ORIGINAL ARTICLE

Potential antimicrobial properties of *streptomyces* isolated from Sammuk Mountain soil, Chonburi Province, Thailand

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J. Adv. Pharm. Technol. Res.

ABSTRACT

An infection of pathogenic microorganisms can create a big problem for human health. This has triggered the need for discovery and development of antibiotic drugs with altered modes of action. Approximately 45% of antibiotic drugs are derived from *Streptomyces*, which are the most commonly isolated actinomycete genera. The present investigation aimed to search for and study antimicrobial-producing actinomycetes isolated from soil samples from Sammuk Mountain, Chonburi province, Thailand. A total of 50 isolates from six soil samples were obtained and their actinomycetes were better isolated using humic acid-vitamin agar medium (64.0%) than starch casein nitrate agar medium (36.0%). In addition, the secondary metabolites produced from 13 isolates (26.0%) exhibited a broad spectrum of antimicrobial activity against Gram-positive bacteria and yeast. Thus, Sammuk Mountain soil is an important source of antibiotic-producing actinomycetes.

Key words: Actinomycetes, antibiotic, antimicrobial activity, multidrug resistant

INTRODUCTION

Diseases caused by infections of pathogenic microorganisms such as diarrhea, gastrointestinal and urogenital diseases, and wound contamination are increasing and becoming a problem for human health because of the emergence of multidrug-resistant pathogens.[1] It has been estimated that the multidrug-resistant Gram-positive and Gram-negative bacteria such as *Enterobacteriaceae* (carbapenem) and *Enterococcus* (vancomycin) together with *Salmonella enterica, Staphylococcus aureus* (methicillin), *Pseudomonas aeruginosa, Clostridium difficile*, and *Escherichia coli* could cause up to 10 million deaths/annum by 2050.[2] This has triggered the need for the discovery and development of antibiotic drugs with altered modes of action. Natural products, including plants, microorganisms, and their compounds, have been utilized to treat and cure several diseases such as diarrhea, cancer, diabetes, Alzheimer's, anti-inflammatory, analgesic and antipyretic solutions, and as alternatives for hormone replacement therapy.[3,4] In particular, microorganisms have an ability to produce a wide variety of bioactive metabolites, especially antibiotic agents. It has been recently reported that over 10,000 bioactive natural antibiotics such as penicillin, tetracycline, gentamicin, vancomycin, and pimaricin obtained from microbes were produced by actinomycetes.[5-7] These are an extensive and diverse group of Gram-positive aerobic bacteria usually grow by filament formation, and they are one of the major microbial populations present

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Access this article online

Quick Response Code:

Website: www.japtr.org

DOI: 10.4103/japtr.JAPTR_55_19

How to cite this article: Sripreechasak P, Athipornchai A. Potential antimicrobial properties of *streptomyces* isolated from Sammuk Mountain soil, Chonburi Province, Thailand. J Adv Pharm Technol Res 2019;10:195-9.

in soils.[8] Moreover, approximately 45% of these agents derived from *Streptomyces* which are the most commonly isolated actinomycete genera. Therefore, actinomycetes play an important natural resources role for producing new antimicrobial agents. In 2014, Sripreechasak *et al*. reported the isolation and identification of *Streptomyces* strains which were isolated from 13 soil samples collected around the Angthong Islands National Park, Thailand. All isolated *Streptomyces* were screened and evaluated for antimicrobial activity. All of these strains showed good inhibitory activity against *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC 9341, *Mucor racemosus* IFO 4581, *Candida albicans* KF1, *E. coli* NIHJ KB213, and *Xanthomonas campestris* pv. oryzae KB88.[9] They also reported the isolation of two new naphthoquinones and 17 known metabolites from *Streptomyces* sp. BCC71188 isolated from soil. Some of the isolated compounds showed strong antimicrobial activities such as antimalarial, anti-tuberculosis and antibacterial activities, and cytotoxicity against MCF-7, KB, and NCI-H187.[10] In an attempt to address the serious public health problem of the infectious disease, the present investigation aimed to search for and study antimicrobial-producing actinomycetes from soil samples from Sammuk Mountain, Chonburi province, Thailand.

MATERIALS AND METHODS

Sample processing and isolation of actinomycetes

Six soil samples were collected around Sammuk Mountain (SM1-6), Chonburi province, Thailand. The samples were dried by heating at 100°C for 1 h, suspended in basic lauryl-sulfate buffer solution and heated at 60°C for 5 min. Serial 10-fold dilutions of the suspension were prepared and 0.1 mL of 10^{-2}, 10^{-3} and 10^{-4} dilutions were spread on the surface of starch casein nitrate agar (SCN agar: starch 0.1%, sodium caseinate 0.03%, KNO_3 0.2%, and agar 1.5%, pH 7.0) and humic acid-vitamin agar (HV agar: humic acid 0.1%, Na_2HPO_4 0.05%, KCl 0.17 g, $MgSO_4.7H_2O$ 0.005%, $FeSO_4.7H_2O$ 0.001 g, $CaCl_2$ 0.1%, B-Vitamins including 0.05% each of thiamine-hydrochloride, riboflavin, niacin, pyridoxine, capantothenate, inositol, *p*-aminobenzoic acid, and 0.025% of biotin and agar 1.8% g, pH 7.4). Each agar plate was supplemented with antibiotics, 50 mg/L of cycloheximide and 20 mg/L of nalidixic acid. The different colonies were picked up after incubation at room temperature for 14 days, and these plates were streaked for further purification on yeast extract-malt extract agar (International *Streptomyces* Project (ISP) medium no. 2).[11] The pure isolates were observed for their cultural characteristics after cultivation on ISP 2 agar (yeast extract 4.0%, malt extract 10.0%, dextrose 4.0%, and agar 20.0%, pH 7.3) at 28°C for 2 weeks. All pure isolates were kept in 20% (v/v) glycerol solution at −20°C until further use.

Screening of antimicrobial activity of pure isolates

The pure isolates were screened for antimicrobial activity against six microorganisms, including *B. subtilis* ATCC 6633, *Micrococcus luteus* ATCC 9341, *S. aureus* ATCC 25925, *P. aeruginosa* ATCC 27853, *C. albicans* ATCC 10231, and *E. coli* ATCC 25922 using the perpendicular streak method on ISP 2 agar medium.[12,13] The results were conducted in triplicate and averaged. All pathogenic microorganisms were kindly supplied by Prof. Dr. Somboon Tanasupawat, Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Science, Chulalongkorn University.

Morphological characterization and identification of active isolates

All pure isolates were inoculated on ISP 2 media and incubated for 14 days at room temperature. Colony morphology was observed under a microscope and color, aerial and substrate mycelium were noted.[14,15] The 16S rRNA gene was amplified using the primers described by Takahashi *et al*.[16] The polymerase chain reaction products were checked by agarose gel electrophoresis and directly sequence using a BigDye® Terminator V3.1 cycle sequencing kit (Applied Biosystems), according to the manufacturer's instructions.

Antimicrobial metabolites production of pure isolates

Each isolate was cultured in ISP 2 medium and shaken (150 rpm) at room temperature for 3 days. Two percent of the ISP 2 culture was transferred into 200 mL of new ISP 2 medium and also cultivated on a shaker for 9 days. The cultured broth was then evaluated for antimicrobial activity using an agar well diffusion assay.[17,18] All analyses in this study were performed in three replicates.

RESULTS AND DISCUSSION

Isolation and characterization of the isolates

As shown in the supporting information [Table S1], a total of 50 isolates obtained from six soil samples collected from Sammuk Mountain were studied. Actinomycetes cultures were isolated more using HV agar medium (64.0%) than SCN agar medium (36.0%). Thus, HV agar medium was found to be suitable for isolating actinomycetes from these soil samples. Table 1 summarizes the morphological patterns of the active isolates actinomycetes. All of the isolates growth was found to be in good condition and showed the color appearance of individual isolates. The 16S rDNA sequence was generated for most potent isolates. Comparison of this nucleotide sequence with members of actinomycetes clearly showed that these strains belong to the genus *Streptomyces*.

Antimicrobial activity of the isolates

On screening all of the isolates for antimicrobial activity, using the perpendicular streak method on ISP 2 agar medium, 13 isolates (26.0%) showed strong inhibitory activity against human pathogens [Table 2]. The isolate SM2-HV8 showed good inhibition against *C. albicans* with

Table 1: Morphological analysis of active actinomycetes isolates

Numbers	Isolates	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
1	SM1-HV5	Good	Light gray	Light olive brown - moderate olive brown	Dark yellow
2	SM1-SCN8	Good	Light purplish gray - light gray	Deep brown - deep yellowish brown	Strong yellowish brown
3	SM2-HV1	Good	Medium gray	Grayish yellow - dark grayish yellow	None
4	SM2-HV4	Good	Light brownish gray	Moderate olive	Grayish greenish yellow
5	SM2-HV8	Good	Greenish white - medium gray	Light yellow	Grayish greenish yellow
6	SM2-SCN5	Good	Moderate olive brown	Moderate olive brown	Light greenish yellow
7	SM3-HV2	Good	Greenish white	Deep yellowish brown	Dark yellow
8	SM3-SCN7	Good	Medium gray	Moderate olive	Grayish greenish yellow
9	SM4-HV1	Good	Yellowish gray	Light orange yellow - strong yellowish brown	Moderate yellow
10	SM4-HV2	Good	Bluish gray	Moderate olive brown	Grayish greenish yellow
11	SM4-HV5	Good	Strong yellow	Brilliant yellow - strong yellow	Brilliant greenish yellow
12	SM4-HV7	Good	Light gray - olive gray	Grayish olive - dark olive	Grayish olive
13	SM4-HV8	Good	Yellowish white	Strong yellowish brown	Dark orange yellow
14	SM4-SCN9	Good	Medium gray	Light grayish olive - moderate olive	None
15	SM6-HV1	Good	Pale blue - blackish purple	Blackish purple	Very dark reddish purple
16	SM6-SCN4	Good	Light bluish gray - greenish gray	Dark grayish yellow - moderate olive brown	None
17	SM6-SCN5	Good	Pale blue - blackish purple	Blackish purple	Very dark reddish purple

SCN: Starch casein nitrate, HV: Humic vitamin

Table 2: Antimicrobial activity of the isolates using the perpendicular streak method

Numbers	Isolates	Zone of inhibition (mm)					
		Gram-positive bacteria			Gram-negative bacteria		Yeast
		Bacillus subtilis	*Micrococcus luteus*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Candida albicans*
1	SM2-HV1	-	16.7±0.58	-	-	-	-
2	SM2-HV4	45.0±1.00	12±1.00	-	-	-	-
3	SM2-HV8	-	-	-	-	-	15.0±0.00
4	SM2-SCN5	33.0±1.00	25.0±1.00	-	-	-	-
5	SM3-SCN7	38.3±4.04	41.3±3.06	27.3±2.08	-	-	-
6	SM4-HV1	22.3±2.51	22.3±1.53	18.3±3.51	-	-	-
7	SM4-HV2	12.7±0.58	16.0±1.00	10.0±1.00	-	-	-
8	SM4-HV5	37.3±6.66	37.3±9.45	43.0±12.12	-	-	-
9	SM4-HV7	49.0±3.60	-	42.3±2.52	-	-	-
10	SM4-HV8	29.0±1.00	37.3±2.51	38.3±4.72	-	-	-
11	SM4-SCN9	21.0±1.00	28.0±1.00	-	-	-	-
12	SM6-SCN4	45.0±1.00	40.0±1.00	45.0±1.00	-	-	-
13	SM6-SCN5	5.0±1.00	11.7±1.53	-	8.0±1.00	-	-

SCN: Starch casein nitrate, HV: Humic vitamin

the inhibition zone being 15.0 ± 0.00 mm, whereas SM6-SCN5 showed inhibitory activity against Gram-negative bacteria, *P. aeruginosa* (8.0 ± 1.00 mm).

The isolates were then fermented in ISP 2 medium to produce the secondary metabolites. The cultured broths were evaluated for their antimicrobial activity using an agar well diffusion assay and the results are shown in Table 3. Thirteen isolates (26.0%) out of 50 actinomycetes exhibited a broad spectrum of antimicrobial activity against Gram-positive bacteria. The isolates SM1-HV5 and SM1-SCN8 specifically inhibited against *C. albicans* with the inhibition zone being 8.7 ± 0.58 and 10.0 ± 1.00 mm, respectively.

In addition, all isolates could not inhibit Gram-negative bacteria, *P. aeruginosa* and *E. coli*. As shown in Figure 1, all isolates showed moderate-to-high inhibitory activity to *M. luteus* (79.92%), *B. subtilis* (69.23%) and *S. aureus* (46.15%), whereas their isolates showed moderate activity against *C. albicans* (15.38%). The isolates obtained from HV agar medium were more active than from SCN agar medium, as shown in Figure 2. Thus, actinomycetes isolated from HV agar medium could be suitable for producing bioactive metabolites against Gram-positive bacteria. This result was confirmed by Hayakawa and Nonomura[19] who found that HV agar medium supported adequate growth and good sporulation for these actinomycetes

Table 3: Antimicrobial activity of secondary metabolites produced from actinomycetes

Numbers	Isolates	Zone of inhibition (mm)					
		Gram-positive bacteria			Gram-negative bacteria		Yeast
		Bacillus subtilis	*Micrococcus luteus*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Candida albicans*
1	SM1-HV5	16.3±0.58	20.3±0.58	15.3±0.58	-	-	8.7±0.58
2	SM1-SCN8	16.7±0.58	20.0±0.00	15.8±0.29	-	-	10.0±1.00
3	SM2-HV1	-	12.0±0.00	-	-	-	-
4	SM2-HV4	9.2±0.29	-	-	-	-	-
5	SM2-SCN5	9.3±0.58	-	14.3±1.15	-	-	-
6	SM3-HV2	-	17.0±0.00	18.0±0.00	-	-	-
7	SM3-SCN7	-	9.0±0.00	-	-	-	-
8	SM4-HV2	13.0±0.00	14.7±0.58	13.0±0.00	-	-	-
9	SM4-HV5	12.7±0.58	12.2±0.29	-	-	-	-
10	SM4-HV8	-	11.2±0.29	-	-	-	-
11	SM4-SCN9	8.8±0.29	12.0±0.00	-	-	-	-
12	SM6-HV1	8.8±0.29	-	-	-	-	-
13	SM6-SCN4	9.0±0.00	9.0±0.00	11.5±0.50	-	-	-

SCN: Starch casein nitrate, HV: Humic vitamin

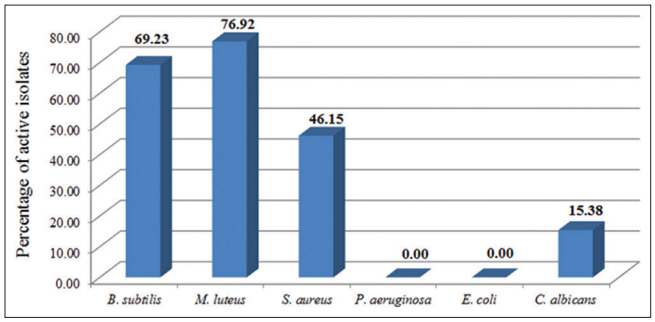

Figure 1: Antimicrobial activity of secondary metabolites produced from actinomycetes

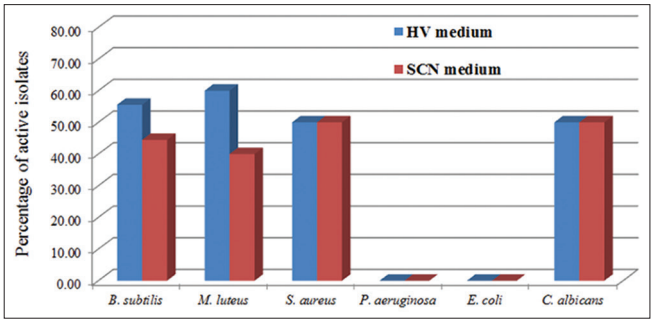

Figure 2: Antimicrobial activity of actinomycetes isolated by using humic vitamin and starch casein nitrate medium

because it contained soil humic acid as the sole source of carbon and nitrogen.

CONCLUSIONS

Of the 50 isolates obtained from six soil samples, 64.0% of their actinomycetes were isolated using HV agar medium compared to 36.0% using SCN agar medium. The HV agar medium contained soil humic acid as the sole source of carbon and nitrogen. In addition, the secondary metabolites produced from 13 isolates (26.0%) exhibited a broad spectrum of antimicrobial activity against Gram-positive bacteria and yeast. Thus, Sammuk Mountain soil from Chonburi province, Thailand, is an important source for exploration of antibiotic-producing actinomycetes.

Acknowledgments

The authors extend their appreciation to the Faculty of Science, Burapha University, for funding the work through the research group project No.: 20/2560. We thank the Center of Excellence for Innovation in Chemistry (PERCH-CIC) and Department of Chemistry and Department of Biotechnology, Faculty of Science, Burapha University for providing research facilities. Special thanks to Professor Dr. Ronald Beckett for comments and grammatical suggestions on the manuscript.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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Table S1: Morphological analysis of all actinomycetes isolates

Numbers	Isolates	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
1	SM1-HV1	Good	Pale yellowish pink	Vivid orange yellow	None
2	SM1-HV2	Good	Light orange	Strong orange yellow	None
3	SM1-HV3	Good	Yellowish white	Moderate yellow	None
4	SM1-HV4	Good	Olive black	Dark grayish olive	Grayish greenish yellow
5	SM1-HV5	Good	Light gray	Light olive brown - moderate olive brown	Dark yellow
6	SM1-HV6	Good	Blackish purple	Very dark reddish purple	Grayish reddish brown
7	SM1-HV7	Good	Pale yellow	Light yellow	None
8	SM1-SCN8	Good	Light purplish gray - light gray	Deep brown - deep yellowish brown	Strong yellowish brown
9	SM1-SCN9	Good	Grayish yellow - dark grayish yellow	Grayish yellow - dark grayish yellow	None
10	SM1-SCN10	Good	Medium gray	Moderate brown - light grayish olive	None
11	SM1-SCN11	Good	Light gray - medium gray	Dark yellow	None
12	SM2-HV1	Good	Medium gray	Grayish yellow - dark grayish yellow	None
13	SM2-HV2	Good	White - bluish gray	Strong grayish yellow - deep grayish yellow	None
14	SM2-HV4	Good	Light brownish gray	Moderate olive	Grayish greenish yellow
15	SM2-HV8	Good	Greenish white - medium gray	Light yellow	Grayish greenish yellow
16	SM2-SCN5	Good	Moderate olive brown	Moderate olive brown	Light greenish yellow
17	SM2-SCN6	Good	grayish yellow - medium gray	Moderate greenish yellow	None
18	SM3-HV1	Good	Greenish white - medium gray	Grayish yellow - light grayish olive	None
19	SM3-HV2	Good	Greenish white	Deep yellowish brown	Dark yellow
20	SM3-HV3	Good	Moderate olive brown	Light olive brown	Light olive brown
21	SM3-HV4	Good	Greenish white - medium gray	Pale yellowish green - light grayish olive	Pale yellowish green
22	SM3-SCN5	Good	Medium gray	Dark grayish yellow	Light yellowish brown
23	SM3-SCN6	Good	Medium gray	Light grayish olive and brownish orange	Grayish greenish yellow
24	SM3-SCN7	Good	Medium gray	Moderate olive	Grayish greenish yellow
25	SM3-SCN8	Good	Light brownish gray	Dark brown	Deep yellowish brown
26	SM3-SCN10	Good	Grayish greenish yellow	Dark grayish yellow	None
27	SM3-SCN11	Good	Grayish yellow	Dark yellow	Grayish greenish yellow
28	SM4-HV1	Good	Yellowish gray	Light orange yellow - strong yellowish brown	Moderate yellow
29	SM4-HV2	Good	Bluish gray	Moderate olive brown	Grayish greenish yellow
30	SM4-HV4	Good	White - medium gray	Pale yellowish green - moderate olive	Grayish greenish yellow
31	SM4-HV5	Good	Strong yellow	Brilliant yellow - strong yellow	Brilliant greenish yellow
32	SM4-HV6	Good	Yellowish white - pale orange yellow	Moderate yellow	None
33	SM4-HV7	Good	Light gray - olive gray	Grayish olive - dark olive	Grayish olive
34	SM4-HV8	Good	Yellowish white	Strong yellowish brown	Dark orange yellow
35	SM4-SCN9	Good	Medium gray	Light grayish olive - moderate olive	None
36	SM4-SCN10	Good	Light orange	Moderate orange yellow	None
37	SM4-SCN11	Good	Pinkish white	Moderate yellow	None
38	SM4-SCN12	Good	Greenish white	Deep yellow	None
39	SM5-HV1	Good	White - greenish white	Dark yellow	None
40	SM5-HV2	Good	Medium gray	Light grayish orange	None
41	SM5-HV3	Good	Light orange yellow	Moderate yellow	None
42	SM5-HV4	Good	Medium gray	Dark grayish yellow	Grayish greenish yellow
43	SM5-HV5	Good	Vivid orange	Strong orange yellow	None
44	SM5-HV7	Good	Bluish gray	Grayish olive green	Light grayish olive
45	SM5-HV8	Good	Bluish gray	Grayish olive green	Light grayish olive
46	SM6-HV1	Good	Pale blue - blackish purple	Blackish purple	Very dark reddish purple

Contd...

Table S1: Contd...

Numbers	Isolates	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
47	SM6-HV2	Good	White - greenish white	Pale yellowish green	None
48	SM6-HV3		Light olive gray - olive gray	Moderate olive brown	Moderate olive brown
49	SM6-SCN4		Light bluish gray - greenish gray	Dark grayish yellow - moderate olive brown	None
50	SM6-SCN5		Pale blue - blackish purple	Blackish purple	Very dark reddish purple

SCN: Starch casein nitrate, HV: Humic vitamin