In vitro evaluation of antimicrobial activities and antibiotic susceptibility profiling of culturable actinobacteria from fresh water streams

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Actinobacteria are major producers of antibiotics, industrially significant enzymes and many pharmaceutically important biologically active compounds. Twenty two actinobacterial strains were isolated from fresh water stream sediment samples of Murlen National Park, Mizoram, India. The actinobacterial strains were screened against antifungal pathogens (Fusarium oxysporum, Fusarium udum, Fusarium proliferatum, Fusarium oxysporum ciceri and Fusarium graminearum), and antibacterial activities against five bacterial pathogens (Staphylococcus aureus, Pseudomonas aeruginosa, Micrococcus luteus, Bacillus subtilis and Escherichia coli) and a yeast pathogen Candida albicans. All strains showed antibacterial activity against E. coli and F. proliferatum. Based on the results of antagonistic, antibacterial and anti-yeast, two most potent strains Kocuria sp. and Streptomyces intermidias were further evaluated for their antibiotics susceptibility activity against 21 different antibiotics. Kocuria sp. showed resistance to 10 antibiotics whereas Streptomyces intermidas was resistance to 15 antibiotics. Modular genes Polyketide Synthase (PKS II) and Nonribosomal Peptide Synthetase (NRPS) were also detected in these two strains, which might be responsible for the production of secondary metabolites. Two volatile compounds, Di-N-octyl phthalate and 1-Bromo-3, 7-Dimethyloctane were identified from the extract of Streptomyces intermidas BPSWAC29 strain using Gas chromatography Mass spectrometry (GC-MS). This study highlights the promise of discovering novel actinobacteria with antimicrobial activity from underexplored niche biotopes such as fresh water stream sediments.

Keywords: 16S rRNA gene, Antibacterial, Antifungal, 1-bromo-3,7-Dimethyloctane, Fusarium spp., Kocuria sp., Nonribosomal peptide synthetase, Di-N-Octyl phthalate, Polyketide synthase, Streptomyces intermidas

Actinobacteria are a class of filamentous, Gram positive bacteria, having high GC (guanine and cytosine) content in their DNA and are widely distributed in nature including terrestrial and aquatic habitats1. Actinobacteria has important role in carbon cycle and in the breakdown of organic matter into easily accessable nutrients. Majority of them are found in soil, marine/fresh water and are an important source of biotechnologically, commercially and therapeutically useful bioactive compounds2. They are well-known antibiotic producer, producing more than two-thirds of all identified antibiotics3,4. Among them, genus Streptomyces accounts for the largest antibiotic producer with more than 70% of all commercially valuable antibiotics viz. tetracycline, streptomycin, nystatin, etc.5,6. The study of actinobacteria and their potential to produce antimicrobials in general, from aquatic systems have been somewhat neglected compared to terrestrial ecosystems3,8. The profusion of antibiotics produced by terrestrial actinobacteria is known but, there has been a drop in unearthing of new compounds from terrestrial sources9.

Antimicrobial resistance is a grave challenge to public health worldwide with the spread of multiple antibiotic resistant strains. Therefore, there is need to search novel antimicrobials compounds and their sources. It has been emphasized that new group of microorganism from untapped habitats can assist as sources of novel pharmaceutical agents10. According to the review on actinobacteriological research in India11, various habitats have been screened for the study of actinobacteria, of which soil habitats have been largely surveyed followed by marine water while fresh water have been neglected and most of which remains unexplored. Exploration of unexplored habitats such as fresh water in search of potential microbes in modern era is in advance interest to
isolate novel biologically active compounds. However, scientists were becoming more aware of the potential assessment of fresh water habitats as a source of actinobacteria that yield useful metabolic products. All water bodies in Mizoram are fresh water which is a promising habitat for the isolation of new and novel antimicrobials producing actinobacteria. In this study, we isolated potential actinobacteria producing biologically active compounds from the sediments of fresh water, identified using 16S rRNA gene sequencing and screened them for the production of antimicrobial agents and evaluated their antibiotics susceptibility profiling.

Materials and Methods

Sample collection

Sediment samples were collected from fresh water streams inside Murlen National Park, located near Myanmar border in Champhai District, India (23°37′N, 93°18″E). The park is situated about 245 km East of Aizawl (Fig. 1). Several streams flow through this park providing copious water to its existing wildlife. Collection was done using sterile screw capped tubes and transported to laboratory and stored at 4°C until processed for isolation. Samples were subjected to pre-treatment method by keeping the samples in water bath at 50°C for 6 min in order to simplify the isolation of actinobacteria and to avoid the growth of eubacteria.

Isolation of actinobacteria

Isolation was done using serial dilution method and spread plate technique using starch casein agar media (SCA). The culture media were supplemented with cyclohexamide (30 µg/mL) and nalidixic acid (30 µg/mL) antibiotics to inhibit the growth of fungi and fast growing bacteria respectively. The plates were incubated for around 15-30 days at 28°C. The colonies were purified by streaking and sub-culturing on fresh culture media which were then stored and maintained at 4°C in slants of yeast extract malt extract agar (ISP2) and 30% glycerol at –80°C for further studies.

Preliminary identification of actinobacterial strains

Preliminary identification of the isolates was performed as depicted in the International Streptomyces Project (ISP). All the morphological and biochemical characters were recorded as described earlier.

Determination of antimicrobial activity

In vitro antimicrobial activity of the isolates was checked against five pathogenic bacterial strains (gram positive bacteria viz., Staphylococcus aureus (MTCC-96), Bacillus subtilis (NCIM-2097), and Micrococcus luteus (NCIM-2170); gram negative bacteria viz., Pseudomonas aeruginosa (MTCC-2453) and Escherichia coli (MTCC-739)) and a yeast Candida albicans (MTCC-3017). Actinobacterial isolates were grown in tryptone yeast extract broth medium (ISP1), incubated at 28°C, 150 rpm for 7-15 days. The supernatant obtained after centrifugation of the grown cultures at 8000 rpm for 5 min was used for determining the antimicrobial activity following agar well diffusion method. The testing bacteria were spread on LB modified agar plate, and 6 mm diameter cork borer was used to prepare the wells, where 50 µL of the harvested cell free supernatant was added. The plates were kept at 37°C for 24 h and the antimicrobial activity of the isolates was recorded by measuring the inhibition zone around each well. The experiments were done in triplicate and mean value was taken.

Determination of antifungal activity

The isolates were assessed for their antifungal activity against five pathogenic fungi viz. Fusarium oxysporum (CABI-293942), Fusarium udum (MTCC-2755), Fusarium proliferatum (MTCC-286), Fusarium oxysporum ciceri (MTCC-2791), Fusarium graminearum (MTCC-1893) by dual culture in vitro assay. Test was performed by keeping the fungal pathogens at the centre of potato dextrose agar (PDA) plates while actinobacterial isolates were streaked 3 cm away from the fungal strains on opposite sides. The pathogens grown in the absence of actinobacteria is used as control. They were incubated at 28°C for 14 days and percentage of inhibition (%) were
calculated using the formula: C–T/C x 100 where C is the control growth of fungal pathogens without actinobacteria and T is test in which fungal pathogens and actinobacteria were grown together in a single plate. All the experiments were performed in triplicate and mean values was calculated.

**Antibiotic sensitivity profiling**

Twenty one standard antibiotics were tested against the actinobacterial strains to determine using Muller Hinton agar (MHA) medium. The isolates were grown in ISP-1 broth and incubated at 28°C, 150 rpm for 10-15 days (depends on the growth). The harvested cells were spread with a sterilized L-shaped spreader on MHA plates and different standard antibiotic discs were placed on the spread plates which were then incubated at 37°C for 24 h.

Sensitivity of the antibiotics against different isolates was observed by measuring zone of inhibition\(^{19}\). Actinobacterial strains were either considered as sensitive (S), intermediate (I) or resistant (R) to an antibiotic\(^ {20}\).

**Molecular identification using 16S rRNA gene amplification**

Genomic DNA of the selected strains was isolated using DNA extraction kit (Invitrogen, USA) according to the manufacturer’s protocol, DNA quantity was checked by measuring optical density (OD) at 260/280 nm. 16S rRNA gene was amplified by using universal bacterial primers\(^ {21}\). PCR conditional were used as described earlier\(^ {20}\). The obtained PCR product was purified by quick PCR purification kit (Invitrogen) and sequenced commercially at SciGenome Pvt. Ltd. Kochin, India. The spore chain morphology was examined using field emission gun scanning electron microscope (FEG-SEM).

**Detection and PCR amplifications of biosynthetic gene fragments (PKSII and NRPS):**

The potential antagonistic isolates were subjected for the amplification of genes for KS domains of Polyketide synthase (PKS), the adenylation domains of non-ribosomal peptide synthetase (NRPS). The following degenerate primers were used for detection of PKS-II: KSFI 5′-TSGCSTGCTTGGAYGCSATC-3′ as forward primer and KSIR – 5′TGGANCCG CCGAACCTCTT-3′ as reverse primer\(^ {22}\). NRPS gene fragments were amplified using degenerate primers: A3F 5′-GCSTACSYTATSTACACTCSC-3′ and A7R 5′-SASGTCCCGTGCTGAT-3′\(^ {23}\). The PCR amplification of PKSII and NRPS was carried out as described earlier\(^ {20}\).

**Gas chromatography mass spectroscopy (GC-MS) analysis**

The selected isolates were grown in ISP1 broth and incubate at 28°C in a shaker for 7 days, methanol extracts of the samples were prepared using rotary evaporator. The volatile compounds were detected and identified using GC-MS as described previously\(^ {24}\).

**Statistical analysis**

All experiments were performed in triplicate. One Way analysis of variance (ANOVA) was used to analyzed significant differences (\(P = 0.05\)) between antimicrobial and antifungal activities of different isolates using SPSS software version 20.0.

**Results**

**Isolation of actinobacteria**

A total of 22 pure actinobacteria strains were recovered from the sediments samples. The pure isolates were initially identified on the basis of morphological characteristics after 2-3 weeks of incubation on SCA medium (Fig. 2 A\(_1\) & A\(_2\)). Majority of the isolated strains were observed with slow growth on solid culture media showing different range of mycelium colors from yellow, white black, brown to orange. Purple and blackish brown pigments were formed on the media by certain isolates. The SEM results showed the presence of the aerial mycelia with spiral spore chains (Fig. 2 B\(_1\) & B\(_2\)), a typical characteristic of actinobacteria.

**Assessment of antimicrobial activity**

Antimicrobial assessment of the actinobacterial strains was carried out against 5 pathogenic bacteria

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**Fig. 2** — Morphological appearance of Kocuria sp. strain BPSWAC27 (A\(_1\)) and Streptomyces intermidu strain BPSWAC29 (A\(_2\)). Scanning electron microscope showing their spore chain morphology of strain BPSWAC27 (B\(_1\)) and BPSWAC29 (B\(_2\)) and antibiotics sensitivity pattern of selected strains BPSWAC27 (C\(_1\)) and BPSWAC29 (C\(_2\)).
and one yeast pathogen. Out of 22 actinobacteria, 10 isolates (45.4%) inhibited the growth of at least three of the tested six pathogens. Two isolates (BPSWAC27 and BPSWAC29) exhibited positive activity against 4 and 5 pathogens, respectively (Table 1). E. coli was susceptible to all the isolated strains, 19 isolates (86.3%) showed positive activity against P. aeruginosa while no activity of the isolates found against S. aureus and M. luteus. Isolate BPSWAC29 showed highest antibacterial activity against E. coli (14.5 mm), S. aureus (10.1 mm), B. subtilis (9.8 mm) P. aeruginosa (8.2 mm) and C. albicans (9.2 mm). Isolates BPSWAC27 and 29 showed broad spectrum antimicrobial activities and were selected for further investigations (Fig. 3 A₁ & A₂).

**Table 1 — In vitro antibacterial activity of selected 10 actinobacterial isolates against bacterial pathogens**

<table>
<thead>
<tr>
<th>Isolate Code</th>
<th>Antibacterial properties (zone in mm)</th>
<th>Yeast (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPSWAC1</td>
<td>E. coli 09.1±0.16&lt;sup&gt;a&lt;/sup&gt; / P. aeruginosa 09.23±0.03&lt;sup&gt;a&lt;/sup&gt; / S. aureus Nil / M. luteus Nil / B subtilis 12.42±0.02&lt;sup&gt;a&lt;/sup&gt; / C. albicans Nil</td>
<td></td>
</tr>
<tr>
<td>BPSWAC4</td>
<td>10.50±0.05&lt;sup&gt;bc&lt;/sup&gt; / Nil / 12.50±0.16&lt;sup&gt;a&lt;/sup&gt; / 8.37±0.02&lt;sup&gt;bc&lt;/sup&gt; / Nil</td>
<td></td>
</tr>
<tr>
<td>BPSWAC9</td>
<td>09.50±0.01&lt;sup&gt;a&lt;/sup&gt; / 06.40±0.07&lt;sup&gt;bc&lt;/sup&gt; / Nil / Nil / Nil / 12.00±0.09&lt;sup&gt;a&lt;/sup&gt; / Nil</td>
<td></td>
</tr>
<tr>
<td>BPSWAC10</td>
<td>10.70±0.02&lt;sup&gt;bc&lt;/sup&gt; / 10.00±0.02&lt;sup&gt;a&lt;/sup&gt; / Nil / Nil / Nil / 10.35±0.05&lt;sup&gt;de&lt;/sup&gt; / Nil</td>
<td></td>
</tr>
<tr>
<td>BPSWAC17</td>
<td>10.00±0.10&lt;sup&gt;b&lt;/sup&gt; / 09.65±0.02&lt;sup&gt;a&lt;/sup&gt; / Nil / Nil / 10.00±0.1&lt;sup&gt;abe&lt;/sup&gt; / Nil</td>
<td></td>
</tr>
<tr>
<td>BPSWAC19</td>
<td>10.00±0.10&lt;sup&gt;b&lt;/sup&gt; / 10.63±0.03&lt;sup&gt;bde&lt;/sup&gt; / Nil / Nil / Nil / 10.35±0.05&lt;sup&gt;de&lt;/sup&gt; / Nil</td>
<td></td>
</tr>
<tr>
<td>BPSWAC23</td>
<td>08.62±0.02&lt;sup&gt;bde&lt;/sup&gt; / Nil / 08.20±0.05&lt;sup&gt;bc&lt;/sup&gt; / 10.40±0.05&lt;sup&gt;bd&lt;/sup&gt; / Nil</td>
<td></td>
</tr>
<tr>
<td>BPSWAC27</td>
<td>10.06±0.05&lt;sup&gt;bc&lt;/sup&gt; / 10.43±0.05&lt;sup&gt;bdfg&lt;/sup&gt; / 09.00±0.10&lt;sup&gt;bc&lt;/sup&gt; / Nil</td>
<td></td>
</tr>
<tr>
<td>BPSWAC29</td>
<td>14.5±0.1&lt;sup&gt;bc&lt;/sup&gt; / 08.20±0.20&lt;sup&gt;b&lt;/sup&gt; / 10.17±0.1&lt;sup&gt;bd&lt;/sup&gt; / Nil / 09.80±0.04&lt;sup&gt;bd&lt;/sup&gt; / 09.25±0.05&lt;sup&gt;bdfh&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>BPSWAC30</td>
<td>10.62±0.02&lt;sup&gt;bc&lt;/sup&gt; / 10.62±0.02&lt;sup&gt;bc&lt;/sup&gt; / Nil / 08.30±0.05&lt;sup&gt;bd&lt;/sup&gt; / Nil</td>
<td></td>
</tr>
</tbody>
</table>

[Mean (±SD) followed by the same letter(s) in each column are not significantly different at P <0.05 using Duncan’s new multiple range test]

**Evaluation of antifungal activity**

The antifungal activity of the isolates was evaluated against five fungal pathogens viz. F. oxysporum, F. udum, F. proliferatum, F. oxy. ciceri and F. graminearum. A total of 10 strains, out of 22 isolates showed inhibition of at least two tested pathogens. All the isolates inhibited the growth of F. proliferatum, with percentage of inhibition ranging from 47-73%. Three actinobacterial strains (BPSWAC19, BPSWAC27 and BPSWAC29) exhibited significant growth inhibition against almost all the tested fungal pathogens except F. graminearum with percentage of inhibition ranging from 31-73% whereas all the isolates were susceptible to F. graminearum. Isolates BPSWAC27 and BPSWAC23 exhibited highest percentage of inhibition against F. udum (Fig 3 B₁ & B₂) and F. proliferatum (Fig 3 C₁ & C₂), respectively (67.68%) followed by isolate BPSWAC19 against F. udum (52%) as shown in Table 2.

**Antibiotic susceptibility profiling**

Isolates BPSWAC27 and BPSWAC 29 which showed antimicrobial activity against almost all the tested bacterial and fungal pathogens were selected and screen their antibiotic sensitivity pattern against 21 standard antibiotics viz. kanamycin (K<sup>3</sup>), ampicillin (Amp<sup>10</sup>), streptomycin (S<sup>10</sup>), erythromycin (E<sup>15</sup>), ketoconazole (KT<sup>50</sup>), norfloxacin (NX<sup>15</sup>), tetracycline (TE<sup>50</sup>), gentamycin (Gen<sup>50</sup>), nystatin (NS<sup>10</sup>), penicillin G (P<sup>5</sup>), fluconazole (FLC<sup>25</sup>), miconazole (MIC<sup>50</sup>), neomycin (N<sup>10</sup>), chloramphenicol (C<sup>10</sup>), polymixin B (PB<sup>10</sup>), voriconazole (VRC<sup>1</sup>), ciprofloxacin (CIP<sup>10</sup>), vancomycin (VA<sup>5</sup>), trimethoprim (TR<sup>10</sup>), and others.

**Fig. 3 — Antibacterial activity of four different isolates against P. aeruginosa (A₁) and E. coli (A₂); C represents control. B₁: Control growth of F. udum and B₂: Growth of pathogen (F. udum) inhibited by BPSWAC27. C₁: Control growth of F. proliferatum and C₂: Growth of pathogen (F. proliferatum) inhibited by BPSWAC29.**


orfloxacin; rythromycin;

Table 2 — In vitro antagonistic activity of selected 10 actinobacterial isolates against fungal pathogens

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>F. udum</th>
<th>F. oxysporum</th>
<th>F. graminearum</th>
<th>F. proliferatum</th>
<th>F. oxy cieri</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPSWAC1</td>
<td>Nil</td>
<td>60.50±0.01</td>
<td>Nil</td>
<td>55.26±0.01a</td>
<td>Nil</td>
</tr>
<tr>
<td>BPSWAC4</td>
<td>47.36±0.03a</td>
<td>50.00±0.00bc</td>
<td>Nil</td>
<td>Nil</td>
<td>57.89±0.15bfg</td>
</tr>
<tr>
<td>BPSWAC9</td>
<td>Nil</td>
<td>47.36±0.12bc</td>
<td>Nil</td>
<td>47.36±0.14de</td>
<td>Nil</td>
</tr>
<tr>
<td>BPSWAC10</td>
<td>52.63±0.00bc</td>
<td>Nil</td>
<td>Nil</td>
<td>57.89±0.15bfg</td>
<td>Nil</td>
</tr>
<tr>
<td>BPSWAC17</td>
<td>47.36±0.03bc</td>
<td>Nil</td>
<td>Nil</td>
<td>67.12±0.18inh</td>
<td>Nil</td>
</tr>
<tr>
<td>BPSWAC19</td>
<td>71.79±0.47bde</td>
<td>31.83±0.33bde</td>
<td>Nil</td>
<td>57.89±0.36bfg</td>
<td>61.64±0.02a</td>
</tr>
<tr>
<td>BPSWAC23</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>73.68±0.12bths</td>
<td>57.89±0.36bc</td>
</tr>
<tr>
<td>BPSWAC27</td>
<td>73.68±0.01bths</td>
<td>45.20±0.09bths</td>
<td>Nil</td>
<td>48.65±0.51bths</td>
<td>61.64±0.02a</td>
</tr>
<tr>
<td>BPSWAC29</td>
<td>57.89±0.36bths</td>
<td>35.48±0.28bths</td>
<td>Nil</td>
<td>52.63±0.00bths</td>
<td>44.73±0.09bde</td>
</tr>
<tr>
<td>BPSWAC30</td>
<td>52.63±0.00bths</td>
<td>Nil</td>
<td>Nil</td>
<td>61.64±0.02bths</td>
<td>45.20±0.02bef</td>
</tr>
</tbody>
</table>

[Mean (±SD) followed by the same letter(s) in each column are not significantly different at P < 0.05 using Duncan’s new multiple range test]

Table 3 — Antibiotic sensitivity profile of actinobacteria isolates against 21 tested standard antibiotics

<table>
<thead>
<tr>
<th>Strain</th>
<th>K5</th>
<th>Amp10</th>
<th>S10</th>
<th>E15</th>
<th>KT10</th>
<th>TX10</th>
<th>TE10</th>
<th>Gen10</th>
<th>NS10</th>
<th>FLC10</th>
<th>P2</th>
<th>Mic50</th>
<th>N10</th>
<th>C10</th>
<th>PB50</th>
<th>Cip10</th>
<th>Va10</th>
<th>Tr10</th>
<th>CD10</th>
<th>VRC1</th>
<th>LE5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kocuria sp. 9(1)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>11(1)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>14(1)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>10(1)</td>
<td>8.5(1)</td>
<td>0(1)</td>
<td>13(1)</td>
</tr>
<tr>
<td>Streptomyces sp. 10(1)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>0(1)</td>
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<td>0(1)</td>
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<td>0(1)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>0(1)</td>
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</tr>
</tbody>
</table>

[Degree of susceptibility: >10 mm: sensitive; 5.0-9.9 mm: intermediate; 0.0-4.9 mm: resistant. K5, kanamycin; Amp10, ampicillin; S10, streptomycin; E15, erythromycin; KT10, ketoconazole; TX10, norfloxacin; TE10, tetracycline; Gen10, gentamicin; NS10, nystatin; P2, penicillin G; FLC10, fluconazole; Mic50, miconazole; N10, neomycin; C10, chloramphenicol; PB50, polymyxin B; VRC1, voriconazole; Cip10, ciprofloxacin; Va10, vancomycin; Tr10, trimethoprim; CD10, clindamycin; and LE5, levofloxacin]

Table 4 — Morphological characteristics of actinobacterial isolates followed by identification using 16S rRNA gene sequencing and their accession no.

<table>
<thead>
<tr>
<th>Isolate name and code</th>
<th>Closest relative species in BLAST and accession no.</th>
<th>% Similarity</th>
<th>Gene bank accession number</th>
<th>Colony nature</th>
<th>Aerial mycelium</th>
<th>Substrate mycelium</th>
<th>Pigmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kocuria sp. strain BPSWAC27</td>
<td>Kocuria sp.(KF951262)</td>
<td>98%</td>
<td>KM405312</td>
<td>sticky</td>
<td>white</td>
<td>yellow</td>
<td>yellow</td>
</tr>
<tr>
<td>Streptomyces intermedius strain BPSWAC29</td>
<td>Streptomyces intermedius (KJ571077)</td>
<td>100%</td>
<td>KM406396</td>
<td>hard</td>
<td>white</td>
<td>brown</td>
<td>brown</td>
</tr>
</tbody>
</table>

clindamycin (CD10), levofloxacin (LE5) (Table 3). The two isolates showed high sensitivity against chloramphenicol, ciprofloxacin, clindamycin and levofloxacin. BPSWAC27 showed resistance activity against 10 out of 21 antibiotics and showed intermediate activity against 6 antibiotics and susceptible to only 5 antibiotics. BPSWAC29 showed resistance to 15 antibiotics, susceptible to 5 and intermediate to one antibiotic (Fig. 2 C1 & C2).

Amplification of 16S rRNA gene and phylogenetic analysis

The two potent isolates BPSWAC27 and BPSWAC29 demonstrated good antimicrobial activity and antibiotic resistance capability were identified by the amplification of 16S rRNA gene as Kocuria sp. and Streptomyces intermedius respectively. Partial 16S rRNA gene sequences (820 - 980 bp) of the two strains exhibited high level of sequence similarity (98 and 100%) with Genbank sequences. The nucleotide sequences were deposited to NCBI GenBank and accession number was obtained as KM405312 and KM406396 (Table 4). 16S rRNA gene sequences were aligned by BLAST analysis with five type-strains obtained from EzTaxon database. Phylogenetic tree was constructed (Fig. 4) based on Maximum Likelihood method with
Tamura 3-parameter model (R=1.36) according to lowest BIC values using Mega 5.05. Gaps were treated by pair wise deletion and the estimated Transition/Transversion bias (R) is 1.36. The topology of the phylogenetic tree shows that two actinobacterial genera along with their type strains were separated into two different clades (clade I and clade II). The entire Kocuria genus forms a major clade I, and all the genus of Streptomyces forms a separate clade II. The phylogenetic tree demonstrates that BPSWAC27 (Kocuria sp.) was clustered together with a type strain of Kocuria palustris and Kocuria assamensis under a bootstrap support value of 99%. BPSWAC29 (Streptomyces intermidus) was very close to the type strains of other Streptomyces genus with a bootstrap value of 100%.

**Detection of PKS-II and NRPS genes**

The presence of PKS type II and NRPS genes were evaluated in two selected strains (Kocuria sp. and Streptomyces intermidus). The PCR amplification results showed a specific DNA band of 600 bp for PKS type II and 750 bp for NRPS (Fig. 5).

**Gas chromatography-mass spectrometry (GC-MS) analysis**

Methanolic crude extract of the potential strain BPSWAC29 was further analyzed for their chemical constituents and two volatile compounds were determined by comparison of their mass spectra with the NIST library. The two compounds are (1) di-n-octyl phthalate (2) 1-bromo-3, 7-dimethyloctane (Table 5). The peak area and the quantity of compound present in the sample are directly proportional to each other.

**Discussion**

Actinobacteria remain one of the most potential sources of useful bioactive products including secondary metabolites with an inconceivable variety of important biological activities, antimicrobials, and valuable different group of enzymes. In the present study, 22 actinobacterial strains were isolated from fresh water streams inside Murlen National Park, India. The morphological identification showed strains species, which were further verified by using FEG-SEM. The SEM analysis showed that the aerial mycelia of most of the actinobacterial strains displayed a long spore chains with smooth surface, similar character were reported earlier by Zothanpuia et al. Among the 22 isolates, two isolates BPSAWAC27 and BPSWAC29 demonstrated a significant antimicrobial activity against both bacteria and fungi pathogen. The BPSAWAC27 and BPSWAC29 isolates were identified as Kocuria sp. and Streptomyces intermidus respectively using 16S rRNA gene sequencing. These results showed that actinobacteria such as Kocuria sp. and Streptomyces intermidus isolated from sediments possess potential for the production of antimicrobial compounds supported by the findings of Baskaran et al. Similarly, Sibanda et al. also described the antimicrobial potential of freshwater actinomycetes. Our results showed that fresh water actinobacteria of streams at Murlen National Park possess a significant antifungal activity by inhibiting the growth of four out of five fungal phytopathogens tested, several species of Streptomyces have earlier been reported to have
antifungal activity\textsuperscript{29,31}. These findings are also in agreement with the results that demonstrated the antifungal activity of fresh water actinobacteria isolated from river Nile\textsuperscript{7}. Furthermore, the recent findings of Saravanan et al.\textsuperscript{32} and Zothanpuia et al.\textsuperscript{12} also demonstrated the antimicrobial activity of the fresh water isolates of Pudukkottai and Tuichang river, India. Here, the two potent strains were confirmed as \textit{Kocuria} sp. and \textit{Streptomyces intermidus} using 16S rRNA gene sequencing. And it is evident that the \textit{Kocuria assamensis}, a novel actinobacterium was isolated from the river Brahmaputra, India\textsuperscript{33} and various species of \textit{Streptomyces} have been reported earlier from freshwater\textsuperscript{34,35}. Therefore, our result of this study clearly indicates that fresh water streams at Murlen National Park habits actinobacteria with potential antimicrobial activity.

The \textit{Kocuria} sp. and \textit{Streptomyces intermidus} strains were evaluated for their antibiotic resistance pattern using 21 standard antibiotic discs. \textit{Kocuria} sp. showed resistance activity against 10 out of 21 antibiotics and were susceptible to 5 antibiotics, while \textit{Streptomyces intermidus} showed resistance for 15 antibiotics and found susceptible for 5 antibiotics. The two isolates were susceptible to tetracycline (85%) followed by gentamicin (50%) and ciprofloxacin (45%). All the isolates showed resistance to two types of antibiotics viz. penicillin G and ampicillin (100% each). Gousterova et al.\textsuperscript{36} also reported the biosynthetic potential of twenty six actinobacteria and their antibiotic sensitivity profiling against 12 antibiotics were also described which is in accordance with the present study. These results according to previous reports indicate that these two isolates could be a good candidate for the discovery of antibiotics\textsuperscript{12,37}.

Detection of genes encoding polyketide synthases and nonribosomal peptide synthetases is crucial to comprehend the biosynthetic potential of the isolates involved in the synthesis of bioactive secondary metabolites polyketide and peptide and commonly employed for evaluating the biosynthetic potential of culturable and nonculturable microorganisms\textsuperscript{38}. In this study, the two potential isolates \textit{Kocuria} sp. and \textit{Streptomyces intermidus} which showed antimicrobial activity against most of the tested pathogens also showed the presence of these genes and indicates that PKSII and NRPS genes are vital for antimicrobial activity. Similar studies were earlier described by Yuan et al.\textsuperscript{13}.

Gas chromatography mass spectrometry is one of the most consistent techniques for determination of volatile compounds described by a different scientists worldwide\textsuperscript{24,39}. The GC-MS chromatogram of \textit{Streptomyces intermidus} BPSWAC29 extract exhibited two major peaks with different retention time. Di-n-octyl phthalate (DOP) was detected in maximum amount constituted 71.31\% of the total compounds present in the sample. DOP has already been reported by several researchers in plants for its antimicrobial activity\textsuperscript{40,42}. To best of our knowledge, 1-bromo-3, 7-dimethyloctane has not been reported for its antimicrobial activity.

This study revealed that freshwater actinobacteria possess enormous potential as a source of antimicrobial compounds which was supported by the isolation of several novel species and other potential actinobacteria from fresh water as previously described\textsuperscript{32,43,46}.

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