List of Publications


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Under Minor Revision

Antimicrobial and Cytotoxicity Properties of 2,3-Dihydroxy-9,10-Anthraquinone Isolated from *Streptomyces galbus* (ERINLG-127)

C. Balachandran · Y. Arun · V. Duraipandiyan · S. Ignacimuthu · K. Balakrishna · N.A. Al-Dhabi

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**Abstract** *Streptomyces galbus* ERINLG-127 was isolated from the soil samples of the Marapalam forest, Nilgiris, India. The ethyl acetate extract was subjected to activity-guided fractionation by column chromatography over silica gel. This led to the isolation of 2,3-dihydroxy-9,10-anthraquinone as the active principle. The compound showed good antimicrobial activity against tested bacteria and fungi. The minimum inhibitory concentration values of isolated compound were 12.5 μg/mL against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* (ESBL-3971), *K. pneumoniae* (ESBL-3894) and *Staphylococcus aureus* (MRSA). The compound showed prominent cytotoxic activity in vitro against A549 lung adenocarcinoma cancer cell line. It showed 75.1 % activity at the dose of 100 μg/mL with IC50 value of 60 μg/mL. The isolated compound was subjected to molecular docking studies for the inhibition of TtgR and Topoisomerase IV enzymes which are targets for antimicrobials. Docking studies of the compound showed low docking energy indicating its usefulness as antimicrobial agent.

**Keywords** *Streptomyces galbus* · Antimicrobial · 2,3-Dihydroxy-9,10-anthraquinone · Molecular docking · Cytotoxicity

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**Introduction**

Natural products have provided many promising leads for drugs of clinical and industrial use; they are widely used as pharmaceutical agents or agrochemicals, and they still do represent a major source for structurally novel bioactive molecules. They are widely distributed not only in fungi, lichens, and higher plants but also in animals, and, in particular, in soils, where they are mainly formed by microorganisms [1]. Extremophilic actinomycetes constitute an increasingly important and highly productive source of bioactive secondary metabolites [2]. Actinomycetes are Gram-positive bacteria that are widely spread in nature and play a pivotal role in the production of bioactive metabolites [3]. Among Actinomycetes, the members of the genus *Streptomyces* are considered economically important because they alone constitute 50 % of the soil actinomycete population, and 75 % of total bioactive molecules are produced by this genus [4]. Majority of the *Streptomyces* and other *Actinomyces* produce a diverse array of antibiotics including aminoglycosides, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes and tetracyclines [5]. As a result of the increasing prevalence of antibiotic resistant pathogens and the pharmacological limitations of antibiotics, there is an exigency for new antimicrobial substances [6].

Among the naturally occurring quinones, the anthraquinones form the largest group of plant and microbial secondary metabolite. They belong to the group of microbial metabolites which have been investigated in the very early studies on microbial secondary metabolism. Anthraquinone derivatives, including emodin, physcion, catenarin, rubrocristin, rubrocristin-6-methyl ether and rubrocristin-6-acetate and questin, are nowadays well recognized as important biologically active components from microbes [7]. The anthraquinones are effective against certain diseases and are reported to possess wide range of biological activities including antifungal [8], antimicrobial [9], anticancer [10], antioxidant [11] and antihuman cytomegalovirus [12]. On the other hand, anthraquinones act as metabolic precursors of mycotoxins such as sterigmatocystin and aflatoxins. Both facts show that anthraquinones are a group with potent biological activities [13]. In the present study, antibacterial and cytotoxic activities of 2,3-dihydroxy-9,10-anthraquinone isolated from *Streptomyces galbus* (ERINLG-127) were assessed. The binding efficiency of the molecule was checked using docking studies.

**Materials and Methods**

**Sample Collection**

The soil samples were collected from the depth of 5–15 cm at Marappalam forest, Nilgiris (Southwestern Ghats), Tamil Nadu, India.

**Isolation of *Streptomyces galbus***

The isolation of *S. galbus* was performed by serial dilution using dilution plate technique. One gram of soil was suspended in 9 mL of sterile distilled water. The dilution was carried out up to $10^{-6}$ dilutions. Aliquots (0.1 mL) of $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$ and $10^{-6}$ were spread on the isolation plates containing starch casein agar (HiMedia, Mumbai). To minimize the bacterial and fungal growth, 30 mg/L actidione and 40 mg/L nalidixic acid were added. The plates were incubated at 28 °C for 7 to 20 days. The pure colonies were transferred to ISP-2 medium and incubated at 27 °C for 5 days.
Morphological and Biochemical Observations

Cultural and morphological features of ERINLG-127 were characterized following the directions given by the International *Streptomyces* Project (ISP) [14] and the Bergey’s Manual of Systematic Bacteriology. Cultural characteristics of pure isolates in various media (ISP 1–7) were recorded after incubation at 30 °C for 7–14 days. Morphology of spore-bearing hyphae with entire spore chain was observed with a light microscope (model SE, Nikon) using coverslip method in ISP medium (ISP 3–6). The shape of cell, Gram stain, color, the presence of spores, and colony morphology were assessed on solid ISP agar medium. Biochemical reactions, different temperatures, NaCl concentration, pH level, pigment production, enzyme reaction and acid or gas production were done following the methods of Balachandran et al. (2012) [15] and Valanarasu et al. (2009) [16].

Molecular Analysis

*16S rRNA Gene Amplification*

Genomic DNA of ERINLG-127 was isolated by the methods of HiPurA *Streptomyces* DNA Spin Kit MB527-20PR from HiMedia. The 16S ribosomal RNA gene was amplified by PCR method using primers 27f (5′AGTTTGATCCTGGCTCAG3′) and 1492r (5′ACGGCTACCTTGTTCAGACTT3′). Each PCR mixture in a final volume of 20 μL contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 μM of each dNTP, 10 pmol of each primer, 50 ng of genomic DNA and 1 U of Taq DNA Polymerase (New England Biolabs Inc). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94 °C for 4 min followed by 30 cycles at 94 °C for 1 min, primer annealing at 52 °C for 1 min and primer extension at 72 °C for 1 min. At the end of the cycling, the reaction mixture was held at 72 °C for 10 min and then cooled to 4 °C. PCR amplification was detected by 1 % agarose gel electrophoresis and was visualized by ultraviolet (UV) fluorescence after ethidium bromide staining. The PCR product obtained was sequenced by an automated sequencer (Genetic Analyser 3130, Applied Biosystem, USA). The same primers as above were used for this purpose. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks using the NCBI BLAST available at [http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/).

*Nucleotide Sequence Accession Number*

The partial *16S rRNA* gene sequences of isolate ERINLG-127 have been deposited in the GenBank database under accession number KC820652. A phylogenetic tree was constructed using the neighbour-joining DNA distance algorithm using software MEGA [17] (version 4.1).  

*Extraction and Purification*

Primary antimicrobial activity was evaluated on modified nutrient glucose agar (MNGA) medium by the cross streak method against various pathogenic microorganisms [18]. Culture inoculate of the isolate ERINLG-127 was taken in 500 mL Erlenmeyer flasks containing 150 mL of MNGA medium and incubated at 30 °C in a shaker (200 rpm) for 12 days. After the 12th day, the culture broth was centrifuged at 8000×g for 20 min to remove the biomass. The supernatant pH was adjusted to 2 by using 0.1 N HCl. Equal volume of ethyl acetate (1:1 v/v) was added and shaken in a separating funnel. The ethyl acetate layer was removed. The process was repeated three times...
and the combined ethyl acetate extract was washed with little water. The extract was dried over anhydrous sodium sulphate and distilled in a rotary evaporator, and the brown residue obtained was finally dried in vacuum (yield 7 g). The ethyl acetate extract was subjected to silica gel column chromatography (Acme’s 100–200 mesh). The column was successively eluted with chloroform, chloroform/ethyl acetate mixtures with increasing polarity and finally with ethyl acetate/methanol and methanol. Based on thin layer chromatography (TLC) profiles, the fractions were combined to give 21 fractions. Active fraction 13 (yield 350 mg) was further purified using preparative HPLC by Isocratic elution capability (Waters Alliance System). A column length of 250 mm, with internal diameter of 6.0 mm, was filled with porous silica particles of 15-μm diameter bonded with octadecylsilane (YMC pack ODS A (250×6.0 mm), 15 μm). The mobile phase was composed of acetonitrile and aqueous acetic acid (15:85, v/v). Elution was monitored at 254 nm and peak fraction was collected according to the elution profile.

Microbial Organisms

The following Gram-negative and Gram-positive bacteria and fungi were used for the experiment. Seven Gram-negative bacteria: Enterobacter aerogenes MTCC 111, Shigella flexneri MTCC 1457, Salmonella paratyphi B, Klebsiella pneumoniae MTCC 109, P. aeruginosa MTCC 741, Proteus vulgaris MTCC 1771 and S. typhimurium MTCC 1251; four Gram-positive bacteria: Bacillus subtilis MTCC 441, Micrococcus luteus MTCC 106, Staphylococcus aureus MTCC 96 and S. epidermidis MTCC 3615; and seven clinical isolates: Escherichia coli (ESBL-3984, extended-spectrum beta-lactamase), E. coli (ESBL-3904), K. pneumoniae (ESBL-3971), K. pneumoniae (ESBL-75799), K. pneumoniae (ESBL-3894), K. pneumoniae (ESBL-3967) and S. aureus (methicillin-resistant S. aureus (MRSA), clinical pathogen). The reference cultures were obtained from the Institute of Microbial Technology (IMTECH), Chandigarh 160 036, India. Three fungi were used: Candida albicans MTCC 227, Malassesia pachydermatis and Aspergillus flavus. All the fungal cultures were obtained from the Department of Microbiology, Christian Medical College, Vellore, Tamil Nadu, India. Bacterial inoculums were prepared by growing cells in Mueller-Hinton broth (MHB, HiMedia) for 24 h at 37 °C. The filamentous fungi were grown on sabouraud dextrose agar (SDA) slants at 28 °C for 10 days, and the spores were collected using sterile double-distilled water and homogenized. Yeast was grown on sabouraud dextrose broth (SDB) at 28 °C for 48 h.

Antimicrobial Assay

Antibacterial and antifungal activities were carried out using disc diffusion method [19]. Petri plates were prepared with 20 mL of sterile Mueller-Hinton agar (MHA, HiMedia, Mumbai). The test cultures were swabbed on the top of the solidified media and allowed to dry for 10 min, and a specific amount of ethyl acetate extract was added to each disc separately. The loaded discs were placed on the surface of the medium and left for 30 min at room temperature for compound diffusion. Negative control was prepared using respective solvents. Streptomycin (10 μg/disc) was used as positive control for bacteria. Ketoconazole was used as positive control for fungi. The plates were incubated for 24 h at 37 °C for bacteria and for 48 h at 28 °C for fungi. Zones of inhibition were recorded in millimetres and the experiment was repeated twice.

Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration studies of the isolated compound were performed according to the standard reference methods for bacteria [20], for filamentous fungi [21] and yeasts [22,23].

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The required concentrations (100, 75, 50, 25, 12.5, 6.25 and 3.125 μg/mL) of the compound were dissolved in dimethyl sulfoxide (DMSO, 2 %) and diluted to give serial twofold dilutions that were added to each medium in 96 well plates. An inoculum of 100 μL from each well was inoculated. The antifungal agent ketoconazole and antibacterial agent streptomycin were included in the assays as positive controls. For fungi, the plates were incubated for 48 to 72 hours at 28 °C, and for bacteria, the plates were incubated for 24 h at 37 °C. The MIC for fungi was defined as the lowest-extract concentration, showing no visible fungal growth after incubation time. Five microliters of tested broth was placed on the sterile MHA plates for bacteria and incubated at respective temperature. The MIC for bacteria was determined as the lowest concentration of the compound inhibiting the visual growth of the test cultures on the agar plate.

**Cytotoxic Properties**

A549 lung adenocarcinoma cancer cell line was obtained from National Institute of Cell Sciences, Pune. The A549 cell line was maintained in a complete tissue culture medium, Dulbecco's Modified Eagle's Medium, with 10 % fetal bovine serum and 2 mM L-glutamine, along with antibiotics (about 100 international unit/mL of penicillin, 100 μg/mL of streptomycin) with the pH adjusted to 7.2. The cytotoxicity was determined according to the method of Balachandran et al. [20] with some changes. Cells (5000 cells/well) were seeded in 96 well plates containing medium with different concentrations such as 100, 80, 60, 40, 20, and 10 μg/mL. The cells were cultivated at 37 °C with 5 % CO₂ and 95 % air in 100 % relative humidity. After various durations of cultivation, the solution in the medium was removed. An aliquot of 100 μL of medium containing 1 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide was loaded in the plate. The cells were cultured for 4 h and then the solution in the medium was removed. An aliquot of 100 μL of DMSO was added to the plate, which was shaken until the crystals were dissolved. The cytotoxicity against cancer cells was determined by measuring the absorbance of the converted dye at 540 nm in an enzyme-linked immune sorbant assay reader. Cytotoxicity of each sample was expressed as the half maximal inhibitory concentration (IC₅₀) value. The IC₅₀ value is the concentration of the test sample that causes 50 % inhibition of cell growth, averaged from three replicate experiments.

**Molecular Docking Studies**

The docking analysis was developed by AutoDock Tools (ADT) [24] version 1.5.6 and AutoDock version 4.2.5.1 programs (AutoDock, AutoGrid, Copyright 1989–2012) from the Scripps Research Institute [http://www.scripps.edu/mb/olson/doc/autodock].

**Ligand Preparation**

Ligand 2,3-dihydroxy-9,10-anthraquinone 2D structure was drawn using ChemDraw Ultra 7.0 (ChemOffice 2002). Chem3D Ultra 7.0 was used to convert 2D structure into 3D, and the energy was minimized using semi-empirical AM1 method. Minimized energy to minimum RMS gradient of 0.100 was set in each iteration then the structure was saved as pdb file format for input to ADT. In ADT, ligand bond torsion was assigned and saved in PDBQT file format for input into docking.

**Protein Preparation**

Three-dimensional structures of topoisomerase IV (PDB entry code 3LPS) and TtgR (PDB entry code 2UXO) were retrieved from the Protein Data Bank (PDB) [http://www.pdb.org]. For
the protein structure, polar hydrogen atoms were added, lower occupancy residue structures were deleted, and any incomplete side chains were replaced using the ADT. Further, ADT was used to remove crystal water, add Gasteiger charges to each atom, and merge the non-polar hydrogen atoms to the protein structure. The distance between donor and acceptor atoms that form a hydrogen bond was defined as 1.9 Å with a tolerance of 0.5 Å, and the acceptor–hydrogen–donor angle was not less than 120°. The structures were then saved in PDBQT file format for input into docking.

Docking Analysis

A grid box with dimensions of $126 \times 126 \times 126$ Å$^3$ centred on 0.383, 34.758, 45.087, and $104 \times 126 \times 106$ Å$^3$ centred on 7.983, 30.454, 4.809 was created around the binding site on 3LBS and 2UXO protein, and grid energy calculations were carried out using ADT. 2,3-dihydroxy-9,10-anthraquinone was docked to target protein complexes 3LPS and 2UXO with the protein molecule considered as a rigid body and the ligands being flexible. The search was carried out with the Lamarckian Genetic Algorithm [25]; populations of 150 individuals with a mutation rate of 0.02 evolved for 10 generations. Evaluation of the results was done by sorting the different complexes with respect to the predicted binding energy. A cluster analysis based on root-mean-square deviation values, with reference to the starting geometry, was subsequently performed, and the lowest-energy conformation of the more populated cluster was considered as the most trustable solution. The outputs were exported to Chimera [26] and PyMol [27] for visual inspection of the binding modes and interactions of the compounds with amino acid residues in the active sites.

Statistical Analysis

Antimicrobial and cytotoxic activities of 2,3-dihydroxy-9,10-anthraquinone were statistically analyzed by Duncan multiple range test at $P=0.05$ with the help of SPSS version 11.5 software package.

Results and Discussion

We have isolated 54 isolates from different soil samples of the Marapalam forest, Nilgiris (Southwestern Ghats), Tamil Nadu, India, using starch casein agar medium (data not showed). Among the 54 isolates, the ERINLG-127 strain showed good antimicrobial activity in our preliminary screening. This strain was a Gram-positive filamentous bacterium. The color of the substrate mycelia was white. The spore chains were white (Table 1). These characteristic morphological properties strongly suggested that the isolate belonged to the Streptomyces genus. ERINLG-127 showed good growth on medium amended with sodium chloride up to 8%; no growth was seen at 11%. The temperature for growth ranged from 25 to 37 °C with the optimum of 30 °C, and the pH range was 6–10 with normal pH of 7. Utilization of various carbon sources by ERINLG-127 indicated a wide pattern of carbon source assimilation. Arabinose, ribose, lactose, mannitol and xylose did not support the growth of the isolate. ERINLG-127 showed sensitivity towards ciprofloxacin, gentamicin, ampicillin, cephalexin, erythromycin, vencomycin, amikacin, penicillin, rifamycin and norfloxacin (Table 2). The culture, morphological characteristics and antimicrobial activities of different Streptomyces isolates have been reported by several investigators [15,28].
The result of the sequencing of ERINLG-127 was obtained in the form of rough electrophoregrams. The total nucleotide sequence was 1221 bp. The phylogenetic tree obtained by applying the neighbour-joining method is illustrated in Fig. 1. Culture characteristics and \textit{16S rRNA} studies strongly suggested that our isolate, ERINLG-127, belonged to the genus \textit{Streptomyces}. Studies on the microbial diversity by \textit{16S rRNA} gene analysis showed that a group of high-GC Gram-positive bacteria (actinomycetes) was dominant in the soil [29]. The identification of isolate ERINLG-127 was confirmed as \textit{S. galbus} with homology of 100%. The universal primers seem to be sufficient for identifying the genus, but not the species.

The active principle obtained by preparative HPLC from fraction 13 as the major compound gave pale yellow crystals from methanol (yield 70 mg); it gave a blue color with alcoholic FeCl$_3$ for phenol and pink color with alcoholic NaOH. On TLC over silica gel with ethyl acetate, methanol 4:1 as the developing system gave a single spot ($R_f=0.43$), pale yellow in color which on exposure to ammonia vapour turned pink. The compound was C$_{14}$H$_8$O$_4$ [$M + H$]$^+$, M/Z 241, on the basis of $^1$H NMR and $^{13}$C NMR (DEPT) and mass. UV$_{max}$ MeOH nm: 225, 249, 278, 322 and 441. IR, $\nu_{max}$KBr cm$^{-1}$: 3,409 (hydroxyl), 2,920, 2,849, 1,667 (quinone carbonyl), 1,612, 1,563, 1,522, 1,450 (aromatic), 1,383, 1,320, 1,263, 1,200, 1,169, 1,132, 1,015, 864, 843, 825, 796, and 722 (aromatic). $^1$H NMR ($\delta$, CDCL$_3$, 400 MHz): 8.32 (2H, m (multiplet proton), H-5 and H-8), 7.83 (2H, m, H-6 and H-7), 7.12 (2H, s, (singlet proton) H-1and H-4), 5.12 and 5.14 (1H each, s, 2xOH). $^{13}$C NMR ($\delta$, CDCL$_3$, 100 MHz): 183.2 (C-9, C-10), 155.3 (C-2, C-3), 134.2 (C-6, C-7), 133.2 (C-8a, C-10a), 127.3 (C-9a, C-4a), 130.3 (C-5, C-8), 117.2 (C-1, C-4). EI-MS, (m/z) 241 [M$^+$H]$^+$, 100 % (Fig 2). The IR spectrum showed peaks for hydroxyl (3,409 cm$^{-1}$), quinone carbonyl (1,667 cm$^{-1}$) and aromatic system (1,612, 1,563, 1,522, 1,450, 1,383, 1,320, 1,263, 1,200, 1,169, 1,132, 1,015, 864, 843, 825, 796, and 722 cm$^{-1}$). The $^1$H and $^{13}$C NMR showed the compound to be 2,3-dihydroxy-9,10-anthraquinone. H-5 and H-8 appeared to as low-proton multiplet at $\delta$ 8.32. H-6 and H-7 appeared as two proton multiplets at $\delta$ 7.83. H-1 and H-4 appeared as low-proton singlets at $\delta$ 7.12. The two phenolic hydroxyl protons appeared as a singlet at $\delta$ 5.12 and 5.14 exchangeable with D$_2$O. The $^{13}$C NMR also confirmed the structure of the compound to be 2,3-dihydroxy-9,10-anthraquinone (see experimental). On the basis of the physical and spectroscopic data, the compound was identified as 2,3-dihydroxy-9,10-anthraquinone. The values were comparable with those reported in the literature [30].

\begin{table}
\begin{tabular}{|l|l|l|l|l|l|}
\hline
Medium & Growth & Substrate mycelium & Aerial mycelium & Spores & Reverse \\
\hline
ISP 1 & Good & Present & White & White & Yellowish orange \\
ISP 2 & Good & Present & White & White & Orange \\
ISP 3 & Good & Moderate & White & Poor white & Yellowish orange \\
ISP 4 & Good & Present & White & White & Orange \\
ISP 5 & Good & Present & White & White & Yellowish orange \\
ISP 6 & Good & Present & White & White & Orange \\
ISP 7 & Poor & Poor & White & Poor & Moderate \\
MNGA & Good & Present & White & White & Yellowish orange \\
Gauze & Poor & Poor & White & Poor & Yellowish orange \\
\hline
\end{tabular}
\caption{Culture characteristics of \textit{Streptomyces galbus} (ERINLG-127) in different media}
\end{table}

\textit{ISP1–7} International \textit{Streptomyces} Project, \textit{MNGA} modified nutrient glucose agar
**Streptomyces galbus** (ERINLG-127) was extracted using ethyl acetate. Ethyl acetate extract showed antibacterial and antifungal activities against bacteria and fungi (2.5 mg/mL; Table 3). Ethyl acetate extract showed potent antibacterial activity against Gram-negative bacteria. The

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram staining</td>
<td>Positive</td>
</tr>
<tr>
<td>Shape and growth</td>
<td>Filamentous aerial growth</td>
</tr>
<tr>
<td>Production of diffusible pigment</td>
<td>+</td>
</tr>
<tr>
<td>Range of temperature for growth</td>
<td>25 to 37 °C</td>
</tr>
<tr>
<td>Optimum temperature</td>
<td>30 °C</td>
</tr>
<tr>
<td>Range of pH for growth</td>
<td>6 to 11</td>
</tr>
<tr>
<td>Normal pH</td>
<td>7</td>
</tr>
<tr>
<td>H₂S production</td>
<td>–</td>
</tr>
<tr>
<td>Amylase</td>
<td>+</td>
</tr>
<tr>
<td>Chitinase</td>
<td>–</td>
</tr>
<tr>
<td>Protease</td>
<td>+</td>
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<tr>
<td>Gelatinase</td>
<td>+</td>
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<tr>
<td>Indole production</td>
<td>+</td>
</tr>
<tr>
<td>Growth in the presence of NaCl</td>
<td>1 to 8 %</td>
</tr>
<tr>
<td><strong>Sugar analysis</strong></td>
<td></td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>–</td>
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<tr>
<td>Sucrose</td>
<td>+</td>
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<tr>
<td>Glucose</td>
<td>+</td>
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<tr>
<td>Galactose</td>
<td>+</td>
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<tr>
<td>Starch</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>–</td>
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<tr>
<td>Arabinose</td>
<td>–</td>
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<td>Xylose</td>
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<tr>
<td>Rhamnose</td>
<td>+</td>
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<tr>
<td>Ribose</td>
<td>–</td>
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<tr>
<td><strong>Standard antibiotics</strong></td>
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<tr>
<td>Ciprofloxacin</td>
<td>S</td>
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<tr>
<td>Gentamicin</td>
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<td>Streptomycin</td>
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<td>Penicillin</td>
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<tr>
<td>Rifamycin</td>
<td>S</td>
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<tr>
<td>Norfloxacin</td>
<td>S</td>
</tr>
</tbody>
</table>

+ presence, – absence, S sensitive, R resistant

*Streptomyces galbus* (ERINLG-127) was extracted using ethyl acetate. Ethyl acetate extract showed antibacterial and antifungal activities against bacteria and fungi (2.5 mg/mL; Table 3). Ethyl acetate extract showed potent antibacterial activity against Gram-negative bacteria. The
zones of inhibition were 23 mm against *P. aeruginosa*, 21 mm against *S. typhimurium*, 20 mm against *S. flexneri*, 19 mm against *K. pneumoniae* and 18 mm against *S. paratyphi B.* The isolated compound was also tested against bacteria and fungi. The compound showed potent antibacterial and antifungal activities (Table 4). The minimum inhibitory concentration values of isolated compound were Gram-negative bacteria 12.5 μg/mL against *P. aeruginosa*, *K. pneumoniae* and *S. typhimurium*. The isolated compound showed good activities against some clinical isolates: 12.5 μg/mL against *K. pneumoniae* (ESBL-3971), *K. pneumoniae* (ESBL-3894) and *S. aureus* (MRSA) and fungi 50 μg/mL against *C. albicans*. Primary screening revealed that MNGA medium was a very good base for the production of antibacterial compounds. Maximum growth and pigment production were observed in glucose as the sole source of carbon. The optimum temperature of 30 °C was found to be effective for growth and pigment production. Maximum antimicrobial compound was obtained at pH 7.0. Earlier

Fig. 1 Phylogenetic tree derived from 16S rRNA gene sequences showing the relationship between *Streptomyces galbus* (ERINLG-127) and the other species belonging to the genus *Streptomyces* constructed using the neighbour-joining method. Bootstrap values were expressed as percentages of 1000 replications.
report showed that 12 actinomycetes strains were isolated from the soil samples of the Himalaya, and ERIH-44 showed both antibacterial and antifungal activities [18]. Normally, antibiotic production was higher in medium having glucose (1 %) as carbon source. *S. galbus* (ERINLG-127) showed good antimicrobial activity in MNGA medium and indicated that the antimicrobial compounds were extracellular. Most of the secondary metabolites and antibiotics were extracellular in nature, and extracellular products of actinomycetes showed potent antimicrobial activities [31,32]. The study of the influence of different nutritional media and culture conditions on antimicrobial compound production indicated that the highest biological activities were obtained when MNGA medium was used as a base. In fact, it has been shown that the nature of carbon and nitrogen sources strongly affected antibiotic production in different organisms, and the antibiotic production was increased by glucose-rich medium [33]. *P. aeruginosa* has emerged as one of the most problematic Gram-negative pathogen, with an alarmingly high antibiotic resistance rate [34]. Even with the most effective antibiotics

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**Table 3** Antimicrobial activity of ethyl acetate extract from *Streptomyces galbus* (ERINLG-127) using disc diffusion method (zone of inhibition in millimeters) (2.5 mg/disc)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Ethyl acetate</th>
<th>Streptomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram negative</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. flexneri</em></td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td><em>S. paratyphi B</em></td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>23</td>
<td>30</td>
</tr>
<tr>
<td><em>P. vulgaris</em></td>
<td>-</td>
<td>30</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>21</td>
<td>24</td>
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<tr>
<td><em>E. aerogenes</em></td>
<td>-</td>
<td>22</td>
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<tr>
<td><strong>Gram-positive</strong></td>
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<tr>
<td><em>B. subtilis</em></td>
<td>18</td>
<td>22</td>
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<tr>
<td><em>M. luteus</em></td>
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<td><em>S. aureus</em></td>
<td>10</td>
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<tr>
<td><em>S. epidermidis</em></td>
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<td><strong>Fungi</strong></td>
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<td><em>C. albicans</em></td>
<td>15</td>
<td>28</td>
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<tr>
<td><em>A. flavus</em></td>
<td>12</td>
<td>26</td>
</tr>
<tr>
<td><em>M. pachydermatis</em></td>
<td>14</td>
<td>24</td>
</tr>
</tbody>
</table>

Values in each column followed by the same alphabets are not significantly different by Duncan test at *P*<0.05. Streptomycin is the standard antibacterial agent and ketoconazole is the standard antifungal agent.

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Fig. 2 Structure of 2,3-dihydroxy-9,10-anthraquinone
against this pathogen, namely, carbapenems (imipenem and meropenem), the resistance rate was found to be 15–20.4 % amongst 152 \textit{P. aeruginosa} strains [35]. Our study showed that the isolated compound was active against \textit{P. aeruginosa}. Candida albicans, causing candidiasis, is increasingly becoming an important species worldwide, due to the fact that it is among the opportunistic pathogens frequently found in AIDS patients [36]. It has been reported that the environmental factors like temperature, pH and incubation have profound influence on antibiotic production. This activity might be due to their ability to complex with bacterial cell wall [36]; thus, inhibiting the microbial growth and the membrane disruption could be suggested as the mechanism of action [37]. The antimicrobial compound from \textit{S. galbus} (ERINLG-127) was recovered using ethyl acetate solvent. Most of the antimicrobial compounds are extracted using ethyl acetate [38]. Narayana et al. (2008) [39] reported that three bioactive compounds were purified and identified as 3-phenylpropionic acid, anthracene-9,10-quinone and 8-hydroxyquinoline. Among these metabolites, 8-hydroxyquinoline exhibited strong antibacterial and antifungal activity as compared to phenylpropionic acid and anthracene-9,10-quinone.

The isolated compound showed cytotoxic activity in vitro against A549 lung adenocarcinoma cancer cell line. It showed 75.1 % activity at the dose of 100 \( \mu \text{g/mL} \) with an IC\textsubscript{50} value of 60 \( \mu \text{g/mL} \). All concentrations used in the experiment decreased the cell viability significantly \((P<0.05)\) in a concentration-dependent manner (Fig 3). A number of anthraquinones have been reported to possess tumor cell inhibitory effects and are currently utilized as clinical

| Table 4 | Minimum inhibitory concentration of 2,3-dihydroxy-9,10-anthraquinone from \textit{Streptomyces galbus} (ERINLG-127) against tested bacteria and fungi |
|----------------|---------------------------------|-----------------|
| Organism | Compound | Streptomycin |
| Gram negative | | |
| \textit{S. flexneri} | 25 | 6.25 |
| \textit{K. pneumoniae} | 12.5 | 6.25 |
| \textit{S. typhimurium} | 12.5 | 6.25 |
| \textit{S. paratyphi-B} | 25 | 12.5 |
| \textit{P. aeruginosa} | 12.5 | 25 |
| Gram-positive | | |
| \textit{B. subtilis} | 25 | 12.5 |
| \textit{M. luteus} | >100 | 6.25 |
| \textit{S. aureus} | >100 | 6.25 |
| \textit{S. epidermidis} | >100 | 12.5 |
| Clinical isolates | | |
| \textit{E. coli} (ESBL-3984) | 50 | 25 |
| \textit{E. coli} (ESBL-3904) | 50 | 25 |
| \textit{K. pneumoniae} (ESBL-3971) | 12.5 | 6.25 |
| \textit{K. pneumoniae} (ESBL-75799) | 50 | 25 |
| \textit{K. pneumoniae} (ESBL-3894) | 12.5 | 6.25 |
| \textit{K. pneumoniae} (ESBL-3967) | 75 | 25 |
| \textit{S. aureus} (MRSA) | 12.5 | 6.25 |
| Fungi | Ketoconazole |
| \textit{C. albicans} | 50 | 25 |
| \textit{A. flavus} | >100 | 12.5 |
| \textit{M. pachydermatis} | 75 | 15 |

Streptomycin is the standard antibacterial agent and ketoconazole is the standard antifungal agent.
anticancer agents. Anthraquinones have been shown to inhibit cancer cells through a variety of mechanisms including induction of apoptosis [40,41], intercalation and binding with cellular DNA [42], redox-cycling radical formation [43,44], and inhibition of topoisomerase [45]. The new, highly oxygenated angucyclinone gephyromycin was isolated from an extract of a Streptomyces griseus strain. Gephyromycin exhibited glutaminergic activity towards neuronal cells [46].

The docking simulations in the active sites of topoisomerase IV (PDB: 3LPS) and TtgR (PDB: 2UXO) were performed by the AutoDock Tools (ADT) version 1.5.6 and AutoDock

![Fig. 3](image3.png) MTT assay showing the cytotoxicity of 2,3-dihydroxy-9,10-anthraquinone

![Fig. 4](image4.png) Putative binding pose of compound 2,3-dihydroxy-9,10-anthraquinone with topoisomerase. Docking of compound 2,3-dihydroxy-9,10-anthraquinone with topoisomerase (a, b and c), where hydrogen bonding interactions are shown in dotted lines
version 4.2.5.1 programs. The target protein structures were docked with 2,3-dihydroxy-9,10-
anthraquinone which provided least values of the binding energy. The binding profile of the
2,3-dihydroxy-9,10-anthraquinone docking with topoisomerase IV showed interaction with
ILE314, TYR310 and EU313 amino acids which resulted in the lowest free binding energy of
$-6.39$ kcal/mol (Fig 4). Docked orientations of 2,3-dihydroxy-9,10-anthraquinone with TtgR
showed interactions with LEU66, GLU99 and HIS67 amino acids with lowest free binding
energy of $-6.67$ kcal/mol (Fig 5). TtgR is a multidrug-binding protein and regulates one of the
key mechanisms in antibiotic resistance by active extrusion of toxic compounds through the
membrane-bound efflux pumps. Paul and Choudhury (2010) [47] reported that TtgR is a
multidrug-binding repressor that negatively controls the transcription of the TtgABC operon as
well as its own expression. TtgR belongs to the TetR family of proteins, which are character-
ized by a conserved DNA-binding domain but not a variable ligand binding domain. The
TtgR-TtgAB system has been shown to recognize and extrude compounds that belong to
different functional classes including antibiotics, flavonoids and organic solvents [48–50]. All
of these compounds are toxic to the bacterial cell. Parvathy et al. (2013) [51] reported that eight
different novel benzothiazole analogues were subjected to in silico molecular modelling. These
compounds showed good docking score for topoisomerase IV (PDB entry code 3LPS), gyrase
(PDB entry code 3TTZ) and N-myristoyl transferase (PDB entry code 1IYL).

**Conclusion**

*Streptomyces galbus* (ERINLG-127) was isolated from the soil samples of the Marapalam
forest, Nilgiris, Tamil Nadu, India. The bioactivity-guided fractionation of the ethyl acetate led
to the isolation of 2,3-dihydroxy-9,10-anthraquinone as the active principle. The compound
also showed good antimicrobial activity against tested bacteria and fungi. The compound was

![Fig. 5 Putative binding pose of compound 2,3-dihydroxy-9,10-anthraquinone with TtgR. Docking of compound 2,3-dihydroxy-9,10-anthraquinone with TtgR (a, b and c), where hydrogen bonding interactions are shown in dotted lines](image-url)
also tested against the A549 lung adenocarcinoma cancer cell line. It showed a 75.1% activity at the dose of 100 μg/mL with an IC50 value of 60 μg/mL. Molecular docking studies of isolated compound 2,3-dihydroxy-9,10-anthraquinone with enzyme TtgR and topoisomerase IV showed low binding energy. This is the first report for the antimicrobial and cytotoxic properties of 2,3-dihydroxy-9,10-anthraquinone isolated from *S. galbus*.

**Acknowledgments** The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the research group project no. RGP-VPP-213.

**Reference**


Petroleum and polycyclic aromatic hydrocarbons (PAHs) degradation and naphthalene metabolism in *Streptomyces* sp. (ERI-CPDA-1) isolated from oil contaminated soil

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**A B S T R A C T**

Petroleum and polycyclic aromatic hydrocarbons (PAHs) degrading *Streptomyces* sp. isolate ERI-CPDA-1 was recovered from oil contaminated soil in Chennai, India. The degradation efficiencies were examined by GC-FID and the results showed that the isolate could remove 98.25% diesel oil, 99.14% naphthalene and 17.5% phenanthrene in 7 days at 30 °C (0.1%).

ERI-CPDA-1 was able to degrade naphthalene, phenanthrene and diesel oil and grow on petrol, diesel, kerosene, benzene, pyridine, methanol, ethanol, cyclohexane, tween-80, xylene, DMSO and tolune using them as sole carbon source. Effects of environmental condition on degradation of hydrocarbons (diesel oil, naphthalene and phenanthrene) were also studied at different pH, NaCl, temperature, concentration and incubation time. Degradation pathway for naphthalene has been proposed. Degradation metabolites were identified using GC–MS analysis of ethyl acetate extract of the cell free culture. The degradation products detected were benzaldehyde, catechol, phenylacetic acid and protocatechuc acid.

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1. Introduction

*Streptomyces* are typical soil bacteria which are widely distributed in a variety of natural and manmade environments constituting a significant component of the microbial population in most soils (hot deserts, marsh areas and cold Alpine slopes). There are few reports which indicate that *Streptomyces* flora could play a very important role in degradation of hydrocarbons ([Barabas et al., 2001; Radwan et al., 1998]). In the present era petroleum hydrocarbon contamination is considered a major widespread environmental problem distributed in atmosphere, terrestrial soil, marine waters and sediment. The persistence of a pollutant in the environment is influenced by the nature of the contaminant, the amount of the contaminant present and the interplay between chemical, geological, physical and biological characteristics of the contaminated site. Polycyclic aromatic hydrocarbons (PAHs) were, perhaps, the first recognized environmental carcinogens. They do not degrade easily under natural conditions. Persistence increases with increase in molecular weight. Though they are the chief pollutants of air, soil acts as the ultimate repository of these chemicals. PAHs are reported to be highly toxic, mutagenic and carcinogenic chemicals that are ubiquitous in the environment ([Yucheng et al., 2008]). They have a detrimental effect on the flora and fauna of affected habitats, resulting in the uptake and accumulation of toxic chemicals in food chains and in some instances, in serious health problems and/or genetic defects in humans ([Samanta et al., 2002]). Therefore, US Environmental Protection Agency (EPA) has identified 16 kinds of PAHs as priority pollutants ([Yucheng et al., 2008]). PAHs released into the environment could be removed through many processes, including volatilization, photo-oxidation, chemical oxidation, bioaccumulation, biodegradation and adsorption.

Biodegradation is recognized as an efficient, economic and versatile alternative to physicochemical treatment of oil contaminants. In the past decade, biodegradation techniques have been developed to clean up the soils polluted with hazardous chemicals ([Alexander, 1994; Romantschuk et al., 2000]). Large numbers of microorganisms play a major role to remove low molecular and high molecular weight PAHs from contaminated sites as the sole source of carbon and energy ([Bamforth and Singleton, 2005]). Barabas et al. (2001) have reported three *Streptomyces* strains (*S. griseoflavus, S. parvus, S. plicatus*) from the Kuwait Burgan oil field with the ability to utilize n-hexadecane, n-octadecane, kerosene and crude oil as sole carbon and energy sources. In the last 20 years much has been published about the ability of microorganisms to utilize hydrocarbons, especially n-alkanes as carbon and energy sources. These microorganisms belong to *Pseudomonas* sp. ([Naas et al., 2009]), *Alcaligenes* sp. ([Weissenfels et al., 1990]), *Mycobacterium* sp. ([Pagnout et al., 2007]) and *Rhodococcus* sp. ([Martinikova et al., 2009]). Bacterial degradation represents a significant pathway for the removal of PAHs...
from the environment (Bouwer and Zehnder, 1993). During the past decade a variety of bacteria have been isolated and characterized for the ability to degrade different PAHs and new pathways for PAH degradation have been elucidated (Anweiler et al., 2000; Zeinali et al., 2008; Lin et al., 2010). Only few data are available about the role of Streptomyces in the bioremediation of oil polluted soils (Barabas et al., 2001). The present study was aimed at assessing the petroleum and PAHs degradation potential and naphthalene metabolic pathway in Streptomyces sp. isolate (ERI-CPDA-1) recovered from oil contaminated soil.

2. Methods

2.1. Chemicals and media

Naphthalene (molecular formula: C10H8; molecular weight: 128.17052 g/mol; melting point: 80.26 °C, 353 K, 176 F; boiling point: 218 °C, 491 K, 424 F) and phenanthrene (molecular formula: C14H10; molecular weight: 178.23; melting point: 99 °C, 176 F; boiling point: 340 °C) and Starch casein agar (SCA) were purchased from Himedia (Mumbai). Petroleum products and other chemicals used were of analytical grade or HPLC reagent grade and purchased locally. All chemicals were of the highest purity available, generally greater than 98% as listed by the manufacturers. Modified nutrient local media or other chemicals contained g/L, 10 g of glucose, 5 g of peptone, 3 g of beef extract, 3 g of dry yeast, 3 g of NaCl and 3 g of glucose agar (MNGA) medium contained g/L, 10 g of glucose, 5 g of peptone, 3 g of beef extract, 3 g of dry yeast, 3 g of NaCl and 3 g of CaCO3. The inorganic medium (IM) consisted of the following composition in g per liter: 0.85 NaNO3; 0.56 KH2PO4; 0.86 Na2HPO4; 0.17 K2SO4; 0.37 MgSO4 7H2O; 0.007 CaCl2 6H2O; 0.004 Fe (III) EDTA; 2.5 ml of trace element solution consists of (g per liter): 2.32 ZnSO4 7H2O; 1.78 MnSO4 4H2O; 0.56 H3BO3; 1.0 CuSO4 5H2O; 0.39 Na2MoO4 2H2O; 0.66 KI; 1.0 EDTA; 0.4 FeSO4 7H2O; 0.004 NiCl2 6H2O (Radwan et al., 1998).

2.2. Sample collection

The soil samples were collected from oil spilled areas in petrol bunk in Chetpet (13°4’12”N 80°13’48”E), Chennai, Tamil Nadu, India. Soil samples were collected from the surface layer (5–15 cm) and transported aseptically in a sterile plastic container to the laboratory; they were air dried and stored at 4°C for 2 months and in a freezer at 48°C. Enrichment cultures were prepared in ISP-2 slant culture at 4°C for 7–14 days. Morphology of spore bearing hyphae with entire spore chain was observed with a light microscope (Model SE; Nikon) using cover-slip method in ISP media (ISP 3–6). The cell’s morphological properties were examined by scanning electron microscopy (SEM). The shape of cell, Gram-stain, color determination, the presence of spores, and colony morphology were assessed on solid ISP agar medium. Biochemical reactions, different temperatures, NaCl concentration, pH level, pigment production, enzyme reaction and acid or gas production were done following the method of Valanarasu et al. (2009).

2.2.1. 16S rRNA gene amplification

Genomic DNA of ERI-CPDA-1 was isolated by the methods described in earlier reports (Enticknap et al., 2006). The 16S ribosomal RNA gene was amplified by PCR method using primers 27f (5’AGTGTTGATCCTTCGGCTCAG-3’) and 1492r (5’ACGCTACCTTGTTACGACTT-3’). Each PCR mixture in a final volume of 20 μL consisted of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 μM of each dNTP, 10 pmol of each primer, 50 ng of genomic DNA and 1 U of Taq DNA Polymerase (New England Biolabs Inc.). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94°C for 4 min followed by 30 cycles at 94°C for 1 min, primer annealing at 52°C for 1 min and primer extension at 72°C for 1 min. At the end of the cycling, the reaction mixture was held at 72°C for 10 min and then cooled to 4°C. PCR amplification was detected by 1% agarose gel electrophoresis and was visualized by ultraviolet (UV) fluorescence after ethidium bromide staining. The PCR product obtained was sequenced by an automated sequencer (GeneMarker 3130, Applied Biosystem, and USA). The same primers as above were used for this purpose. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks using the NCBI BLAST available at http://www.ncbi.nlm.nih.gov/.

2.4. Morphological, physiological and biochemical observations

Cultural and morphological features of ERI-CPDA-1 were characterized following the directions given by the International Streptomyces Project (ISP) (Shirling and Gottlieb, 1966) and the Bergey’s Manual of Systematic Bacteriology. Cultural characteristics of pure isolates in various media (ISP 1–7) were recorded after incubation at 28°C for 7–14 days. Morphology of spore bearing hyphae with entire spore chain was observed with a light microscope (Model SE; Nikon) using cover-slip method in ISP media (ISP 3–6). The cell’s morphological properties were examined by scanning electron microscopy (SEM). The shape of cell, Gram-stain, color determination, the presence of spores, and colony morphology were assessed on solid ISP agar medium. Biochemical reactions, different temperatures, NaCl concentration, pH level, pigment production, enzyme reaction and acid or gas production were done following the method of Valanarasu et al. (2009).

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2.5. Nucleotide sequence accession number

The partial 16S rRNA gene sequences of isolate ERI-CPDA-1 have been deposited in the GenBank database under Accession No.: HQ385919. A phylogenetic tree was constructed using the neighbor-joining DNA distance algorithm using software MEGA (Tamura et al., 2007) (version 4.1).

2.6. Carbon source utilization

Various aromatic compounds were added as carbon sources to liquid IM media. The purified isolate ERI-CPDA-1was grown in 100 mL IM medium containing the appropriate PAHs or other carbon source; it was inoculated on one of the following compounds at 0.1%, and incubated at 30°C at 200 rpm for 7 days in an orbital shaker. The compounds were: Petrol, diesel, diesel oil, kerosene, naphthalene, phenanthrene, anthracene, benzene, pyridine, xylene, phenol, dimethyl sulphoxide (DMSO), ethanol, methanol, tween 80, cyclohexane, salicylic acid and tolune. Growth was measured on the 7th day by increase of OD600 nm (Optical Density) of the culture. ERI-CPDA-1 biomass during PAHs degradation was also determined; culture broth was filtered on Whatman No. 1 filter paper, washed with distilled water, and then dried at 60°C overnight until constant weight was achieved.
2.7. Degradation experiments of PAHs

Utilization of naphthalene and phenanthrene as a sole source of carbon and energy was determined. PAHs-degradation in liquid culture was performed using washed cell suspensions pre-grown (culture of Streptomyces sp. ERI-CPDA-1 isolate grown in MNGA medium for 3 days were harvested, washed and resuspended in IM medium) with corresponding PAHs. The liquid culture (100 mL of IM with 100 mg naphthalene and phenanthrene, respectively) was inoculated by transferring 3 mL of pre-culture of isolate ERI-CPDA-1 (3%, v/v). The flasks were incubated in darkness at 200 rpm at 30 °C for 7 days. Uninoculated flasks and flasks without PAHs served as controls. All treatments including control were in triplicate. The growth was monitored by OD600nm. After 7th day the culture broth was centrifuged at 8000g for 15 min to remove the biomass. The cell free supernatant was acidified with 1 N HCl to pH 2.0 and then extracted with an equal volume of ethyl acetate (Zeinali et al., 2008). The combined ethyl acetate layer was dried over anhydrous Na2SO4 and distilled in rotary vacuum evaporator to dryness at 40 °C.

2.8. Gas chromatography (GC-FID) of naphthalene and phenanthrene degradation products

PAHs degradation efficiencies were detected on 7th day by gas chromatography (GC). The concentration of naphthalene and phenanthrene (1 mg/1 mL) in samples were analysed using a CP-3800 series GC equipped with a flame ionization detector (FID) and CP-Sil 8 CB column of 30 m, i.d. 0.32 mm and 0.25 μm film thicknesses. The initial column temperature was 50 °C and temperature was increased from 50 to 250 °C, with column temperature being held for 10 min. Detector and injector temperatures were 300 and 250 °C, respectively. The degradation of PAHs as a whole was expressed as the percentage of PAHs degraded in relation to the amount of the remaining fractions in the appropriate abiotic control samples (external standard technique). The degradation efficiency (DE) based on the decrease in the total concentration of hydrocarbons, was evaluated using the following equation:

\[ \text{DE} (\%) = 100 - \left( \frac{A_t}{A_{ac}} \times 100 \right) \]

where \( A_t \) is the total area of peaks in each sample, \( A_{ac} \) is the total area of peaks in the appropriate abiotic control, DE (%) is the efficiency of degradation.

2.8.1. Biodegradation of diesel oil

The ERI-CPDA-1 isolate washed cell suspension was transferred into 250 mL Erlenmeyer flasks with stoppers each containing 100 mL of IM supplemented with 100 mg diesel oil and cultured at 200 rpm for 7 days. After incubation, the cell density was measured at OD600nm and the culture broth was extracted twice with equal volume of ethyl acetate (Zeinali et al., 2008). The combined ethyl acetate layer was dried over anhydrous Na2SO4 and distilled in rotary vacuum evaporator to dryness at 40 °C.

2.8.2. Effect of environmental condition on degradation of hydrocarbons

Various conditions for degradation of naphthalene, phenanthrene and diesel oil were as follows: Incubation temperatures of 20, 25, 30 and 37 °C; autoclaved medium initial pH of 6, 7, 8, 9 and 10 using 1 M HCl or 1 M NaOH; the salinity of 1, 3, 5, 7, 9 and 11 g/L with NaCl, concentrations of 0.01%, 0.05%, 0.1%, 0.25% and 0.5% and incubation time of 0–7th day. Uninoculated flasks and flasks without PAHs served as controls. Each test flask was prepared in triplicate. After incubation the cell density was measured at OD600nm.

2.8.3. Gas chromatography–mass spectrometry (GC–MS)

Naphthalene metabolites were identified using GC–MS, GC–MS–QP 2010 [SHIMADZU]. For GC–MS analysis, a 30 m × 0.25 mm TR-5MS capillary column with a film thickness of 0.25 μm was used. The carrier gas was helium maintained at a column flow of 1.51 mL/min (at a pressure of 105 kPa). A 1.0 μL sample of the extract was injected and the column temperature was maintained at 70 °C for 2 min followed by temperature programming at 10 °C/min to 200 °C for 2 min. This was raised to 240 °C at a rate of 5 °C/min for 2 min, and finally to 300 °C at a rate of 35 °C/min for 2 min (scan range: 40–1000 m/z). The mass spectrometer and transfer line were held at 290 °C.

3. Results and discussion

3.1. Isolation and identification of hydrocarbons degrading Streptomyces sp

Petroleum and PAHs hydrocarbons degrading Streptomyces sp. isolate ERI-CPDA-1 was recovered from the soil of oil spilled petrol bunk, Chetpet, Chennai, Tamil Nadu, India. The strain was Gram-positive filamentous bacterium. The characteristic properties of strain ERI-CPDA-1 were observed after 7, 14 and 21 days of incubation on different media (Table 1). The spore chains were white in color and 0.814 μm in diameter. The spore was non motile and aerobic. These characteristic morphological properties strongly suggested that ERI-CPDA-1 isolate belonged to the genus Streptomyces. ERI-CPDA-1 showed good growth on medium amended with sodium chloride up to 8%; poor growth was observed at 9% and no growth was seen at 10%. The temperature ranges for growth of ERI-CPDA-1 were 25–37 °C with optimum of 30 °C and the pH range was 6–9 with normal pH of 7. Utilization of various carbon sources (1 g/100 mL) by ERI-CPDA-1 indicated a wide pattern of carbon source assimilation such as, rhamnose, mannose, maltose, lactose, sucrose, glucose, galactose, fructose, mannitol and xylose (Table 2). The total nucleotide sequence of 1450 bp of the 16S rRNA gene of Streptomyces sp.

ERI-CPDA-1 was determined in both strands. Alignment of this sequence through matching with reported 16S rRNA gene sequences in the Genbank showed high similarity (100%) to Streptomyces 16S rRNA genes. Highest similarity (100%) was obtained with the 16S rRNA gene of Streptomyces tiritus (Fig. 1).

3.2. Utilization of carbon source

More than 18 types of carbon source were tested as sole carbon substrate of ERI-CPDA-1 including various low and high molecular weight PAHs and some kinds of n-alkanes. All of them existed ubiquitously in the oil contaminated soil. Strain ERI-CPDA-1 was found to have the ability to degrade naphthalene, phenanthrene and diesel oil and grow on petrol, diesel, kerosene, benzene, pyridine, methanol, ethanol, cyclohexane, tween-80, xylene, DMSO and toluene, thus exhibiting a very broad substrate profile. But the strain was not able to grow anthracene, phenol and salicylic acid. The dry cell mass was also recorded (Table 3).

3.3. Biodegradation of PAHs

The efficiency of the selected isolate on PAHs degradation was individually evaluated by gas chromatography. The results of GC
analyses demonstrated that the isolate ERI-CPDA-1 degraded almost 99.14% naphthalene and 17.5% phenanthrene within 7 days (concentration-0.1%); this showed that phenanthrene was not an efficient carbon source for the strain ERI-CPDA-1 (data not shown). Present results were slightly similar to Lin et al. (2010) who reported that the degradation of naphthalene rose to 99.1% within 4 days under optimum conditions (temperature 30 °C, pH 7.0, inoculum concentration of 0.2% and C/N ratio of 1 when the initial naphthalene concentration was 50 mg/L). In previous results Zhao et al. (2009) reported that nearly 90% of phenanthrene was degraded within 6 days by the strain ZP2. Many different species of bacteria, mostly from soil environments, have the ability to degrade naphthalene and other PAHs. However, there is little information on Gram-positive naphthalene degrading bacteria in marine environments, although PAH-degrading bacteria belonging to the Gram-positive Nocardioides and spore-forming Paenibacillus groups have been isolated from the rhizosphere of salt marsh plants (Daane et al., 2001).

The naphthalene and phenanthrene (0.1%) degradation at a range of culture medium pH values (pH 6–10) was carried out. After 7th day more than 99.14% of naphthalene and 17.5% of phenanthrene were degraded at the pH of the culture medium 7 (Fig. 2a). These results are in agreement with the report by Chen et al. (2006) who found that microorganisms favoured growth at pH level ranging from 6 to 8. The optimum salinity for the Streptomyces used for degradation of naphthalene and phenanthrene ranged from 1 to 5 g/L of NaCl; further increase in the concentration of NaCl from 6 to 11 g/L reduced the growth correspondingly (Fig. 2b). This indicated that high salinity affected cell numbers and distribution, resulting in reduced microbial metabolic rates. Similar result was reported for the biodegradation of phenanthrene using Sphingomonas sp. (Chen et al., 2008). A series of degradation test was carried out at various optimum temperatures from 20 to 37 °C (Fig. 2c). The strain showed good effect at the optimum temperature ranging from 25 to 30 °C for the degradation of naphthalene and phenanthrene (0.1%). Effect of initial naphthalene and phenanthrene concentrations on degradation was also examined to assess the capacity of isolate ERI-CPDA-1 to tolerate different concentrations of naphthalene and phenanthrene. The concentration of naphthalene and phenanthrene increased from 0.01% to 0.5% (Fig. 2d). This suggested that isolate ERI-CPDA-1 could survive and rapidly degrade from 0.01% to 0.1%. The degradation test of naphthalene and phenanthrene was carried out at 30 °C, pH 7 and an initial concentration of 0.1% individually. In the test, none of other carbon sources or surfactants was added to the medium. Fig. 2e presents the degradation results. The strain could remove 99.14% of naphthalene and 17.5% of phenanthrene with an initial concentration of 0.1% in 7 days without adding co-metabolism substrates and surfactants. Pathak et al. (2009) also reported that Pseudomonas sp. (HOB1) degraded 97% of naphthalene within 24 h.

### Table 1

<table>
<thead>
<tr>
<th>Medium</th>
<th>Growth</th>
<th>Substrate mycelium</th>
<th>Aerial mycelium</th>
<th>Reverse</th>
<th>Pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISP 1</td>
<td>Good</td>
<td>Present</td>
<td>White</td>
<td>Brown</td>
<td>+</td>
</tr>
<tr>
<td>ISP 2</td>
<td>Good</td>
<td>Present</td>
<td>White</td>
<td>Brown</td>
<td>+</td>
</tr>
<tr>
<td>ISP 3</td>
<td>Good</td>
<td>Present</td>
<td>Poor</td>
<td>Poor</td>
<td>–</td>
</tr>
<tr>
<td>ISP 4</td>
<td>Good</td>
<td>Present</td>
<td>White</td>
<td>Brown</td>
<td>+</td>
</tr>
<tr>
<td>ISP 5</td>
<td>Good</td>
<td>Present poor</td>
<td>White</td>
<td>Poor</td>
<td>–</td>
</tr>
<tr>
<td>ISP 6</td>
<td>Good</td>
<td>Present</td>
<td>White</td>
<td>Brown</td>
<td>+</td>
</tr>
<tr>
<td>ISP 7</td>
<td>Moderate</td>
<td>Present poor</td>
<td>White</td>
<td>Light brown</td>
<td>–</td>
</tr>
<tr>
<td>MHA</td>
<td>Poor</td>
<td>Poor</td>
<td>Poor</td>
<td>Poor</td>
<td>–</td>
</tr>
<tr>
<td>SDA</td>
<td>Poor</td>
<td>Poor</td>
<td>Poor</td>
<td>Poor</td>
<td>–</td>
</tr>
</tbody>
</table>

ISP1–7: International Streptomyces Project; MHA: Muller Hinton agar; SDA: Sabouraud Dextrose agar; +: present; –: absent.

### Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Growth characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram staining</td>
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<tr>
<td>Shape and growth</td>
<td>Filamentous aerial growth</td>
</tr>
<tr>
<td>Anaerobic growth</td>
<td>–</td>
</tr>
<tr>
<td>Range of temperature for growth</td>
<td>25–37 °C</td>
</tr>
<tr>
<td>Optimum temperature</td>
<td>30 °C</td>
</tr>
<tr>
<td>Range of pH for growth</td>
<td>6–9</td>
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<tr>
<td>Optimum pH level</td>
<td>7</td>
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<tr>
<td>Indole production</td>
<td>–</td>
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<tr>
<td>Amylase</td>
<td>+</td>
</tr>
<tr>
<td>Protease</td>
<td>+</td>
</tr>
<tr>
<td>Chitinase</td>
<td>+</td>
</tr>
<tr>
<td>Gelatinase</td>
<td>–</td>
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<tr>
<td>Growth in the presence of NaCl</td>
<td>1–8%</td>
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<tr>
<td>Sugar analysis</td>
<td></td>
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<tr>
<td>Rhamnose</td>
<td>++</td>
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<tr>
<td>Mannose</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>±</td>
</tr>
<tr>
<td>Lactose</td>
<td>±</td>
</tr>
<tr>
<td>Sucrose</td>
<td>±</td>
</tr>
<tr>
<td>Glucose</td>
<td>±</td>
</tr>
<tr>
<td>Galactose</td>
<td>±</td>
</tr>
<tr>
<td>Fructose</td>
<td>±</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
</tr>
<tr>
<td>Arabinose</td>
<td>–</td>
</tr>
<tr>
<td>Xylose</td>
<td>–</td>
</tr>
</tbody>
</table>

+: Presence; –: absence.

3.4. Biodegradation of diesel oil

The efficiency of the strain ERI-CPDA-1 on diesel oil degradation was individually evaluated by gas chromatograph. The results of GC analyses showed that the isolate ERI-CPDA-1 degraded 98.25% of diesel oil within 7 days (concentration 0.1%). Marchal et al. (2003) reported gasoline and diesel oil biodegradation by micro flora from an activated sludge. Mukherji et al. (2004) also reported that nearly 90% of diesel oil was degraded. Moreover, among 38 bacteria isolated from oil contaminated soils of an oil field in Daqing, China, more than 22 strains utilized diesel oil and 11 strains degraded the hydrocarbons of diesel oil by more than 70% in 7th day (Zhang et al., 2010). In the present study 0.1% diesel oil was degraded (98.25%) by ERI-CPDA-1 isolate in 7 days at pH 7 (Fig. 3a). The optimum salinity for biodegradation of diesel oil ranged from 1 to 5 g/L of NaCl (Fig. 3b). The growth was stopped when the concentration of NaCl increased from 6 to 11 g/L. The effect of initial diesel oil concentrations on biodegradation capacity of ERI-CPDA-1 was also examined from 0.01% to 0.5%. 98.25% diesel oil was degraded on 7th day when the concentration of diesel oil ranged from 0.01% to 0.1%. No effective degradation was found for diesel oil concentration at 0.5% (Fig. 3c). A series of degradation tests were carried out at various temperatures from...
The temperature to degrade the diesel oil ranged from 25 to 30 °C (Fig. 3d). The degradation test of diesel oil was carried out at 30 °C, pH 7 and an initial concentration of 0.1% individually. To illustrate the relationship between growth of ERI-CPDA-1 in IM and the biodegrading diesel oil in the culture medium with diesel oil as a sole source of carbon and energy in the best conditions, the growth of ERI-CPDA-1 in IM was done by measuring biomass (OD600nm) (Fig. 4). The remaining concentration of diesel oil declined in response to increased cell numbers, indicating that diesel oil promoted cell growth. A short time lag was observed during the initial incubation phase, but the biomass growth and degradation of diesel oil rapidly increased after 7 day incubation. Diesel oil at 0.1% was completely degraded (>98.25%) after 7 days of incubation. Several studies have indicated that the extent of oil and total petroleum hydrocarbon biodegradation are closely linked to the type of oil and its molecular composition; the alkanes are more susceptible to biodegradation (Huesemann, 1995; Sugiura et al., 1997).

Ijah (1998) reported that bacteria and yeast isolates from tropical soils were capable of degrading 52% and 69% of crude oil in 16 days. The best conditions for diesel oil degradation by Streptomyces sp. (ERI-CPDA-1) were 30 °C, pH7, NaCl concentration 1–3 g/L and 7 days.

### 3.5. Metabolic pathway of naphthalene

GC–MS profile of the cell free culture extract with ethyl acetate. The degradation products identified were benzoic acid, catechol, phenylactic acid and protocatechuic acid (3,4-dihydroxybenzoic acid). The identification of the compounds was by comparison with...
authentic spectra from the GC–MS library (Wiley.275L, NIST08s and FAME mass spectra data base) and also by direct comparisons with authentic samples. The fragments detected for each of the identified metabolites are given below (m/z, rel int,%). Benzaldehyde (a) 106(M+, 100), 107[M+H]+ (7.85), 105(45.70), 78(18.34), 77[M/C0]+ (99.88), 75(4.43), 74(9.04), 52(12.29), 51(47.21) and SI-97%. Catechol (b) 110(M +, 100), 92(11.47), 81(13.86), 64(41.34), 63(41.34), 53(21.66) and SI-98%. Phenylacetic acid (c) 136(M+, 19.88), 92[M–CO2]+ (19.48), 91[M–COOH]+ (100%), 89(4.65), 85(4.03), 77[M–CH2COOH]+ (1.98), 65(16.66), 63(6.87) and SI-97%. Protocatechuic acid (d) 154(M+, 100), 137[M–OH]+ (69.05), 125(4.23), 110[M–CO2]+ (3.12), 109[M–COOH]+ (17.87), 77(Ph+, 7.88), 107(58), 70(14.78) and SI-92%. The initial step involves hydroxylation by 1,2-dioxygenase to give cis 1,2-naphthalenedihydriodiol. This on dehydrogenation gives 1,2-dihydroxynaphthalene. This undergoes extra diol cleavage and further degradation to give cinnamic acid via 4-(4-hydroxyphenyl)-2oxobut-3-enoic acid and 2-hydroxycinnamic acid. Cinnamic acid gives phenylacetic acid which undergoes further degradation to give benzaldehyde. This on oxidation gives benzoic acid. Hydroxylation at 3,4 position gives protocatechuic acid (3,4-dihydroxybenzoic acid); this on decarboxylation gives catechol. Catechol degrades to muconic acid. Further degradation takes place by TCA cycle (Fig. 5). Dioxygenase and monooxygenase enzymes were reported as major degrading enzymes in the oxidizing degradation of PAHs (Nievas et al., 2006). The degradation pathway of naphthalene is

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**Fig. 2.** Factors affecting the biodegradation of naphthalene and phenanthrene Streptomyces sp. isolate (ERI-CPDA-1): (a) pH; (b) salinity g/L; (c) temperature and (d) concentrations (%). (e) Streptomyces sp. (ERI-CPDA-1) growth and degradation of naphthalene and phenanthrene (absorbance value at OD600nm in 7 days).
similar to that reported by Zeinali et al. (2008), by the bacterium
Nocardia otitidiscaviarum strain TSH1 a moderately thermophilic
microorganism as the bacterium used in the present work. The
degradation from cinnamic acid is similar to that reported by Streptomyces setonii (Sutherland et al., 1983). The occurrence of benzoic
acid in naphthalene metabolism has been reported by Annweiler
et al. (2000). Benzoic acid was proposed to be formed by decarbox-
ylation of phthalic acid. But present study neither phthalic acid nor
its preceding metabolite 2-carboxy cinnamic acid were detected.
Yen and Serdar (1988) reported the metabolism of naphthalene
by Pseudomonas putida PpG7 in which salicylic acid was proposed
to be formed. But in the present study salicylic acid was not
detected in the culture extract and growth on salicylic acid was also
negative. Therefore, the main route for degradation of naphthalene
in strain ERI-CPDA-1 is dioxygenation by a dioxygenase enzyme.
Phenylacetic acid and its esters are extensively used in plastic, tex-
tile, paper, insect repel-lent, pesticide, and cosmetic industries
(Peakall, 1975). Many microorganisms have been identified from
various sources of environment for their ability to degrade naph-
thalene aerobically or anaerobically. This Streptomyces sp. isolate
ERI-CPDA-1 can be exploited for the development of effective bio-
degradation for PAHs in the environment.

4. Conclusion
Streptomyces sp. isolate ERI-CPDA-1 was able to degrade petroleum and polycyclic aromatic hydrocarbons in in vitro condition.
The degradation result showed that the isolate could remove 98.25% for diesel oil, 99.14% for naphthalene and 17.5% for phenanthrene in 7 days at 30°C. A degradation pathway has been proposed for the degradation of naphthalene, based upon earlier reports. Naphthalene is converted into 1,2-dihydroxynaphthalene by hydroxylation with 1,2 dioxygenase followed by dehydrogenation. This is converted into benzoic acid which undergoes hydroxylation to give protocatechuic acid which is degraded into catechol and muconic acid. This is followed by TCA cycle.

Acknowledgement

The authors are grateful to Entomology Research Institute, Loyola College, Chennai, for financial assistance.

Appendix A. Supplementary data


References


ISP (International Streptomyces Project) Medium

ISP-1
Pancreatic digest of casein - 5 g
Yeast extract - 3 g
D.H₂O - 1000 mL
pH - 7-7.2

ISP-2 (Growth Media)
Bacto Yeast extract - 4 g
Malt extract - 10g
Glucose - 4 g
D.H₂O - 1000 mL
Agar - 20 g
pH - 7.3

ISP- 3 (Oat meal agar)
Oat meal - 20 g
Agar - 20 g
Trace salt - 1 mL
D.H₂O - 1000 mL

ISP- 4 (Inorganic salt starch agar)
Solution-1
Starch - 10 g

Solution-2
K₂HPo₄ - 1 g
MgSO₄ - 1 g
NaCl - 1 g
NH₄SO₄ - 2 g
CaCO₃ - 2 g
Trace salt - 1 mL
Agar - 20 g
D.H₂O - 1000 mL
pH - 7.2-7.4
ISP-5

- Glycerol: 10 g
- L-Asparagine: 1 g
- K$_2$HPO$_4$: 0.5 g
- Trace salt: 1 mL
- D.H$_2$O: 1000 mL
- pH: 7.4

ISP-6 (Melanin pigment media)

- Peptone Iron agar: 36 g
- Yeast extract: 1 g
- D.H$_2$O: 1000 mL
- pH: 7-7.2

ISP-7 (Tyrosine agar)

- Glycerol: 15 g
- L-Tyrosine: 0.5 g
- L-Asparagine: 1 g
- K$_2$HPO$_4$: 0.5 g
- MgSO$_4$: 0.5 g
- NaCl: 0.5 g
- FeSO$_4$: 0.01 g
- D.H$_2$O: 1000 mL
- Agar: 20 g
- pH: 7.3