Isolation of *Actinomycetes* Indigenous to Guilan Province and Their Effect on Human Pathogens

Abstract

Actinomycetes are known as the largest reservoir 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 actinomycetes 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 done using *16S rRNA* sequencing and phylogenetic analysis. Antimicrobial activity was investigated against pathogenic microorganisms. A total of 14 isolates were identified.2 isolates with more antimicrobial properties were selected. Based on the results of phylogenetic studies and *16S rRNA* sequencing, *Amycolatopsis roodepoortensis* strain EA7 with 99.63% confidence, and *Streptomyces microflaveus* strain EA6 with 93.92% confidence were identified. The isolated bacteria had more antimicrobial activity against Gram-positive pathogenic microorganisms *Staphylococcus aureus* and standard sample *Staphylococcus aureus* PTCC 1112. This research is 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 roodepoortensis* strain EA7 from the northern regions of Iran makes the soils of these regions very valuable.

Keywords: actinomycete, antimicrobial activity, *16S rRNA*, *Amycolatopsis*, *Streptomyces*

Introduction

Actinomycetes are known as the largest reservoir of natural antibiotics in the world, but the abundance of discovery of Bioactive compounds is a new structure (Adamek et al., 2018). Actinomycetes, belonging to the order Actinomycetal, are members of a heterogeneous group of gram-positive bacteria that contain more than 55% GC content in their DNA (Peng et al., 2016). It is believed that actinomycetes are the source of about 61% of all bioactive substances derived from microorganisms that have been discovered so far (Song et al., 2021; Law et al.,2017). Among them, the genus Streptomyces has the largest share in the production of secondary metabolites, which is 16% of all important producers of antibiotics, mainly from *Micromonosporaceae* and with a smaller share of *Pseudonocardiaceae* and *Thermomonosporaceae* from "rare *actinomycetes*".

This shows that rare actinomycetes are a valuable source of new compounds, and improved isolation strategies are needed to increase their isolation frequency (Takahashi and Nakashima, 2018). Due to their ability to produce various antibiotics, anticancer compounds, and other compounds with therapeutic importance, considered the golden microorganisms of the 21st century (Ibnouf et al., 2022; Adamek et al., 2018).

Therefore, there is a need to continue the search for new microorganisms that are able to produce bioactive compounds that can combat emerging and resistant infectious pathogens. Actinomycetes are known as the most economically important microbes. Mainly because they can produce important medical and pharmaceutical products (Takahashi and Nakashima., 2018). The purpose of this study is to isolate actinomycetes and screen them to evaluate their antimicrobial activity and molecular and phylogenetic identification of this group of bacteria from the soil samples of Alborz Mountain in northern Iran. That is why, in the current study, we isolated and screened actinomycetes from the soil samples of Alborz Mountain in northern Iran.

Materials and Methods

Soil sampling

A total of 15 soil samples from 3 different places in Alborz mountains (Roodbar city), in geographical coordinates (east 49/5292° and north 36/8767°) from agricultural areas were collected from a depth of 15 cm with a shovel after discarding the top layer of soil. The samples were placed in clean, sterile containers with lids and immediately transferred to the laboratory (Tan et al., 2019). In the separation step, successive dilution was used 10^{-1} to 10^{-7} dilutions (1 to 7) were prepared from the soil. In this way, 1 gram of dried soil samples was added to 9 ml of sterile distilled water and after pipetting, 1 ml of each of dilutions 3 to 7 was transferred to the starch casein agar (SCA) culture medium by pipette. and it was cultivated as a continuous streak (Kalaba et al., 2021). Tetracycline and nystatin antibiotics were used to prevent contamination. The plates were incubated for 4 days in an incubator at a temperature of 28°C (Law et al., 2017). After incubation, to determine the morphological characteristics of the selected isolates, they were inoculated in the standard culture medium ISP2 (Yeast Malt Extract Agar) for 5 days at a temperature of 28°C (Ibnouf et al., 2022).

Isolation and identification of *actinomycete* strains

The isolated strains were selected by standard microbiology method in terms of morphology, biochemical, and fermentation of sugars. For primary diagnosis, warm staining methods, soil smell, and colony observation were used. All isolated actinobacterial strains were compared with actinobacterial morphology presented in Bergey's manual of systematic bacteriology for possible identification of isolates (Ranjitha and Ravishankar, 2017). Antibiotic sensitivity test against commercial antibiotics (ampicillin, tetracycline, vancomycin, chloramphenicol, imipenem, ceftazidime, piperacillin, and ciprofloxacin) was investigated using the Kerby-Bauer disc diffusion method (Fahmy et al., 2021). Bacterial cultures were spread on Muller-Hinton Agar (MHA) plates with 0.5 McFarland turbidity. Eight antibiotic discs were placed on the inoculated plates. They were incubated for 2 days at 28°C. The diameter of the halo around the disk indicated sensitivity to antibiotics and vice versa. It was measured in millimeters (Ansari et al., 2019). The ability of superior strains to grow at different temperatures (27-37°C), different pHs (4-10), and the ability to tolerate sodium chloride at different concentrations (0-7%) were 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 together with 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. (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 assay of actinomycete isolates was performed by well diffusion agar method on Mueller Hinton agar medium. First, from the tested pathogens, 0.5 McFarland's turbidity was prepared and cultured on Mueller Hinton agar medium with sterile swap. Then, wells were created on the medium with a sterile dropper and 50 µL of actinomycete strains grown (0.5 McFarland turbidity) in TSB broth medium were transferred to the wells. Then they were incubated in an incubator at 37°C for 24-48 hours. After this time, the result was reported (Oliveros et al., 2021). Based on the presence and absence of the zone of inhibition, the actinomycete with the most antimicrobial activity was selected for further studies.

Molecular identification using *16S rRNA* gene sequencing

Molecular identification of isolated isolates was done using *16S rRNA* gene sequencing and phylogenetic analysis (Tan et al., 2019). In order to extract the DNA of the selected isolates, a DNA extraction kit (Sinaclon kit) was used. Extraction was done by the mini-column method. Actinobacteria general primers (Table 1) were used to isolate and amplify the *16S rRNA* gene (Osama et al., 2022). After ethidium bromide staining, polymerase chain reaction (PCR) amplification was detected using agarose gel electrophoresis (Tan et al., 2019; Li et al., 2021).

The program used in the PCR of actinomycetes isolates was thirty-five cycles as described below (Table 2). After completing the reaction steps and confirming the obtained bands by agarose gel electrophoresis, the reaction product was sent to Pishgam Biotechnology Company for sequencing. Then, the results obtained from sequence alignment were compared with the sequences recorded in the NCBI database using the BLAST program (www.ncbi.nlm.nih.gov/blst). The similarity of *16S rRNA* gene sequences and the phylogenetic tree of these isolates were drawn by the Neighbor-joining method using the NCBI database and BLAST program (Fahmy et al., 2021; Tan et al., 2019).

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, and 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. It can use several carbon sources such as glucose, xylose, mannitol, raffinose, and arabinose. But 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. The 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 given in the table below (Table 4).

Pathogenic bacteria *Staphylococcus aureus*, cocci, catalase positive, oxidase negative, coagulase-positive, growth in mannitol salt agar medium and *Pseudomonas aeruginosa* as gram-negative bacteria, rods, catalase positive, oxidase positive, non-fermenting, motile, able to use citrate and They grow at 42°C.

Antimicrobial assay of *actinomycete* isolates

The isolates were screened for antimicrobial activity. Due to the distinct antimicrobial activity, 2 samples were selected as strains EA7 and EA6 for further identification and phylogenetic investigation. Because they showed broad-spectrum antimicrobial activity. In the stage of the antimicrobial assay by Well diffusion agar method, two strains EA7 and EA6 showed more antimicrobial properties. strains EA7 and EA6 showed the diameter of the antimicrobial halo against *staphylococcus aureus* and the standard strain of *staphylococcus aureus* PTCC 1112 of 25 and 15 mm, respectively. Against *Pseudomonas aeruginosa*, no halo was observed or the diameter of the halo was small (Fig. 4). They were selected as two indicator strains in terms of antimicrobial effect.

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. Also, the alignment of these sequences with the sequences recorded in the NCBI data bank showed that strain EA7 is *Amycolatopsis roodepoortensis* with 99.63% confidence and strain EA6 is Streptomyces microflaveus with 93.92% confidence. Two strains named *Streptomyces microflaveus* strain EA6 and *Amycolatopsis roodepoortensis* strain EA7 were named. The results of PCR and the relationship of *Amycolatopsis roodepoortensis* strain EA7 with other *Amycolatopsis* species and *Streptomyces microflaveus* strains EA6 with other *Streptomyces* species using the Neighbor-joining method are shown in Fig. 5, 6, 7 respectively. Gene registration of these isolates was done as follows.

Discussion

Investigating and discovering new microorganisms that produce new secondary metabolites can be essential for an effective role in the competition with new, increasing diseases and antibiotic-resistant pathogens (Kisil et al., 2021). Actinomycetes are remarkable as a rich source of secondary metabolites, in addition, they play a vital role in the decomposition of organic matter (Kurnianto et al., 2021).

The diversity of actinomycetes and their capacity to produce new materials places this category in a remarkable position. In the last two decades, there has been a decline in the discovery of new vital compounds from soil-derived actinomycetes, which have produced a large number of previously described secondary metabolites (Chen et al., 2018). As a result, this gives rise to new actinomycete species from unusual environments, which in turn creates a new era for medicinal specialists (Kalaba et al., 2021). *Actinomycetes* produce a wide range of biologically active compounds such as 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.2 strains showed more antimicrobial activity against the studied pathogens. These isolates were able to grow in the 3 environments tested (ISP2, SCA, MHA), but showed the most and most appropriate growth in SCA and ISP2 agar environments for the production of antimicrobial compounds. These strains showed broad-spectrum antimicrobial activity against Gram-positive bacteria. It can be said that the antimicrobial effect of the desired strains has a higher effect against gram-positive bacteria. Out of 14 isolates of *actinomycetes*, only strain EA7 was highly resistant to eight commercial antibiotics (including ampicillin, penicillin, chloramphenicol, tetracycline, piperacillin, imipenem, ceftazidime, and ciprofloxacin). While other isolates showed the lowest resistance with a maximum halo diameter of 20 mm.

Other research (Alam and Jha, 2019; Shaik et al., 2017) showed the antibiotic resistance of soil actinomycetes, *Amycolatopsis Balhimycina,* and *Amycolatopsis orientalis* against 4 antibiotics (ampicillin, penicillin, chloramphenicol, tetracycline) as well as antimicrobial activity in They have shown methicillin-resistant *Staphylococcus aureus* strains. which is consistent with the results of our research.

Ansari et al.'s study (Ansari et al., 2019) confirmed the antibiotic resistance 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 another study, Gram-negative bacteria were more resistant to the antimicrobial effect produced by actinomycetes compared to Gram-positive ones (Fahmy et al., 2021).

In research, it was shown that gram-negative bacteria have more resistance to antimicrobial effects compared to gram-positive ones, and in the case of *Staphylococcus aureus*, the diameter of the halo was greater than that of *Pseudomonas aeruginosa* (Abd-Elnaby et al., 2016).It can be attributed to the different structure of the outer membrane in gram-negative bacteria that have lipopolysaccharide compounds, which leads to their impermeability against antimicrobial substances (Nikbakht et al., 2021). It is important to obtain data from both methods based on *16S rRNA* gene sequencing and biochemical characteristics in order to provide a suitable method for the classification of prokaryotes, especially for the genera *Amycolatopsis* and *Streptomyces* in actinomycetes (Osama et al., 2022). Studies have shown that the species in the genus *Amycolatopsis* with the species in the genus *Streptomyces*, which have the similarity of the Yala *16S rRNA* gene, may still show very different phenotypic characteristics according to biochemical characteristics and patterns of carbon source utilization (Sharma and Manhas, 2019). Molecular technique is a common and accurate technique for identifying microorganisms. Among the different molecular methods, measuring *16S rRNA* sequences is a very powerful tool in the classification of *Streptomyces*. Therefore, in this research, in order to identify the isolated bacteria and in-depth evaluation of the biochemical and physiological characteristics of the isolated strains, with the aim of providing a better understanding, the *16S rRNA* gene sequencing method was used (Tan et al., 2019). The *16S rRNA* gene sequence has a highly conserved sequence. So, in bacteria of a very similar species, in bacteria that are in the same strain and there is almost a hundred percent identical sequence, as a result, it is very suitable as a target gene for DNA sequencing in samples containing thousands of different species (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 Psuedenocardiace 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), *Streptomyces microflaveus* strain EA6 (GenBank accession number: OR680713).

CONFLICT OF INTEREST: No conflict of interest declared.

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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 (^{O}c)	level	
1	2	95	denaturation	
33	30 Second	95	denaturation	second stage
	30 Second	55	Annealing	
	45 Second	72	Extension	
1	5	72	The third stage (final extension)	

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

Biochemical tests	ACT 1	ACT 2	ACT 3	ACT 4	ACT 5	ACT 6	ACT 7	ACT 8	ACT 9	ACT 10	ACT 11	ACT 12	ACT 13	ACT 14	STS
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 yellow	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 10mg	11	12	12	13	14	9	R	10	R	R	12	15	11	10	7
Tetracycline 30 mg	11	14	15	15	16	10	R	R	R	10	12	R	11	16	10
Vancomycin 30 mg	21	22	18	21	22	10	R	10	R	10	12	18	12	16	28
Chloramphenicol30mg	15	16	15	15	17	13	R	10	R	R	14	14	13	16	10
Imipenem10 mg	13	14	13	14	12	R	R	R	11	13	12	11	14	12	R
Ciprofloxacin 5 mg	22	19	18	21	21	22	16	17	19	19	21	22	21	23	22
Ceftazidime 30 mg	18	19	17	18	18	R	R	10	15	15	15	17	14	18	R
Piperacillin100 mg	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	+	+	+	+	-	-	-	-	-	+	-	-	+	+	+

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^{0}C	+

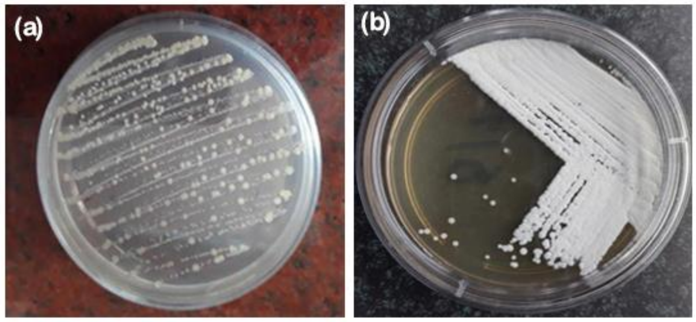

Fig. 1 Macroscopic shape of (a) EA7, and (b) EA6 strains

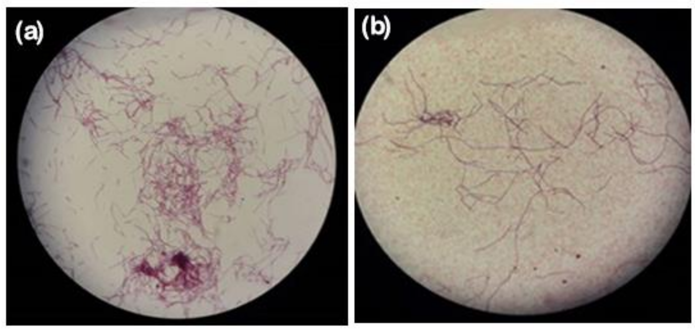

Fig. 2 Microscopic image of (a) EA7, and (b) EA6 strains

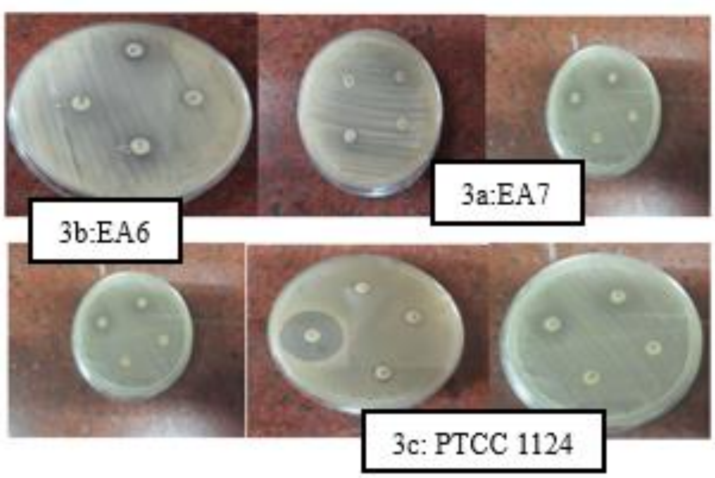

Fig. 3 Antibiotic sensitivity test of sample isolated from soil and standard sample

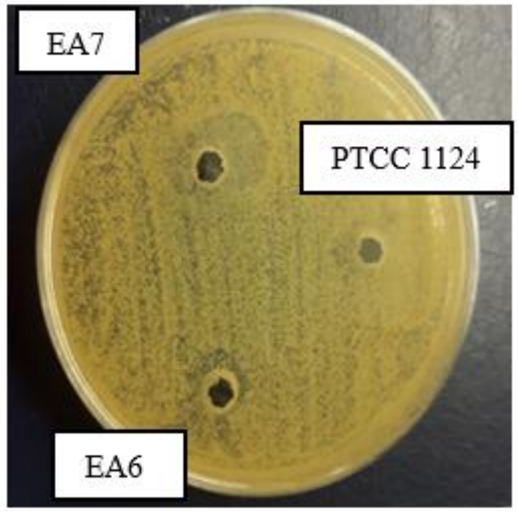

Fig. 4 Antimicrobial assay of actinomycete isolates

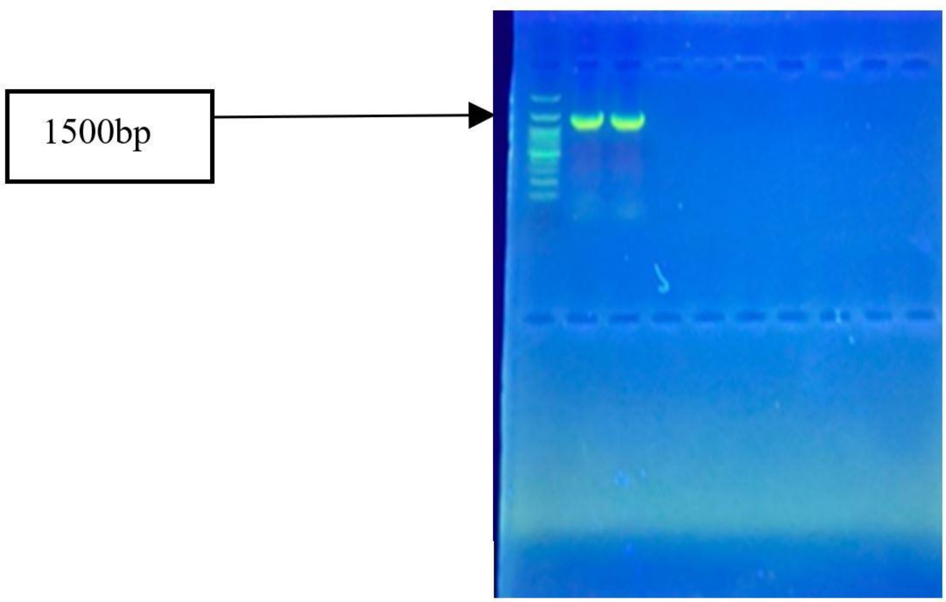

Fig. 5 PCR product electrophoresis results on agarose gel

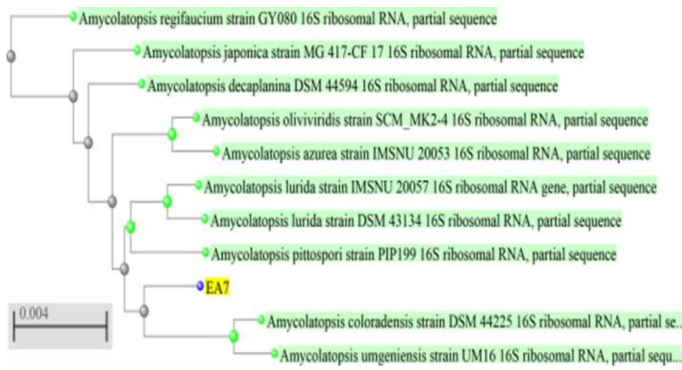

Fig. 6 Phylogenetic tree of strain EA7 based on *16S rRNA* gene sequences with other *Amycolatopsis* species using the Neighbor-joining method. Bootstrap values were expressed as a percentage of 1000 repetitions. Bar, 0.004 substitutions per nucleotide position.

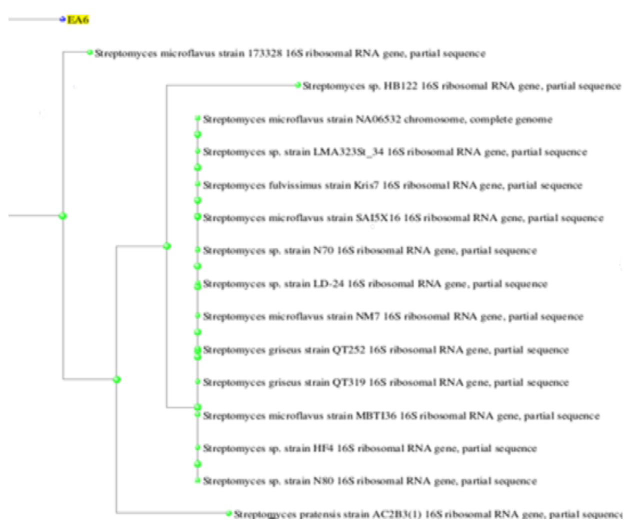

Fig. 7 Phylogenetic tree of strain EA6 based on *16S rRNA* gene sequences with other *Streptomyces* species using the Neighbor-joining method. Bootstrap values were expressed as a percentage of 1000 replicates and a bar of 0.002 substitutions at each nucleotide position.