biosynthetic gene clusters (BGCs) of the most prospective isolate using antiSMASH.

EXPERIMENTAL

Sampling

Sampling was conducted under the SIMAKSI permission number as mentioned in our previous study.[16] Approximately 3 cm fragments of *Porites cylindrica* were collected at a depth of 10 m from Menjangan Besar Island, Karimunjawa National Park, Central Java, Indonesia (Fig.-1). The sample was stored inside a sterile zip lock plastic and kept in a cool box for bacterial isolation. Coral species were identified by comparing the pictures of underwater samples to the database in Coral Finder.

Fig-1: Sampling Location and Coral *P. cylindrica*

Actinobacteria Isolation

The serial dilution method was performed in this study. The dilution of 10^{-2} and 10^{-3} were spread onto ISP 4 (International *Streptomyces* Project) medium (Difco™) consisted nystatin (100 mg/L). The isolation plates were incubated and daily observed at room temperature (27°C). During the bacterial isolation, two Petri discs were opened to let the environmental contamination grow as a control. Subsequently, each isolate from isolation media was compared to the control media based on their macroscopy characters. Then, the isolates with different morphology were purified and inoculated on an ISP4 medium as a single pure isolate.

Agar Plug and Overlay Methods

The multidrug-resistant (MDR) strains of *Staphylococcus aureus*, *S. epidermidis*, and *Propionibacterium acne* was revived from our pathogens collection. For each assay, the pathogens were grown onto Nutrient Agar (NA, HiMedia) and incubated for 24 h. For the agar plug method, all isolates were cultivated for 7×24 h, covering the ISP4 medium (Difco™). On the screening day, all pathogens were inoculated into Nutrient Broth (NB, HiMedia) and the inoculums were standardized using 0.5 McFarland Standard (HiMedia). Each plate containing actinobacteria colonies was cut using a cork borer and the agar was placed onto the test plate. [17] As for the overlay method, all actinobacteria were streaked onto ISP4 medium for 7×24 h. Each adjusted pathogen inoculum (0.5 McFarland) was mixed with a soft agar and poured onto the plates containing actinobacteria colonies on the assay day.[18] All assays were performed with two replicates. After 24 h of incubation, each test plate from each assay was documented. The presence of clear a zone indicates a positive result.

Characterization and Identification of Prospective Isolates

Prospective isolates with antibacterial activity were identified using biochemistry and molecular approach. Three potential isolates were sent to the Fisheries Center for Brackish Water Cultivation, Jepara to investigate their biochemistry profile, such as catalase, oxidase, glucose, and aerobe/anaerobe. Further, the molecular study was conducted following Sibero *et al.* using primers 27F and 1492R. The bacterial homologs were obtained using the Basic Local Alignment Search Tool (BLAST) by comparing the sample

sequence to the GenBank. Maximum-likelihood method on MEGA 11 was utilized to reconstruct the phylogenetic tree.

Cultivation and Metabolites Extraction

Each prospective isolate was cultivated in a K-1 flask containing 100 ml of V22 seed culture medium and incubated at 30°C for 7 days (200 r.p.m.). Afterward, five percent of the seed culture was added into 100 mL modified medium, namely A3, A11, and A16 media inside a K-1 flask at 30°C for 7 days (200 r.p.m.).[19] The composition of each medium is described in our previous studies.[19–21] After the cultivation period, their biological properties were extracted using a 1-butanol solvent.

Antibacterial Assay

The paper disc diffusion method was performed according to Sibero *et al.*[16] All pathogens were prepared as described in the previous step. A variety of extract concentrations was prepared by diluting the crude extracts using dimethyl sulfoxide (DMSO). The concentrations were 1000, 500, 250, 125, and 62.5 µg/mL. The paper disc was injected with each concentration and placed onto the test medium along with amoxicillin 10 µg/disc and DMSO.

Cytotoxicity Assay

The screening was done by diluting all crude extracts into one concentration, 1 mg/mL. Then, the crude extract with cell viability < 50% was continued for IC_{50} determination. For that purpose, the prospective crude extracts were diluted into five concentrations (0.0002, 0.002, 0.02, 0.2, and 2 mg/mL). P388 Murine Leukemia Cells were used in this study in 96-well plates. Doxorubicin and DMSO were used as the controls. After treating the cells with the extracts and controls, they were incubated under exact conditions according to our previous study. [22] Further, each well was added with the 50 µL of XTT and re-incubated for 4 h in the same condition. A microplate reader with 540 nm was used to count the cell viability. The IC_{50} was calculated using the logarithmic chart.

Detection and Analysis of Biosynthetic Gene Clusters (BGCs)

The presence of PKS I, PKS II, and NRPS genes was detected according to Wijaya *et al*.[17] The amplification was done using similar protocols following the DNA barcoding stage. The further analysis of BGCs was done by submitting the whole genome sequence of the most promising isolate to the online server antiSMASH 6.0.[23]

RESULTS AND DISCUSSION

Karimunjawa National Park is one of the productive sampling sites that has been frequently reported as the source of prospective marine microorganisms.[24–29] Our study collected *Porites cylindrica* from Menjangan Besar Island (Fig.-1). The existence of this coral in Menjangan Besar Island has been reported by Solihuddin *et al.*[30] According to Seebauer, *P. cylindrica* was reported as the major reef-building coral as it reproduces asexually and can form a large colony with branching morphology.[31] Even though this coral has been widely studied, particularly for their distribution and metabolic response, there are only a few reports about its associated microorganisms. Hence, our current study successfully isolated fifteen actinobacteria with a different morphology from *P. cylindrica*. The macroscopic characteristics were dominated by circular shape, entire margin, and yellow color (Fig.-2, Suppl. Data-1).

In this study, two different antibacterial assays were conducted to examine the mechanism of each isolate biological properties against three multi-drug resistant (MDR) skin opportunistic bacteria. The purpose of the overlay method is to assess the antibacterial activity due to the growing competition between the isolates and the pathogens. In contrast, the agar plug method aims to evaluate the antibacterial activity that is unconditionally produced by the isolates.[32,33] Hence, we found that CSM 11 and CSM 13 exhibited antimicrobial activity against all pathogens using the agar plug and overlay method. On the other hand, CSM 18 only showed activity against *Staphylococcus aureus* and *S. epidermidis* using the overlay method (Fig.-3, Table-1). There are significant factors that affect the antibacterial activity in a few bioassay procedures, such as the type of pathogens, the difference in growth condition of the inoculum and the pathogens, and the incubation conditions.[32,34]

Three prospective isolates were characterized and identified using biochemical and molecular approaches. In general, CSM 11, CSM 13, and CSM 18 shared similar results in biochemical tests (Suppl. Data-2).

Fig.-2: Coral-Associated Actinobacteria

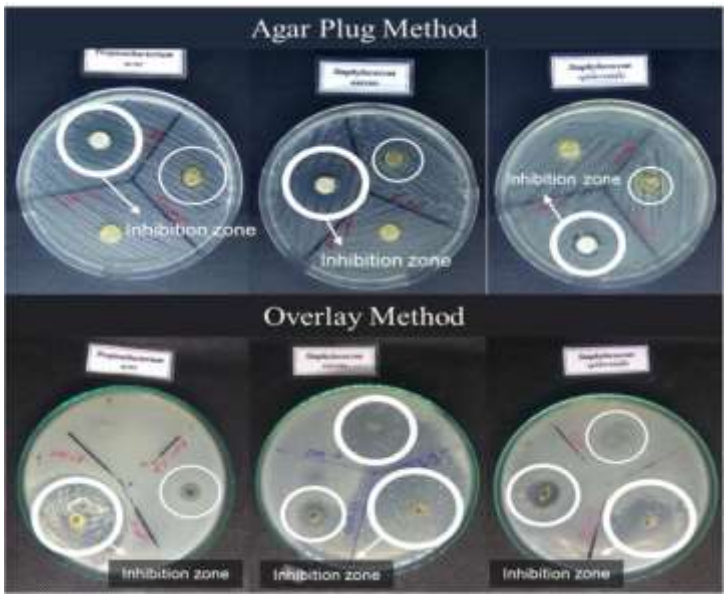

Fig.-3: Antibacterial Activity Exhibited by CSM 11, CSM 13, and CSM 18 Against Three Multidrug-Resistant Skin Opportunistic Bacteria

Table-1: Screening of Antibacterial Activity Using Agar Plug and Overlay Method

No.	Code	Agar Plug Method			Overlay Method		
		P. acne	*S. aureus*	*S. epidermidis*	*P. acne*	*S. aureus*	*S. epidermidis*
1.	CSM 02	-	-	-	-	-	-
2.	CSM 03	-	-	-	-	-	-
3.	CSM 04	-	-	-	-	-	-
4.	CSM 05	-	-	-	-	-	-
5.	CSM 06	-	-	-	-	-	-
6.	CSM 08	-	-	-	-	-	-
7.	CSM 11	+	+	+	+	+	+
8.	CSM 13	+	+	+	+	+	+
9.	CSM 14	-	-	-	-	-	-
10.	CSM 15	-	-	-	-	-	-
11.	CSM 16	-	-	-	-	-	-
12.	CSM 18	-	-	-	-	+	+
13.	CSM 19	-	-	-	-	-	-

14.	CSM 20	-	-	-	-	-	-
15.	CSM 21	-	-	-	-	-	-

Note: (+) indicates the presence of the activity; (-) indicates no activity.

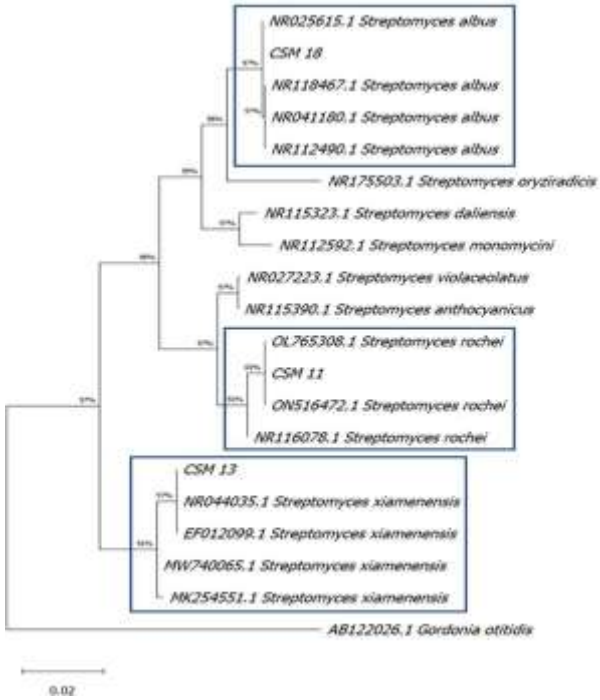

Fig.-4: Phylogenetic Tree of Prospective Coral-Associated Actinobacteria based on 16S rRNA gene sequences

Table-2: Molecular Identification and Detection of Biosynthetic Gene Clusters (BGCs)

Code	Closest Similarity*	Similarity (%)	Accession No.*	BGCs		
				PKS I	PKS II	NRPS
CSM 11	*Streptomyces rochei*	99.93	NR116078.1	-	-	-
CSM 13	*Streptomyces xiamenensis*	99.85	NR044035.1	-	+	+
CSM 18	*Streptomyces albus*	99.59	NR025615.1	-	+	+

Note: *according to NCBI.

However, CSM 13 showed no activity in glucose fermentation. Following the biochemical test, the molecular study found that all isolates belonged to the *Streptomyces* genera. CSM 11, CSM 13, and CSM 18 were identified as *Streptomyces rochei*, *Streptomyces xiamenensis*, and *Streptomyces albus*, respectively (Fig.-4, Table-2). This result was supported by previous studies that isolated *Streptomyces* spp. from corals with biological properties mainly as antibacterial, antifungal, and cytotoxicity.[35–39]

Regretfully, after evaluating all crude extracts, we found that none of the crude extracts demonstrated antibacterial potential. This result unquestionably contradicts the screening result, as each isolate demonstrated an inhibition zone. We suspected that the absence of positive results in antimicrobial activity came from the isolates' incapability to produce desirable compounds in the broth media.[40] Besides, the inability of 1-butanol to extract the desirable compounds is also suspected of affecting this result.[41,42] Consequently, different compounds that were extracted could not act synergistically to demonstrate their antibacterial activity. For the screening of cytotoxicity assay, the CSM 11 crude extracts from A3 and A11 media gave outstanding activities with a low cell viability percentage of <30% (Fig.-5). Interestingly, it was shown that the A11 medium significantly promoted the cytotoxic activity of CSM 11 with a strong IC_{50} value of 4.50 µg/mL, followed by CSM 13 cultivated in the A3 medium with a moderate IC_{50} value of 97.64 mg/mL (Fig.-6). Our previous work successfully isolated a new chlorinated α-lapachone derivative from marine *Streptomyces* sp. that was cultivated in an A11 medium. This compound exhibited a strong

IC_{50} value of 9.8 µM against P388 Murine Leukemia Cells.[43] In another study Zhang *et al* also obtained several novel polyketide compounds namely iseolides A-C from marine *Streptomyces* sp. that was cultivated in an A3 medium.[39] Subsequently, plenty of cytotoxic compounds such as 1-Hydroxy-1-norresistomycin, lobophorin K, piperazimycins A-C, and staurosporine have been reported from marine actinobacteria.[36,44–46] Instead of reporting the compounds, this study analyzed the biosynthetic gene clusters (BGCs) using antiSMASH.

As described in Table-2, CSM 13 and CSM 18 contained PKS II and NRPS, while CSM 11 did not contain any BGCs. Regardless of the result, this research analyzed the BGCs of *Streptomyces rochei* CSM 11 using an antiSMASH platform as it demonstrated the best bioactivity. The complete genome of *S. rochei* was obtained from Nindita *et al.* with accession number AP018517.[47] Based on the analysis, a total of 33 secondary metabolite regions were identified (Table-3). Among them, we found three regions with a 100% similarity, such as ectoine BGC from *Streptomyces anulatus*, geosmin BGC from *Streptomyces coelicolor* A3(2), and pentamycin BGC from *Streptomyces* sp. S816. This result was supported by Ningsih *et al.* who also reported similar ectoine BGC after identifying the secondary metabolites of *Gandjariella thermophila* SL3-204^T.[48] Microorganisms utilized ectoine to protect them from unfavorable conditions, such as high osmotic pressure, cell rupture, overhydration, and osmosis caused by high salinity.[49,50] On the other hand, geosmin has been frequently reported from *Streptomyces* spp. as it is responsible for the earthy odor, produced during secondary mycelial growth coinciding with sporulation.[51] In addition, pentamycin is isolated from *Streptomyces* sp. and reported for its antimicrobial activity against several pathogenic fungi and bacteria.[52,53] Hence, it is concluded that all analyzed secondary metabolites describe biological activities, particularly antimicrobial and antitumor, which are generally classified into several classes of biosynthetic gene clusters.[54–56]

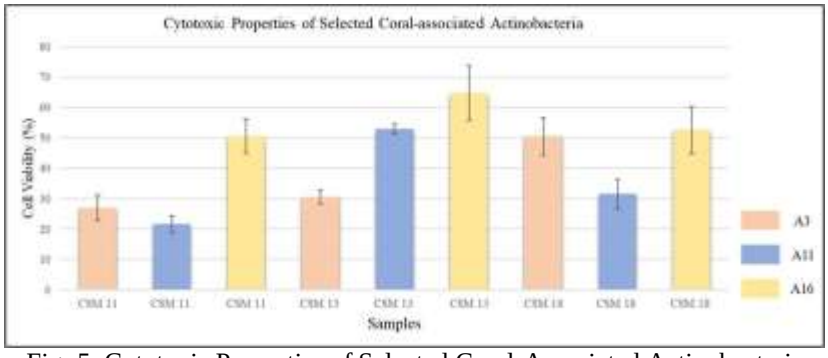

Fig.-5: Cytotoxic Properties of Selected Coral-Associated Actinobacteria

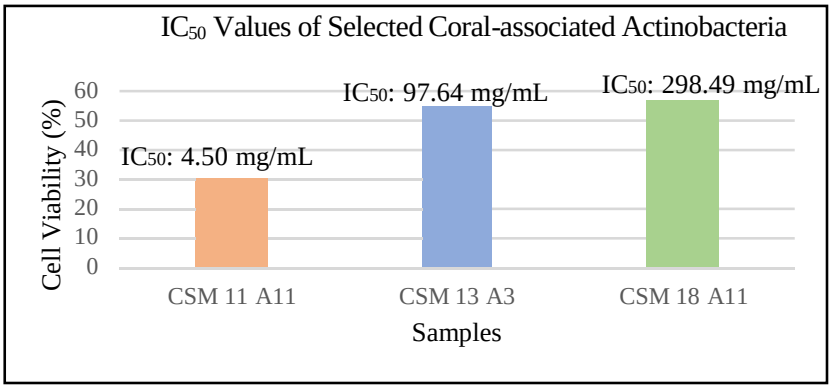

Fig.-6: IC_{50} Values of Prospective Isolates According to Their Cultivation Medium

Table-3: AntiSMASH analysis of *Streptomyces rochei*

Region	Position	Types of Secondary Metabolite Clusters	Most Similar Known Cluster and Its Biological Activities	Similarity (%)	Reference
1	111,485-176,700	T1PKS; NRPS; NRPS-like	carbapenem MM4550 BGC from *Streptomyces argenteolus* BP: antibacterial	10	57-59
2	288,029-321,412	NAPAA	lavendiol BGC from *Streptomyces lavendulae* BP: cholesterol biosynthesis inhibitory activity, immunoregulatory activity, and antitumor activity	6	60
3	344,141-354,959	Siderophore	natamycin BGC from *Streptomyces gilvosporeus* BP: antifungal	9	61
4	365,133-404,510	Other; Lanthipeptide-class-iv	A-503083 A BGC from *Streptomyces* sp. SANK 62799 BP: antibiotic	7	62
5	445,916-465,824	Terpene	ebelactone BGC from *Kitasatospora aburaviensis* BP: cutinase inhibitor	5	63
6	465,914-549,750	NRPS-like; NRPS; T1PKS	bleomycin BGC from *Streptomyces verticillus* BP: antitumor	6	64-66
7	715,438-723,342	RiPP-like	informatipeptin BGC from *Streptomyces viridochromogenes* DSM 40736 BP: antimicrobial	28	67
8	763,520-804,817	T1PKS	tetronasin BGC from *Streptomyces longisporoflavus* BP: antibiotic, anticoccidant	3	68
9	875,926-936,699	Lanthipeptide-class-i; Lanthipeptide-class-ii; NRPS	ansacarbamitocin A BGC from *Amycolatopsis alba* DSM 44262 BP: antitumor	2	69
10	1,087,049-1,136,199	T1PKS; NRPS-like; transAT-PKS-like	cinnabaramide A BGC from *Streptomyces cinnabarigriseus* BP: antifungal, antitumor	18	70
11	1,298,191-1,350,386	Thiopeptide; LAP; Terpene	hopene BGC from *Streptomyces coelicolor* A3(2) BP: antibiotic	92	71,72
12	1,435,740-1,522,951	NRPS; NRPS-like; Betalactone	kirromycin BGC from *Streptomyces collinus* Tu 365 BP: antibiotic	16	73-75
13	1,721,731-1,783,829	NRPS; Siderophore	friulimicin A BGC from *Actinoplanes friuliensis* BP: antibacterial	21	76
14	1,787,822-1,847,859	RRE-containing; NRPS; T1PKS	meilingmycin BGC from *Streptomyces nanchangensis* BP: antibiotic	2	77,78
15	1,956,165-1,977,245	Terpene	geosmin BGC from *Streptomyces coelicolor* A3(2)	100	79
16	1,988,106-1,999,410	RiPP-like	-	-	-
17	2,049,086-2,106,642	PKS-like; T1PKS; Other	meilingmycin BGC from *Streptomyces nanchangensis* BP: antibiotic	5	77,78
18	2,302,916-2,350,417	Siderophore; NRPS	-	-	-
19	2,914,026-2,934,037	Terpene	julichrome Q3-3 BGC from *Streptomyces afghaniensis* 772 BP: antibacterial, antitumor, antioxidant	25	80
20	3,760,217-3,771,149	Butyrolactone	thioviridamide BGC from *Streptomyces olivoviridis* BP: antibiotic	10	81
21	4,262,238-4,287,500	Lanthipeptide-class-i	-	-	-
22	4,442,341-4,514,852	T2PKS	spore pigment BGC from *Streptomyces avermitilis*	83	82
23	5,158,506-5,170,275	Siderophore	desferrioxamin B BGC from *Streptomyces coelicolor* A3(2) BP: Fe (III) chelator	83	83-85
24	5,242,901-5,253,497	Melanin	melanin BGC from *Streptomyces avermitilis* BP: antioxidant, ultraviolet protector	60	82
25	5,522,754-5,565,091	NRPS-like	SCO-2138 BGC from *Streptomyces coelicolor* A3(2)	64	86
26	6,124,810-6,134,899	Ectoine	ectoine BGC from *Streptomyces anulatus* BP: ultraviolet protector	100	87
27	6,725,230-6,777,021	hglE-KS; T1PKS	cinnamycin BGC from *Streptomyces cinnamoneus* BP: antibiotic	19	88,89
28	6,814,586-6,855,650	T3PKS	herboxidiene BGC from *Streptomyces chromofuscus* BP: anticholesterol, antitumor	7	90
29	6,859,541-6,973,901	NRPS; NAPAA; Ectoine	stenothricin BGC from *Streptomyces filamentosus* NRRL 15998 BP: antibacterial	27	91
30	6,992,281-7,038,863	T1PKS	-	-	-
31	7,885,650-8,041,334	NRPS; T1PKS	pentamycin BGC from *Streptomyces* sp. S816 BP: antibiotic	100	92
32	8,114,731-8,162,377	T1PKS	carbapenem MM4550 BGC from *Streptomyces argenteolus* BP: antibiotic	10	57-59
33	8,248,179-8,273,870	Terpene	carotenoid BGC from *Streptomyces avermitilis* BP: antioxidant	63	82

Notes: BGC: biosynthetic gene cluster; BP: biological properties; NRPS: non-ribosomal peptide synthase; NAPAA: non-alpha poly-amino acids like e-Polylysin; RiPP-like: other unspecified ribosomally synthesized and post-translationally modified peptide product; hglE-KS; heterocyst glycolipid synthase-like PKS; LAP: linear azol(in)e-containing peptides; T1PKS: type I polyketide synthase (PKS); T2PKS: type II PKS; T3PKS: type III PKS.

CONCLUSION

Fifteen isolates were isolated from coral *Porites cylindrica*. Hence, three isolates with given codes CSM 11, CSM 13, and CSM 18 demonstrated antibacterial activity against several MDR skin opportunistic

bacteria through the agar plug and overlay method. CSM 11, CSM 13, and CSM 18 were identified respectively as *Streptomyces rochei*, *Streptomyces xiamenensis*, and *Streptomyces albus*. Regretfully, their crude extracts did not show potential as antibacterial agents. On the other hand, CSM 11, demonstrated potential as an anticancer agent with a strong IC_{50} value of 4.50 µg/mL. Based on its antiSMASH analysis, *Streptomyces rochei* contained 33 secondary metabolite regions dominated by antimicrobial compounds.

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