https://doi.org/10.48048/tis.2024.8234

Antibacterial Compounds and Draft Genome Sequence of *Streptomyces* sp. PA5.6 Isolated from Deciduous Dipterocarp Forest Soils, University of Phayao, Thailand

Piyawan Amimanan[1,*], Wajeeorn Ouancharee[2], Noppadon Muangsue[1], Supakanya Lasom[1] and Nicha Charoensri[2]

[1]Department of Medical Technology, School of Allied Health Sciences, University of Phayao, Phayao 56000, Thailand
[2]Centre for Research and Development of Medical Diagnostic Laboratories, Department of Microbiology, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand

([*]Corresponding author's e-mail: piyawan.am@up.ac.th)

Received: 25 March 2024, Revised: 20 May 2024, Accepted: 27 May 2024, Published: 20 September 2024

Abstract

Antibiotic resistance is a major problem in the control of infectious diseases. To overcome this obstacle, a common approach is to search for novel antimicrobial drugs from natural sources, especially microorganisms such as *Streptomyces*. *Streptomyces* has long been demonstrated to produce bioactive secondary metabolites with antimicrobial activities. The objectives of this research were to isolate and characterize *Streptomyces* strains from the deciduous dipterocarp forest soils in the University of Phayao, and to evaluate their metabolites for antibacterial activities. The results show that the morphological and physiological characteristics of PA5.6 are consistent with those of the genus *Streptomyces*. Furthermore, a comparative analysis of the whole genome sequence verified that PA5.6 represents a novel species within the genus *Streptomyces*. The genome sequence analysis of *Streptomyces* sp. PA5.6 revealed a total length of 9,146,340 bp, a G + C content of 71.1 %, and an N50 scaffold of 320,345 bp. The antiSMASH analysis revealed putative secondary metabolite Biosynthetic Gene Clusters (BGCs) involved in the biosynthesis of antimicrobial metabolites, including terpene, Type I PKS, NRPS and lassopeptide gene clusters. Functional prediction of these gene clusters indicates that they are involved in the biosynthesis of several antimicrobial-associated metabolites, such as albaflavenone, monensin and citrulassin D. *Streptomyces* sp. PA5.6 culture supernatant and its crude ethyl acetate extract exhibited antibacterial activity against several drug-resistant, bacterial pathogens, including *S. aureus*, *E. faecalis*, *E. coli*, *P. aeruginosa*, *A. baumannii*, MRSA, VREF, ESBL-*E. coli* and CRPA. The GC-MS analysis of the crude ethyl acetate extracts revealed benzeneacetic acid, benzeneacetic acid, 4-hydroxy and benzeneacetamide as major active compounds metabolites. In conclusion, *Streptomyces* sp. PA5.6 exhibited promising potential for producing bioactive compounds against bacterial pathogens.

Keywords: *Streptomyces*, Genome sequencing, Secondary metabolites, AntiSMASH, Antimicrobial activity, GC-MS, Soil

Introduction

Antibiotic resistance remains a global challenge in treating infectious diseases, especially in developing countries [1]. Currently, research is being conducted to discover new types of antibiotics from natural sources such as plants, fungi and bacteria due to their safety and effectiveness against drug-resistant bacteria [2]. Approximately 80 % of antibiotics are produced by bacteria of the genus *Streptomyces*, such as Streptomycin, Tetracycline and Chloramphenicol, which are used against both Gram-positive and Gram-negative bacteria [3].

Streptomyces is a filamentous, Gram-positive bacteria, that contains DNA with a high G + C content [4]. It possesses BGCs with potential antibacterial properties, such as polyketides (PKS), non-ribosomal peptide synthetase (NRPs), terpenes and the lassopeptide gene [2]. These compounds are utilized in the synthesis of antibiotics such as Tetracycline [5], Daptomycin [6], Phenalinolactones [7] and Aborycin [8], respectively. *Streptomyces* found in different soil environments and diverse habitats can give rise to new species of the genus *Streptomyces* and produce bioactive compounds with antimicrobial potential [9]. Therefore, there has been exploration and isolation of strains from soil in various locations worldwide, such as *Streptomyces* sp. SM01, isolated from soil in Kashmir Himalayan (India), which is capable of producing a novel antibiotic, Picolinamycin, with potential against drug-resistant bacteria [10]. Similarly, *Streptomyces* sp. E23-4 OM883990, isolated from soil in a location with extremely cold temperatures in Fez-Meknes, Morocco, exhibits potential in resisting Gram-positive and Gram-negative bacteria, including drug-resistant strains [11]. Additionally, *Streptomyces acidicola* sp. nov. has been isolated from soil in a peat swamp forest in Thailand [12].

The soil in the deciduous dipterocarp forest in the northern region of Thailand has an acidic condition, sandy and granular soil characteristics, and high temperatures during the dry season [13]. Soils with dry characteristics and high temperatures can harbor *Streptomyces* due to the exospores of the microorganism, which can survive for extended periods [14,15]. Additionally, *Streptomyces* strains isolated from soil with extreme conditions have the potential to produce secondary metabolites with effective antimicrobial properties [16]. However, there has been limited exploration and investigation into the potential antibacterial activities of *Streptomyces*. The objective of the study was to isolate *Streptomyces* from the soil of deciduous dipterocarp forests located at the University of Phayao in Northern Thailand. The study aimed to determine phenotype characteristics, including morphological and physiological traits, to confirm their classification within the genus *Streptomyces*. Additionally, the research involved screening the potential antibacterial activity of *Streptomyces* isolates using cross-streak and well-diffusion methods with their crude ethyl acetate extracts. The active compounds in the crude ethyl acetate extract were identified for antibacterial activity by gas chromatography-mass spectrometry (GC-MS) analysis. Furthermore, the genotype characteristics of the *Streptomyces* isolate were analyzed through whole-genome sequencing, combined with bioinformatics, to confirm the genus *Streptomyces* and identify putative bioactive compounds with potential antibacterial activity.

Materials and methods

Samples collection and isolation of *Streptomyces* sp. PA5.6

The soil samples were collected at a depth of 10 - 15 cm from the upper surface of topsoil in the deciduous dipterocarp forest at the University of Phayao in Northern Thailand (latitude, 19°01'40.3"N; longitude, 99°54'23.9"E). Approximately 50 g of soil was collected in sterile plastic bags and transported to the laboratory. The soil was dried in a hot air oven at 60 °C for 2 h. 1 g of dry soil was dissolved in 9 mL sterile distilled water and a serial dilution up to 10^{-4}. After that 100 µL of each dilution was spread onto

Actinomycetes Isolation Agar (AIA) (Himedia Laboratories Pvt. Limited, Mumbai, India) [17]. supplemented with Nalidixic acid (20 µg/mL) and Nystatin (25 µg/mL) to inhibit gram negative bacteria and fungal growth, respectively [18]. The plates were incubated at 30 °C for 3 days. The *Streptomyces* sp. PA5.6 colony was picked and purified by streaking on AIA. The pure cultures were preserved for a long time with 50 % (v/v) glycerol at –20 °C [19].

Determination of morphological and physiological characteristics of *Streptomyces* sp. PA5.6

The *Streptomyces* sp. PA5.6 was identified, based on cell morphology, by the Gram staining procedure [20]. The morphology properties of isolating *Streptomyces* sp. PA5.6 were determined according to the International *Streptomyces* Project (ISP) [21]. The aerial and vegetative mycelium of *Streptomyces* sp. PA5.6 was observed on yeast extract-malt extract agar (ISP2), Oat Meal Agar (ISP3) and inorganic Salts-Starch Agar (ISP4). Melanin production was determined on tryptone-yeast extract agar (ISP1). The carbon utilization test was determined on carbon utilizing agar (ISP9) with the addition of 1 sugar from D-glucose (positive control), sucrose, D-fructose, D-arabinose, D-mannitol, D-xylose and no carbon source (negative control) as described in the ISP project [21]. The utilization of nitrogen sources was tested according to Williams *et al*. [22]. The morphology characteristic of *Streptomyces* sp. PA5.6 was measured from cultures on Modified Nutrient Glucose (MNG) agar as recommended by Al-Dhabi *et al*. [23].

Whole genome sequence and phylogenetic characterization

The *Streptomyces* sp. PA5.6 was cultured in an MNG medium as recommended by Al-Dhabi *et al*. [23] and incubated in a shaking incubator (150 rpm) at 30 °C for 7 days. The supernatant was removed by centrifugation at 15,000×g for 15 min. The genomic DNA of *Streptomyces* sp. PA5.6 was extracted by cetyl trimethylammonium bromide (CTAB) [24]. The DNA samples were submitted to NovogeneAIT Genomics Singapore (Singapore). The Whole genome sequence of *Streptomyces* sp. PA5.6 was performed by Illumina NovaSeq 6000 system (Illumina, San Diego, CA) generating 150-bp read lengths with > 100×coverage. The raw paired-end reads were trimmed, assembled de novo, and annotated using Trim Galore [25] and Unicycler [26]. The raw files were assembled using the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) [27]. The 16S ribosomal RNA (rRNA) sequence of *Streptomyces* sp. PA5.6 was compared with the sequence of all published species of the genus *Streptomyces* using the NCBI BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 2 March 2024) [28]. The pairwise alignment analysis, 16S rRNA sequence similarity between species was calculated. The MEGA7 software [29] was used to construct a phylogenetic tree using neighbor-joining [30] and maximum likelihood [31] methods. Whole genome sequences of *Streptomyces* sp. PA5.6 and each closely related strain were analyzed by Digital DNA-DNA hybridization (dDDH) value and a phylogenomic tree produced by the Type (strain) Genome Server (TYGS) (https://tygs.dsmz.de) [32]. The assembled genome sequence was annotated by Rapid Annotation using Subsystem Technology (RAST) server v.2.0 (https://rast.nmpdr.org) [33]. The prediction of potential secondary metabolite was analyzed using antiSMASH (antibiotics & Secondary Metabolite Analysis Shell) v.7.0.1 (https://antismash.secondarymetabolites.org/#/start, accessed on 2 March 2024) [34].

Bacterial strains for antibacterial activity testing

The bacterial strains were used in this study for assessment of antibacterial activity. They were drug-sensitive bacteria strains including *Staphylococcus aureus* (*S. aureus*) DMST 6512, *Enterococcus faecalis* (*E. faecalis*) DMST 2860, *Escherichia coli* (*E. coli*) DMST 4212 and *Pseudomonas aeruginosa* (*P. aeruginosa*) DMST 4739 that were purchased from the Department of Medical Sciences Thailand (DMST). Whereas

drug-resistant bacteria strains include Methicillin-resistant *Staphylococcus aureus* (MRSA) MRSA 167-15, Vancomycin-Resistant *Enterococcus faecium* (VREF) VREF-10_Van A, Extended-spectrum beta-lactamase-producing *E. coli* (ESBL-*E. coli*) ESBL-*E. coli* 164_CTX-M-15, Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) CRPA P11_VIM-2 were kindly provided by Prof. Dr. Aroonlug Lulitanond and Assoc. Prof. Dr. Aroonwadee Chanawong, Faculty of Associated Medical Sciences, Khon Kaen University, Thailand. In addition, clinical strains including *Acinetobacter baumannii* (*A. baumannii*) were isolated from hemoculture at Nan Hospital, approved by the Institutional Ethical Committee of the University of Phayao, Phayao, Thailand (approval no. UP-HEC 1.1/042/64).

Screening for antibacterial activity of *Streptomyces* sp. PA5.6

The antibacterial activity of *Streptomyces* sp. PA5.6 was screened by cross streak following the method described by Al-Dhabi *et al*. [23] with a slight modification (the temperature for *Streptomyces* sp. PA5.6 growth on MNGA medium). The *Streptomyces* sp. PA5.6 was streaked in a single line on MNGA medium and incubated at 30 °C for 7 days. Then the 10^8 CFU/mL of each drug-sensitive, drug-resistant and clinical bacteria strain were streaked perpendicular to the *Streptomyces* sp. PA5.6 growth line. The plate was incubated for 24 h at 37 °C and the distance of the inhibition zone between the margin of *Streptomyces* sp. PA5.6 and each bacterial strain determined.

Extraction of the antibacterial metabolite

The *Streptomyces* sp. PA5.6 was cultured in MNG medium and incubated in a shaking incubator (150 rpm) at 30 °C for 7 days. The supernatant was filtrated using Whatman No.1 filter paper and the pH adjusted to 2 by 0.1 N HCL according to Al-Dhabi *et al*. [23]. The culture filtrate was added with an equal volume of the ethyl acetate (1:1), mixed and the organic and the aqueous layers separated in a separating funnel. The organic layer was collected and concentrated by a rotary evaporator (EYELA@ N-1200A Rotary Evaporator model) at 60 °C and stored at 4 °C.

Antibacterial metabolite activity by agar well diffusion

Antibacterial metabolite activity of the crude ethyl acetate extract of *Streptomyces* sp. PA5.6 was determined by the agar well diffusion method, following the method described by Al-Dhabi *et al.* [23]. The 10^8 CFU/mL of each drug-sensitive, drug-resistant and clinical bacteria strains were speared on Muller Hinton Agar (MHA). The wells were drilled with a sterile cork borer (6 mm in diameter) and filled with 50 µL of the crude extract (200 mg/mL in sterile distilled water). Fifty µL of Amikacin (30 µg/mL) and sterile distilled water were used as positive and negative controls, respectively. (Amikacin has a broad spectrum of activity). The plates were incubated at 37 °C for 24 h and the inhibition zone (mm) was determined.

GC-MS analysis of crude ethyl acetate extract

The crude ethyl acetate extract of *Streptomyces sp.* PA5.6 was determined for chemical composition by Agilent gas chromatography-mass spectrometry (GC-MS) (GC:8890A; MSD 5977B). The fused silica HP-5 capillary column (30 m × 0.25 mm ID, 0.25 µm thickness) was directly coupled to the MS. The carrier gas was helium with a flow rate of 0.5 mL/min. The mass spectrometer was programmed at 42 - 320 °C at a rate of 15 °C/min. The injection volume was 1 µL and the evaporation temperature was at 280 °C, with a flowing velocity of 36 cm/s. GC-MS analysis was performed at ALS Testing services (Thailand) Co., Ltd.

Results and discussion

The study of the phenotypic characteristics, including morphological and physiological characteristics of the PA5.6 strain, isolated from soil in the deciduous dipterocarp forest at the University of Phayao in Northern Thailand, reveals that this strain exhibits positive gram-staining characteristics and has a filamentous shape (**Figure 1**). The colony morphology of the PA5.6 on MNG agar shows a solid cream-colored surface, while on ISP-2, ISP-3 and ISP-4 media, it forms aerial and vegetative mycelium with a white color on the upper and lower colonies. PA5.6 strain produced a reddish-brown melanoid pigment on ISP1. Additionally, the PA5.6 strain demonstrates growth in media containing various carbon sources, such as sucrose, D-fructose, D-arabinose, D-mannitol and D-xylose. It exhibits growth in nitrogen-containing media supplemented with L-arginine, L-phenylalanine, L-serine and L-valine. Therefore, the phenotypic characteristics of the PA5.6 strain indicate its relationship with the genus *Streptomyces*.

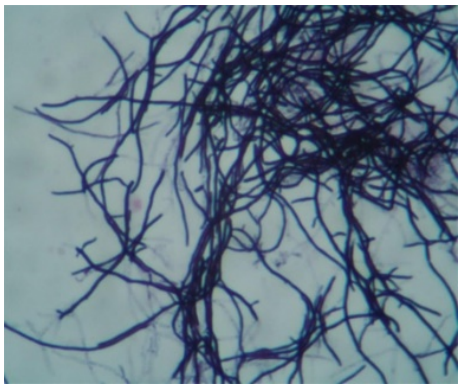

Figure 1 Gram-positive filamentous mycelium of *Streptomyces* sp. PA5.6 under microscopic examination.

The genomic characteristics of *Streptomyces* sp. PA5.6 were analyzed through whole-genome sequencing. The analysis revealed that its genomic sequence has a total length of 9,230,481 bp (69 contigs), GC content of 71.1 %, and an N50 scaffold of 322,909 bp. The genome annotation revealed 8,606 protein-coding sequences (CDS) and a total of 70 RNAs (67 tRNAs and 3 rRNAs) (**Table 1**).

Table 1 Genome information of the assembly *Streptomyces* sp. PA5.6.

Assembly	***Streptomyces* sp. PA5.6**
Total length (bp)	9,230,481
contigs	69
GC content (%)	71.1
N50 (bp)	322,909
N75 (bp)	190,154
CDS	8,606
tRNA	67
rRNA	3

The phylogenetic analyses showed that *Streptomyces* sp. PA5.6 belongs to the genus *Streptomyces* (**Figure 2**) and the 16S rRNA gene has a similarity with *Streptomyces xanthii* strain CRXT-Y-14 (98.43 %), *Streptomyces avermitilis* (97.90 %), *Streptomyces broussonetiae* strain T44 (97.64 %) and *Streptomyces fagopyri* strain QMT-28 (97.51 %) (NCBI Blastn), suggesting that *Streptomyces* sp. PA5.6 is

identified as a new species based on the cutoff value of 16S rRNA sequence similarity < 98.7 % [35]. Furthermore, the genome sequence of *Streptomyces* sp. PA5.6 was submitted to NCBI with the following accession numbers: BioProject - PRJNA946031, BioSample - SAMN33752414, SRA - SRR23901935 and Genbank - JAROKT000000000. The genome phylogeny indicated that *Streptomyces* sp. PA5.6 constituted a member of the genus *Streptomyces* and the digital DNA-DNA hybridization (dDDH) value between *Streptomyces* sp. PA5.6 and *Streptomyces kanamyceticus* ATCC 12853 was 30.4 % (**Figure 3**), which is below the threshold of 70 % used to identify a new species [35]. Thus, based on the information, *Streptomyces* sp. PA5.6 can be classified as a new species.

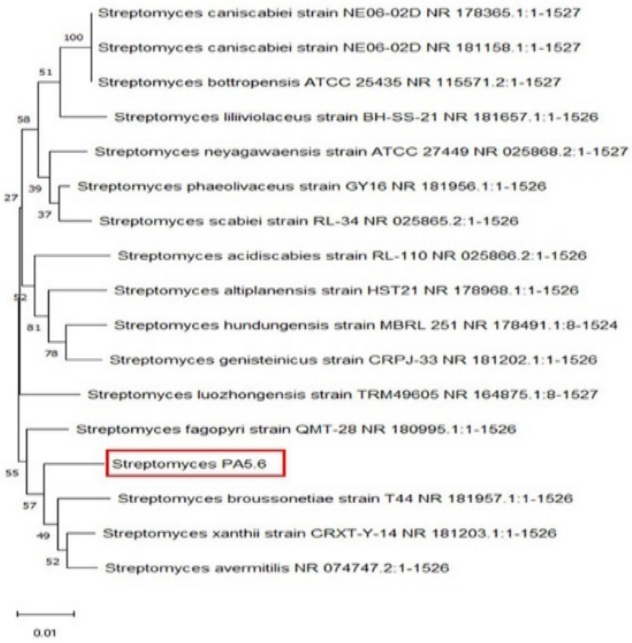

Figure 2 Phylogenetic tree of the *Streptomyces* sp. PA5.6 was constructed using MEGA7 software and the 16 members of *Streptomyces* with close relationships, obtained from the NCBI BLAST database.

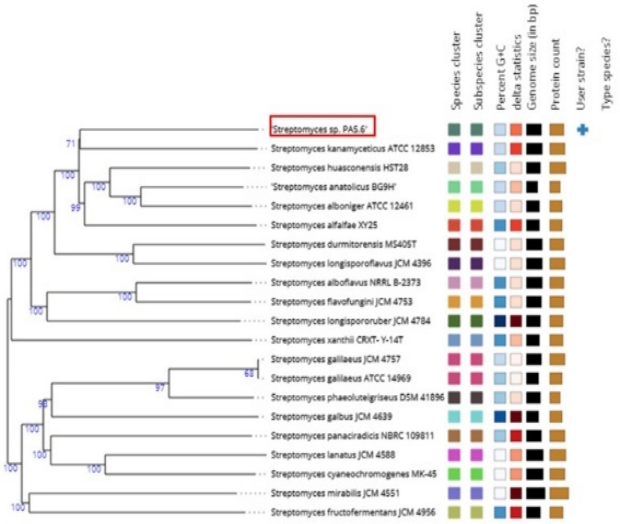

Figure 3 Phylogenomic tree was constructed from the genome sequence of *Streptomyces* sp. PA5.6 and the closely related strains identified by the TYPGS server.

The annotation of the *Streptomyces* sp. PA5.6 genome was predicted by the RAST server. The result revealed a total of 4,025 subsystems, which represented amino acid and derivative metabolisms (18.4 %), protein metabolisms (8.4 %), DNA metabolisms (4.4 %) and secondary metabolisms (0.2 %) (**Figure 4**). Additionally, the analysis of the antiSMASH program (v.7.0.1) predicted 22 putative secondary metabolites Biosynthetic Gene Clusters (BGCs). These clusters include terpene, PKS (Polyketide Synthase), NRPS (Nonribosomal Peptide Synthetase), melanin, lanthipeptide-class-iii, NRPS-like, blactam, lassopeptide, ectoine and hybrids gene cluster (**Table 2**). Interestingly, the gene clusters of terpene, Type I PKS, NRPS and lasso peptide are involved in the biosynthesis of antimicrobial metabolites [36]. These data indicate that *Streptomyces* sp. PA5.6 may have the potential to produce antimicrobial metabolites.

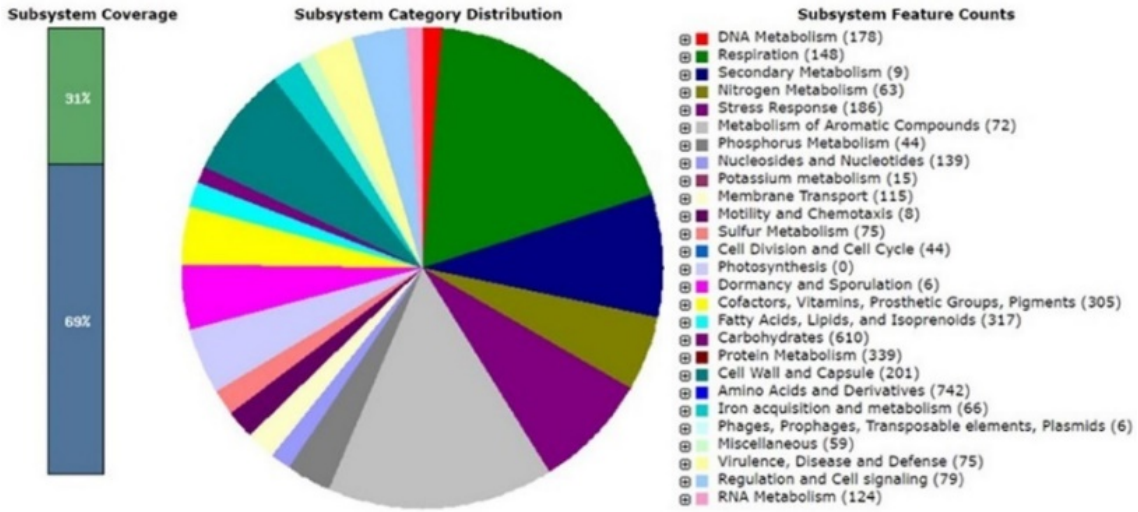

Figure 4 The subsystems category distribution for the genome of *Streptomyces* sp. PA5.6 (accessed on 2 March 2024).

The functional predictions of secondary metabolites BGCs (Type I PKS, terpene and lassopeptide) are related to several antimicrobial-associated metabolites including monensin, albaflavenon and citrulassin D. The monensin was produced by *Streptomyces* spp. and belongs to the Type I PKS. It exhibits antibacterial properties against Gram-positive bacteria. Monensin disrupts ionic gradients and alters the cell physiology of bacteria, leading to cell death [37]. The albaflavenone is classified as a terpene and is produced by *Streptomyces albidoflavus* [38]. Previous studies have reported that albaflavenone inhibits *Bacillus subtilis* [34]. The citrulassin D belongs to the lassopeptides and displays antibacterial activity against *Listeria monocytogenes* and *Klebsiella pneumoniae* [39]. However, the mechanism of antibacterial activity of albaflavenone and citrulassin D is still unclear.

Table 2 The secondary metabolites producing Biosynthetic Gene Clusters as predicted by antiSMASH.

Contig	Region	Position (bp)	Type	Most similar known cluster	Similarity (%)
1	1.1	133475-154380	Terpene	Cosmomycin C	3 %
	1.2	382904-409564	Terpene	Hopene	84 %
	1.3	431790-557986	T1PKS	Monensin	79 %

Contig	Region	Position (bp)	Type	Most similar known cluster	Similarity (%)
	1.4	709035-741119	T3PKS, NRPS	Corbomycin	59 %
2	2.1	224309-234686	Melanin	Istamycin	5 %
3	3.1	44613-94629	NRPS	Mirubactin	50 %
	3.2	196779-240843	NRPS-like	Foxicin A/Foxicin B/Foxicin C/Foxicin	39 %
4	4.1	1-18870	Blactam	valclavam/(-)-2-(2-hydroxyethyl)clavam	50 %
	4.2	346560-369136	Lassopeptide	Citrulassin D	100 %
5	5.1	182693-205329	Lanthipeptide-class-iii	Informatipeptin	42 %
6	6.1	352562-435108	T3PKS, NRPS	Corbomycin	48 %
11	11.1	1657-12061	Ectoine	Ectoine	100 %
	11.2	176677-198899	Terpene	Geosmin	100 %
15	15.1	227957-250273	NRPS	Bosamycin A/Bosamycin B/BosamycinC/ Bosamycin D/Bosamycin E/Bosamycin F	33 %
18	18.1	169254-207579	T1PKS	α-lipomycin	18 %
19	19.1	62867-142734	NRP-metallophore, NRPS, NI-siderophore	Coelichelin	100 %
24	24.1	1-25106	Terpene	Isorenieratene	100 %
27	27.1	52209-73246	Terpene	Cyclooctatin	50 %
28	28.1	8743-81231	T2PKS	Nenestatin	55 %
32	32.1	22753-43703	Terpene	Albaflavenone	100 %
36	36.1	1-60164	T1PKS	Macrotermycins	53 %
38	38.1	33851-55050	Terpene	Leucomycin	11 %

The antimicrobial activity of crude ethyl acetate extracts from *Streptomyces* sp. PA5.6 against bacteria was demonstrated by the cross-streak method and agar well diffusion. The results revealed that *Streptomyces* sp. PA5.6 can inhibit drug-sensitive, drug-resistant and clinical bacteria strains, except for *P. aeruginosa* and CRPA (**Figure 5**). Furthermore, the results on the efficacy of the crude ethyl acetate extract from *Streptomyces* PA5.6 revealed that the crude extract exhibited high activity against VREF (17.0 mm) followed by *E. faecalis* (15.0 mm), CRPA (14.0 mm), *A. baumannii* (13.0 mm), *P. aeruginosa* (12.5 mm), MRSA (11.5 mm), ESBL-*E. coli* (11.5 mm), *E. coli* (10.0 mm) and *S. aureus* (9.0 mm) (**Figure 6**). These data indicate that the *Streptomyces* sp. PA5.6 strain isolated from soil in the deciduous dipterocarp forest has antimicrobial activity.

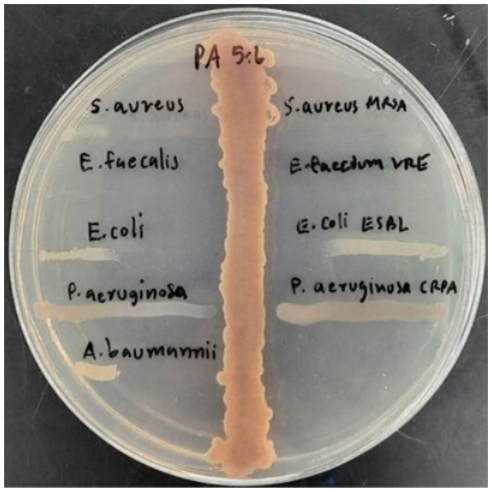

Figure 5 Antibacterial activity of *Streptomyces* sp. PA5.6 by the cross-steak method.

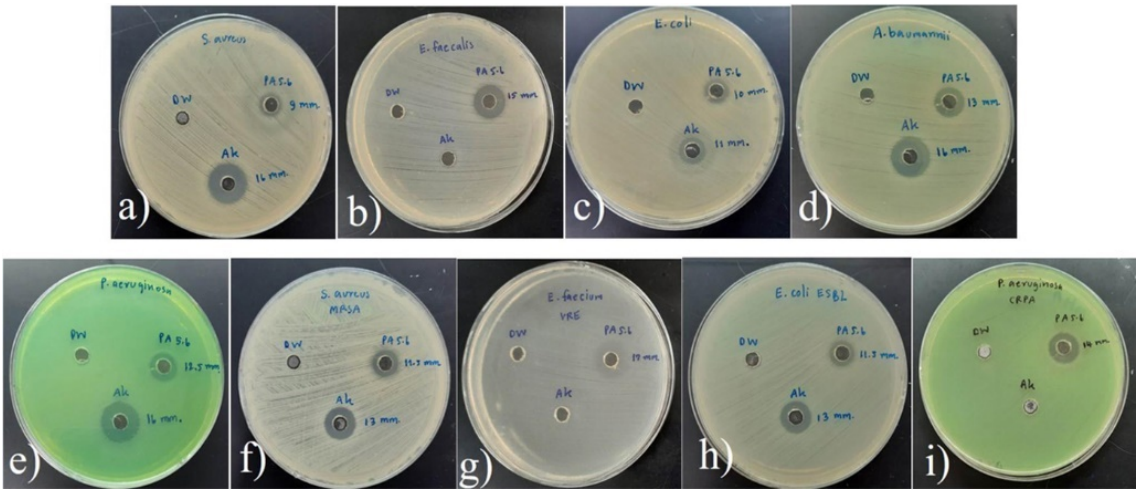

Figure 6 Inhibition zones of crude ethyl acetate extract of *Streptomyces* sp. PA5.6 against drug-sensitive, drug-resistance and clinical bacteria strains: (a) *S. aureus*, (b) *E. faecalis*, (c) *E. coli*, (d) *A. baumannii*, (e) *P. aeruginosa*, (f) MRSA, (g) VREF, (h) ESBL-*E. coli* and (i) CRPA.

Further study of the crude ethyl acetate extract of *Streptomyces* sp. PA5.6 by gas chromatography-mass spectrometry (GC-MS) analysis revealed 26 compounds. However, a literature review supported only 16 compounds that showed bacterial activity and their mechanisms of action (**Table 3**). Additionally, the data in **Table 3** show the mechanisms of antibacterial activity for each compound, compiled from various studies. The GC-MS analysis revealed that the major active compounds include Benzeneacetic acid (2.14 %), benzeneacetic acid, 4-hydroxy (0.98 %) and benzeneacetamide (0.43 %). Previous studies have reported that Benzeneacetic acid and Benzeneacetamide were detected in the ethyl acetate extract of *Streptomyces* sp. Al-Dhabi-1, *Streptomyces* sp. strain EA-PWS52 and *Streptomyces achromogenes* TCH4 and exhibited potential antibacterial activity [11,23,40]. Benzeneacetic acid inhibits protein synthesis and affects the integrity of the cell membrane, leading to the leakage of substances and eventual cell death [41], while Benzeneacetamide inhibits topoisomerase IV (ParE), resulting in the inhibition of bacterial cell

proliferation [42]. Benzeneacetic acid, 4-hydroxy, exhibited activity against *Listeria monocytogenes*, inducing cell death by disrupting the cell membrane and reducing the expression of three virulence factors that affect its interaction with host cells [43]. Additionally, there are 13 other minor compounds (ranging from 0.12 to 0.35 %) recognized for their antibacterial properties due to their effects on the cell membrane, fatty acid synthesis, DNA replication and inhibition of Quorum Sensing; these minor compounds result in the leakage of substances and the inhibition of bacterial cell division, leading to cell death [44-56]. Therefore, our results propose that both major and minor compounds in the crude ethyl acetate extract of *Streptomyces* sp. PA5.6 own antimicrobial activity.

Table 3 Compounds in crude ethyl acetate extract of *Streptomyces* sp. PA5.6 by GC-MS analysis and their mechanism of antibacterial activity.

No.	Retention time (RT)	Compounds	Quality	Peak aera (%)	Mechanism of antibacterial activity
1	6.89	Benzeneacetaldehyde	93	0.13	Damage cell membrane [44]
2	8.41	Butanedioic acid	80	0.14	Damage cell membrane [45]
3	8.90	Benzothiazole	89	0.17	Inhibit DNA replication [46]
4	9.17	Benzeneacetic acid	91	2.14	Damage cell membrane and inhibit protein synthesis [41]
5	9.29	Propanoic acid	53	0.30	Damage cell membrane [47]
6	9.87	Benzenepropanoic acid	97	0.22	Damage cell membrane [48]
7	9.91	Benzamide	93	0.19	Inhibit cell division protein FtsZ [49]
8	9.96	3,5-Dihydroxytoluene	74	0.13	Inhibit fatty acid attach to the cell wall [50]
9	10.04	Propanoic acid, 2-methyl-, 2,2 dimethyl-1-(2-hydroxy-1-methylethyl)propyl ester	53	0.35	Break down the DNA structure [51]
10	10.45	Benzeneacetamide	91	0.43	Inhibit DNA replication (topoisomerase IV: ParE) [42]
11	10.69	Benzeneethanol, 4-hydroxy-	90	0.25	Inhibit Quorum Sensing regulated virulence factor production [52]
12	11.38	Phenol, 2,4-bis(1,1 dimethylethyl)-	94	0.13	Inhibit Quorum Sensing regulated virulence factor production [53]
13	11.88	Benzeneacetic acid, 4-hydroxy-	87	0.98	Damage cell membrane [43]
14	16.02	Octadecanoic acid	97	0.12	Damage cell membrane, inhibit transcription and translation [54]
15	16.85	Tricosane	87	0.14	Inhibit fatty acid synthesis [55]
16	17.95	Pentacosane	87	0.19	Damage cell membrane [56]

Conclusions

The *Streptomyces* sp. PA5.6 isolated from the soil in the deciduous dipterocarp forest at the University of Phayao in Northern, Thailand, is identified as a new species. The genome sequence of *Streptomyces* sp. PA5.6 has been submitted to GenBank under the accession number JAROKT000000000. AntiSMASH analysis predicts 22 putative secondary metabolites BGCs. The gene clusters of terpene, Type I PKS, NRPS and lassopeptide are involved in the biosynthesis of antimicrobial metabolites. Additionally, functional predictions are related to the biosynthesis of antimicrobial-associated metabolites, including albaflavenon, monensin and citrulassin D. The screening for antimicrobial activity of *Streptomyces* sp. PA5.6 by the cross-streak method showed that it can inhibit drug-sensitive, drug-resistant and clinical bacteria strains. Furthermore, crude ethyl acetate extract from *Streptomyces* PA5.6 exhibits antibacterial activities. GC-MS analysis of crude ethyl acetate extract showed 3 major compounds: Benzeneacetic acid (2.14 %), benzeneacetic acid, 4-hydroxy (0.98 %) and benzeneacetamide (0.43 %), and 13 minor compounds that exhibit antimicrobial activity. Therefore, *Streptomyces* sp. PA5.6 should be considered a promising organism for the efficient production of metabolites against drug-sensitive, drug-resistant and clinical bacteria strains.

Acknowledgements

This research was funded by the Thailand Science Research and Innovation funds and the University of Phayao, Thailand (Grant No. FF65-RIM129).

References

[1] JA Ayukekbong, M Ntemgwa and AN Atabe. The threat of antimicrobial resistance in developing countries: Causes and control strategies. *Antimicrob. Resist. Infect. Contr*. 2017; **6**, 47.

[2] K Alam, J Hao, L Zhong, G Fan, Q Ouyang, MM Islam, S Islam, H Sun, Y Zhang, R Li and A Li. Complete genome sequencing and in silico genome mining reveal the promising metabolic potential in *Streptomyces* strain CS-7. *Front. Microbiol*. 2022; **13**, 939919.

[3] HS Chaudhary, B Soni, AR Shrivastava and S Shrivastava. Diversity and versatility of *Actinomycetes* and its role in antibiotic production. *J. Appl. Pharm. Sci*. 2013; **3**, S83-S94.

[4] S Miyadoh. Research on antibiotic screening in Japan over the last decade: A producing microorganism approach. *Actinomycetologica* 1993; **7**, 100-6.

[5] C Risdian, T Mozef and J Wink. Biosynthesis of polyketides in *Streptomyces*. *Microorganisms* 2019; **7**, 124.

[6] V Miao, MFCL Gal, K Nguyen, P Brian, J Penn, A Whiting, J Steele, D Kau, S Martin, R Ford, T Gibson, M Bouchard, SK Wrigley and RH Baltz. Genetic engineering in *Streptomyces roseosporus* to produce hybrid lipopeptide antibiotics. *Chem. Biol*. 2006; **13**, 269-76.

[7] C Dürr, HJ Schnell, A Luzhetskyy, R Murillo, M Weber, K Welzel, A Vente and A Bechthold. Biosynthesis of the terpene phenalinolactone in *Streptomyces* sp. Tü6071: Analysis of the gene cluster and generation of derivatives. *Chem. Biol*. 2006; **13**, 365-77.

[8] M Shao, J Ma, Q Li and J Ju. Identification of the anti-infective aborycin biosynthetic gene cluster from deep-sea-derived *Streptomyces* sp. SCSIO ZS0098 enables production in a heterologous host. *Mar. Drugs* 2019; **17**, 127.

[9] M Chanama, C Suriyachadkun and S Chanama. *Streptomyces antimicrobicus* sp. nov., a novel clay soil-derived actinobacterium producing antimicrobials against drug-resistant bacteria. *PLoS One* 2023; **18**, e0286365.

[10] PK Maiti, S Das, P Sahoo and S Mandal. *Streptomyces* sp SM01 isolated from Indian soil produces a novel antibiotic picolinamycin effective against multi drug resistant bacterial strains. *Sci. Rep*. 2020; **10**, 10092.

[11] S Rammali, L Hilali, K Dari, B Bencharki, A Rahim, M Timinouni, F Gaboune, ME Aalaoui and A Khattabi. Antimicrobial and antioxidant activities of *Streptomyces* species from soils of three different cold sites in the Fez-Meknes region Morocco. *Sci. Rep*. 2022; **12**, 17233.

[12] K Lipun, T Chantavorakit, R Mingma and K Duangmal. *Streptomyces acidicola* sp. nov., isolated from a peat swamp forest in Thailand. *J. Antibiot.* 2020; **73**, 435-40.

[13] L Panadda, E Marit and W Panya. Diversity and uses of tree species in the deciduous diptherocarp forest, Mae Chaem district, Chaing Mai province, Northern Thailand. *Naresuan Univ. J. Sci. Tech*. 2017; **25**, 43-55.

[14] J Bobek, K Šmídová and M Čihák. A waking review: Old and novel insights into the spore germination in *Streptomyces*. *Front. Microbiol*. 2017; **8**, 2205.

[15] ST Williams, M Shameemullah, ET Watson and CI Mayfield. Studies on the ecology of *Actinomycetes* in soil-VI. The influence of moisture tension on growth and survival. *Soil Biol. Biochem*. 1972; **4**, 215-25.

[16] P Sivalingam, K Hong, J Pote and K Prabakar. Extreme environment *Streptomyces*: Potential sources for new antibacterial and anticancer drug leads? *Int. J. Microbiol*. 2019; **2019**, 5283948.

[17] L Pine and SJ Watson. Evaluation of an isolation and maintenance medium for *Actinomyces* species and related organisms. *J. Lab. Clin. Med*. 1959; **54**, 107-14.

[18] NS Webster, KJ Wilson, LL Blackall and RT Hill. Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*. *Appl. Environ. Microbiol*. 2001; **67**, 434-44.

[19] A Uzel, EEH Kocabaş and E Bedir. Prevalence of *Thermoactinomyces thalpophilus* and *T. sacchari* strains with biotechnological potential at hot springs and soils from West Anatolia in Turkey. *Turk. J. Biol*. 2011; **35**, 195-202.

[20] L Prescott, J Harley and D Klein. *Procaryotic cell structure and function*. 5th eds. McGraw-Hill, New York, 1999, p. 41-73.

[21] EB Shirling and D Gottlieb. Methods for characterization of *Streptomyces* species. *Int. J. Syst. Evol. Bacteriol*. 1966; **16**, 313-40.

[22] ST Williams, M Goodfellow, EM Wellington, JC Vickers, G Alderson, PH Sneath, MJ Sackin and AM Mortimer. A probability matrix for identification of some *Streptomycetes*. *J. Gen. Microbiol*. 1983; **129**, 1815-30.

[23] NA Al-Dhabi, GA Esmail, V Duraipandiyan, MV Arasu and MM Salem-Bekhit. Isolation, identification and screening of antimicrobial thermophilic *Streptomyces* sp. Al-Dhabi-1 isolated from Tharban hot spring, Saudi Arabia. *Extremophiles* 2016; **20**, 79-90.

[24] K Wilson. Preparation of genomic DNA from bacteria. *Curr. Protocol. Mol. Biol*. 2001; **56**, 2.4.1-2.4.5.

[25] F Krueger and G Trim. A wrapper tool around Cutadapt and FastQC to consistently apply quality and adapter trimming to FastQ files, Available at: https://www.bioinformatics.babraham.ac.uk/projects/trim_galore, accessed December 2023.

[26] RR Wick, LM Judd, CL Gorrie and KE Holt. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput. Biol*. 2017; **13**, e1005595.

[27] AR Wattam, JJ Davis, R Assaf, S Boisvert, T Brettin, C Bun, Neal Conrad, EM Dietrich, T Disz, JL Gabbard, S Gerdes, CS Henry, RW Kenyon, D Machi, C Mao, EK Nordberg, GJ Olsen, DE Murphy-Olson, R Olson, … and RL Stevens. Improvements to PATRIC, the all-bacterial bioinformatics database and analysis resource center. *Nucleic Acids Res*. 2017; **45**, D535-D542.

[28] M Johnson, I Zaretskaya, Y Raytselis, Y Merezhuk, S McGinnis and TL Madden. NCBI BLAST: A better web interface. *Nucleic Acids Res*. 2008; **36**, W5-W9.

[29] S Kumar, G Stecher and K Tamura. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 2016; **33**, 1870-4.

[30] N Saitou and M Nei. The neighbor-joining method: A new method for reconstructing phylogentic trees. *Mol. Biol. Evol*. 1987; **4**, 406-25.

[31] J Felsenstein. Evolutionary trees from DNA sequences: A maximum likelihood approach. *J. Mol. Evol*. 1981; **17**, 368-76.

[32] JP Meier-Kolthoff and M Göker. TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. *Nat. Commun*. 2019; **10**, 2182.

[33] RK Aziz, D Bartels, AA Best, M Dejongh, T Disz, RA Edwards, K Formsma, S Gerdes, EM Glass, M Kubal, F Meyer, GJ Olsen, R Olson, AL Osterman, RA Overbeek, LK McNeil, D Paarmann, T Paczian, B Parrello, … and O Zagnitko. The RAST Server: Rapid annotations using subsystems technology. *BMC Genomics* 2008; **9**, 75.

[34] K Blin, S Shaw, AM Kloosterman, Z Charlop-Powers, GPV Wezel, MH Medema and T Weber. AntiSMASH 6.0: Improving cluster detection and comparison capabilities. *Nucleic Acids Res*. 2021; **49**, W29-W35.

[35] J Chun, A Oren, A Ventosa, H Christensen, DR Arahal, MSD Costa, AP Rooney, H Yi, XW Xu, SD Meyer and ME Trujillo. Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. *Int. J. Syst. Evol. Microbiol*. 2018; **68**, 461-6.

[36] M Wang, J Li, W Cong and J Zhang. Antimicrobial mechanism and secondary metabolite profiles of biocontrol agent *Streptomyces lydicus* M01 based on ultra-high-performance liquid chromatography connected to a quadrupole time-of-flight mass spectrometer analysis and genome sequencing. *Front. Microbiol*. 2022; **13**, 908879.

[37] JB Russell. A proposed mechanism of monensin action in inhibiting ruminal bacterial growth: Effects on ion flux and protonmotive force. *J. Anim. Sci.* 1987; **64**, 1519-25.

[38] H Gürtler, R Pedersen, U Anthoni, C Christophersen, PH Nielsen, EM Wellington, C Pedersen and K Bock. Albaflavenone, a sesquiterpene ketone with a zizaene skeleton produced by a *Streptomycete* with a new rope morphology. *J. Antibiot.* 1994; **47**, 434-9.

[39] JI Tietz, CJ Schwalen, PS Patel, T Maxson, PM Blair, HC Tai, UI Zakai and DA Mitchell. A new genome-mining tool redefines the lassopeptide biosynthetic landscape. *Nat. Chem. Biol*. 2017; **13**, 470-8.

[40] J Tangjitjaroenkun, W Pluempanupat, R Tangchitcharoenkhul, W Yahayo and R Supabphol. Antibacterial, antioxidant, cytotoxic effects and GC-MS analysis of mangrove-derived *Streptomyces achromogenes* TCH4 extract. *Arch. Biol. Sci*. 2021; **73**, 223-35.

[41] H Pan, Y Xiao, A Xie, Z Li, H Ding, X Yuan, X Yuan, R Sun and Q Peng. The antibacterial mechanism of phenylacetic acid isolated from *Bacillus megaterium* L2 against *Agrobacterium tumefaciens*. *PeerJ* 2022; **10**, e14304.

[42] V Yele, BKR Sanapalli, AD Wadhwani and AA Mohammed. Benzohydrazide and phenylacetamide scaffolds: New putative ParE inhibitors. *Front. Bioeng. Biotechnol*. 2021; **9**, 669728.

[43] MK Zinn and D Bockmühl. Did granny know best? Evaluating the antibacterial, antifungal and antiviral efficacy of acetic acid for home care procedures. *BMC Microbiol*. 2020; **20**, 265.

[44] XN Yang, I Khan and SC Kang. Chemical composition, mechanism of antibacterial action and antioxidant activity of leaf essential oil of *Forsythia koreana* deciduous shrub. *Asian Pac. J. Trop. Med.* 2015; **8**, 694-700.

[45] S Huang, X Chen, R Yan, M Huang and D Chen. Isolation, identification and antibacterial mechanism of the main antibacterial component from pickled and dried mustard (*Brassica juncea* Coss. var. *foliosa Bailey*). *Molecules* 2022; **27**, 2418.

[46] P Kashyap, S Verma, P Gupta, R Narang, S Lal and M Devgun. Recent insights into antibacterial potential of benzothiazole derivatives. *Med. Chem. Res*. 2023; **32**, 1-31.

[47] N Liang, V Neužil-Bunešová, V Tejnecký, M Gänzle and C Schwab. 3-Hydroxypropionic acid contributes to the antibacterial activity of glycerol metabolism by the food microbe *Limosilactobacillus reuteri*. *Food Microbiol*. 2021; **98**, 103720.

[48] M Sousa, AC Afonso, LS Teixeira, A Borges, MJ Saavedra, LC Simões and M Simões. Hydrocinnamic acid and perillyl alcohol potentiate the action of antibiotics against *Escherichia coli*. *Antibiotics* 2023; **12**, 360.

[49] V Straniero, L Suigo, A Casiraghi, V Sebastián-Pérez, M Hrast, C Zanotto, I Zdovc, CDG Morghen, A Radaelli and E Valoti. Benzamide derivatives targeting the cell division protein FtsZ: Modifications of the linker and the benzodioxane scaffold and their effects on antimicrobial activity. *Antibiotics* 2020; **9**, 160.

[50] JG Zorrilla, T D'addabbo, E Roscetto, C Varriale, MR Catania, MC Zonno, C Altomare, G Surico, PL Nimis and A Evidente. Antibiotic and nematocidal metabolites from two lichen species collected on the Island of Lampedusa (Sicily). *Int. J. Mol. Sci*. 2022; **23**, 8471.

[51] MN Haque, R Chowdhury, KM Islam and MA Akbar. Propionic acid is an alternative to antibiotics in poultry diet. *Bangladesh J. Anim. Sci*. 2009; **38**, 115-22.

[52] A Chang, S Sun, L Li, X Dai, H Li, Q He and H Zhu. Tyrosol from marine fungi, a novel quorum sensing inhibitor against *Chromobacterium violaceum* and *Pseudomonas aeruginosa*. *Bioorg. Chem*. 2019; **91**, 103140.

[53] AR Padmavathi, B Abinaya and SK Pandian. Phenol, 2,4-bis(1,1-dimethylethyl) of marine bacterial origin inhibits quorum sensing mediated biofilm formation in the uropathogen *Serratia marcescens*. *Biofouling* 2014; **30**, 1111-22.

[54] SC Karthikeyan, S Velmurugan, MBS Donio, M Michaelbabu and T Citarasu. Studies on the antimicrobial potential and structural characterization of fatty acids extracted from Sydney rock oyster *Saccostrea glomerata*. *Ann. Clin. Microbiol. Antimicrob*. 2014; **13**, 332.

[55] SH Kim, R Khan, K Choi, SW Lee and S Rhee. A triclosan-resistance protein from the soil metagenome is a novel enoyl-acyl carrier protein reductase: Structure-guided functional analysis. *FEBS J*. 2020; **287**, 4710-28.

[56] WC Lin, KC Hsu, MF You, KH Lee, CH Chi and JY Chen. Octanoic acid promotes clearance of antibiotic-tolerant cells and eradicates biofilms of *Staphylococcus aureus* isolated from recurrent bovine mastitis. *Biofilm* 2023; **6**, 100149.