IJSRM
INTERNATIONAL JOURNAL OF SCIENCE AND RESEARCH METHODOLOGY
An Official Publication of Human Journals

Human Journals
Research Article
May 2018 Vol.:9, Issue:3

Isolation and Characterization of Nocardia from Laiha Cave Soil in Hadhramout of Yemen as a Source of Antibiotic

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Submission: 19 April 2018
Accepted: 25 April 2018
Published: 31 May 2018

HUMAN JOURNALS
www.ijsrm.humanjournals.com

Keywords: *Nocardia asteroids*, Cave soil, Antibacterial, Antibiotic and Pathogenic bacteria.

ABSTRACT

Introduction: Actinomycetes are indigenous to soil and other extreme environments resembling both bacteria and fungi in their morphology. They are highly exploited for antibiotics and other bioactive compounds since ages. **Methods:** An actinomycete was isolated from the soil sample of Laiha cave, Hadhramout, Yemen and was identified as *Nocardia asteroids* based on morphology, physiology and biochemical properties. Its antibacterial activity was tested against both Gram-positive (*Escherichia coli* and *Pseudomonas aeruginos*a) and Gram negative (*Staphylococcus aureus* and *Bacillus subtilis*) bacteria through agar disc diffusion method. The isolate was screened for various enzyme activities-amylase, chitinase, urease, gelatinase, caseinase, DNase, keratinase, lipase, pectinase, lecithinase and catalase. **Results:** *Nocardia asteroids* was an aerobic, non-motile, partially acid-fast, rod-shaped, beaded, branched filamentous bacterium. The colony was irregular, hard and grew at 120h. The spore was straight chain, loop flexible and brown. It grew well in Starch Casein Agar (SCA), Nutrient Agar (NA), SDA, Potato Dextrose Agar (PDA), Yeast-Malt Extract Agar (YMA), Glycerol Asparagine Agar(GAA), Czapeck's Dox Thom Agar (CDTA), Tyrosine Agar (TA), Glycerol Yeast Extract Agar (GYA) and Starch Yeast Extract Agar (SYA) media having a range of substrates and produced aerial mycelia with relatively few diffusible pigments. The isolate showed broad spectrum antibacterial activity against all tested bacteria. The isolate also exhibited positive results for catalase and urease enzymes, with varied results for other enzymes. The strain was resistant to most antibiotics and sensitive to gentamycin at 10µg, nalidixic acid, tetracycline and novobiocin at 30 µg each. **Conclusion:** *Nocardia asteroids* showed antibacterial activity against various pathogenic bacteria. The strain may be further validated to authenticate its potentiality for the elaboration of antimicrobials and enzymes as its bioactive principles.

1. INTRODUCTION

Actinomycetes (Nocardia) produce many secondary metabolites including various biologically active compounds such as antimicrobial, cytotoxic and immunosuppressant agents. As the ability to isolate new antibiotics useful from Streptomyces sp. is now low and shrinking all the time (Berdy,2005), our focus is to search for other sources such as caves which are considered novel and poor environments for microbes(Barton, 2006).

Isolation of Nocardia from caves is limited and also their antibiotic- production because the environment is limited with organic materials. It is also difficult to isolate microbes from these caves in Hadhramout as these caves are with solid rocks with very small quantities of soil. The isolates may be considered among the rare actinomycetes and represent a reliably unexplored resource for the discovery of new biologically active compounds. Many isolates of actinomycetes from caves in Hadhramout, Yemen have been collected and identified as Nocardia.

Nocardia is a genera of actinomycetes with weakly staining Gram-positive, catalase-positive, rod-shaped bacteria. It forms partially acid-fast beaded branching filaments (resembling fungi, but being truly bacteria). It contains a total of 85 species. Some species are nonpathogenic, while others are responsible for Nocardiosis (Ryan and Ray, 2004). Nocardia species are found worldwide in soil rich in organic matter. In addition, they are also found as oral microflora in healthy gingiva, as well as periodontal pockets. Most Nocardia infections are acquired by inhalation of the bacteria or through traumatic introduction.

In the course of an investigation directed towards the discovery of new antibiotics, aminoglycoside, macrolide, ansamacrolide, ß-lactam, peptide, glycopeptides, anthracycline, tetracycline, nucleotide, quinine (Okami and Hotta, 1988) and nocardicin (Aoki *et al.*, 1976). The discovery of nocardicin produced by a Nocardia strain shows that the search for new and novel chemotherapeutic agents in microbial products is still promising. At the same time, the rarity of certain types of synthetic abilities is shown by the fact that only strains of Nocardia have been found to produce these antibiotics.

The primary aim of the present study was to isolate Nocardia strains from different sites of Hadhramout caves in Yemen and screen the isolates for the strong antibacterial activity. In the present study, taxonomic characterization was done based on the biochemical and

morphological data and the isolated strain was identified as *Nocardia asteroids.* Further, its ability to produce antibacterial substance was also investigated.

2. MATERIALS AND METHODS

2.1 Sample collection, isolation and screening of Nocardia

The soil sample was collected from cave of Laiha located in the region of Kaasa, Rahba Bin Junaid, Directorate Ghail Bin Yamen in Hadhramout Governorate of Yemen. The upper 5 cm layer of cave soil was collected in sterile plastic bags and transported to the laboratory. Soil was air dried at room temperature before grinding with mortar and pestle and then sieved. The pH of each soil sample was determined by the procedure described by Reed and Cummings (1945), using a glass electrode pH meter and expressed as an average of triplicate readings. SCA of pH 7 was used for the isolation of Nocardia (Nawani, 2002). Isolation of Nocardia was done by suspending 1 g of soil sample in 10 ml of sterile distilled water, which was vigorously shaken and allowed to settle for 5 min. The supernatant was serially diluted and incubated at 37°C for 14 days (Kanavade, 2003).

2.2 Identification of Nocardia

Nocardia was characterized by morphological and biochemical methods. Morphological characterizations were done microscopically by coverslip culture method (Kawato and Shinobu,1959). The mycelial structure, color and arrangement of conidiospore and arthrospore on the mycelium were observed using oil immersion (100X) objective. The observed structure was compared with Waksman (1954) and Berd (1973), to identify the organism. Various biochemical and physicochemical tests were performed for its identification.

Nocardia isolates were screened for their antibacterial activity by Agar Disc Diffusion (ADD) method against several targets bacterial cultures such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa.* Nocardia isolates were plated on starch casein agar of pH 7 and incubated at 28°C for 7 days. The target bacterial strains were seeded on NA plates of pH 7 and 1.0 cm diameter disc of the actinomycetes was transferred on to the surface of NA plates. These plates were kept for one hour in refrigerator to facilitate diffusion and then incubated at 37°C for 24 hours for the growth of bacteria. Antimicrobial

activity was noted in terms of zone of inhibition around the agar disc of actinomycetes (Pisano *et al.*, 1992).

2.3 Media used for the maintenance of Nocardia

Isolates of Nocardia were maintained on SCA at 4°C. They were also preserved at -20°C in 20% glycerol in distilled water, where glycerol acted as a cryo-protectant (Kanavade, 2003).

3. RESULTS

3.1 Isolation of Nocardia (La5/3)

The isolated strain was identified based on their colony morphology, mycelium structure, color, arrangement of spore and microscopic morphology. The pure strain was named as La5/3 and the isolate was maintained in SCA medium.

3.2 Screening of Nocardia (La5/3) isolated

Nocardia La5/3 activity was screened against four pathogenic bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa)* by ADD method and turned out to be active against these pathogenic bacteria Fig. 2.

3.3 Morphological characterization

The colony morphology of Nocardia *(La5/3)* was observed with respect to color, aerial and substrate mycelium, soluble pigment, colony margin, Gram staining, and growth of colony. The results are given in Table 1, 2 and Fig.1.

3.4 Biochemical characterization

The Nocardia (La5/3*)* was analyzed by biochemical studies. The results are summarized in Table 3.

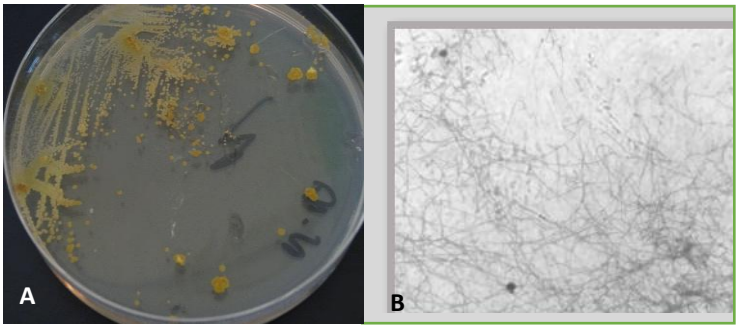

Fig 1: A: Colony morphology on Nutrient Agar. B: Microscopic view of spore morphology of Lah5/3 isolate

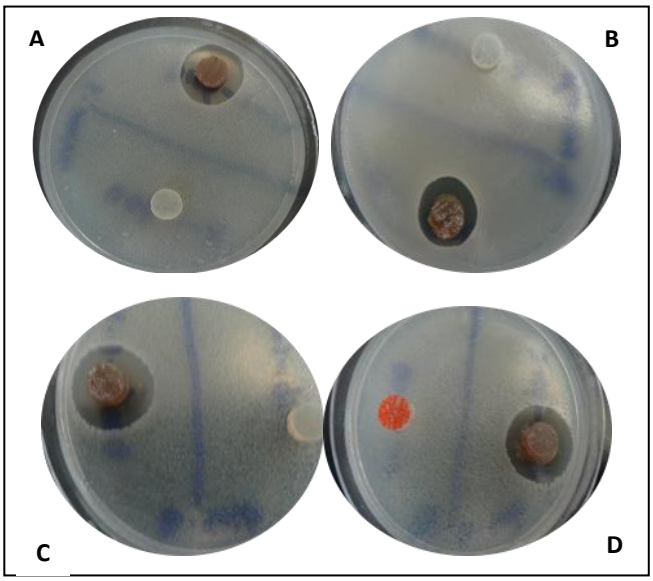

Fig 2: Antibacterial activity by ADD method from SCA against A: *Staphylococcus aureus*, B: *Bacillus subtilis*, C: *Escherichia coli*, D: *Pseudomonas aeruginosa*

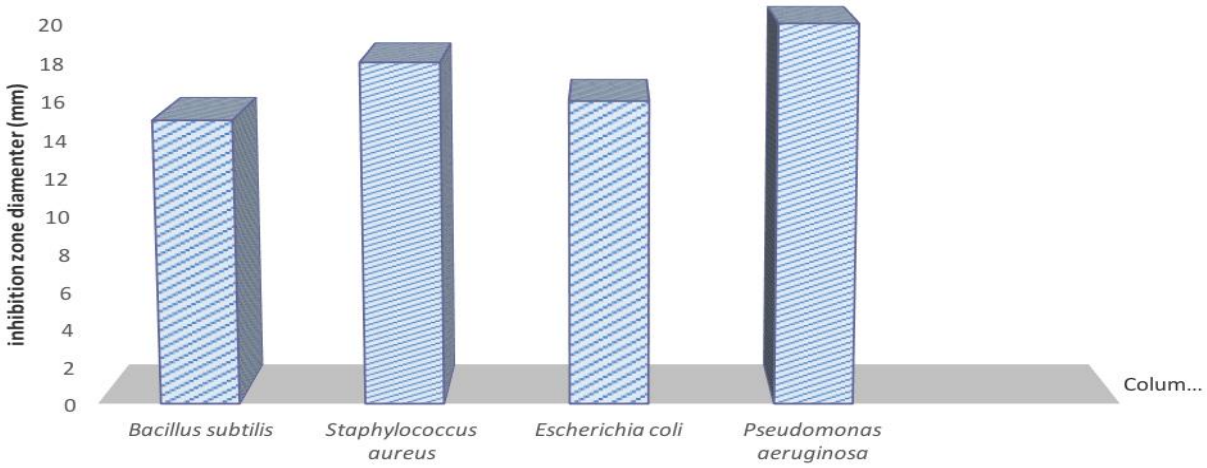

Fig 3: Inhibition zone of antibacterial activity of Lah5/3 isolate by ADD

Table 1: Colony and spore morphology of Lah 5/3 isolate

Colony and spore characters	Colony shape	Colony consistency	Colony elevation	Time of Colony visible
Lah 5/3	Irregular	Hard	Convex	120 hours
	Spore grouping	**Spore motility**	**Spore color**	**Spore chain**
	Chain	Non motile	Brown	Straight, loop and flexible

Table 2: Growth and cultural characteristics of the strain Lah 5/3 on different media

Culture medium	Growth	Arial mycelium	Substrate mycelium	Soluble pigment
SCA	Good	None	Ochre brown	Honey yellow
NA	Poor	None	Yellow	None
SDA	Poor	None	Brown	None
YMA	Moderate	None	Yellow	None
GAA	Moderate	None	Light brown	Yellow
TA	Good	None	Ochre brown	Honey yellow
CDTA	Poor	None	Yellow	None
GYA	Moderate	None	Yellow	None
SYA	Good	Yellow Orange	Deep orange	Sun yellow

Table 3. Physiological and biochemical characteristics of strain Lah5/3

Test	Result	Test	Result
Carbon sources		**Physiological characters**	
D- glucose	+	Temperature	
Citric acid	+	20°C.	+
Lactic acid	-	28°C.	+
Cellulose	+	37°C.	+
Lactose	+	45°C.	+
Dextrose	+	60°C.	-
D – mannose	+	pH:	
D- fructose	+	3.5	-
Sucrose	+	4.5	+
L- arabinose	+	7.0	+
D- mannitol	+	10.0	+
Sodium acetate	+	NaCl Tolerance:	
Starch	+	0.0%	+
Glycerol	+	2%	+
D- maltose	+	4%	+
Nitrogen source		6%	-
L-asparagine	+	8%	-
L-tyrosine	-	10%	-
Glycine	+	**Enzymes**	
Ammonium sulphate	+	Amylase	_
Sodium nitrate	+	Caseinase	+
Antibiotics resistance		Catalase	+
Gentamicin (10 μg)	-	Chitinase	+
Nalidixic acid (30 µg)	-	Gelatinase	-
Tetracycline (30 µg)	-	Keratinase	_
Co-trimazole (25 µg)	-	Lecithinase	-
Amphotericin B (100 µg)	+	Lipase	-
Fluconazole (10 µg)	+	Pectinase	-
Novobiocin (30µg)	-	DNase	+
Penicillin G (10 µg)	+	Urease	+
Nystatin (10 µg)	+	H_2S production	
Clotrimazole (10µg)	-	Acid fast staining	

4. DISCUSSION

Nocardia sps. La5/3 was isolated from the soil sample of Laiha cave. Isolated strain was identified based on its colony morphology and microscopic characteristics. The mycelial structure, color and arrangement of spores were observed by coverslip technique. Similar method has been followed by Berd (1973) and Mansour (2003). In this study, taxonomy of the isolate was based on the morphological, physiological and biochemical properties according to Waksman (1954) and the isolate *La5/3* was identified as *Nocardia asteroids*. The colony shape was irregular, hard and grew at 120 hrs. The spore morphology was chain,

straight and loop flexible while the spore color was brown and non-motile (Table 1 and Fig 1). These results agree with the reports of Eshraghi (2015) and Nasab *et al.*, (2017).

The *Nocardia asteroids* grew well on SCA, NA, SDA, PDA, YMA, GAA, CDTA, TA, GYA and SYA plates producing a range of substrate and aerial colored mycelia with relatively few diffusible pigments (Table 2). Similar results were also shown by Bady *et al*., (2014) It was grown at 20, 28, 37 and 45°C. On the other hand, it failed to grow at 60°C (Table 3).

Nocardia asteroids grew at different pH and different percentage of NaCl. Zhao *et al*., (2011) found similar result that the growth of Nocardia sps occurred with 0-7% (w/v) NaCl (optimum 0- 3 %), pH 5.0- 9.0 (optimum pH 6.0) and temperature between 10 and 37°C (optimum 20–28°C). In the present study, Nocardia isolates were acid fast (Table 3). These results agree with AL-Mahdi (2005) and Bawazir *et al., (2017)* who found that *Nocardia otitidiscaviarum and Nocardia brasiliensis* were acid fast.

The ability of *Nocardia asteroids* in utilizing various carbon and nitrogen compounds as source of energy (Table 3) was done by following the method recommended in International Streptomyces Project. The result showed that *Nocardia asteroids* were resistant to most of the antibiotics and sensitive to gentamycin (10 µg), nalidixic acid (30 µg), tetracycline (30 µg), and novobiocin (30 µg). These results agree with Kim (2010) who found that some isolates were resistant to tetracycline, gentamycin, Novobiocin and Nalidixic acid. Bawazir *et al.*, (2017) found *Nocardia otitidiscaviarum* resistant *to* amphotericin B (100µg), fluconazole (10 µg) and nystatin (10 µg). In this study, *Nocardia asteroids* were screened for various enzyme activities and the results showed positive for catalase and urease enzymes (Table 3). These results agree with Biehle *et al*., (1996); Ara *et al*., (2010) and Deepa *et al.*, (2012). Most of the *Nocardia* isolates have been shown to be positive for DNase enzyme (Oskay *et al.*, 2004).

In the present study, the isolated strain of *Nocardia* was also screened for antibacterial activity against four pathogenic bacteria (*E. coli, S. aureus, B. subtilis* and *P. aeruginosa*). The result showed that the isolate was active against all the tested pathogenic bacteria (Fig 2 and 3).

5. CONCLUSION

An Actinomycete characterized as *Nocardia asteroids* were isolated from the soil of Lahia caves of Hadhramout, Yemen, where there is less involvement by human for agriculture or

other purpose. The isolate showed antibacterial activity against four pathogenic bacteria tested in the present investigation. This may be attributed to the antibiotics elaborated by *Nocardia asteroids* that need to be further examined. Thus from the present work, it may be concluded that actinomycetes isolated from sources far from human and animal intervention may contribute to the production of antibiotics that helps in the eradication of intractable diseases for the human welfare.

6. ACKNOWLEDGMENTS

Our special thanks to Mangalore University and Department of Studies in Biochemistry, Post Graduate Centre, Jnana Kaveri, Chikka Aluvara, Kodagu for the support and encouragement. We also take great pleasure in expressing thanks to our parents and friends who helped us.

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