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Isolation and Characterization of Antimicrobial Activities of Native *Actinomycete* Strains from Agricultural Soils in Guilan province

Elham Amiri[1], Mirsasan Mirpour[2]*, Khosro Essazadeh[3], Behnam Rasti[4]

[1]Department of Microbiology, Faculty of Basic Sciences, Lahijan Branch, Islamic Azad University, Lahijan, Iran
[2]Department of Microbiology, Faculty of Basic Sciences, Lahijan Branch, Islamic Azad University, Lahijan, Iran
[3]Department of Microbiology, Faculty of Basic Sciences, Lahijan Branch, Islamic Azad University, Lahijan, Iran
[4]Department of Microbiology, Faculty of Basic Sciences, Lahijan Branch, Islamic Azad University, Lahijan, Iran
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Abstract

*Actinomycete*s are known as the largest Source of natural antibiotics in the world. For this reason, due to their ability to produce various antibiotics and other compounds of therapeutic importance, they are considered the golden microorganisms of the 21st century. The purpose of this research is the isolation and molecular identification of *Actinomycete* with antimicrobial properties from agricultural soils in the native areas of Guilan province. Soil samples were collected from the southwestern agricultural areas of Guilan province. Serial dilution was used to isolate *Actinomycetes*. Then the morphological, physiological, and biochemical identification of the samples was done, and finally, the molecular identification of the isolates was conducted using 16S rRNA sequencing and phylogenetic analysis. Antimicrobial activity was investigated against pathogenic microorganisms. A total of 14 isolates were identified, and two isolates with stronger antimicrobial properties were selected. Based on the results of phylogenetic studies and 16S rRNA sequencing, *Amycolatopsis roodepoortensis* strain EA7 was identified with 99.63% confidence, and *Streptomyces microflaveus* strain EA6 with 93.92% confidence. The isolated bacteria exhibited higher antimicrobial activity against Gram-positive pathogenic microorganisms such as *Staphylococcus aureus* and the standard sample *Staphylococcus aureus* PTCC 1112. This research represents the first report on the identification of *Actinomycetes* with antimicrobial properties in the agricultural soils of the southwestern regions of Guilan province, located in the Alborz mountains. The identification of the rare strain of *Amycolatopsis roodepoortensi*s strain EA7 from the northern regions of Iran highlights the significant value of the soils in these regions.

Key words: *Actinomycete*, Antimicrobial activity, 16S rRNA, *Amycolatopsis roodepoortensis*, *Streptomyces*

*Corresponding Author: E-mail: sassan47@gmail.com

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Introduction

Actinomycetes are known as the largest Source of natural antibiotics in the world, but the rate of discovery of bioactive compounds with new structures is increasing (Adamek et al., 2018). *Actinomycetes*, belonging to the order Actinomycetales, are members of a diverse group of gram-positive bacteria with over 55% GC content in their DNA (Peng et al., 2016). It is estimated that *Actinomycetes* are the origin of approximately 61% of all bioactive substances derived from microorganisms discovered to date (Song et al., 2021; Law et al., 2017). Among them, the genus *Streptomyces* is the primary contributor to the production of secondary metabolites, accounting for 16% of all significant antibiotic producers, predominantly from *Micromonosporaceae*, with smaller contributions from *Pseudonocardiaceae* and *Thermomonosporaceae*, known as "rare *Actinomycetes*". This highlights the value of rare *Actinomycetes* as a source of novel compounds, necessitating enhanced isolation techniques to increase their frequency of discovery (Takahashi and Nakashima, 2018). Due to their capacity to generate various antibiotics, anticancer agents, and other therapeutically important compounds, *Actinomycetes* are regarded as the key microorganisms of the 21st century (Ibnouf et al., 2022; Adamek et al., 2018). Therefore, there is a pressing need to continue exploring new microorganisms capable of producing bioactive compounds to combat emerging and resistant infectious pathogens. *Actinomycetes* are recognized as the most economically significant microbes, primarily due to their ability to produce crucial medical and pharmaceutical products (Takahashi and Nakashima, 2018). The aim of this study is to isolate *Actinomycetes*, screen them for antimicrobial activity, and conduct molecular and phylogenetic identification of this bacterial group from soil samples collected from the Alborz Mountain in northern Iran. Hence, in this study, *Actinomycetes* were isolated and screened from soil samples obtained from the Alborz Mountain in northern Iran.

Materials and Methods

Soil sampling

A total of 15 soil samples were collected from three different locations in the Alborz Mountains (Roodbar city) at geographical coordinates (east 49.5292° and north 36.8767°) from agricultural areas. The samples were collected from a depth of 15 cm using a shovel after removing the top layer of soil. They were then placed in clean, sterile containers with lids and promptly transported to the laboratory (Tan et al., 2019). During the separation process, successive dilutions ranging from 10^{-1} to 10^{-7} (1 to 7) were prepared from the soil. Specifically, 1 gram of dried soil samples was mixed with 9 ml of sterile distilled water. Subsequently, 1 ml of each dilution from 3 to 7 was transferred to the starch casein agar (SCA) culture medium using a pipette and cultivated as a continuous streak (Kalaba et al., 2021). Tetracycline and nystatin antibiotics were employed to prevent contamination. The plates were then placed in an incubator at 28°C for 4 days (Law et al., 2017). Following the incubation period, the morphological characteristics of the selected isolates were determined by inoculating them in the standard culture medium ISP2 (Yeast Malt Extract Agar) for 5 days at 28°C (Ibnouf et al., 2022).

Isolation and identification of *Actinomycete* strains

The isolated strains were selected using standard microbiology methods based on morphology, biochemical characteristics, and sugar fermentation. For initial diagnosis, warm staining methods, observation of soil smell, and colony appearance were employed. All isolated actinobacterial strains were compared with actinobacterial morphology described in Bergey's Manual of Systematic Bacteriology for potential identification of isolates (Ranjitha and Ravishankar, 2017). Antibiotic sensitivity tests were conducted against various commercial antibiotics (ampicillin, tetracycline, vancomycin, chloramphenicol, imipenem, ceftazidime, piperacillin, and ciprofloxacin) using the Kirby-Bauer disc diffusion method (Fahmy et al., 2021). Bacterial cultures were plated on Muller-Hinton Agar (MHA) plates with a 0.5 McFarland turbidity. Eight antibiotic discs were then positioned on the inoculated plates, which were subsequently incubated for

2 days at 28°C. The diameter of the halo around the disc indicated sensitivity to antibiotics, and vice versa, and was measured in millimeters (Ansari et al., 2019). The ability of superior strains to grow at different temperatures (27-37°C), various pH levels (4-10), and their tolerance to different concentrations of sodium chloride (0-7%) were also investigated (Nabila and Kannabiran, 2018).

Preparation of standard strain

The standard strain of *Streptomyces griseus* PTCC 1124 was purchased from the Scientific and Industrial Research Organization of Iran. It was cultivated alongside the samples isolated from the soil.

Identification of Test Organisms

The studied organisms for the antimicrobial assay of *Actinomycetes* were collected from the infectious department of Razi Hospital in Rasht. According to the protocol of Ansari et al. (2019), pathogenic bacteria were identified and isolated by biochemical methods. In this study, Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Pseudomonas aeruginosa*) pathogenic bacteria were used as target organisms (Oliveros et al., 2021). *Pseudomonas aeruginosa* standard strain PTCC 1565 and *Staphylococcus aureus* standard strain PTCC 1112 were purchased from the Iran Scientific and Industrial Research Organization.

Antimicrobial Assay of *Actinomycete* Isolates

Antimicrobial assays of *Actinomycete* isolates were conducted using the well diffusion agar method on Mueller-Hinton agar medium. Initially, 0.5 McFarland turbidity of the tested pathogens was prepared and cultured on Mueller-Hinton agar medium using a sterile swab. Subsequently, wells were created on the medium with a sterile dropper, and 50 µL of *Actinomycete* strains grown in TSB broth medium (0.5 McFarland turbidity) were transferred to the wells. The plates were then incubated in an incubator at 37°C for 24-48 h. Following incubation, the results were recorded (Oliveros et al., 2021). Based on the presence or absence of the zone of inhibition, the *Actinomycete* exhibiting the highest antimicrobial activity was chosen for further investigations.

Molecular identification using 16S rRNA gene sequencing

Molecular identification of isolated strains was conducted through 16S rRNA gene sequencing and phylogenetic analysis (Tan et al., 2019). To extract the DNA of the selected strains, a DNA extraction kit (Sinaclon kit) was utilized, employing the mini-column method. Actinobacteria general primers (refer to Table 1) were employed to isolate and amplify the 16S rRNA gene (Osama et al., 2022). Following ethidium bromide staining, polymerase chain reaction (PCR) amplification was confirmed using agarose gel electrophoresis (Tan et al., 2019; Li et al., 2021). The PCR program for *Actinomycetes* strains consisted of thirty-five cycles, as detailed in Table 2. After completing the reaction steps and validating the obtained bands through agarose gel electrophoresis, the reaction product was forwarded to Pishgam Biotechnology Company for sequencing. Subsequently, the results from sequence alignment were compared with the sequences stored in the NCBI database using the BLAST program (www.ncbi.nlm.nih.gov/blast). The similarity of 16S rRNA gene sequences and the phylogenetic tree of these strains were constructed using the Neighbor-joining method with the NCBI database and BLAST program (Fahmy et al., 2021; Tan et al., 2019).

Table 1. Primers used in polymerase chain reaction

Primer type	Primer sequence	Number of nucleotides
27F	5'- AGAGTTTGATCCTGGCTCAG -3'	20
1492R	5' -GGTTACCTTGTTACGACTT-3'	19

Table 2. Adjusted temperature schedule for polymerase chain reaction

Number	Time	Temperature (°C)	level	
1	2	95	denaturation	
33	30S	95	denaturation	second stage
	30S	55	Annealing	
	45S	72	Extension	
1	5min	72	The third stage (final extension)	

Results

Isolation and identification of *Actinomycete strains*

During our research on *Actinomycetes* from Alborz Mountains (Roodbar city), 14 isolates with the morphology of *Actinomycetes* were identified using Gram staining, biochemical tests, and fermentation of sugars. The results of biochemical investigations, fermentation of sugars, and antibiotic sensitivity tests are shown in Table 3. The macroscopic shape of the strains in ISP2 solid medium was observed as dry, chalky appearance (Fig. 1a, b), and the microscopic shape of the strains was observed as filaments (Fig. 2a, b). The strains are Gram-positive, aerobic, and filamentous. These strains grew well in all tested solid media with variable colony colors. Biochemical characteristics showed that these strains were catalase-positive, oxidase-negative, and grew well in the temperature range of 27 to 37°C and the pH range between 7 and 8. Strain EA7 failed to hydrolyze starch and urea but hydrolyzed gelatin and casein. Simmons Citrate agar test, oxidase, movement test, sulfide production, and indole production were also negative. They can use several carbon sources such as glucose, xylose, mannitol, raffinose, and arabinose. However, strain EA6 hydrolyzed starch and urea and could not ferment galactose, sucrose, lactose, and arabinose sugars. For strain EA6, EA7, and the standard strain, movement test, and indole production were also negative. But strain EA6 was able to produce sulfide. The rest of the strains and the standard strain did not produce sulfide. Strain EA6 did not use several carbon sources such as sucrose, galactose, lactose, and arabinose, and the standard strain did not use sucrose, mannitol, and raffinose. Strain EA7 was resistant to eight antibiotics used in this study. Two strains, EA6 and EA7, showed sensitivity to piperacillin and ciprofloxacin antibiotics in this study. However, the standard strain also showed sensitivity to vancomycin antibiotics (Fig. 3).

Identification of test organisms

A total of 6 pathogenic bacteria, *Staphylococcus aureus* as gram-positive bacteria, and *Pseudomonas aeruginosa* as gram-negative bacteria, were isolated and identified from the infectious department of Rasht hospitals. The test results are provided in the table below (Table 4). Pathogenic bacteria *Staphylococcus aureus* are cocci, catalase-positive, oxidase-negative, coagulase-positive, grow in mannitol salt agar medium. *Pseudomonas aeruginosa*, as gram-negative bacteria, are rods, catalase-positive, oxidase-positive, non-fermenting, motile, able to use citrate, and grow at 42°C.

Antimicrobial Assay of *Actinomycete* Isolates

The isolates were screened for antimicrobial activity. Due to their distinct antimicrobial activity, two samples were selected as strains EA7 and EA6 for further identification and phylogenetic investigation. They exhibited broad-spectrum antimicrobial activity. During the antimicrobial assay using the Well diffusion agar method, strains EA7 and EA6 demonstrated significant antimicrobial properties. Specifically, strains EA7 and EA6 exhibited antimicrobial halos with diameters of 25 mm and 15 mm, respectively, against *Staphylococcus aureus* and the standard strain of *Staphylococcus aureus* PTCC 1112. No halo was observed against *Pseudomonas aeruginosa*, or the halo diameter was minimal (Fig. 4). These strains were chosen as indicator strains based on their antimicrobial effectiveness.

Biotechnological Journal of Environmental Microorganisms (BJEM)

Biotechnological Journal of Environmental Microorganisms(BJEM) 2(5) 2023 233-243

Table 3. Morphological, biochemical, and physiological characteristics of *Actinomycetes*

Biochemical tests	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14	S
Warm coloring	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid-fast staining	+	+	-	+	+	-	-	+	-	-	-	-	+	+	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis	+	+	+	+	+	+	-	+	-	-	+	-	+	+	+
Simon Citrate	-	+	+	-	+	+	-	-	-	-	+	-	-	+	+
urea	+	-	+	-	-	+	-	+	-	+	-	+	+	+	+
Nitrate reduce	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin hydrolysis test	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
Casein hydrolysis test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Test (SIM) motility Sulfide indole	- - -	- - -	- - -	- - -	- - -	- + -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
Vegetative mycelium	white	white	white	white	white	White	cream	cream	cream	white	white	white	white	cream	white
Salt tolerance	-	-	-	-	4%	2%	7%	2%	4%	4%	2%	2%	2%	4%	2%
temperature	27-30	27-32	27-30	27-30	27-32	27-37	27-37	27-30	27-30	27-30	27-30	27-30	27-32	27-32	27-37
Different pH	4-10	4-7	4-6	4-6	4-5	4-10	4-10	4-7	4-7	4-7	4-7	4-7	4-7	4-7	4-10
Colony color	white	white	white	white	white	white	cream	cream	cream	cream	cream	cream	cream	cream	white
Spore wall	+	+	+	-	-	+	-	-	-	-	-	-	+	+	-
Antibiotic sensitivity test															
Ampicillin	11	12	12	13	14	9	R	10	R	R	12	15	11	10	7
Tetracycline	11	14	15	15	16	10	R	R	R	10	12	R	11	16	10
Vancomycin	21	22	18	21	22	10	R	10	R	10	12	18	12	16	28
Chloramphenicol	15	16	15	15	17	13	R	10	R	R	14	14	13	16	10
Imipenem	13	14	13	14	12	R	R	R	11	13	12	11	14	12	R
Ciprofloxacin	22	19	18	21	21	22	16	17	19	19	21	22	21	23	22
Ceftazidime	18	19	17	18	18	R	R	10	15	15	15	17	14	18	R
Piperacillin	19	22	21	24	26	22	15	16	25	25	22	24	25	25	22
Sugar fermentation test															
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
fructose	+	+	-	-	-	+	-	+	-	+	-	-	+	-	+
Sucrose	-	-	+	+	-	-	-	-	-	+	-	-	-	+	-
Galactose	+	+	+	+	-	-	-	-	-	+	-	-	+	-	+
Raffinose	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Maltose	-	-	+	-	-	+	-	+	-	+	-	-	+	+	+
xylose	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+
Arabinose	-	+	+	-	-	-	+	-	+	+	+	+	+	+	+
Lactose	+	+	+	+	-	-	-	-	-	+	-	-	+	+	+

• **A**: Actinomycete isolates isolated from soil and S: The standard strain of *Streptomyces griseus* PTCC 1124

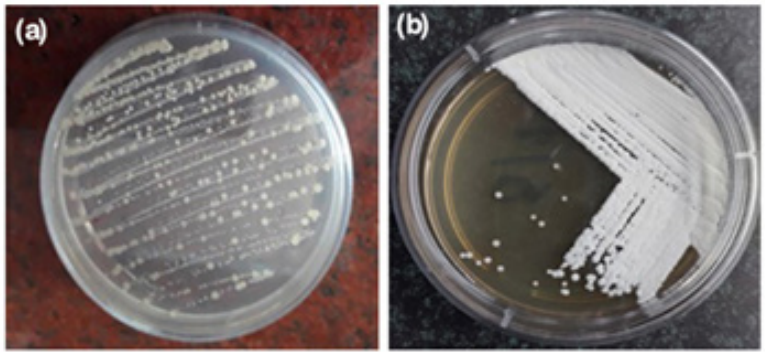

Fig. 1 Macroscopic shape of (a) EA7 and (b) EA6 strains

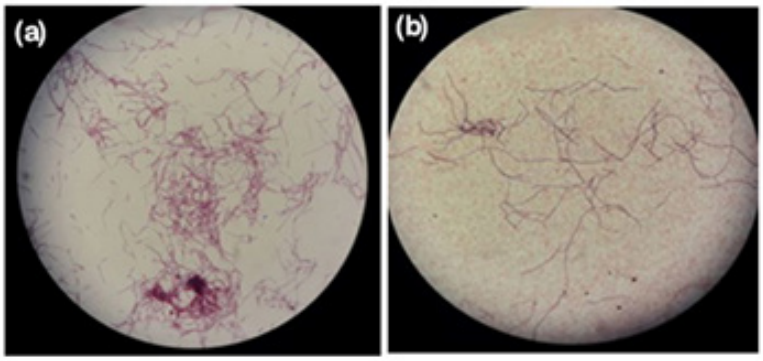

Fig. 2 Microscopic image of (a) EA7 and (b) EA6 strains

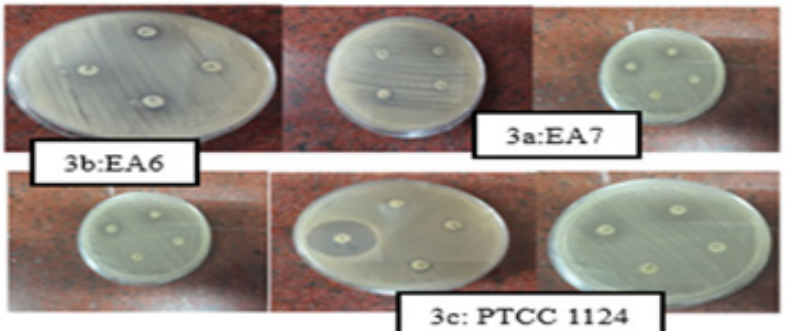

Fig. 3 Antibiotic sensitivity test of sample isolated from soil and standard sample

Table 4 *Staphylococcus aureus* and *Pseudomonas aeruginosa* identification tests

Staphylococcus aureus	Results	*Pseudomonas aeruginosa*	Results
Catalase	+	Catalase	+
oxidase	-	oxidase	+
coagulase	+	SIM	Motility
Mannitol Salt Agar	+	Simmons Citrate agar	+
		TSI	
		Growth at 42^0C	-
			+

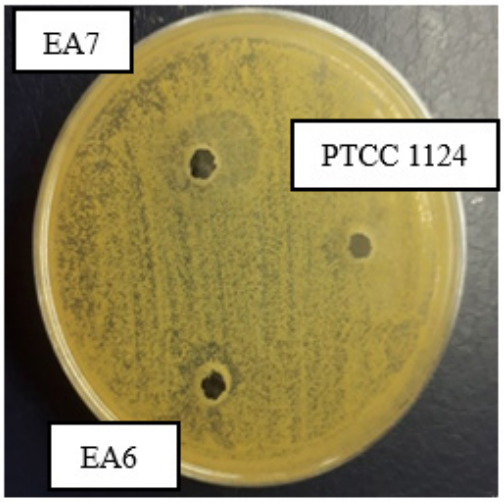

Fig. 4 Antimicrobial assay of *Actinomycete* isolates

Molecular Identification

The phylogenetic position of strains EA7 and EA6 was determined based on the 16S rRNA gene sequence. Analysis of the 16S rRNA gene using BLAST software with other bacteria in the genetic database (NCBI) showed that the strains consist of 16S rRNA and 1500 bp gene sequences. Additionally, the alignment of these sequences with the sequences recorded in the NCBI database showed that strain EA7 is *Amycolatopsis roodepoortensis* with 99.63% confidence and strain EA6 is *Streptomyces microflaveus* with 93.92% confidence. Two strains were named: *Streptomyces microflaveus* strain EA6 and *Amycolatopsis roodepoortensis* strain EA7. The results of PCR and the relationship of *Amycolatopsis roodepoortensis* strain EA7 with other Amycolatopsis species and *Streptomyces microflaveus* strain EA6 with other *Streptomyces* species using the Neighbor-joining method are shown in Figures 5, 6, and 7, respectively. Gene registration of these isolates was completed as follows. *Amycolatopsis roodepoortensis* Strain EA7 (GenBank accession number: OR680714) *Streptomyces* microflavus Strain EA6 (GenBank accession number: OR680713)

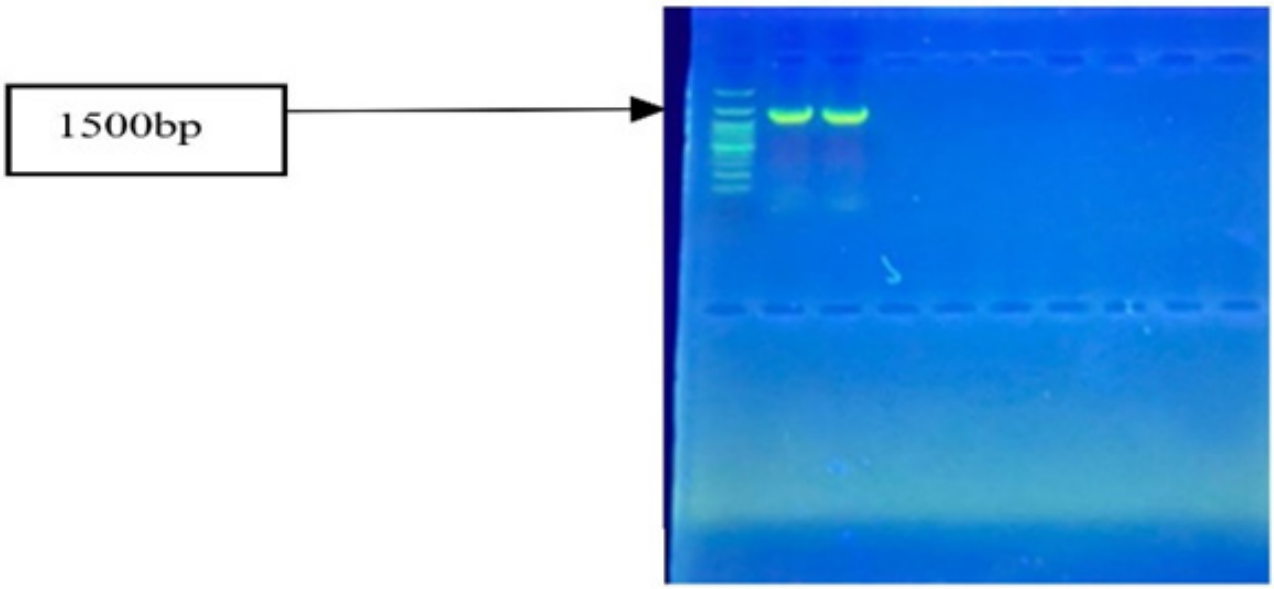

Fig. 5 PCR product electrophoresis results on agarose gel

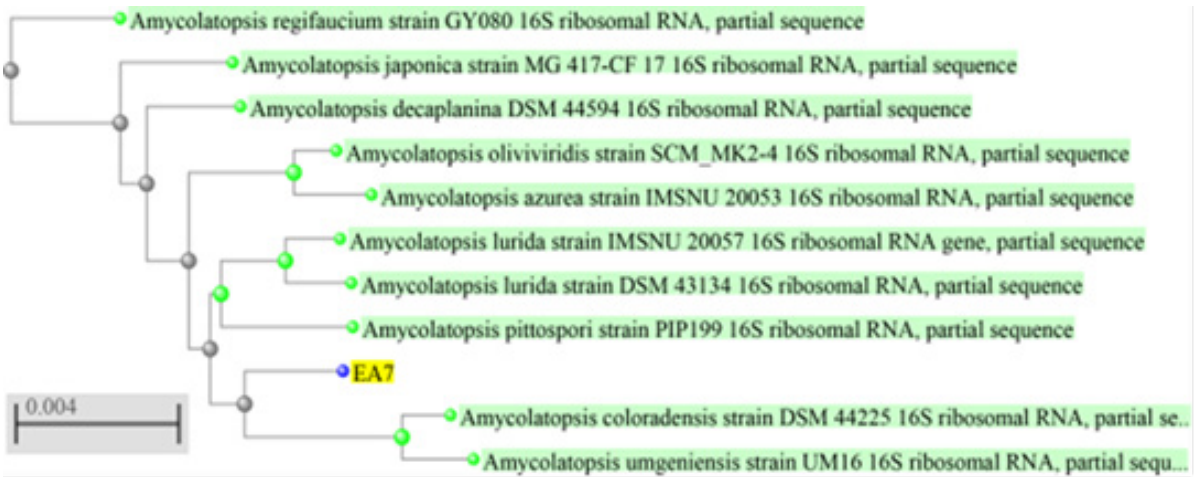

Fig. 6 Phylogenetic tree of strain EA7 based on 16S rRNA gene sequences with other Amycolatopsis species using the Neighbor-joining method. Bootstrap values were expressed as a percentage of 1000 repetitions. Bar, 0.004 substitutions per nucleotide position.

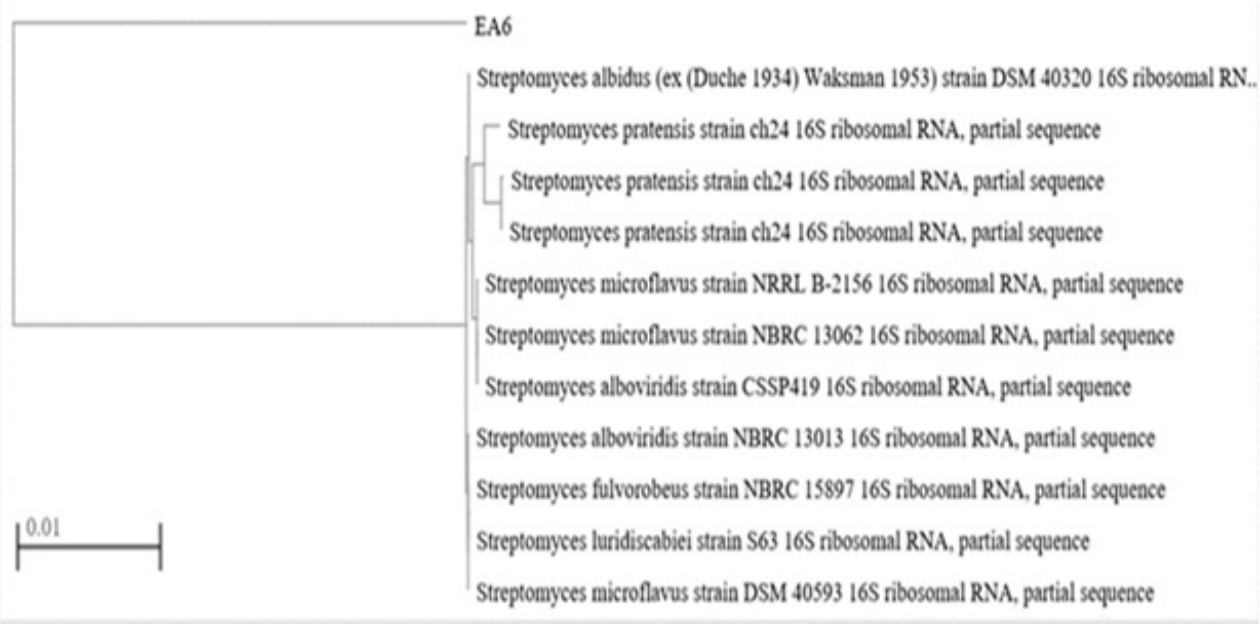

Fig. 7 Phylogenetic tree of strain EA6 based on 16S rRNA gene sequences with other *Streptomyces* species using the Neighbor-joining method. Bootstrap values were expressed as a percentage of 1000 replicates and a bar of 0.01 substitutions at each nucleotide position.

Discussion

Investigating and discovering new microorganisms that produce novel secondary metabolites can be crucial for effectively combating emerging diseases and antibiotic-resistant pathogens (Kisil et al., 2021). *Actinomycetes* are notable for being a rich source of secondary metabolites and also play a vital role in organic matter decomposition (Kurnianto et al., 2021).

The diversity of *Actinomycetes* and their ability to produce novel materials position this category remarkably. In the last two decades, there has been a decline in the discovery of new essential compounds from soil-derived *Actinomycetes*, which have already produced a significant number of previously identified secondary metabolites (Chen et al., 2018). Consequently, this leads to the emergence of new *Actinomycete* species from unusual environments, paving the way for a new era for medical experts (Kalaba et al., 2021). *Actinomycetes* generate a wide array of biologically active compounds, including antibiotics, enzymes, and enzyme inhibitors (Law et al., 2017).

In this research, 14 strains of *Actinomycetes* were isolated from the southwestern regions of Guilan and identified through biochemical, morphological, physiological, and molecular methods. Two strains showed higher antimicrobial activity against the studied pathogens. These isolates were able to grow in the three environments tested (ISP2, SCA, MHA), but exhibited the most robust and suitable growth in SCA and ISP2 agar environments for the production of antimicrobial compounds. These strains demonstrated broad-spectrum antimicrobial activity against Gram-positive bacteria. It can be concluded that the antimicrobial effect of the selected strains is more pronounced against gram-positive bacteria. Out of the 14 *Actinomycete* isolates, only strain EA7 exhibited high resistance to eight commercial antibiotics (including ampicillin, penicillin, chloramphenicol, tetracycline, piperacillin, imipenem, ceftazidime, and ciprofloxacin). In contrast, other isolates displayed lower resistance with a maximum halo diameter of 20 mm.

Other research (Alam and Jha, 2019; Shaik et al., 2017) has demonstrated the antibiotic resistance of soil *Actinomycetes*, specifically *Amycolatopsis Balhimycina* and *Amycolatopsis orientalis*, against four antibiotics (ampicillin, penicillin, chloramphenicol, tetracycline), as well as antimicrobial activity against methicillin-resistant *Staphylococcus aureus* strains. These findings align with the results of our research. Ansari et al.'s study (2019) confirmed the antibiotic resist-

ance of *Streptomyces* sp. to ampicillin and ciprofloxacin. Significant differences in antibiotic susceptibility patterns and nutritional resource utilization within and among *Actinomycete* species may be related to local adaptations (Kisil et al., 2021).

In research, it was shown that gram-negative bacteria have more resistance to antimicrobial effects compared to gram-positive ones. In the case of *Staphylococcus aureus*, the diameter of the halo was greater than that of *Pseudomonas aeruginosa* (Abd-Elnaby et al., 2016). This difference can be attributed to the unique structure of the outer membrane in gram-negative bacteria, which contains lipopolysaccharide compounds, making them impermeable to antimicrobial substances (Nikbakht et al., 2021). Obtaining data from both 16S rRNA gene sequencing and biochemical characteristics is crucial for accurately classifying prokaryotes, especially for the genera Amycolatopsis and *Streptomyces* in *Actinomycetes* (Osama et al., 2022). Studies have revealed that although species in the genus *Amycolatopsis* share similarities in the Yala 16S rRNA gene with species in the genus *Streptomyces*, they may exhibit distinct phenotypic characteristics based on biochemical traits and carbon source utilization patterns (Sharma and Manhas, 2019). Molecular techniques, such as measuring 16S rRNA sequences, are powerful tools for identifying microorganisms. Therefore, in this research, the 16S rRNA gene sequencing method was utilized to identify isolated bacteria and thoroughly evaluate their biochemical and physiological characteristics, aiming to enhance understanding (Tan et al., 2019). The 16S rRNA gene sequence is highly conserved, making it a suitable target gene for DNA sequencing in samples containing a wide array of species, even those with nearly identical sequences within the same strain (Kurnianto et al., 2021).

The strains identified in the 16S rRNA gene sequence determination method differed from each other in terms of their kinship relationships with the closest strains in biochemical characteristics and fermentation of sugars. This issue is related to the factors that affect the behavior of microorganisms, which are still not well understood (Kawuri and Darmayasa, 2018). However, changes, such as the concentration of nutrients and how to access these substances, the occurrence of competitors in the environment, metabolites, and cell density, can play a role in the gene expression and enzyme complex inside the cell (Kumar and Jadeja, 2016). It is hypothesized that *Actinomycetes* from different environments may have different characteristics with unique structural elements due to changes in their environment, including competition for survival, predation, available nutrients, light, oxygen, and pressure (Gupta et al., 2019). In this study, we showed that actinobacteria present in different environmental conditions have diverse characteristics and can form new species that produce new and biologically active compounds. In this research, *Amycolatopsis roodepoortensis* strain EA7 is one of the rare *Actinomycetes* and belongs to the *Pseudonocardiaceae* family. This strain is one of the important producers of antibiotics. The identification of this rare strain from the northern regions of Iran makes the soils of these regions very valuable because this can lead to the production of useful bioactive compounds for future pharmaceutical applications and fight against multidrug resistance and pathogens becoming resistant to antibiotics.

Data availability: *Amycolatopsis roodepoortensis* strain EA7 (GenBank accession number: OR680714) and *Streptomyces microflaveus* strain EA6 (GenBank accession number: OR680713).

Conflict of interest : No conflict of interest declared.

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