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Screening for PKS-I gene cluster from endophytic actinomycetes residing in *Ocmium tenuiflorum* (Tulsi) and *Azadirachta indica* (Neem)

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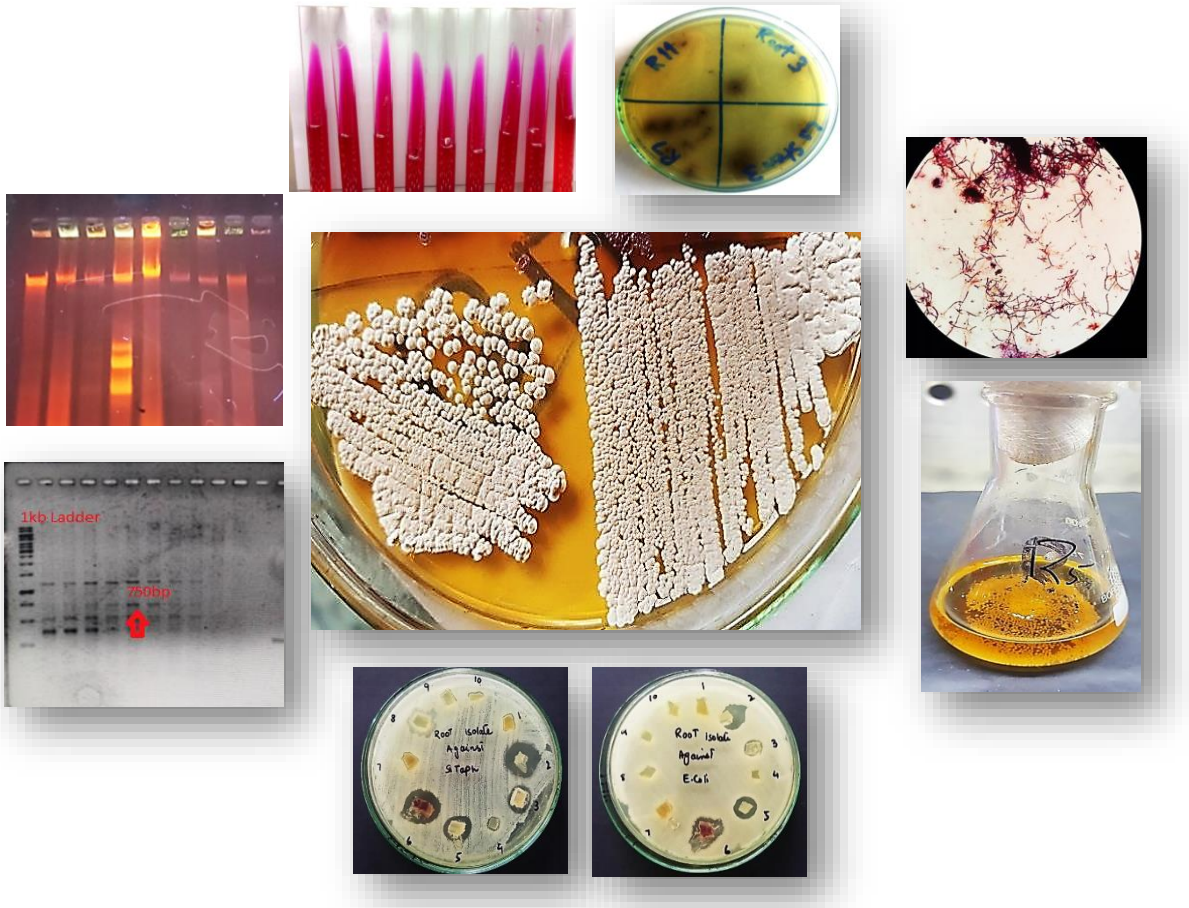
Graphical Abstract



Abstract:

Polyketide synthases type I (PKS-I) gene cluster is responsible for the synthesis of highly assorted group of secondary metabolites such as antimicrobial and anticancer agents. In our study, screening was carried out using degenerate primers to determine the presence of PKS-I gene cluster in endophytic actinomycetes isolated from two medicinal plants *Ocmium teniflorum* (Tulsi) and *Azadirachta indica* (Neem). A total of 28 endophytes that were isolated and identified from our previous study were further confirmed through 16S rRNA gene sequencing to exhibit a 99% similarity with *Streptomyces sp.* The molecular screening using PCR revealed the presence of PKS- I gene with a product size of 750bps in the isolates, FHK-1, FHK-2, FHK-3, FHK-4, FHK-5, FHK-6, FHK-7, FHK-8, FHK-9, FHK-11, FHK-13, FHK-16, FHK-18, FHK-20, FHK-21, FHK-23, FHK,25 and FHK-28. These isolates were further checked for their antimicrobial potential using their crude extracts. They displayed prominent bioactivity against ATCC pathogens, *Escherichia coli*, *Proteus vulgaris, Rhodococcus equi, Staphlococcus epidermidis, Enterococcus faecalis* and *Acinetobacter baumanii.* Our study revealed that the endophytes from *O. tenuiflorum* and *A. indica* are bioactive and versatile haboring the PKS-I gene cluster.

Keywords: Actinomycetes; Endophytes; Polyketide Synthases type I

Introduction

- Traditional medicine
  - Essential role in the healthcare systems
  - In Asian countries
    - 80% of the population dependent on medicinal practices [1]

- Medicinal plants
  - Attractive targets for discovering novel therapeutic agents [1]

[1] Miller KI, Ingrey SD, Alvin A, Sze MYD, Roufogalis BD, Neilan BA. Endophytes and the microbial genetics of traditional medicines. Microbiology Australia. 2010;31(2):60-3.

Introduction (Cont.)

- *Ocmium teniflorum*

- Locally known as ‘Tulsi’
- Widespread throughout the Southeast Asian tropics [2]
- Clinical pharmacological properties of oils and extracts from leaves
  - Antiemetic, antipyretic, anti-inflammatory, anti diabetic, antifungal, antibacterial, analgesic and anti stress effects [3]

Ocmium tenuiflorum growing in the nursery of University of Veterinary and Animal Sciences (UVAS), Lahore Pakistan. Photo courtesy: Ms. Fatima Nawaz

[2] Warrier P, Nambiar V, Ramankutty C. Indian Medicinal Plants: A Compendium of 500 Species, vol. III, Orient Longman Pvt. Ltd, Anna Salai, Chennai, India. 1995:38-42.

[3] Singh E, Sharma S, Dwivedi J, Sharma S. 2012. Diversified potentials of *Ocimum sanctum* Linn (Tulsi): An exhaustive survey. J Nat Prod Plant Resour. 2(1): 39-48.

Introduction (Cont.)

- *Azadirachta indica* A. Juss

- Locally known as ‘Neem’
- Native to Pakistan, India, Bangladesh, Burma, Malaysia and Sri Lanka [4]
- Clinical pharmacological properties
  - Antifungal, antibacterial, anti-diabetic, antiviral, anthelmintic and sedative properties [5]

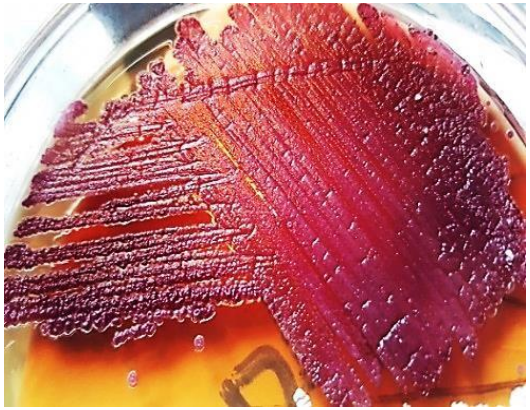
Azadirachta indica growing in the nursery of University of Veterinary and Animal Sciences (UVAS), Lahore Pakistan. Photo courtesy: Ms. Fatima Nawaz

[4] Babu KS, Naik VKM, Latha J, Ramanjaneyulu K. 2016. Pharmacological review on natural products (*Azadirachta indica* Linn). IJCS. 4(5): 01-04.

[5] Agrawal D. Medicinal properties of neem: new findings. History of Indian science and. 2001.

Introduction (Cont.)

- Actinomycetes
  - Greek ‘aktis’ (a ray) and ‘mykes’ (fungus)
  - Producers of a broad array of secondary metabolites
    - Useful applications in veterinary and human medicine [6]



Endophytic actinomycetes strain FHK-6 isolated from *Ocmium tenuiflorum* (Tulsi). Photo courtesy: Ms. Fatima Nawaz

- Endophytic Actinomycetes
  - Starting platform
  - Antibiotics, enzyme, anticancer agents, immunomodulators, anthelminthic agents,
  - Long-held alliance, plants and endophytic microorganisms develop good information transfer [7]

[6] Janso JE, Carter GT. 2010. Biosynthetic potential of phylogenetically unique endophytic actinomycetes from tropical plants. J Appl Environ Microbiol 76(13): 4377-4386.

[7] Zhao K, Penttinen P, Guan T, Xiao J, Chen Q, Xu J, Lindström K, Zhang L, Zhang X, Strobel GA. 2011. The diversity and anti-microbial activity of endophytic actinomycetes isolated from medicinal plants in Panxi plateau, China. Curr Microbiol. 62(1): 182-190.

Introduction (Cont.)

- Polyketides
  - Consequence of the successive condensation of carboxylic acid units [8]

- Polyketide synthases (PKS)
  - Multifunctional enzymes
  - Responsible for the biosynthesis of secondary metabolites
  - Comprising of antibacterial, antifungal, anticancer [8]

[8] Tanvir, R, I Sajid, and S Hasnain. "Screening for Type I Polyketide Synthases Genes of Endophytic Streptomycetes Isolated from Parthenium Hysterophorus L." *Molecular Genetics, Microbiology and Virology* 28, no. 1 (2013): 32-39.

Results and discussion

- Total 28 endophytic *actinomycetes* were isolated [9]

- Frequency of isolation
  - *Ocimum tenuiflorum* (Tulsi)
    - Roots = 03
    - Roots slurry= 10
    - Shoots = 02
    - Shoot slurry = 06
    - Leaves slurry = 01

    - Total = 22

[9] Tanvir, R., I. Sajid, and S. Hasnain, *Screening of endophytic Streptomycetes isolated from Parthenium hysterophorus L. against nosocomial pathogens.* Pak J Pharm Sci, 2013. **26**: p. 277-283

Results and discussion (Cont.)

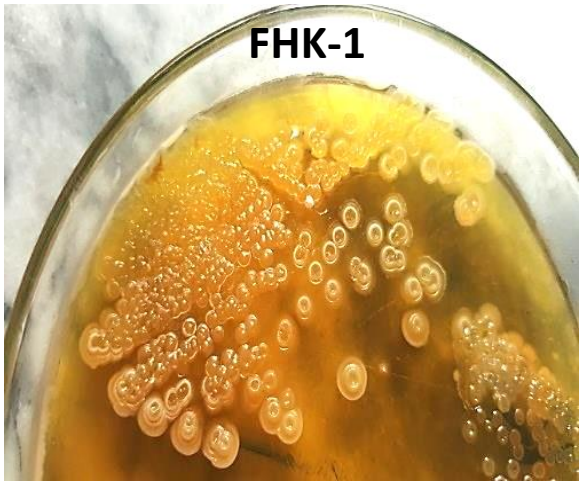
- Total 28 endophytic *actinomycetes* were isolated [9]

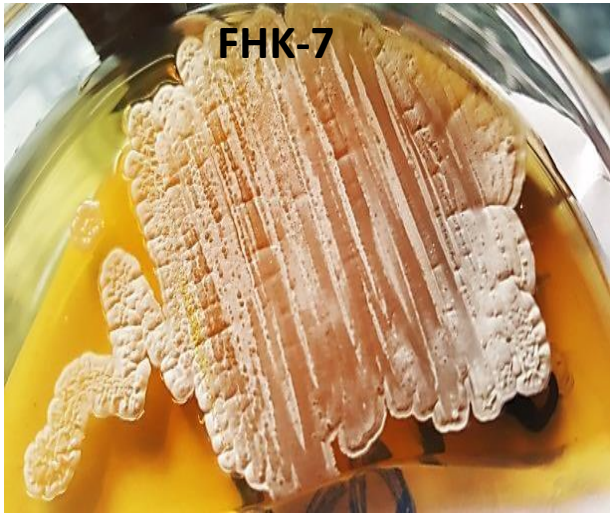
- Frequency of isolation
  - *Azadirachta indica* (Neem)
    - Roots slurry= 05
    - Shoot slurry = 01

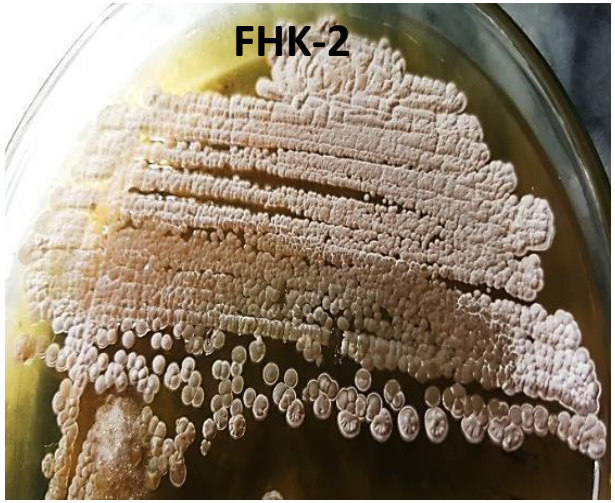
    - Total = 06

[9] Tanvir, R., I. Sajid, and S. Hasnain, *Screening of endophytic Streptomycetes isolated from Parthenium hysterophorus L. against nosocomial pathogens.* Pak J Pharm Sci, 2013. **26**: p. 277-283

Results and discussion (Cont.)







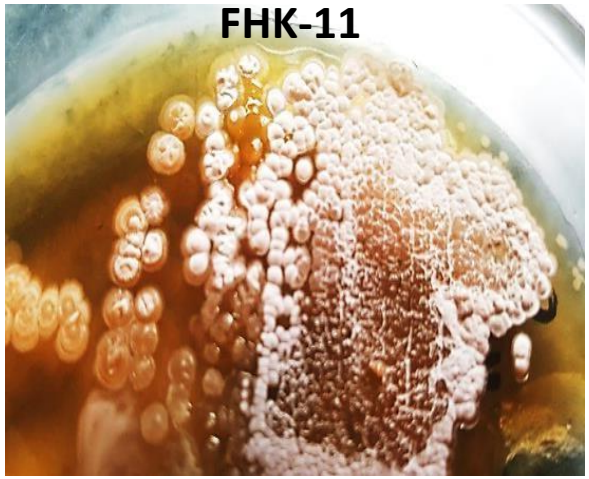
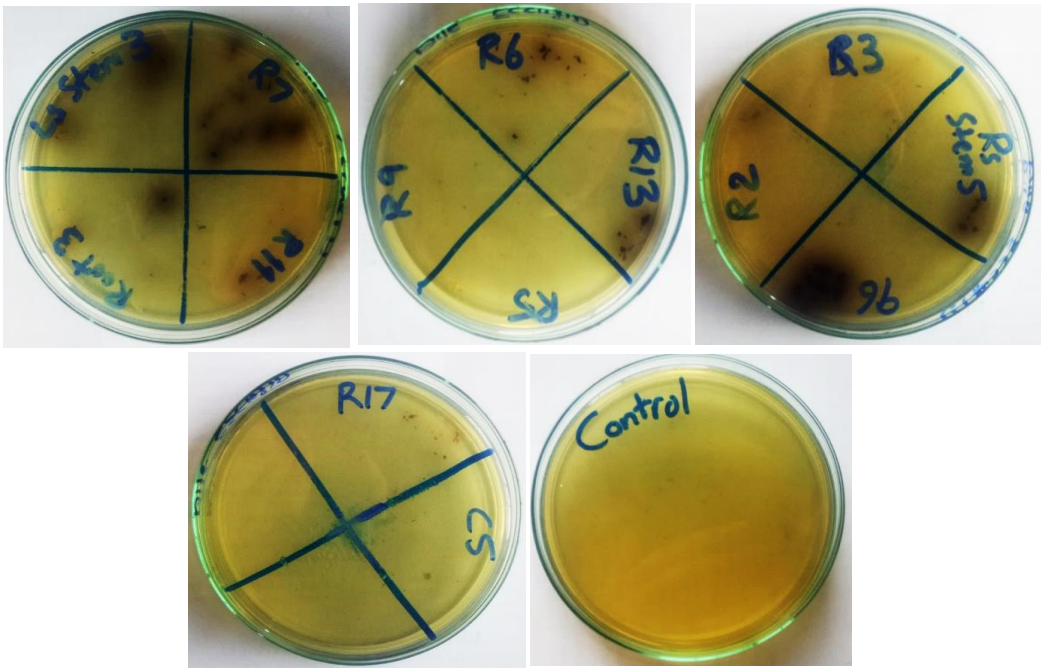


Photo courtesy: Ms. Fatima Nawaz

Results and discussion (Cont.)

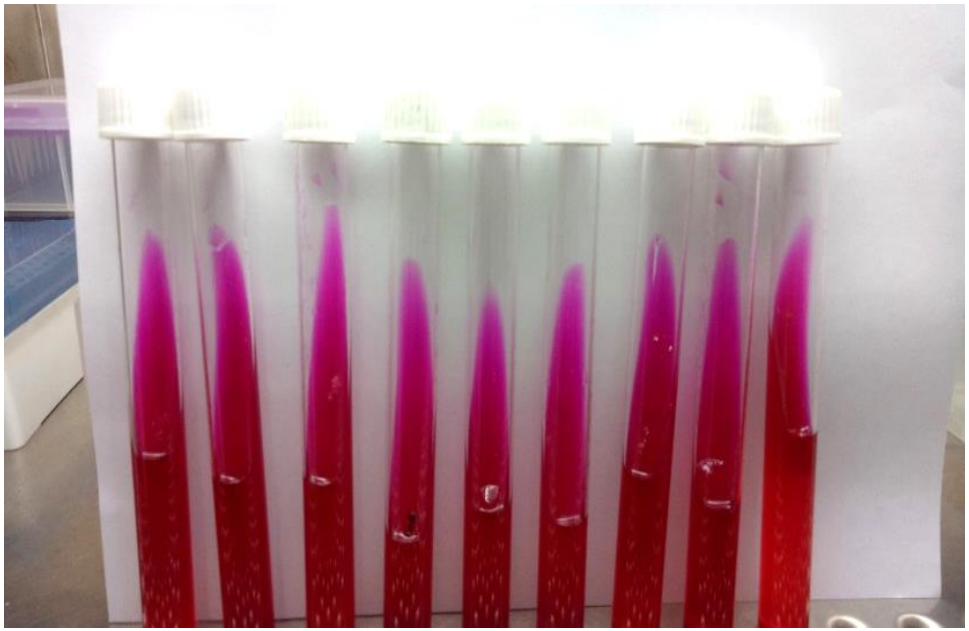
- Physiological characterization
  - Esculin hydrolysis [9]
    - Esculin producers = 27
    - Non esculin producers = 01



[9] Tanvir, R., I. Sajid, and S. Hasnain, *Screening of endophytic Streptomycetes isolated from Parthenium hysterophorus L. against nosocomial pathogens.* Pak J Pharm Sci, 2013. **26**: p. 277-283

Results and discussion (Cont.)

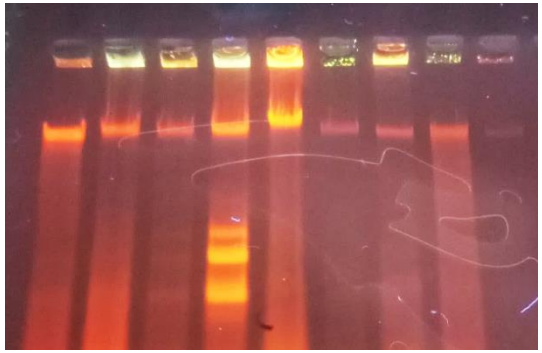
- Physiological characterization
  - Hydrolysis of urea [9]
    - Positive = 27
    - Negative = 01

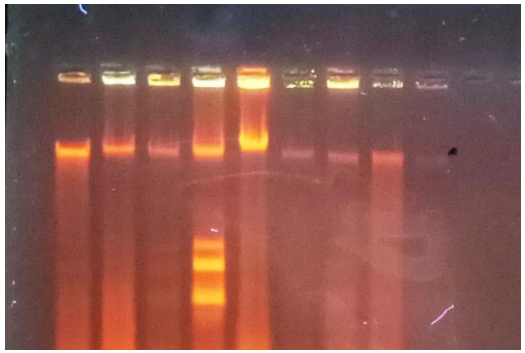


[9] Tanvir, R., I. Sajid, and S. Hasnain, *Screening of endophytic Streptomycetes isolated from Parthenium hysterophorus L. against nosocomial pathogens.* Pak J Pharm Sci, 2013. **26**: p. 277-283

Results and discussion (Cont.)

- Genomic DNA isolation
  - Manual method [10]
  - 28 selected actinomycetes strains

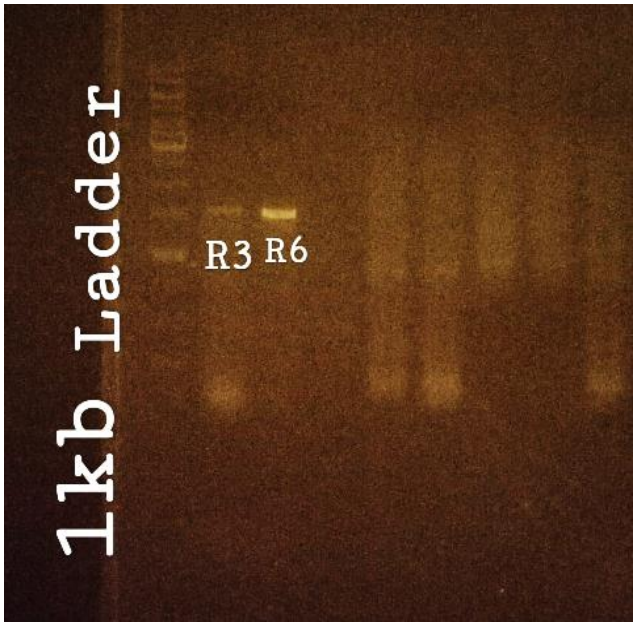




[10] Sajid I, Yao CBFF, Shaaban KA, Hasnain S, Laatsch H. 2009. Antifungal and antibacterial activities of indigenous Streptomyces isolates from saline farmlands: prescreening, ribotyping and metabolic diversity. World J Microbiol Biotechnol. 25(4): 601.

Results and discussion (Cont.)

- 16s Ribosomal RNA gene sequencing
  - FHK-3 and FHK-6
  - 27f and 1522r universal primers [9]
  - 1.5 kb gene sequenced



[9] Tanvir, R., I. Sajid, and S. Hasnain, *Screening of endophytic Streptomycetes isolated from Parthenium hysterophorus L. against nosocomial pathogens.* Pak J Pharm Sci, 2013. **26**: p. 277-283

Results and discussion (Cont.)

- 16s Ribosomal RNA gene sequencing
  - BLAST analysis for FHK-3 and FHK-6
  - 99% homology with *Streptomyces* sp.

Sequences producing significant alignments:

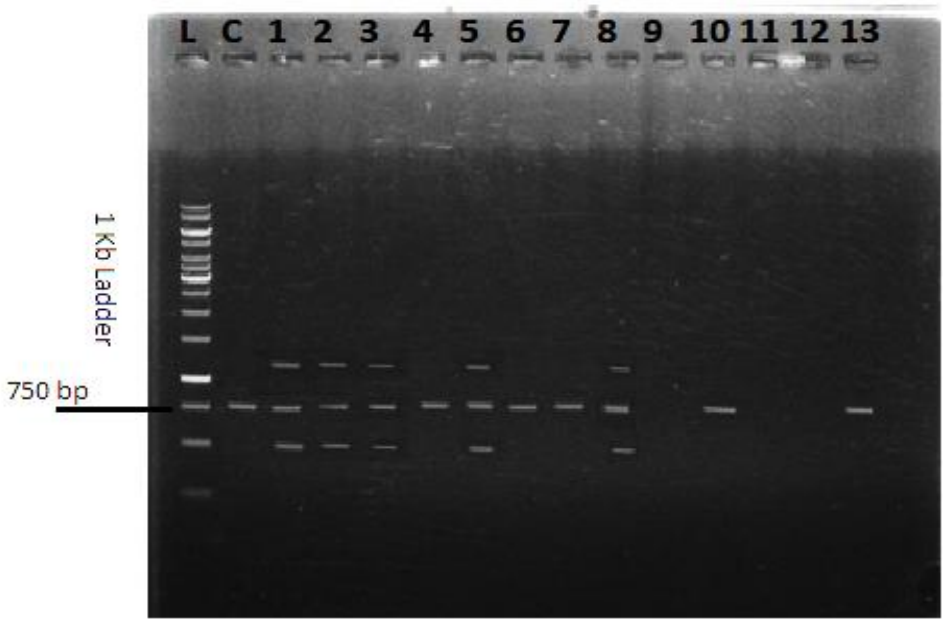
Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptomyces sp. strain RB110 16S ribosomal RNA gene, partial sequence	2361	2361	89%	0.0	99.09%	KY558688.2
Streptomyces sp. strain G7 16S ribosomal RNA gene, partial sequence	2361	2361	89%	0.0	99.09%	MK134588.1
Streptomyces puniceus strain NBRC 12811 (T) 16S ribosomal RNA gene, partial sequence	2361	2361	89%	0.0	99.09%	MK424311.1
Streptomyces mediolani strain RHPR25 16S ribosomal RNA gene, partial sequence	2361	2361	89%	0.0	99.09%	MH209253.1
Streptomyces sp. strain sdu6 16S ribosomal RNA gene, partial sequence	2361	2361	89%	0.0	99.09%	MH410521.1
Streptomyces sp. strain MJM16129 16S ribosomal RNA gene, partial sequence	2361	2361	89%	0.0	99.09%	MF344817.1
Streptomyces sp. strain WA23-2-1 16S ribosomal RNA gene, partial sequence	2361	2361	89%	0.0	99.09%	KY206812.1
Streptomyces californicus strain TY004-069 16S ribosomal RNA gene, partial sequence	2361	2361	89%	0.0	99.09%	MF582317.1
Streptomyces sp. strain Yifu69 16S ribosomal RNA gene, partial sequence	2361	2361	89%	0.0	99.09%	KX900591.1
Streptomyces violaceoruber strain S21, complete genome	2361	14166	89%	0.0	99.24%	CP020570.1
Streptomyces sp. strain RB90 16S ribosomal RNA gene, partial sequence	2361	2361	89%	0.0	99.09%	KY558671.1
Streptomyces sp. strain 3C-HV2 16S ribosomal RNA gene, partial sequence	2361	2361	89%	0.0	99.09%	KX777595.1
Streptomyces microflavus strain nenu_DS_25 16S ribosomal RNA gene, partial sequence	2361	2361	89%	0.0	99.09%	KY007180.1
Streptomyces cavourensis strain nenu_DS_16 16S ribosomal RNA gene, partial sequence	2361	2361	89%	0.0	99.09%	KY007172.1
Streptomyces sp. NEAU-JF7 16S ribosomal RNA gene, partial sequence	2361	2361	89%	0.0	99.09%	KP267995.1
Streptomyces sp. NEAU-JF11 16S ribosomal RNA gene, partial sequence	2361	2361	89%	0.0	99.09%	KP267994.1
Streptomyces puniceus strain EB120 16S ribosomal RNA gene, partial sequence	2361	2361	89%	0.0	99.09%	KP209438.1
Streptomyces sp. ZZ036 16S ribosomal RNA gene, partial sequence	2361	2361	89%	0.0	99.09%	KJ995742.1
Streptomyces sp. KhCrAH269 16S ribosomal RNA gene, partial sequence	2361	2361	89%	0.0	99.09%	KJ889182.1

Results and discussion (Cont.)

- Screening for PKS-I gene cluster
  - Degenerate primers MDPQQRf and HGTGTr [11]
  - 750 bp product size
  - 18 strains positive (64.2%)



[11] Ayuso-Sacido A, Genilloud O. 2005. New PCR primers for the screening of NRPS and PKS-I systems in actinomycetes: detection and distribution of these biosynthetic gene sequences in major taxonomic groups. Microbiol Ecol. 49(1): 10-24.

Results and discussion (Cont.)

- Antimicrobial activity of strains containing the PKS-1 gene against ATCC pathogens using agar well method [12]
  - Crude extraction
    - 1:1 ethyl acetate [13]
    - Extracts stored in absolute methanol

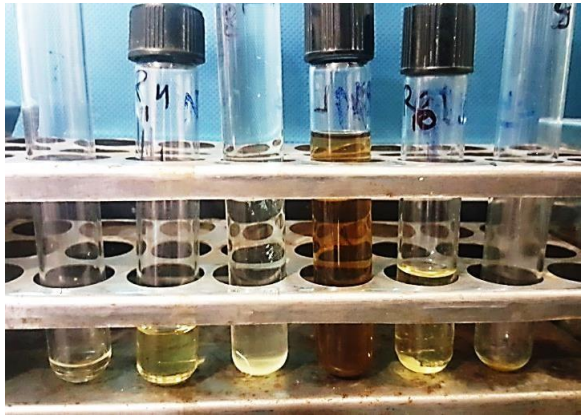


Photo courtesy: Ms. Fatima Nawaz

[12] Gebreyhannes, Gebreselema, Feleke Moges, Samuel Sahile, and Nagappan Raja. "Isolation and characterization of potential antibiotic producing actinomycetes from water and sediments of Lake Tana, Ethiopia." *Asian Pacific Journal of Tropical Biomedicine* 3, no. 6 (2013): 426-35.

[13] Tanvir, Rabia, Imran Sajid, Shahida Hasnain, Andreas Kulik, and Stephanie Grond. "Rare actinomycetes *Nocardia caishijiensis* and *Pseudonocardia carboxydivorans* as endophytes, their bioactivity and metabolites evaluation." *Microbiological Research* 185 (2016): 22-35.

Results and discussion (Cont.)

- Preliminary antimicrobial activity against Gram positive and Gram negative ATCC pathogens
  - Broad spectrum activity
  - Maximum zones of inhibition of 18mm

Photo courtesy: Ms. Fatima Nawaz

Results and discussion (Cont.)

- *In vitro* antimicrobial testing for the strains containing PKS-1 gene

Strain code	Zone of inhibition (mm)					
	E. coli	*P. vulgaris*	*R. equi*	*S. epidermidis*	*E. faecalis*	*A. baumanii*
FHK-1	-	-	-	11.6	10.6	-
FHK-2	6.3	-	6.3	10	17.6	-
FHK-3	6.3	11	8.6	11	13	6
FHK-4	5.3	9.6	8.3	9	17	6
FHK-5	6.6	10.6	6.6	6.6	10.3	-
FHK-6	10.6	13.6	8.3	8.3	12.3	-
FHK-7	4	6	6.6	6.6	5.3	5
FHK-8	6	10	11.3	11.3	7.6	4.3
FHK-9	11.3	9.6	8.6	8.6	9.6	11.3
FHK-11	6.6	9.6	10	10	8	6.6
FHK-13	3.6	12.3	12.6	12.6	10.6	5.6
FHK-16	7.8	-	8.7	13	-	10

Key: *E. coli= Escherichia coli*; *P. vulgaris = Proteus vulgaris; R. equi = Rhodococcus equi; S. epidermidis = Staphlococcus epidermidis; E. faecalis = Enterococcus faecalis; A. baumanii = Acinetobacter baumanii*

Results and discussion (Cont.)

- *In vitro* antimicrobial testing for the strains containing PKS-1 gene

Strain code	Zone of inhibition (mm)					
	E. coli	***P. vulgaris***	***R. equi***	***S. epidermidis***	***E. faecalis***	***A. baumanii***
FHK-18	7.8	-	8.7	13	-	10
FHK-20	6	3	-	17	10.3	7.6
FHK-21	9.3	8	7	9	8	8.4
FHK-23	7.9	7.3	-	10.5	-	7
FHK-25	6.7	12	9	-	16	-
FHK-28	8	11	8.3	-	12	6.7

Key: *E. coli= Escherichia coli*; *P. vulgaris = Proteus vulgaris; R. equi = Rhodococcus equi; S. epidermidis = Staphlococcus epidermidis; E. faecalis = Enterococcus faecalis; A. baumanii = Acinetobacter baumanii*

Conclusions

- Endophytic actinomycetes from *Ocmium teniflorum* (Tulsi) and *Azadirachta indica* (Neem)
  - Rarely studied before

- Our study
  - Diversity of actinomycetes reside in different plant parts
  - Biological screening revealed
    - Broad spectrum activity
    - Bioactive against gram positive and gram negative pathogens
      - » *Escherichia coli, Proteus vulgaris, Rhodococcus equi, Staphlococcus epidermidis, Enterococcus faecalis, Acinetobacter baumanii*

Conclusions (Cont.)

- Contain PKS-1 gene cluster
  - 18 strains
  - Potential for novel antibiotics

- Further exploration of these strains
  - Large scale study
  - Further bioactivity screening
  - HPLC-MS and NMR