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ISOLATION, PHYLOGENETIC CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF *STREPTOMYCES* STRAINS ISOLATED FROM AIR AT WALAILAK UNIVERSITY

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ABSTRACT

Emergence of drug resistance among *Staphylococcus aureus* to currently used antibiotics has made the search for novel bioactive compounds from natural and unexplored habitats a necessity. In this study, isolation, characterization and antimicrobial activity of an actinomycete strain isolated from air at Scientific and Technological Equipments Building, Walailak University, located in Southern Thailand. Eight strains were done and identified as *Streptomyces* spp. using 16S rDNA sequence analysis. *Streptomyces lydicus* A2 exhibited antibacterial activity against Gram positive bacteria, Gram negative bacteria. Partial characterization of the active substance (resistance to heat) showed that it would be of non-protein nature. The production of the active substance showed that the production occurs between the 4^{th} and 7^{th} day of fermentation. This will open the way to further investigations to demonstrate their potential importance in combating pathogenic bacteria, methicillin resistant *S. aureus*.

KEYWORDS: **Phylogenetic tree, 16s rRNA, anti-MRSA, *Streptomyces* sp.**

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INTRODUCTION

The actinomycetes especially the strains of *Streptomyces* are the most common antibiotic producing microorganisms. They are represented in nature by the largest number of species and varieties, widely recognized as sources of antibiotics and other important novel metabolites, including antifungal agents[1], antitumor agents[2], antihelminthic agents[3] and herbicides[4]. Although heavily studied over the past three decades, actinomycetes continue to prove themselves as reliable sources of novel bioactive compounds and richest source of secondary metabolites. Among the well-characterized pharmaceutically relevant microorganisms, actinomycetes remain major sources of novel, therapeutically relevant natural products[5]. Bacteria belonging to the genus *Streptomyces* could produce approximately 6,550 antibiotics[6]. *Streptomyces* are prokaryotic gram-positive, aerobic bacteria which produce an extensive branch mycelia that rarely fragment[7]. Several antibiotics such as actinomycin, amphotericin, chloramphenicol, geldanamycin, nystatin, streptomycin, and streptothricin are produced by *Streptomyces*[8,9]. At least 70 of the approximately 100 marketed antibiotics used for the treatment of infections in humans are derived from substances produced by *Streptomyces* sp.[10]. Since fewer antibiotics are being discovered now than in the 1950s and1960s, nevertheless these microorganisms remain a rich source of novel bioactive compounds; one study estimates that the number of antibiotics produced by the genus *Streptomyces* is of the order of 100,000, thus many more antibiotics remain to be identified[11]. Discovery of new antibiotics produced by actinomycetes, *Streptomyces*, still continues as an essential component in natural product-based drug discovery. Exploring new habitats is one of the most promising ways to isolate new strains of actinomycetes endowed with antimicrobial activity[12-14]. This has resulted in isolation of a promising new strain of *Streptomyces* species from an air sample collected at the Scientific and Technological Equipments Building, Walailak University. Herein, report the isolation and molecular identification of a *Streptomyces* strains endowed with anti-*Staphylococcus* activity. Antimicrobial and cytotoxic activities of the bioactive compounds in culture broth against some pathogenic bacteria are also described

MATERIALS AND METHODS

1. Isolation of Streptomyces strains

The organisms were isolated from an air sample collected from Walailak University, Thailand, using Biosampler apparatus, Microflow 90, with the flow rate 100 l/min for 30 min according to the manufacture's instruction. The phenotypic characteristics were determined by the methods as previously described[15,16]. The colonies of actinomycetes were recognized according to their macroscopic and microscopic characteristics (optical microscopy and Gram stain), embedded colony and Gram positive branching bacteria were selected. Eight isolates showing different morphological characteristics were obtained, named as A2, AB, AG, AH, AL10, AL15, AL18, and S2. Primary screening of antimicrobial activities was performed on Yeast extract-Malt extract agar (YMA) plates[17] (Himedia) or half-formula Luria-Bertani agar (LB/2) (2.5 g/l yeast extract, 5 g/l tryptone, 5 g/l NaCl : Oxoid) plates by cross streak method[18]. Presence of reduced growth of test bacteria near the growth of actinomycetes was considered as positive for antagonistic activity. Secondary screening of the strains was examined by the agar well diffusion method[19] against *Staphylococcus aureus* TISTR 517, methicillin-resistant *S. aureus* (142, 1096, 2499, 2559, 7645, 1424), *Escherichia coli* TISTR 887, *Pseudomonas aeruginosa* TISTR 1467 and *Salmonella typhimurium* TISTR 292. The detail was described in the topic "Antimicrobial activity". Pure culture of the strains were maintained on LB (Oxoid) agar slant and conserved at 4°C for short periods and at -20°C in glycerol stock (20%, v/v) for a longer period. The organisms were identified on the basis of 16S rDNA studies. The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strains A2, AB, AG, AH, AL10, AL15, AL18 and S2 are KF12383.1, KF712384.1, KF712385.1, KF712386.1, KF712387.1, KF712388.1, KF712389.1 and KF712390.1, respectively.

2. 16S rDNA sequencing

PCR amplification of 16S rDNA

DNA coding for 16S rRNA regions was amplified by PCR as previously described[20-22]. Briefly, DNA templates were prepared by using "Genomic DNA mini kit (Blood/culture cell)" (Geneaid Biotech Ltd., Taiwan). A PCR product for sequencing 16S rDNA regions was prepared by using the following two primers, 20F (5´-GAG TTT GAT CCT GGC TCA G-3´) and 1500R (5´-GTT ACC TTG TTA CGA CTT-3´)[23]. The PCR amplification was carried out with DNA Engine Dyad Thermal Cycler (Bio-Rad Laboratories). One hundred µl of a reaction mixture contained 15-20 ng of template DNA, 2.0 µmoles each of the two primers, 2.5 units of Taq polymerase, 2.0 mM $MgCl_2$, 0.2 mM dNTP and 10 µl of 10xTaq buffer, pH 8.8, containing $(NH_4)_2SO_4$, which was comprised of 750 mM Tris-HCl, 200 mM $(NH_4)_2SO_4$ and 0.1% Tween 20. The PCR amplification was programmed to carry out an initial denaturation step at 94 ^oC for 3 min, 25 cycles of denaturation at 94 ^oC for 1 min, annealing at 50 ^oC for 1 min and elongation at 72 ^oC for 2 min, followed by a final amplification step at 72 ^oC for 3 min. The PCR product was analyzed by 0.8% (w/v) agarose gel electrophoresis and purified with a QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany). The purified PCR product was stored at -20 ^oC for further step.

Direct sequencing of 16S rDNA

Direct sequencing of the single-banded and purified PCR products (approximately, 1500 bases on 16S rDNA by the *E. coli* numbering system was carried out. Sequencing of the purified PCR products was carried out with an ABI PRISM BigDye™ Terminator Ready Reaction Cycle Sequencing Kit (version 3.1, Applied Biosystems, Foster City, California, USA). The primers 518F (5´-CCA GCA GCC GCG GTA ATA CG-3´) and 1492R (5´-TAC GGY TAC CTT GTT ACG ACT T-3´) for full length sequencing were used for sequencing of 16S rDNA. Ten µl of a sequencing reaction mixture contained 5–20 ng of template DNAs, 2.0 µl of BigDye™ terminator ready reaction mixture, 5–20 ng of DNA template, 1.6 pmole of sequencing primer, 1.5 µl of 5×BigDyeTM sequencing buffer and deionized water. The PCR reactions were carried out as follows: an initial denaturation step at 96 ^oC for 30 sec, 25 cycles of denaturation at 96 ^oC for 10 sec, annealing at 50 ^oC for 5 sec and elongation at 60 ^oC for 4 min. Eighty µl of freshly prepared ethanol/acetate solution was added to the sequencing reaction mixture in 1.5 ml microcentrifuge tube, and mixed well with a brief vortex. The mixture was left to stand at room temperature for 15 min and centrifuged at the maximum speed or 14,500 rpm for 20 min at room temperature. The ethanol solution was immediately removed carefully from the tube with an aspirator equipped with a fine tip. The resulting DNA pellets were washed by adding 250 µl of 70% ethanol to the tube, and vortexed briefly. The precipitated DNA was collected by centrifugation for 5 min at the maximum speed. The remaining ethanol was carefully removed from the tube with an aspirator equipped with a fine tip. The DNA obtained was dried in a heat box at 90^oC for 1 min, and the dried DNA was stored at either 4 ^oC or -20 ^oC. The DNA pellets were suspended in 20 µl of a terminator sequencing reagent, mixed on a vortex and spun down. The double-stranded DNA was completely separated by heating at 95^oC for 2 min, and immediately placed on ice, until ready to load on the instrument. The DNA sequencing was performed on an ABI Prism 3730XL DNA Sequence (Applied Biosystems, Foster City, California, USA).

Sequence analyses

The nucleotide sequences obtained from all primers were assembled using Cap contig assembly program, an accessory application in BioEdit (Biological sequence alignment editor) Program (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). The identification of phylogenetic neighbors was initially carried out by the BLASTN[24] program against the database containing type strains with validly published prokaryotic names[25]. The top thirty sequences with the highest scores were then selected for the calculation of pairwise sequence similarity using global alignment algorithm[26], which was implemented at the EzTaxon-e server (http://eztaxone ezbiocloud.net)[25].

Phylogenetic analysis

The DNA sequences determined and obtained from databases were aligned with a program CLUSTAL X (version 1.8)[26] in BioEdit

Program[27]. Alignment gaps and unidentified bases were eliminated. The evolutionary distances were computed using the Maximum Composite Likelihood method[28]. Phylogenetic trees of 16S rRNA genes were constructed by the neighbor-joining method described by Saitou and Nei[29]. The robustness for individual branches was estimated by 1000 replications bootstrapping[30] with the program MEGA Version 5.2.1[31]. 16S rDNA sequence of *Actinomaedura madurae* DSM 43067^T was used as an out group. Gaps and ambiguous nucleotides were eliminated from the calculations.

3. Antimicrobial activity

By cylinder agar method (agar plug method)

Firstly, the actinomycete isolates were sown in scratches tightened in two agar media YMA, and LB/2 to show which medium stimulates maximum antimicrobial activity. After incubation for 7 days at 30 °C, agar cylinders were then taken with hollow punch[32] and placed on Mueller Hinton (MH) agar plates, previously seeded with the test microorganisms (10^5-10^6 CFU/ml). The Plates were kept at 4°C for 2 h, and then incubated at 37 °C and observed for antibiosis, inhibition zone, after 24 to 48 h. The bacteria used as targets were *Staphylococcus aureus* TISTR517 and MRSA (142, 1096, 2499, 2559, 7645, 1424).

By agar well diffusion

A loopful of each selected strain (total 8 strains) was inoculated into a 25×125-mm screw-capped test tube containing 10 ml of YM broth or LB/2 broth and incubated on a rotary shaker at 200 rpm, 30^{o}C for 7 days. The culture broth was used to test the antimicrobial activity by agar well diffusion method[19]. All tested bacteria were cultivated on MH (HiMedia) agar slants at 37° C for 24 h. Using sterile swabs, the broth cultures of test bacteria were swabbed on sterile MH agar plates followed by punching wells of 6 mm diameter using sterile cork borer. 100 µl of culture broths were transferred into labeled wells and the plates were incubated at 37 ^{o}C for 24 hours. The plates were observed for zone of inhibition formed, if any, and measured using a ruler. The experiment was carried in replicate and the average value was recorded.

5.Partial characterization of the antimicrobial products

The sensitivity to heat was examined by boiling the culture broth of each isolate to 60 for 30 h and 121° C for 15 min then the treated culture broth was tested against *S. aureus* to determine its anti *Staphylococcus* activity by the agar well diffusion method. After incubation for 24 h at 37°C, the inhibition zone was measured.

6.Cytotoxicity against primate cell line (Vero)[33]

The GFP-expressing Vero cell line was generated in-house by stably transfecting the African green monkey kidney cell line (Vero, ATCC CCL-81), with pEGFP-N1 plasmid (Clontech). The cell line is maintained in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/l sodium bicarbonate and 0.8 mg/ml geneticin, at 37°C in a humidified incubator with 5% CO_2. The assay was carried out by adding 45 µl of cell suspension at $3.3x10^4$ cells/ml to each well of 384-well plates containing 5 µl of test compounds were previously diluted with 0.5% DMSO, and then incubated for 4 days in 37^{o}C incubator with 5% CO_2. Fluorescence signals are measured by using SpectraMax M5 microplate reader (Molecular Devices, USA) in the bottom reading mode with excitation and emission wavelength of 485 and 535 nm, respectively. Fluorescence signal at day 4 is subtracted with background fluorescence at day 0. The percentage of cytotoxicity is calculated by the following equation, where FUT and FUC represent the fluorescence units of cells treated with test compound and untreated cells, respectively: **%** cytotoxicity = $[1-(FU_T / FU_C)] \times 100$, where where FU_T and FU_C are the mean fluorescent intensity from treated and untreated conditions respectively. IC_{50} values are derived from dose-response curves, using 6 concentrations of 3-fold serially diluted samples, by the SOFTMax Pro software (Molecular device). Ellipticine and 0.5% DMSO are used as a positive and a negative control, respectively.

RESULTS AND DISCUSSION

1.Isolation and selection of the actinomycete strains

Based on the results, the embedded colonies on media agar plate showed the fungal-like formation (aerial mycelium, substrate mycelium) were selected for Gram staining. 8 selected colonies are Gram positive branching formation, known as actinomecetes. Actinomycetes have evolved as a group with greatest genomic and metabolic diversity[34], indeed well adapted and are functional members of the environmental microbial community. Actinomycetes account of the earth's surface and represent an attractive source for isolation of novel microorganisms and production of potent bioactive compounds (secondary metabolites). The majority of these compounds demonstrate one or more bioactivities, many of them developed into drugs for treatment of a wide range of diseases in human, veterinary and agriculture sectors[35]. Searching for novel actinomycetes constitutes an essential component in natural product-based drug discovery. Herein, a total of 8 selected actinomycete strains were isolated from air samples collected from Scientific and Technological Equipments Building, Walailak University, Nakhon Si Thammarat, Thailand based on their morphological and cultural characteristics. On primary screening, most of these strains showed the anti-*S. aureus* TISTR 517 and MRSA 142 (Table 1). The cross-streaking is an easy and relatively rapid method for screening cultures in search for new antibiotics and thus establish a spectrum of inhibiting properties of any bacterium, mold, or actinobacteria which will grow discretely on an agar plate. As a result, the inhibitory activity on tested bacteria by cross streak method or agar plug method as preliminary screening for antimicrobial activity is seen as better than those obtained by agar well diffusion method[18]. However, in bacteriocin or secondary metabolites investigations, the cross streak method should be controlled by the agar well diffusion method, it could be caused of the major drawback of the 'cross streak method' was difficulty in obtaining quantitative data, since the margins of the zone of inhibition were usually very fuzzy and indistinct.On the secondary screening of the strains by agar well diffusion, 8 strains exhibited antibacterial activity against *S. aureus* TISTR 517 and MRSA 142, 3 strains against *E. coli* TISTR 887, 1 strain against *S. typhimurium* TISTR 292 (Table 1). These finding indicates that strain A2 showed more effective against both *S. aureus* TISTR 517 and MRSA (142, 1096, 2499, 2559, 7645, 1424). Novel antibiotics against drug resistant bacteria are urgently needed. Microbial natural products have been one of the major sources of novel drugs. In this study showed that exploring new habitats is one of the most promising ways to isolate new strains of actinomycetes endowed with antimicrobial activity, especially anti-MRSA activity. In several previous studies, the *S. antibioticus* and *S. flaveolus* were antibacterial substances producing strains, actinomycins and polycyclic ethers, respectively. These antibacterial substances were inhibited Gram positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis*[36-39]. It was also reported that *S. psammoticus* produced polyketide antibiotics effective against MRSA[40]. In this study, an actinomycete isolate named A2 was isolated from air in Scientific and Technological Equipments Building 7th, Walailak University. The isolate was Gram positive and formed tough and filamentous colonies that were hard to pick from the culture media as a characteristic of actinomycetes. It also produced colored pigments (greenish black) after growing on LB/2 agar plate for at least 7 days, which were secreted into the culture media.

Table 1
Strain number and antibacterial activity of Streptomyces strains

Strain no.	S. *aureus* TISTR 517	*E. coli* TISTR 887	*Ps. aeruginosa* TISTR 1467	*S. typhimurium* TISTR 292	MRSA (s)
A2	+	+	-	+	+
AB	+	+	-	-	+
AG	+	+	-	-	+
AH	+	-	-	-	+
AL10	+	-	-	-	+
AL15	+	-	-	-	+
AL18	+	-	-	-	+
S2	+	-	-	-	+

+, have antibacterial activity; -, have no antibacterial activity

2. 16S rDNA sequence and phylogenetic analyses

On the basis of morphological, cultural, physiological, biochemical and chemotaxonomic characteristics including phylogenetic analyses of 16S rDNA sequences, strains (A2, AB, AG, AH, AL10, AL15, AL18, and S2) from air in Scientific and Technological Equipments Building 7th, Walailak University, Nakhon Si Thammarat, Thailand. All were identified as *Streptomyces*. Spore chain morphology of 8 selected strains were rectiflexibiles or spiral. They showed the same chemotaxonomic pattern which were similar to the member of the genus *Streptomyces*[41]. Phylogenetic analysis of 8 selected strains as previously described revealed that they were belonged to the genus *Streptomyces* comparing with some of the type strains validly described, and *Actinomaedura madurae* DSM 43067^T was selected as an outgroup (Figure 1). They were close to the type strains of *S. lydicus* NBRC 13058^T, *S. rochei* NBRC 12908^T, *S. gancidicus* NRBC 15412^T, *S. longispororuber* NBRC 13488^T, *S. coerulescens* NBRC 12758^T, and *S. angustmycinicus* NBRC 3934^T with sequence similarity ranged from 99.42-100.00% as shown in Table 3. Therefore, strain A2 should be identified as *S. lydicus*, AG as *S. gancidicus*, AH as *S. longispororuber*, AL10 as *S. coerulescens* and strain S2 as *angustmycinicus*. Strains AB, AL15 and AL18 should be identified as *S. rochei* group. The review articles related about *Streptomyces* and antibiotics produced by *Streptomyces* have been reported[42,43]. For example, natamycin, also known as pimaricin, is a polyene macrolide antibiotic, produced in submerged cultures of certain *Streptomyces* strains such as *S. natalensis*, *S. chattanoogensis*, and *S. lydicus*[43]. Strain A2 which was identified as *S. lydicus* (Tables 2) was selected for bioactive compound or secondary metabolite fermentation study because this strain exhibited good antimicrobial activity against *S. aureus* TISTR 517, *E. coli* TISTR 887, *S. typhimurium* TISTR 292 and 6 tested MRSA strains (Tables 1). Moreover, there were no reports on an antimicrobial substances study from *S. lydicus* in Thailand.

Table 2
Similarity (%) of 16S rDNA sequences and identification of Streptomyces strains

Strains	Sequence length (bp)	Closest species	Accession number	% Similarity of 16S rDNA	Diff/Total nt
A2	1,434	*S. lydicus* NBRC 13058^T	AB184281	99.58	6/1431
AB	1,430	*S. rochei* NBRC 12908^T	AB184237	99.93	1/1418
AG	1,427	*S. gancidicus* NRBC 15412^T	AB184660	100.00	0/1411
AH	1,407	*S. longispororuber* NBRC 13488^T	AB184440	99.42	8/1377
AL10	1,425	*S. coerulescens* NBRC 12758^T	AB184122	99.79	3/1420
AL15	1,421	*S. rochei* NBRC 12908^T	AB184237	99.93	1/1417
AL18	1,424	*S. rochei* NBRC 12908^T	AB184237	100.00	0/1417
S2	1,452	*S. angustmycinicus* NBRC 3934^T	AB184817	99.93	1/1443

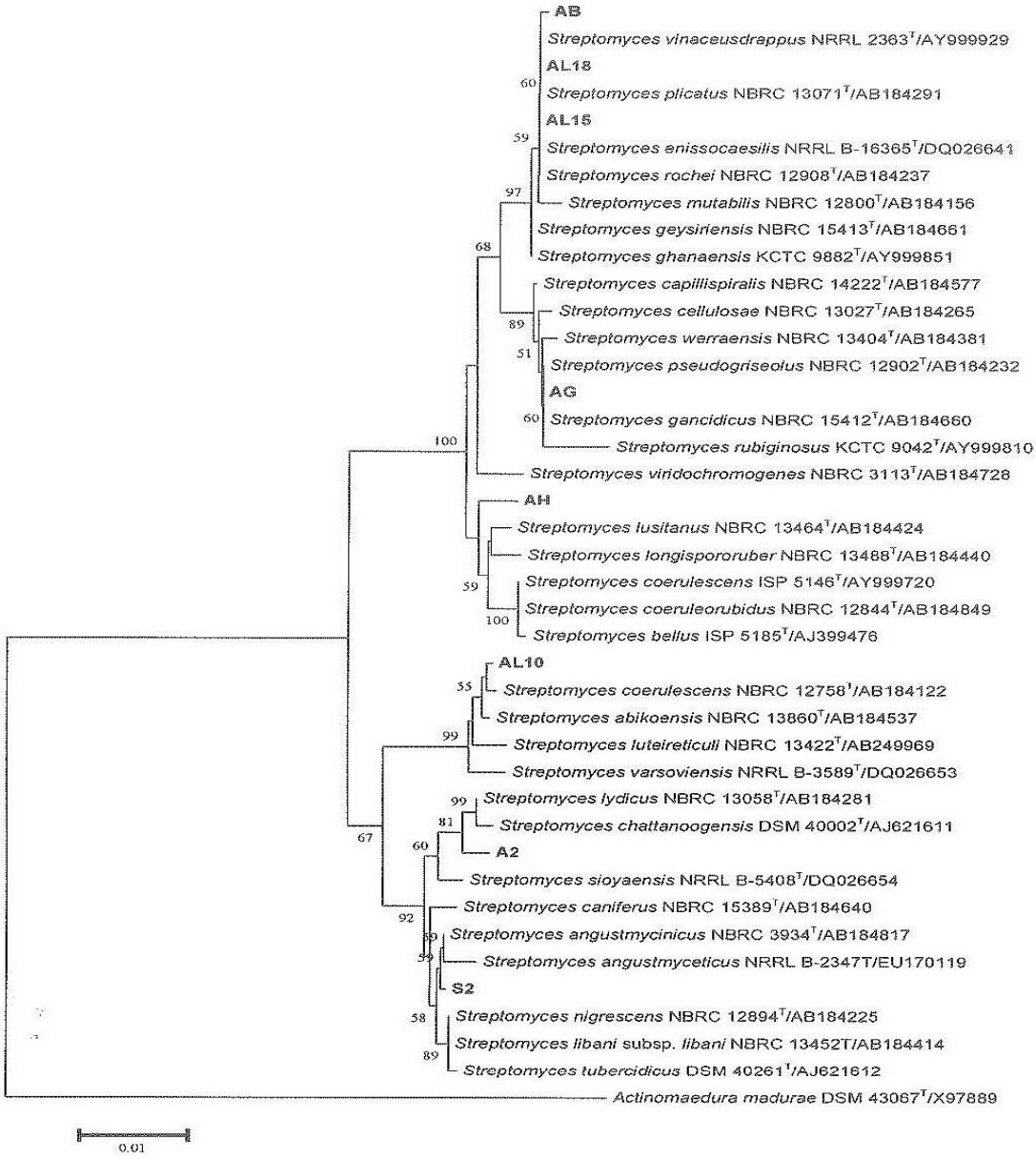

Figure 1
Neighbour-joining tree of the bacterial isolates and representative species of the genus Streptomyces based on partial 16S rRNA gene sequences. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

2. Antimicrobial activity according to the culture medium

Antibiotic production by actinomycetes is dependent on the composition of the medium[44] and especially on carbon and nitrogen sources. It was noticed that the spectrum of activity was different depending on the culture medium used. Moreover, there is a review article which explains the negative effects of carbon catabolites on antibiotic production[45-47]. Interestingly, YMA medium could induce spore formation, resting/productive stage, of isolate A2 as shown in Figure 2. No activity was observed on both media for strains *P. aeruginosa* TISTR 1467 indicating that this strain might be resistant to the substances produced by the isolate A2. These substances were active on *S. aureus* TISTR 517 (Figure 3) and all test MRSA (Table 1) on LB/2. It is interesting to note that *S. typhimurium* TISTR 292 was sensitive only on LB/2 medium (Table 1) (data not shown). So, in the following, LB/2 medium was used as the production medium.

Figure 2

Colonial morphology of isolate A2 grew on LB/2 (left) and YMA (right) for 7 days.

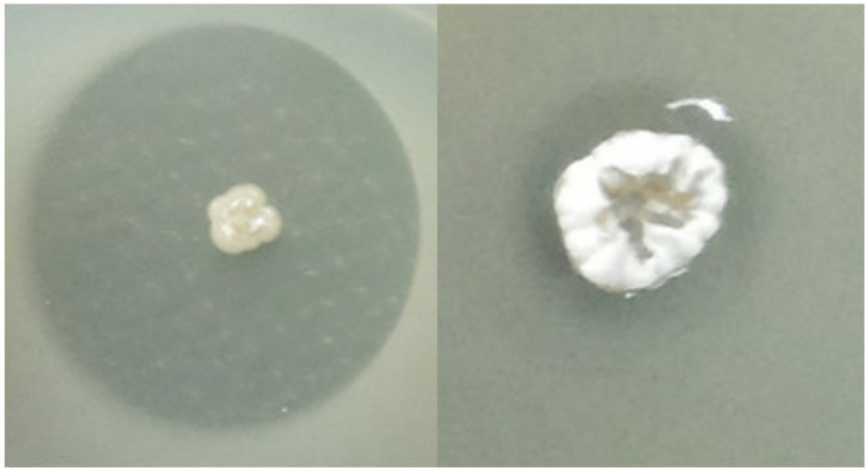

Figure 3

Anti Staphylococcus activity of isolate A2 cultured on LB/2 (left) plate and YMA (right) plate tested by overlay method. The cell suspension of S. aureus was mixed in 0.7% softed Mueller Hinton (MH) agar and then overlayed on colony of isolate A2 (5^{th} days of incubation). The plate was incubated at 37 ^{o}C for 24 h, and then the inhibition zone was observed.

3. Partial characterization of the antimicrobial product

The bioactive compounds of the isolate A2 inhibited *S. aureus* growth creating an inhibition zone around the wells. This activity was not eliminated upon treatment by temperature (60, and 121°C) (Table 3). It is concluded that the active substance might be of non protein nature.

Table 3
Sensitivity of the active substance from the isolate A2 to temperature.

	Inhibition diameter against *S. aureus* (mm.)		
	Not subjected to heat treatment	After heat treatment	
		60 ^{o}C	121 ^{o}C
Culture broth of the isolate A2	19±0.14	19±0.14	16±0.00

Values are means of two replicates ± standard deviation.

4. Cytotoxicity against primate cell line (Vero)

The effect of bioactive compounds in culture broth of isolate A2 after grew in LB/2 liquid medium for 7 days at the condition as previously described in Materials and Methods. The protein concentration was determined by Bradford's method, as 1.04 mg protein/ml, acting as 100% concentration of bioactive compounds. The bioactive compounds on cytotoxicity on Vero cells showed a concentration and time dependent activity. The IC50 value against Vero cells was found to be 3.28%. At 10% concentration more than 60% cell death was observed. The results suggest that the culture broth may be toxic to normal cells. However, notable activity was seen against *Mycobacterium tuberculosis* (Anti -TB) H37Ra (data not shown) at 10% concentration of bioactive compounds in culture broth.

CONCLUSION

Isolation of 8 actinomycetes from air samples collected by Microflow 90 apparatus. They all showed antimicrobial activity. The actinomycetes have been identified as *Streptomyces* sp. based on morphological, and 16s rRNA gene sequencing. Interestingly, strain A2 showed much more active activity against antibiotic resistant bacteria (MRSA), and then identified as *Streptomyces lydicus*. *Streptomyces lydicus* has been reported to produce natamycin, a polyene macrolide antibiotic,[48] and a strain WYEC 108 was used to pesticide agent[49]. Unfortunately, these findings found that the bioactive compounds in culture broth may be toxic to normal cell, Vero cell line. It should be tested the cytotoxicity to Brine shrimp or other cell lines for confirmation the data. By the way, it suggests that the strain could be clinically important need to be investigated further for the anti antibiotic resistant bacteria properties.

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CONFLICT OF INTERESTS

The author has declared not any conflict of interests.

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