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Isolation, Identification and Molecular Characterization of Nocardiopsaceae Producing Novel Antibiotics From Soil Samples in Bingol, Turkey

Samer Abuzerr (Corresponding author)

School of Public Health, Department of Social and Preventive Medicine, University of Montreal, Montréal, Canada

Quality Improvement and Infection Control Unit, Ministry of Health, Gaza, Palestine

Kate Zinszer

School of Public Health, Department of Social and Preventive Medicine, University of Montreal, Montréal, Canada

Syamand Ahmed Qadir

Medical laboratory science department, Sulaimani Polytechnic University, Sulaymaniyah, Iraq

Ekrem Atalan

Molecular Biology and Genetic Department, Inonu University, Malatya, Turkey

Halgord Ali M. Farag

Nursing Department, Sulemani Polytechnic University (SPU), Kurdistan, Iraq

Abdel Hamid el Bilbeisi

Department of Clinical Nutrition, Faculty of Pharmacy, Al Azhar University of Gaza, Palestine

Kawa Khwarahm Hamafaraj

Nursing Department, Sulemani Polytechnic University (SPU), Kurdistan, Iraq

Rima Naser

Medical analysis department, Faculty of medicine and health, Jordan University, Amman, Jordan

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Miran Husein Qader
College of agricultural engineering science, University of Raparin, Iraq

Alshaarawi Salem
Optometry and vision sciences,University of Minho, Portugal

Amany Al Afifi
Faculty of Pharmacy, Al Azhar University, Gaza, Palestine

Salem Abu Musleh
College of Basic Sciences, Department of Physics, University of Palestine, Gaza, Palestine;
National Research Center, Director of Applied Sciences, University of Palestine, Gaza, Palestine

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Abstract

There is growing interest in the antimicrobial discovery of life-threatening multidrug-resistant pathogens. The study was undertaken to isolate, identify, and characterize antibiotic-producing *actinomycetaceae*, particularly *nocadisosaceae*, from soil samples of Bingol, Turkey. Soil samples were collected from three different regions of Bingol, Turkey. The physicochemical analysis of the soil samples was immediately measured using standard methods. This was followed by isolation of *Nocardiopsaceae*, nutritional tests, chemotaxonomic analysis, and molecular characterization. The isolated organisms showed morphological properties consistent with the Nocardiopsaceae soil bacteria. The 16s rDNA gene sequence indicated a similarity between the strains with 99.86% which was *Nocardiopsaceae synnemata-formans*. The BLAST hits had a significant e-value of 0.005. The results of the present study revealed that soil Nocardiopsaceae of Bingol appears to have immense potential as a source of antibacterial compounds.

Keywords: *Actinomycetaceae*, *16s rDNA*, Isolation, Identification, Molecular characterization

1. Introduction

In recent years, there are many studies about the biological activities of secondary metabolites isolated from marine microorganisms, and these studies mainly focus on the antibacterial, cytotoxic, antioxidation, and antiviral, immunosuppressant activities (Amann et al., 1995, pp. 143-169). *Actinomycetaceae* have important ecological roles besides their significant influence on human health. The family of *Nocardiopsaceae* has been defined on phylogenetic grounds (Rainey et al., 1996, pp. 1088-1092).

Nocardiopsaceae are aerobic, Gram-positive, non-acid-fast which form an extensively branched substrate mycelium, which may bear single spores, sometimes in clusters or spore chains that terminate in *pseudosporangium*. e.g., *Nocardiopsis* (Sindhuphak et al., 1985, pp. 1332-1334), *Streptomonospora* (Goodfellow et al., 2012), *Haloactinospora* and *Thermobifida* (Golinska et al., 2013, pp. 1079-1088). The aerial hyphae of *Nocardiopsis* strains are either long and moderately branched, straight and flexuous or irregularly zig-zagged, completely fragmenting into an oval to elongated, smooth-surfaced spores rod-shaped (Chen et al., 2008, pp. 699-705; Hozzein & Goodfellow, 2008, pp. 2520-2524; Cui et al., 2001, pp. 357-363). Mycolic acids are absent in their cell walls of *Nocardiopsis* (Sindhuphak et al., 1985, pp. 1332-1334).

The natural habitat of most described strains is soil (Goodfellow & Fiedler, 2010, pp. 119-142). Species of the genus *Nocardiop Nocardiopsis* have also been found in an Antarctic glacier, in marine sediments (Sabry et al., 2004, pp. 453-456), plant rhizosphere soil [14], animal guts (Vasanthi & Hoti, 1992. pp. 312-317) Plant tissue (Qin et al., 2009, pp. 6176-6186) indoor environments (Peltola et al., 2011, pp. 4293-4304) clinical material (Bernatchez & Lebreux, 1991, pp. 174-175; Yassin et al., 1977, pp. 983-988). Novel species of *Nocardiopsaceae* are increasingly being reported, for instance, *Nocardiopsaceae oceani* and *Nocardiopsaceae nanhaiensis* (Pan et al., 2015, pp. 3384-3391) *Nocardiopsaceae mangrovei* (Huang et al., 2015, pp. 1541-1546) *band Nocardiopsaceae algeriensis* (Bouras et al., 2015, pp. 313-320).

In Turkey, no significant studies have been conducted so far to isolate and evaluate *Nocardiopsaceae* from different soils that could produce useful antibiotics. Therefore, the present study is intended to isolate, screen, and characterize antibiotic producing Nocardiopsaceae from soils of Bingol, Turkey.

2. Method

2.1 Study Setting and Sampling Method

Actinomycete strains were isolated from three agricultural soil samples B002, B014, and B016. Soil samples were collected from three different regions of Bingol, Turkey. The samples were taken at a depth of 15 cm then put in a firmly closed labeled sterile container then transferred to the laboratory. The physicochemical analysis of the soil samples was immediately measured using standard methods. This was followed by isolation of *Nocardiopsaceae*, nutritional tests, chemotaxonomic analysis, and molecular characterization according to analytical standard methods (Reed & Cummings, 1945, pp. 97-105).

2.2 Isolation of Nocardiopsaceae

One gram of soil sample was weighed accurately and aseptically put in 9 ml of sterile Ringers solution ¼ strength (Sigma) and agitated by a vortex. The test tube was pretreated using a water bath at 55°C for 10 minutes. The dilution re-agitated again by the vortex for a few seconds. To isolate the *Niocariopsis* and total *actinomycetaceae*, 0.1 ml of each dilution was aseptically inoculated to plates containing sterilized starch casein agar medium (Küster & Williams, 1964, pp. 928-929) and raffinose–histidine agars spread evenly (WILLIAMS, 1984, pp. 553-561). Both Media were supplemented with cycloheximide and nystatin to inhibit fungi and unwanted growth of other bacteria. The desired colonies were distinguished from other bacterial species according to colony morphology, pigmentation. Isolates were stored in a sterile 2 ml Eppendorf tube containing 20% glycerol after coding based on locality. Desired pure colonies were stock at -80ºC in a modified Bennett's agar plates.

2.3 Nutritional Tests for Specified Microorganisms

Forty nutritional tests were carried out including the capability of strains to use different carbon sources, nitrogen compounds for growth and energy, biochemical hydrolysis of urea by testing their ability to Nitrate and H_2S production, degradation of sterilized xanthine, casein, starch and gelatin, temperature susceptibility and capability of isolates to grow in different chemical inhibitors, antibiosis, antimicrobial activity. Besides, their antimicrobial action against six different pathogenic bacteria including Gram-negative bacteria (*Escherichia coli*, *Pseudomonas fluoresces*, and *Klebsiella pneumonia*), Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and fungi (*Candida sp.*) was evaluated.

2.4 Chemotaxonomic Analysis

Each sample of B002, B014, and B016 was inoculated in glucose yeast malt extract broth (ISP6) in shaken flask 180 rpm at 28°C for 1 week for chemotaxonomic study. Thin-layer chromatography following the method of [31, 32] was employed to characterize the samples using diaminopimelic acid (DAP) and a mixture of stereoisomers (Meso > > DD approximately to LL. N-Hydroxy-A2pm). Moreover, the hierarchical cluster analysis dendrogram was used to assess the phylogenetic relationships among three test strains of *Nocardiopsaceae* regarding the base sequence of the 16s rDNA gene.

2.5 Molecular Characterization of Test Strains

Each sample of B002, B014, and B016 was subjected to DNA isolation according to Pitcher's method [33]. Guanidine thiocyanate and DNA isolation kit (Invitrogen, Pure Link (R) Genomic DNA Kit) were used to lyse cells and microorganisms particles in DNA extractions. Agarose gel electrophoreses were used for adopting total genomic DNA. 1% agarose gel (60 ml 1XTBE buffer, 0.6 g agarose) supplemented with ethidium bromide 4 μl was prepared. Pure DNA was obtained from the organisms and the region encoding 16s rRNA gene amplified by two universal primers 27f, (5'-AGA GTT TGA TCM TGG CTC AG-3) and 1525R, (5'- AAG GAG GTG WTC CAR CC-3 ') (Lane 1991). Stock solutions were prepared for the PCR reaction with sterile double-distilled water (ddH_2O). Stock solutions were separated in sterile Eppendorf tubes in small quantities 25-100μl to remove the risk of

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contamination and stored at -20°C until the time of use. Polymerase chain reaction procedures of 16s rDNA were performed in a Thermal Cycler (MyGenie-96 Gradient Thermal Cycler), (Korea) in a 0.2 ml PCR tube. After the preparation of 50 µl PCR mixture, three different PCR programs including denaturation, annealing, and extension with arranged temperatures and time were applied in this study. Pure PCR products whose bands were proven by agarose gel electrophoresis were sent to the MacroGen Inc in order to sequence the 16s rRNA gene region.

3. Results

3.1 Isolation of Nocardiopsaceae

Nocardiopsaceae was isolated and the morphological appearance of isolates is shown in

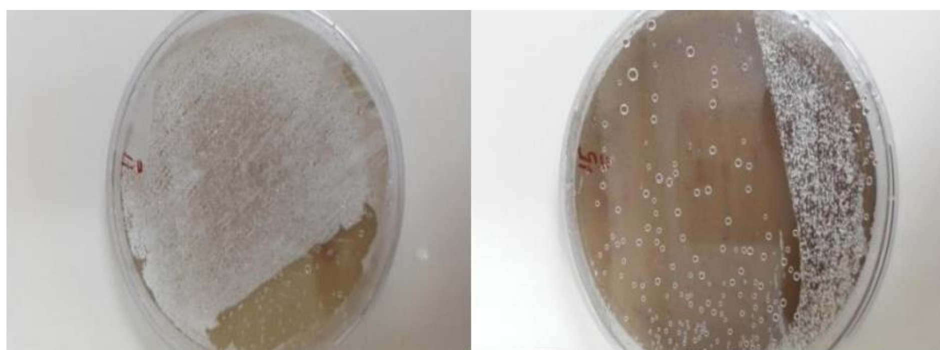

Figure 1. Nocardiopsaceae strains growth on 10 days incubated Bennett's agar plates at 25ºC

3.2 Physicochemical Properties of Soil Sample

The percentage of organic matter in the soil samples was 16.5% (B002), 12.4% (B014), and 18.6% (B016), and the PH values were 7.8 (B002), 7.9 (B014), and 7.8 (B016).

3.3 Phenotypic Characterization

The results of the forty tests including biochemical test, nutritional test degradation, susceptibility test, chemical inhibitor, antibiosis, antimicrobial activity, and growth tests are presented in Table 1. While the outcome of the antibiotic resistance tests, H2S production by test strains, and antimicrobial activity test strains against pathogenic bacteria and fungi are shown in Figure 2a, b, c respectively.

Table 1. Nutritional tests for identifying Nocardiosaceae from the soil sample

Biochemical test		**Sample**	**H2S production**	**Nitrate reduction**	**Urea hydrolysis**				
		B002	-	-	-				
		B014	-	-	-				
		B016	+	-	-				
Nutritional	**Growth on sole carbon source (1%, w/v)**	**Sample**	**Dextran**	**Fructose**	**Lactose**	**Raffinose**	**Sucrose**	**Maltose**	**Mannitol**
		B002	-	-	-	-	-	-	+
		B014	-	+	-	-	-	+	+
		B016	-	+	+	-	-	+	+
Nutritional	**Growth on sole carbon source (0.1%, w/v)**	**Sample**	**Sodium acetate**		**Sodium citrate**		**Sodıum Propıonate**		
		B002	-		-		-		
		B014	-				-		
		B016	-		-		-		
Nutritional l tests	**Growth on sole nitrogen source**	**Sample**	**Hıstıdıne**	**KNO_3**	**Tyrosine**				
		B002	-	-	+				
		B014	-	-	-				
		B016	-	-	-				
Degradation		**Sample**	**Xanthine**	**Casıen**	**Starch**	**Gelatin**	**Lecithin's activity**	**Lipolysis activity**	
		B002	+	-	-	+	+	+	
		B014	+	+	+	+	+	+	
		B016	-	-	-	-	+	+	
Tolerance tests	**Resistance to chemical inhibitors**	**Sample**	**Phenol 0.1%**	**Sodium azide 0.01%**		**Sodium chloride 7%**		**Crystal violet 0.0001%**	
		B002	-	+		+		-	
		B014	-	+		+		-	
		B016	-	+		+		-	

		Sample	25°C	30°C	50°C			
Tolerance	Resistance to temperatures	B002	-	+	+			
		B014	-	+	+			
		B016	-	+	+			
Tolerance	Resistance to antibiotics	Sample	Ampicillin (20 mg)	Vancomycin (30 mg)	Penicillin (30 mg)	Gentamycin (10 mg)	Rifampin (5 mg)	Erythromycin (15mg)
		B002	-	+	-	-	-	+
		B014	-	-	+	+	+	-
		B016	-	-	-	-	+	+
Tolerance tests	Antimicrobial activity tests	sample	*Escherichia coli*	*Pseudomonas fluorescens*	*Bacillus subtitous*	*Klebsiella pneumoniae*	*Staphylococcus aureus*	*Candida sp.*
		B002	-	-	+	+	+	+
		B014	+	-	+	+	+	+
		B016	-	-	-	-	+	-

Note. + and – denote positive and negative, respectively

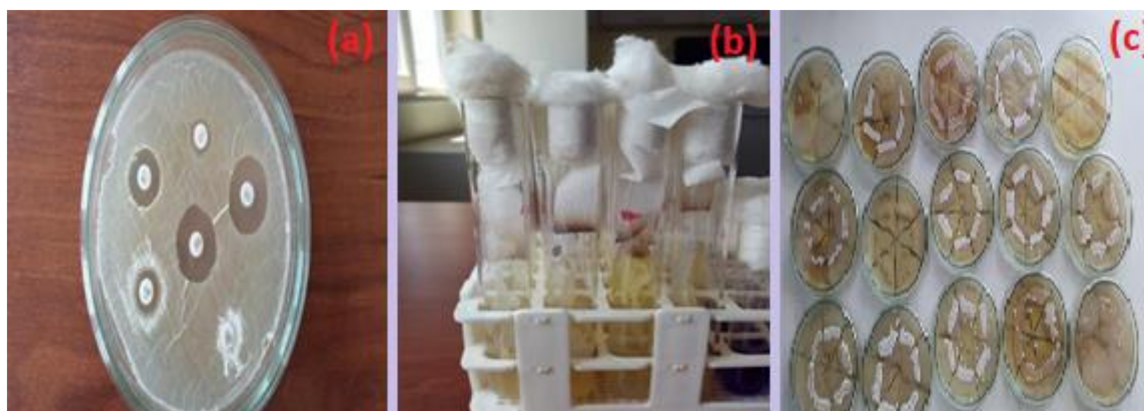

Figure 2. Antibiotic resistance tests (a), H_2S production by test strains (b) and Antimicrobial activity test strains against pathogenic bacteria and fungi (c)

3.4 Genomic DNA

The true amounts of genomic DNA in the B002, B014, and B016 soil samples are presented in Figure 3, which showed clear genomic DNA bands, no nucleic acid degradation, or genomic DNA.

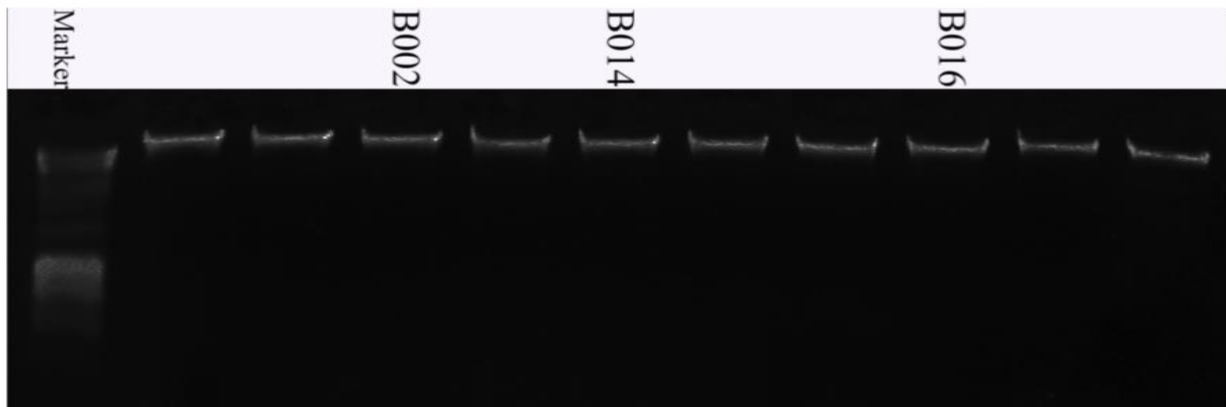

Figure 3. Genomic DNA bands of the three test strains on 1% agarose gel electrophoresis image

3.5 Polymerase Chain Reaction (PCR)

The amplified 16s rDNA gene region with the Gradient PCR using universal primers 27f and 1525r from the genomic DNA of three test strains showed that the average of the 16s rDNA region sizes was 1500 base pairs.

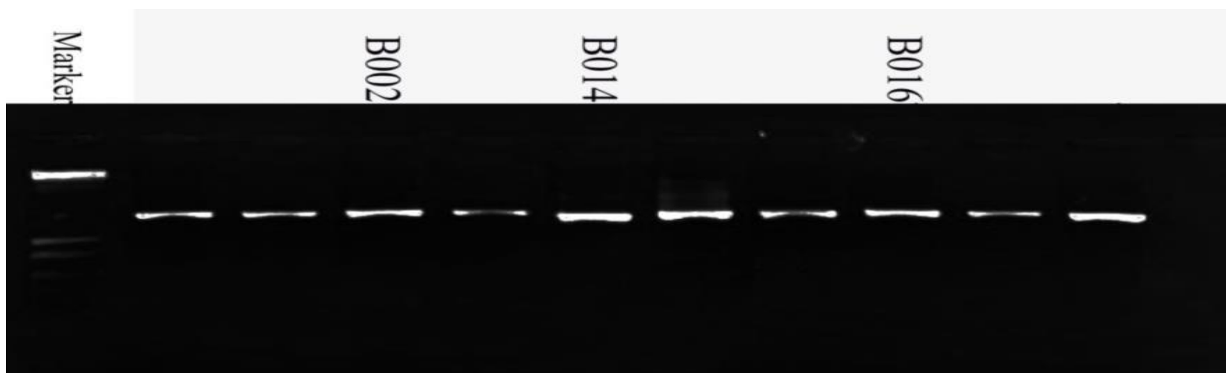

Figure 4. The amplified 16s rDNA gene region of the three test strains using Gradient PCR and using 1.5% agarose gel electrophoresis

3.6 16s rDNA Ribosomal DNA Sequence and Phylogenetic Tree Analysis

The identified sequence showed 99.86% similarity with the gene cluster sequences of Streptomyces species available from the GenBank database. The BLAST hits had a significant e-value of 0.005 (Figure 5).

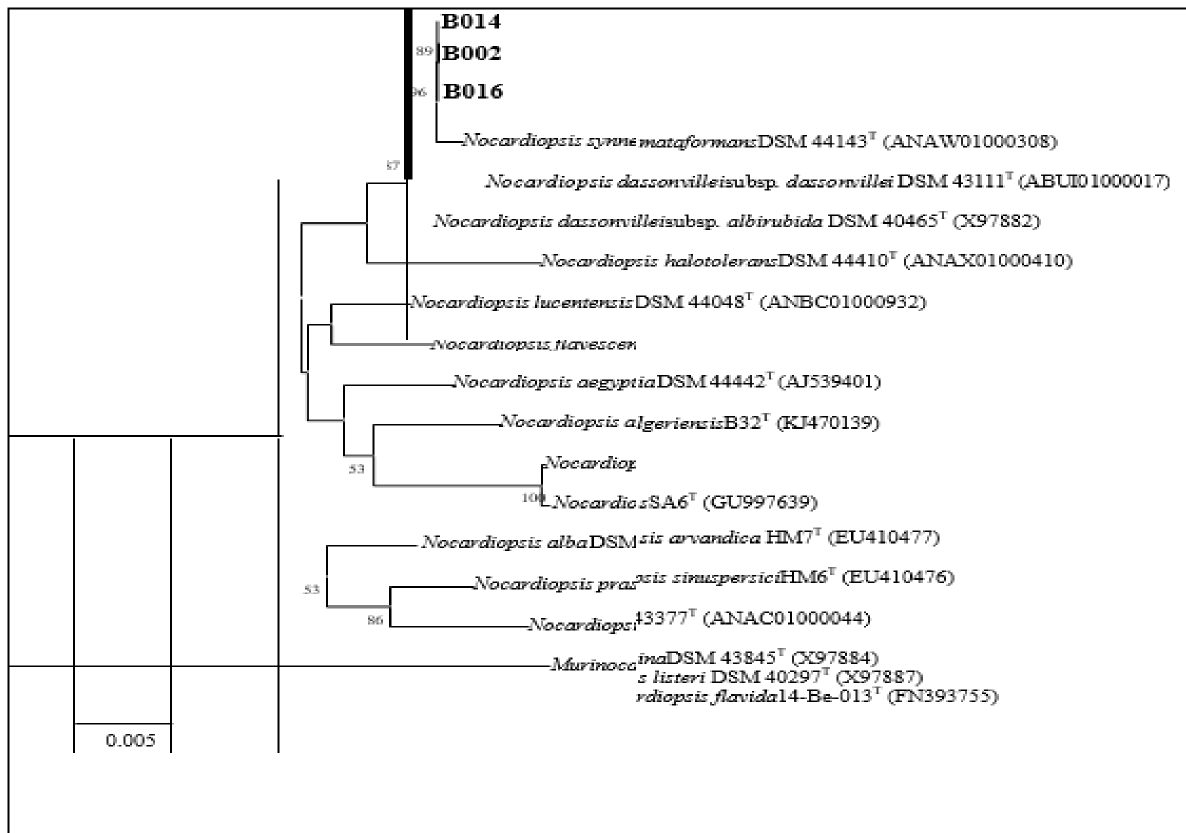

Figure 5. Phylogenetic dendrogram showing the relation of three test strains of Nocardiopsaceae regarding the base sequence of the 16s rDNA gene

3.6.1 Diaminopimelic Acid Test

The DAP type in the cell wall of test strains was recognized, where the spots were determined when the position of the bands formed in one-dimensional thin-layer chromatography (TLC) compared with the standard A2pm solution. A one-dimensional TLC showed that the strain B014 contained Mezo-A2pm, which was identified as *Nocardiopsaceae genus* from 16S rDNA analyses (Figure 6).

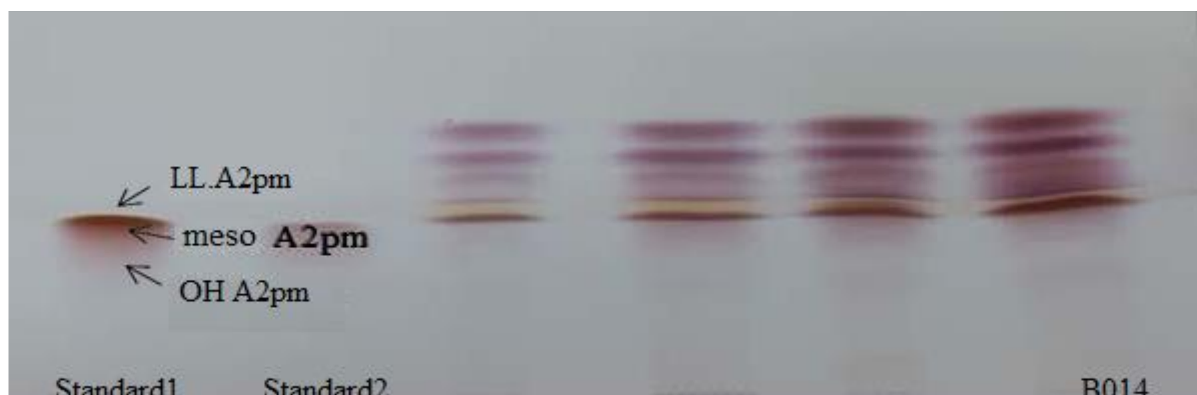

Figure 6. One-dimensional thin-film chromatogram of A2pm isomers of B014 isolate. Two Standard: A2pm (Diaminopimelic acid DAP) – Sigma

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3.6.2 Sugar Analysis

The whole-cell sugar profile in the cell wall chemo-type was determined by comparing two standards that contain seven sugars for test microorganisms in one-dimensional thin-layer chromatography. The results showed that isolates of B014 belonged to *Nocardiopsaceae* that contain galactose and ribose (Figure 7).

Figure 7. One-dimensional thin-layer chromatogram of the sugar profile of one test isolate. Std1=standard 1, Gal=galactose, Ara=arabinose, and Xyl=xylose, Std 2=standard 2, Glu=glucose, Man=mannose, Rib=ribose, and Rham=rhamnose

4. Discussion

Actinobacteria grow in a wide range of environments and they can grow on naturally occurring substrates (Mao et al., 2011, pp. 621-630). In this study, polyphasic taxonomy, which is a genomic, chemotaxonomic, and phenotypic method, was carried out to identify and characterize strains isolated from soil samples of Bingol, Turkey. The physicochemical characteristics of soil samples were within the normal levels and consistent with the European countries' soil (Atalan, 1993). Polyphasic taxonomy is an important approach to identify bacteria at the species level (category) owing to base on three different methods (Coenye et al., 2005, pp. 147-167). Last two decades, before using PCR and DNA sequencing, 16s rDNA sequencing was a widespread technique to identify and characterize microorganisms.

The 16s rDNA sequencing, in particular, has played a pivotal role in the accurate identification of bacterial isolates and the discovery of novel bacteria in microbiology laboratories. The technique helps microbiologists to identify tested organisms accurately. Meantime it helps clinicians in choosing antibiotics and infection control procedures (Woo et al., 2008, pp. 908-934). 16s rDNA gene is stable, conserved, and impossibility of gene transfer. Nevertheless, it is being used in taxonomic study and identification of bacteria (Ramasamy et al., 2014, pp. 384-391).

Antibiotics are one of the most important commercially exploited secondary metabolites produced by the bacteria and employed in a wide range. Therefore, molecular characterizations and novel discovery of *Nocardiopsis* in soil have a promising future for the production of antibiotics. As evidenced by genotypic, chemotaxonomic, and phenotypic results, test strains B002, B014, and B016 were identified as *Nocardiopsis synnemataformans*

sp. nov. Test strains B002, B014, and B016 may be a new species where the percentage of the similarity with their type strain was 99.86%. To confirm this result, DNA-DNA homology is suggested to be carried out with their type strain. Isolated species may have the potential for biotechnological products such as antibiotics and enzymes into new isolate of virgin soil. They could be scan for new antibiotics or other biotechnology in industrial compounds.

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