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Screening for Antimicrobial Activities of *Actinomycetes sp.* isolated from Afyonkarahisar, Turkey

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Abstract
We aimed to determined that characterization of antimicrobial compounds of Actinomycete isolates (A32, A33) which were determined have antimicrobial activities in a previous study of ours. Different fermentation media were used to determination effective antimicrobial compounds production of AA32 and AA33 by fermentation. Fermentation procedure were performed in three major steps: sporulation, inoculation, and fermentation. Effective antimicrobial molecules of AA32 and AA33 to determined as test microorganisms *Staphylococcus aureus* ATCC 25923 and *Esherichia coli* ATCC 25922 were used during fermentation. Raw materials extraction of AA32 and AA33 were performed by solvent extraction and active compound determination were performed by using TLC, biootography, colon chromatography and UV spectrophotometer methods. Also to describe these two isolates, fatty acid profiles were determined using gas chromatography. As conclusion, The isolate AA32 was identified as *Streptomyces-halstedii-olivaceus* at a similarity index of 0.154 and the isolate AA33 was identified as *Streptomyces californicus* at a similarity index of 0.540 by the MIDI system. The antimicrobial compound isolated has O-H and/or N-H rich, having amine groups, has an Rf value as 0.527, its λ_{max} is at 254 nm, and a polar compound. Its UV spectrum peak and, its λ_{max} bring to mind the cephalosporin or cephalosporin like aromatic or polyene compounds.

Key words: Actinomycete, antimicrobial compound, fatty acide prophile

INTRODUCTION

Actinomycetes are filamentous bacteria, and naturally soils inhabiting microorganisms. They are of great importance in biotechnological processes due to their ability to produce a great number of antibiotics and other bioactive secondary metabolites, such as growth promoting substances for plants and animals, immunomodifiers, enzyme inhibitors for use by human. They have provided about two-thirds of the naturally occurring antibiotics including medicinally important, such as aminoglycosides, anthracyclines, chloramphenicol, β-lactams, macrolides, tetracyclines etc. The search for such substances of microbial origin is largely based on the isolation, from diverse sources, of different strains [1-2].Moreover, one of the strategies for enhancing the likelihood of obtaining particular isolates and secondary metabolites is to analyze uncommon ecosystems which exist under extreme conditions and to consider genera of Actinomycetes that have been poorly studied in the past. Many of these organisms may represent new taxa and thus can provide a valuable resource for use in future biotechnological processes [3]. The list of novel Actinomycetes and their products are one of the poorly explored areas in microbiology in all over the world. It is suggested that a careful exploration of new habitats might continue to be useful [4-5].

In the present study, isolation of novel Actinomycetes from Afyonkarahisar, and both determination and partially characterization of their antimicrobial activities were aimed.

MATERIALS AND METHODS

Microbial test strains

Stphylococcus aureus ATCC 25923, *Klebsiella pneumoniae*, *Esherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris*, *Salmonella enteriditis*, *Candida albicans* were kindly obtained from Afyon Kocatepe University, Faculty of Medicine, Microbiology Department. The hospital isolates of bacteria were identified by Phonix BD automatic identification system (Phoenix, BD, USA) [6]. These bacteria were used to determine the anti-microbial activity of the new isolates from this study.

Isolation of Actinomycetes and determination of their antimicrobial activity

Soil samples were collected from different agricultural parts of Afyonkarahisar and sediments of Akarçay stream and then transferred to the laboratory in sterile condition. The soil samples were dried at 30 °C for 2–7 days with 1% $CaCO_3$. 10g of soil samples were ten-fold diluted with sterile distilled water, and homogenized by vortexing. Dilutions were pour platted on Nutrient agar (NA), Malt agar (MA) and Glycerol-arginine agar (GAA). The plates were incubated at 28 and 40°C for 3 weeks. *Actinomycete* colonies were recognized on the basis of macroscopic and morphological characteristics under light microscopy. Antimicrobial activity of the strain was determined by standard Spectrum Plate method using Gram (+) and (-) bacteria, and yeast [7-8].

Morphological, biochemical and physiological characteristics of the isolates

Cultural characteristics were observed on 12 different media at 30ºC for 7–21 days. Micro morphology and sporulation were observed on the GAA media by light microscopy. Various biochemical tests and physiological criteria performed for the identification of the potent isolates are as follows [9-10-11]. gelatin hydrolysis, catalase, starch hydrolysis, lipid hydrolysis, decomposition of hydrocarbon, tyrosine hydrolysis, NaCl resistance, temperature tolerance, growth in the presence of inhibitory compounds, utilization of different carbon and amino acids sources, as well as the production of melanin.

Fermentation and solvent extraction

Isolates were cultured on ISP2 agar (yeast extract 4g/lt, Malt extract 10 g/lt, glucose 4 g/lt, agar 15 g/lt) plates at 28 °C for 7 days and a single colony was inoculated to a 250 ml Erlenmeyer flask containing 100 ml ISP2, broth as a seed medium. The flask was incubated at 28°C for 5 days on a shaking incubator (200 rpm) (N-BIOTEK. INC NB-205) [12]. The seed culture was inoculated (5% v/v) in to flask containing 250 ml glycerol yeast broth (GYB), Fermentation broth A (FBA) (glucose 10g/lt, peptone 5g/lt, meat extract 5g/lt, NaCI 5g/lt) Incubation was carried out at 28°C for 168 hours under the standard condition of aeration and agitation. The formation of isolates was followed by the testing of the antibiotic activity by Agar Well Diffusion method using *S. aureus* as a test organism[13]. and fresh weight was determined at various times during the fermentation.

At the end of the fermentation the aqueous extract was filtered using Whatman filter paper (No. 1). The aqueous solution was extracted with methylene chloride, n-butanol and hexane. After the removal of solvents under vacuum and then concentrated at 40 ºC (50 rpm) using a rotary evaporator (Heidolph2). The extract was dried in vacuum oven at 40°C overnight [12]. This extract was used for TLC and column chromatography.

Separation of antimicrobial compound from solvent

After extracts solubility with DMSO, acetone, methanol, chloroform, hexane, n-butanol, H_2O, DMSO, the extracts were submitted to thin-layer chromatography (TLC). Plates (Merck 5554) were developed with n-Butanol: Acetic acid: Water (3:1:1), Chloroform: Metanol (4:1), Chloroform: Methanol: water (13:6:2) and Ethyl acetate: Methanol: Water (40:5.4:5) which separated components into a wide range of Rf values. The components were visualized by spraying 0.2% acetonic ninhydrin reagent in order to reveal spots of amine groups.

Identifying the bioactive substance via TLC, column chromatography and UV spectra

Bioautography was performed with a culture of S. aureus ATCC 25923 which showed sensitivity to the extracts. Developed TLC plates (Merck 5554) were carefully dried and overlaid by agar seeded with an overnight culture of Staphylococcus aureus ATCC 25923 The plate was incubated for 24 h at 37ºC and then sprayed with a tetrazolium dye is diphenyl tetrazolium bromide, called MTT [14]. 0.2 g extract was dissolved with 1 ml sterile water and centrifuged at 3500g for 2 minutes. Purification of the antibiotic was carried out by column chromatography using Sephacryl S200 column. The crude antimicrobial compound was loaded at the top of the column and eluted using n-butanol:acetic acid: water (3:1:1) as solvent system. The separation was done by using a flow rate of 4.5ml/h. 52 fractions of 1.5 ml each were collected, and then subjected to biological assay against S. aureus and E. coli to detect the active fractions with disk diffusion technique. Each of 52 fractions and TLC bands showing antimicrobial activities was determined by UV spectrophotometer [11]. The fractions showing antimicrobial activity were confirmed using TLC plates. The light brown colored powder obtained was stored at 4°C. [11].

Fatty acid composition

Actinomycetes isolates were inoculated onto Trypticase Soy Broth (TSB) and grown for 24 h at 28 °C. Cellular fatty acids were extracted and derived to their fatty acid methyl esters (FAME) as described by Sasser (1990) [15]. FAMEs were separated by the MIDI (Microbial Identification System) (Microbial ID, Inc. Newark, Del., USA) utilizing an Agilent Technologies 6890N gas liquid chromatography with a G2614A auto sampler and a 6783 injector. After flame ionization, FAME peaks were analyzed by using MIDI Microbial Identification System, software version ACTIN1 3.80.

RESULTS AND DISCUSSION

A total of 52 Actinomycete isolates were recovered from 21 soil samples from 7 different places of Afyonkarahisar region. Antimicrobial activity was exhibited in 29 (55.7%) of all isolates. While 46.1 %, 44.2%, 9.6% isolates were able to inhibit the growth of Gr (-), Gr (+) and *C. albicans*, respectively. 9.6% Actinomycete isolates got antimicrobial activity not only bacteria but also yeast. The antimicrobial activity was exhibited against *S. aureus* ATCC25923 (44.2%). *S. enteriditis* (32%), *P. aeuroginosa* ATCC27853 (23%), *E. coli* (19.2%), *K. pneumoniae* (7.6%) and *P. vulgaris* (3.8%), respectively.

Especially, 3 isolates exhibited a very strong antimicrobial activity against *S. aureus* ATCC25923 (AA33, KA37 and AA32). In contrast, AA34 isolate exhibited maximum antimicrobial activity against *E. coli* ATCC 25922. Only two thermophylic isolates had got antimicrobial activity (AA17, AA21). Thermophylic isolate AA17 exhibited antimicrobial activity against *S. enteriditis* and *S. aureus* ATCC25923. Thermophylic isolate AA21 effected *K. pneumoniae*. Among those isolates, 2 of them (AA33, AA32) showed the best activity against to selected microorganisms (Table1)

Table 1. Antimicrobial activity of *Streptomyces* isolates.

Inhibition Zone Diameters (mm)	*AA33*	*AA32*	*KA11*	*KA37*	*AA34*	*AA21*	*KA39*
Salmonella anteriditis	-	-	-	-	**20***	-	2
K. pneumoniae	**7***	5	-	-	-	1	-
P. aeuroginosa ATTC27853	5	5	3	**12***	-	-	-
E. coli ATCC25922	5	7	5	2	-	**14***	-
P. vulgaris	**5***	4	-	-	-	-	-
C. albicans	2	-	-	-	2	-	**4***
S. aureus ATCC25923	22	**23 ***	12	20	2	-	-

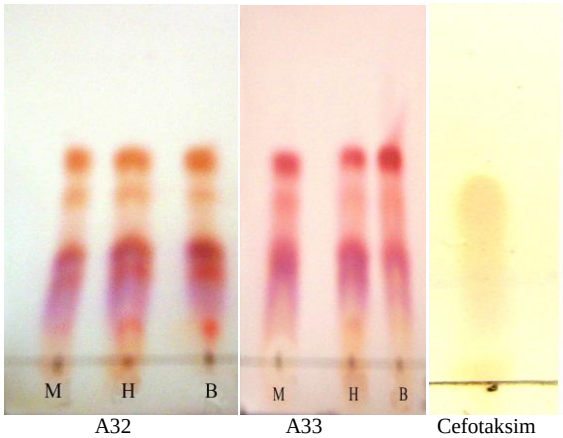
* Maximum inhibition zone in all used isolates.
AA: isolated from Ataköy *Actinomycete*, KA: isolated from ANS *Actinomycete*

The effectively production of antimicrobial metabolite was observed to be at between 20th and 24th h of the incubation in FBA and GYB by both AA33 and AA32 in shaking flasks, respectively. The maximum inhibition zone (25mm) was determinated at between 67th and 72nd h of incubation in AA32 isolate against to *S. aureus*, and these results were 21 mm, 91st and 96th h, respectively for AA33.

Examination of antimicrobial effect of the extracts on *E. coli* was started 20th hours in FBA and 24th hours of incubation in GYB media, respectively. The biggest inhibition zone was measured in AA33 isolate (91st hours, 25 mm diameter) and AA32 isolate (96th hours, 30 mm diameter) in FBA plates. As our observation, antimicrobial effect started at *log* phase and maximum effect was observed before exponential phase against to *S. aureus.* This was observed after exponential phase for *E. coli* in maximum range.

The best growth and antimicrobial activity was observed in FBA grown cultures. The best solvent for extraction the antimicrobial metabolites were determined as n-butanol and methylene chloride.

The antimicrobial compound was light brown, soluble in H_2O and DMSO, and insoluble in acetone, methanol, chloroform, hexane and *n*-butanol. These two compounds were revealed positive by ninhydrin, which gave violet color (Figure 1).



A32 A33 Cefotaksim

Figure 1. TLC profiles of the extract

The UV spectral data and Rf value for the n-butanol extract of selected active fermented broth are shown in Table 4. At AA33 two bioactive regions appeared on the chromatogram. Both regions were inhibitory to *S. aureus* ATCC 25923 in the position of Rf 0.527 and 0.487. AA32 strain's bioactive compound was detected on the TLC plate (Rf 0.527). The bioactive compound exhibited a maximum UV absorption at 254 nm (Table 4, Fig1, 2). Characteristics of two strains namely, AA32 and AA33 which showed the best antimicrobial activity against test microorganisms are listed in Table 2.

Table 2. Culture characteristics of strains isolated

Strain	Agar medium	Growth	Aerial mycelium	Reverse color	Soluble pigment
AA33	Glycerol yeast agar	++	Bg	*Bw*	-
	ISP2	+	Y,	*Y*	-
	ISP4	+++	Iv	*Y*	*Y*
	ISP5	++	Y	*Y*	-
	ISP7	++	W	*Y*	-
	Sucrose nitrate agar	++	W	*W*	-
	Glucose Asparagine agar	+++	W	*Y*	-
	Glyserol nitrate agar	+++	W	*Bw*	-
	Glucose nitrate agar	+++	Iv	*IV*	-
	Nutruent agar	++	W	*Y*	-
	Malt agar	+++	Iv	*Bw*	*Bw*
AA32	Glycerol yeast agar	++	*Y*	*Or*	-
	ISP2	+++	*Y*	*Y*	-
	ISP4	+++	*Y*	*Y*	*Y*
	ISP5	+++	*Y*	*Y*	-
	ISP7	+++	*Iv*	*Y*	-
	Sucrose nitrate agar	++	*W*	*W*	-
	Glucose Asparagine agar	+++	*Iv*	*W*	-
	Glycerol nitrate agar	+++	*Iv*	*Bs*	-
	Glucose nitrate agar	+++	*Iv*	*Bs*	-
	Nutruent agar	++	*W*	*LY*	-
	Malt agar	+++	*Y*	*Gy*	*Bw*

+, weak; ++, normal ; +++, strong; −, negative result.; Bg, beige; Y, yellow, LY light yellow; Bw, brown; Iv, ivory ; Or, orange; Gy, gray; Bs, biscuit

Fatty acid composition varied for 2 Actinomycetes isolates, with 19 different fatty acids being detected. 14 different fatty acids namely, 14:0 ISO, 15:0 ISO, 15:0 ANTEISO, 15:00, 16:1 ISO H, 16:0 ISO, 16:1 CIS 9, 16.00, 16:0 9? METHYL, 17:1 ANTEISO C, 17:0 ANTEISO, 17:1 CIS 9 and 17:0 CYCLO occurred in both isolates. 15:0 ANTEISO was the dominant fatty acid having average value of 44.3%. The relative percentages of each major (>1%) fatty acid occurring in two isolates of Actinomycetes are shown in (Table 5) Minor fatty acids, which were present at lower values (< 1%) were omitted. The major difference between two isolates was found at the following FAME, 17:0 ISO.

The isolate AA32 was identified as *Streptomyces-halstedii-olivaceus* at a Similarity index of 0.154. The isolate AA33 was identified as *Streptomyces californicus* at a similarity index of 0.540 by the MIDI system.

Many bacteria can be characterized and defined on the basis of cellular fatty acid composition [16-17-18]. However, it is very important to prepare and analyze the fatty acid samples to limit test error. Therefore, in this study MIDI system was used as a standardized system.

Fatty acids can be defined as carboxylic acid derivatives of long-chain aliphatic molecules. Taxonomically, fatty acids in the range C10 to C24 have provided the greatest information and are present across a diverse range of microorganisms. The diversity in fatty acid types makes these compounds valuable markers in bacterial systematic [19].

Actinomycetes, like other Gram-positive organisms, contain three main groups of cellular fatty acids, namely straight-chain, branched chain and complex fatty acid types. Numerous Gram positive bacteria including Actinomycetes, have anteiso-branched fatty acids as the predominant fatty acid [20]. In this study, 15:0 ANTEISO was the dominant fatty acid confirming that information. Both isolates (AA32 and AA33) seem to be belong to *Streptomyces* genus however, more studies should be done to confirm their identifications. Actinomycetes have been recognized as the potential producers of secondary metabolites. They produce more than four thousand of the naturally occurring antibiotics, many of those important in medicine, such as aminoglycosides, anthracyclines, chloramphenicol, □- lactams, macrolides, tetracyclines etc. [2].

Saccharothrix species' antibiotics are reported to be highly effective against to Gr (+) bacteria when compared to Gr (-) ones [11-13]. Our isolated compounds have antimicrobial effect on Gr (+), Gr (-), and *C. albicans* as well

Two isolates, AA32 and AA33, were observed to be producing a wide spectrum antimicrobial compound. They grew much better in FBA rather than the other media examined. Their antimicrobial material production began in 20^{th} hour of incubation and it was its maximum level at between 67^{th} and 72^{nd} hours. Some researchers reported the similar findings to our data [11-21].

During the extraction procedures, different solvents (hexane, Me-Cl, n-butanol, etc.) were employed to obtain different compounds. The one, obtained from n-butanol and Me-Cl extraction, dissolved in water and DMSO, had light brown color, was observed to have antimicrobial effect. In general, ionic compounds are more soluble than non-ionic compounds in water [23].

When they subjected to bioautography, AA33 had two and AA32 had one band on TLC plate. The compounds in these bands showed antimicrobial activity. The Rf values of the band belonged to AA33 were 0.527 and 0.487. The band of AA32's Rf was 0.527. The maximum absorbance for all three compounds was at 254 nm. This result suggests that these compounds could be an aromatic compound or be in polyene nature. 17 reported that cefotaximes absorbs the UV light at 256 nm due to their cephem ring in their structure.

Table 3. Comparison of characteristics of strains isolated

Characteristics lysed	**AA33**	**AA32**
Starch hydrolysis	+	+
Lipid hydrolysis	+	+
Gelatin hydrolysis	+	-
Catalase	+	+
Decomposition of	+	+
hydrocarbon	+++	+++
Production of melanin	+	+
Tyrosine hydrolysis	-	+
Inhibition of Penicillin G		
NaCl inhibition	++	-
4%	-	-
7%	-	-
10%	-	-
13%	-	-
Kristal violet inhibition	-	-
(0.0001g/lt)		
Phenol inhibition (0.1 g/lt)		
Sodium azide resistance	-	-
0.01g/lt	-	-
0.02g/ıt		
Temperature tolerance		
10 ºC	-	-
27 ºC	+	+
37 ºC	+	+
40 ºC	-	-
Utilization of different		
carbon	+++	+++
Glucose	++	++
Lactose	++	++
Sucrose	+++	+++
Glycerol	++	++
Inositol	+++	+++
Galactose	++	++
Fructose	+++	+++
Mannitol		
Utilization of different		
amino Acids sources	+++	+++
Potassium nitrate	+++	+++
L- Asparagine	+++	+++
DL-Alanine	+	+++
L-Tyrosine	+++	+++
L-histidine	+++	+++
L- Glutamine	+++	+++
Glisin		
Antimicrobial activity	-	-
Salmonella enteriditis	+	+
Klebsiella pneumoniae	+	+
Pseudomonas aeruginosa		
ATCC27853		
Proteus vulgaris,	+	+
E. coli ATCC 25922		
S. aureus ATCC 25923	+	+
Candida albicans	+	-

Table 4. UV absorption of the bands obtained by TLC.

	AA32		AA33	
	Rf	UV(nm)	Rf	UV (nm)
Extract	0.179 0.230 0.307 0.408 **0.527**	384 **257** 245 211	0.256 0.307 **0.487** **0.527**	282 262
TLC	**0.527**	284 **254** 216	**0.486** **0.527**	282 254 282 256 208
Fraction of 6	**0.527**	**254**		

Table 5. Proportion of fatty acid in Actinomycetes isolates

Isolate	Fatty acisds										
	13:0 ANTEISO	**14:0 ISO**	**14:00**	**15:0 ISO**	**15:0 ANTEISO**	**15:00**	**16:1 ISO H**	**16:0 ISO**	**16:1 CIS 9**	**16:00**	**16:0 9? METHYL**
MA33	0.25	1.85*	0.42	6.52*	42.96*	2.51*	0.53	7.97*	4.44*	8.79*	2.15*
MA32	-	2.66*	-	7.02*	45.81*	3.3*	1.18	8.96*	4.23*	4.61*	2.94*
S. DEV	-	0.57276	-	0.354	2.015254	0.5586	0.46	0.7	0.1485	2.956	0.558614
MEAN	0.25	2.255	0.42	6.77	44.385	2.905	0.855	8.465	4.335	6.7	2.545

* Proportion of major (>1%) fatty aid in Acinomycetes isolates.

Table 5. (continued)

Isolate	Fatty acisds							
	17:1 ANTEISO C	**17:0 ISO**	**17:0 ANTEISO**	**17:1 CIS 9**	**17:0 CYCLO**	**17:00**	**17:0 3OH**	**20:1 CIS 11**
MA33	2.96*	3.65	11.56*	1.09*	1.1*	0.99	0.27	0.01
MA32	4.4*	-	9.16*	1.22*	1.17*	0.9	-	-
S. DEV	1.01823376	-	1.69706	0.09192	0.0495	0.064	-	-
MEAN	3.68	3.65	10.36	1.155	1.135	0.945	0.27	0.01

* Proportion of major (>1%) fatty aid in Acinomycetes isolates.

CONCLUSION

As conclusion, the antimicrobial compound isolated has O-H and/or N-H rich, having amine groups, has an Rf value as 0.527, its λ_{max} is at 254 nm, and a polar compound. Its UV spectrum peak and, its λ_{max} bring to mind the cephalosporin or cephalosporin like aromatic or polyene compounds. Studies on diagnosing the strain isolated and elucidating the compound isolated is now our main goal.

As suggested by [18] obtaining new secondary metabolites from Actinomycetes, it is necessary to isolate them from different environments. From this point of view, Turkey has enormous number of different microclimate and habitats. This is a promising condition and should be evaluated.

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