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Molecular characterization of actinomycetes isolated from Tuichang river and their biosynthetic potential

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Abstract

Actinomycetes are antibiotic producing gram positive bacteria widely distributed in nature including fresh water habitat. The study of fresh water actinomycetes especially from river is still in infancy and has been less explored. In this study, 20 *Streptomyces* sp. were isolated from Tuichang river and were screened for their antimicrobial potential. Antibiotic sensitivity pattern of the isolates against standard antibiotics was also observed. Eight isolates (n=8; 40%) showed significant antibacterial activity against at least two tested bacterial pathogens, while six isolates (n=6; 30%) showed antifungal potential against at least two tested fungal phytopathogens. All the isolates showed resistance to penicillin G and ampicillin. Of all the isolates screened, BPSWAC23 and BPSWAC24 inhibited all the tested fungal and bacterial pathogens and showed resistance to 50% of the antibiotics. BPSWAC23 and BPSWAC24 were identified by using 16S rRNA gene sequencing and found to be *Streptomyces* sp. *Streptomyces parvus* respectively. Further, biosynthetic genes coding for polyketide synthase (PKS II) and nonribosomal peptide synthetase (NRPS) were also detected in these two isolates which indicates a good candidate for the discovery of novel antibiotics.

Key words: Actinomyctes; 16S rRNA gene; antibiotics; polyketide synthase; *Streptomyces*.

Introduction

Actinomycetes are Gram-positive, filamentous, free-living, saprophytic, antibiotics producing bacteria with high GC (51% to 70%) content in their DNA and found in most environments including terrestrial and aquatic habitats.[1-3] They serve as an important source for the production of therapeutically suitable bioactive metabolites. Among the prokaryotes, actinomycetes produce wide range of antibiotics, antitumor agents and enzymes.[4,5] This group is the leading producer of naturally occurring antibiotics.[6] Out of 23,000 bioactive secondary metabolites obtained from

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microbes, around 10,000 (45%) have been produced by actinomycetes. Among actinomycetes, *Streptomyces* species alone produced around 7600 compounds, representing more than 70% of the secondary metabolites.[7-9]

There are several reports of actinomycetes from fresh water worldwide, but in India the fresh water actinomycetes research is still in infancy and has been less explored/neglected as compared to the research on terrestrial and marine actinomycetes.[10,11] Most of the water bodies in Mizoram are fresh water which is a promising habitat for the isolation of secondary metabolites producing actinomycetes. The aim of the present study is to isolate potential actinomycetes from fresh water and to screen them for the production of antimicrobials. The potential isolates were identified by amplifying 16S rRNA gene; a conserved region in prokaryotes, which is widely used for characterization and molecular identification of bacteria.[12] Detection of biosynthetic genes encoding for polyketide synthases (PKS-II) and nonribosomal peptide synthetases (NRPS) have been generally used for assessing the biosynthetic potential of cultivable and non-cultivable microorganism. Such genes are responsible for the production of most biologically active polyketide and peptide compounds.[13]

Materials and Methods

Sample collection and pre-treatment of samples

Water samples were collected from fresh water Tuichang river (23°33' N and 93°06'E), Mizoram, India during summer. Samples were transferred in a sterile screw capped tubes and bought to Molecular Microbiology and Systematics Laboratory, Department of Biotechnology, Mizoram University, for further analysis. Samples were subjected to physical pre-treatment method in order to facilitate the isolation of actinomycetes and to inhibit the growth of gram negative bacteria by keeping in water bath at 50°C for 6 minutes.[14]

Isolation and preliminary identification of Actinomycetes

Isolation of actinomycetes was done by serial dilution method and pour plate technique [27] by using starch casein agar (SCA) and actinomycetes isolation agar (AIA) media. The culture media was supplemented with nalidixic acid (30µg/ml) and cyclohexamide (30µg/ml) to inhibit the growth of gram negative bacteria and fungi respectively. Plates were incubated at 28±1°C for 7 to 30 days and the appearance of colonies was observed periodically. The pure cultures were obtained after three successive sub-culturing on respective media and preserved on slants at 4^0C and 30% glycerol at -80^0C for subsequent studies. The isolates were identified according to their morphological and cultural characteristics as described in the International *Streptomyces* Project (ISP).[15] The cultural characteristics such as the color of aerial and substrate mycelium, characteristics of the colony and production of diffusible pigments were studied.[10]

Screening for antibacterial potential

Antibacterial screening of the selected isolates was performed against three pathogenic bacterial strains; gram positive bacteria *Staphylococcus aureus* (MTCC-96), gram negative bacteria *Pseudomonas aeruginosa* (MTCC-2453) and *Escherichia coli* (MTCC-739), all obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India. Extracts were prepared by inoculating a few colonies of actinomycetes in tryptone yeast extract broth medium (ISP medium 1) and incubated at 28°C, 150 rpm for 7-10 days. The grown cultures were subjected to centrifugation at 8,000 rpm for 3 minutes and the supernatant was used for antimicrobial activity by the agar well diffusion method.[16] The test pathogenic bacteria were spread on nutrient agar plate and wells were prepared by using sterile cork borer of 6 mm diameter. In each of the plates, 50 µl clear supernatant of the actinomycetes were dispensed into each wells and the plates were incubated at 28±2°C for 24 h. The anti-microbial activities of the isolates were ob-

served by measuring the inhibition zone around each well.

Screening for antifungal potential

The actinomycete isolates were evaluated for their antifungal activity towards three pathogenic fungi: *Fusarium oxysporum* (CABI-293942), *Fusarium oxysporum ciceri* (MTCC-2791) and *Fusarium graminearum* (MTCC-1893) by dual-culture *in vitro* assay. Fungal discs (8 mm diam.), from the fully grown pathogen were placed at the centre of PDA plate. Tested isolates were streaked on opposite sides of the plates, 3 cm away from the fungal disc. Plates without the tested actinomycetes served as controls. All plates were incubated at 28°C for 14 days and growth inhibition (%) was calculated by using the formula: C - T/C × 100, where C is the colony growth of pathogen in control, and T is the colony growth of pathogen in dual culture. All isolates were tested in triplicate process.[17]

Antibiotic sensitivity profiling

The antibiotic resistance of the isolates was tested using nine different standard antibiotic discs using Muller Hinton agar media. The isolates were inoculated in tryptone yeast extract broth (ISP 1 broth) and incubated at 28°C, 150 rpm for 10-15 days. The grown cultures were subjected to centrifugation at 8,000 rpm for 3mins and the supernatant was spread with sterile L-shape spreader over the plates of Muller Hinton agar media. The antibiotic discs were placed on the plate and incubated at 30°C for 24h. Antibiotic sensitivity pattern was observed by measuring the diameter of the inhibition zone.[18] Actinomycete isolates were either considered as sensitive (S), intermediate (I) or resistant (R) to the tested antibiotic.

Molecular identification of potential isolates by amplification of 16S rRNA gene

For molecular identification, genomic DNA was isolated by using DNA extraction kit (Invitrogen) according to the manufacturer's protocol and the DNA quantity was checked by taking OD at 260/280 in thermo scientific multi scan GO (Finland). Amplification of 16S rRNA gene was carried out by using universal bacterial primers PA as forward primer (5′-AGAGTTTGATCCTGGCTCAG-3′) and PH as reverse primer (5′-AAGGAGGTGATCCAGCCGCA-3′).[21] Reaction were performed on Veriti thermal cycler (Applied Biosystem, Singapore) in a total volume of 25 µl consisting of 1.0 µl genomic DNA (50 ng), 0.2µl of each primer (10 pmol), 2.0 µl of deoxynucleotide triphosphates (2.5 mM each), 2.5 µl of 1x PCR buffer, 0.2 µl of Taq DNA polymerase (1U/ µl) and 15.9 µl MilliQ grade water. PCR was performed under following conditions: initial denaturation step at 95°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 57.5°C for 40 sec and extension at 72°C for 1.3 min with a final extension step at 72°C for 10 min. A negative control reaction mixture without DNA template of actinomycetes was also included with each set of PCR reactions. The amplified PCR product was checked by using 1.2 % agarose gel electrophoresis using TAE buffer. The PCR bands were analyzed under UV light and documented using a Bio-Rad Gel Doc XR$^+$ system (Hercules, CA, USA) The PCR products of 16S rRNA gene was purified by quick PCR purification kit (Invitrogen) and sequencing was done commercially at SciGenome Pvt. Ltd. Kochin, India. The sequence was compared for similarity level with the reference strains of actinomycetes from genomic database banks, using the NCBI Blast available at the http://www.ncbi.nlm.nih.gov/ blast web site. The structures were analysed using the CLUSTAL W v.1.4 software available at http://www.ebi.ac.uk/clustalw.[22]

Scanning electron microscopy (SEM) of potential isolates

The spore chain morphology of the potential isolates was examined by field emission gun-scanning electron microscope (FEG-SEM).

Detection of biosynthetic gene sequences (PKS II and NRPS)

The potential isolates were subjected for the amplification of genes for KS domains of polyketide synthase (PKS) type I and the adenylation domains of non-ribosomal peptide synthetase (NRPS). The degenerate primers: K1F 5'-TSAAGTCSAACATCCGBCA-3' and M6R 5'-CGCAGGTTSCSGTACCAGTA-3' were used for PKS-I[19] and NRPS gene fragments were amplified using degenerate primers: A3F 5'-GCSTACSYSATSTACACSTCSGG-3' and A7R 5'-SASGTCVCCSGTSGCGTAS-3'.[19]

Results and Discussion

The incidence of multidrug resistant organisms is increasing hence; there is an urgent need to look for efficient source for the discovery of new drugs which are effective against resistant pathogens. Actinomycetes are one of the most potential sources for the isolation of bioactive antibiotics against multidrug resistance organisms.[23] In the present study 30 bacterial strains were isolated from Tuichang river in Mizoram, which were characterized morphologically. From a total of 30 bacterial isolated, 20 isolates were selected as putative actinomycetes based on their morphological appearance (Table 1; Fig 1). Among the two media used for the isolation, SCA was more suitable for isolation and yield more number of isolates which was also reported by various researchers.[22-24] The FEG-SEM result showed that the aerial mycelia displayed the long spore chains with smooth surface (Fig 2).

All the 20 isolates were screened for their antimicrobial activity against three bacterial and fungal pathogens. Among them eight isolates exhibited antibacterial activity against at least two tested bacterial pathogens. In total, 14 isolates (70%) showed positive result against gram negative bacteria *E. coli* and nine isolates (45%) exhibited significant activity against *P. aeruginosa.* This finding were in accordance with the

Table 1. Morphological characteristics of actinomycetes and media used for isolation.

Isolates	Aerial mycelia	Reverse color	Colony type	pigmentation	Media used
BPSWAC2	brown	brown	sticky	-	AIA
BPSWAC5	Light yellow	Light yellow	soft	-	SCA
BPSWAC7	white	Light yellow	hard	-	SCA
BPSWAC9	white	brown	hard	-	SCA
BPSWAC11	white	Light brown	soft	-	SCA
BPSWAC23	brown	brown	hard	+	SCA
BPSWAC24	white	Yellow	soft	-	SCA
BPSWAC27	White	brown	hard	-	AIA
BPSWAC29	white	brown	hard	+	SCA
BPSWAC31	White	brown	hard	-	SCA
BPSWAC34	white	Pale yellow	hard	-	AIA
BPSWAC37	white	Pale yellow	hard	-	SCA
BPSWAC41	white	light yellow	hard	-	SCA
BPSWAC42	white	brown	hard	-	SCA
BPSWAC43	white	Brown	hard	-	SCA
BPSWAC45	brown	Pale yellow	hard	-	SCA
BPSWAC47	white	brown	hard	-	AIA
BPSWAC48	white	brown	hard	-	AIA
BPSWAC50	white	White	soft	-	SCA
BPSWAC51	white	Pale yellow	hard	-	AIA

Table 2. In vitro antagonistic activity of actinomycete isolates against fungal pathogens and bacterial pathogens.

Isolate No.*	Percentage of Inhibition [PI±(SD)][a] against			Zone of inhibition in mm ±SD		
	F. oxysporum (CABI 293942)	*F. oxy. ciceri* (MTCC 2791)	*F. proliferatum* (MTCC 286)	*E. coli* (MTCC739)	*P. aeruginosa* (MTCC 2453)	*S. aureus* (MTCC 96)
BPSWAC2	60.50±0.01a	00.00±0.00a	55.26±0.01a	09.10±0.16a	09.23±0.03a	00.00±0.00a
BPSWAC5	47.36±0.03bc	00.00±0.00a	00.00±0.00bc	00.00±0.00bc	00.00±0.00bc	8.37±0.02bc
BPSWAC7	47.36±0.12bc	00.00±0.00a	00.00±0.00bc	09.25±0.05bde	00.00±0.00bc	00.00±0.00a
BPSWAC9	00.00±0.00bde	00.00±0.00a	57.89±0.15bde	10.50± 0.05bdfg	00.00±0.00bc	00.00±0.00a
BPSWAC11	47.36±0.03bc	00.00±0.00a	00.00±0.00bcf	09.50±0.01bdfhi	00.00±0.00bc	10.00±0.1bd
BPSWAC23	73.68±0.01bdfg	61.64±0.02bc	57.89±0.36bde	10.40±0.05bdfhjk	11.00±0.01bde	8.34±0.03bc
BPSWAC24	50.68±0.00bdfhi	53.42±0.00bde	67.12±0.18bdfg	10.62±0.02bdfhjlm	10.00±0.10bdf	8.62±0.02bde
BPSWAC27	57.80±0.01bdfhjk	00.00±0.00a	73.68±0.12bdfhi	09.50±0.10bdfhi	00.00±0.00bc	00.00±0.00a
BPSWAC29	52.63±0.00bdfhjlm	00.00±0.00a	00.00±0.00bc	00.00±0.00bc	06.40±0.07bdfgh	00.00±0.00a
BPSWAC31	00.00±0.00bde	45.20±0.02bdfg	00.00±0.00bc	10.70±0.02bdfhjlno	00.00±0.00bc	10.00±0.02bdfg
BPSWAC34	52.63±0.00bdfhjlm	00.00±0.00a	00.00±0.00bc	12.00±0.09bdfhjlnp	12.42±0.02bdfgi	00.00±0.00a
BPSWAC37	00.00±0.00bde	00.00±0.00a	00.00±0.00bc	10.62±0.02bdfhjlm	00.00±0.00bc	00.00±0.00a
BPSWAC41	51.31±0.09bdfhjlno	00.00±0.00a	50.00±0.00bdfhjk	00.00±0.00bc	08.20±0.05bdfgjk	00.00±0.00a
BPSWAC42	00.00±0.00bde	00.00±0.00a	44.73±0.09bdfhjlm	09.80±0.04bdfhjlnq	00.00±0.00bc	00.00±0.00a
BPSWAC43	60.50±0.00a	00.00±0.00a	47.36±0.14bdfhjlno	08.62±0.02bdfhjlnrs	00.00±0.00bc	00.00±0.00a
BPSWAC45	00.00±0.00bde	00.00±0.00a	47.36±0.14bdfhjlno	00.00±0.00bc	00.00±0.00bc	10.35±0.05bdfhi
BPSWAC47	39.47±0.05bdfhjlnp	00.00±0.00a	00.00±0.00bc	10.00±0.10bdfhjlnrt	09.65±0.02bdfgjlm	00.00±0.00a
BPSWAC48	00.00±0.00bde	00.00±0.00a	52.63±0.09bdfhjlnp	00.00±0.00bc	00.00±0.00bc	12.50±0.16bdfhj
BPSWAC50	00.00±0.00bde	45.20±0.09bdfg	00.00±0.00bc	10.00±0.10bdfhjlnrt	10.63±0.03bdfgjlno	00.00±0.00a
BPSWAC51	47.36±0.02bc	00.00±0.00a	00.00±0.00bc	00.00±0.00bc	08.30±0.05bdfgjlnp	00.00±0.00a

Figure 1. Morphological appearance of BPSWAC23 and BPSWAC24 isolates on starch casein agar.

Table 3. Antibiotic sensitivity profile of actinomycete isolates against nine tested standard antibiotics.

Isolate No.	E	S	P	N	A	T	Amy	G	Cf
BPSWAC2	*I*	*S*	*R*	*I*	*R*	*S*	*R*	*I*	*S*
BPSWAC5	*I*	*S*	*R*	*R*	*R*	*S*	*R*	*S*	*I*
BPSWAC7	*I*	*I*	*R*	*R*	*I*	*S*	*R*	*I*	*S*
BPSWAC9	*R*	*I*	*R*	*R*	*S*	*S*	*R*	*R*	*I*
BPSWAC11	*I*	*R*	*R*	*I*	*S*	*S*	*R*	*I*	*I*
BPSWAC23	*R*	*I*	*R*	*I*	*R*	*S*	*R*	*R*	*I*
BPSWAC24	*R*	*I*	*R*	*R*	*R*	*I*	*R*	*I*	*I*
BPSWAC27	*S*	*I*	*R*	*R*	*S*	*S*	*R*	*I*	*S*
BPSWAC29	*R*	*S*	*R*	*I*	*R*	*S*	*R*	*S*	*S*
BPSWAC31	*I*	*S*	*R*	*I*	*R*	*S*	*R*	*S*	*S*
BPSWAC34	*I*	*I*	*R*	*R*	*R*	*S*	*R*	*I*	*I*
BPSWAC37	*S*	*S*	*R*	*R*	*R*	*S*	*R*	*S*	*S*
BPSWAC41	*I*	*I*	*R*	*R*	*I*	*I*	*R*	*I*	*I*
BPSWAC42	*I*	*I*	*R*	*I*	*R*	*I*	*R*	*S*	*S*
BPSWAC43	*R*	*I*	*R*	*R*	*R*	*S*	*R*	*I*	*S*
BPSWAC45	*I*	*R*	*R*	*I*	*I*	*S*	*R*	*S*	*I*
BPSWAC47	*I*	*I*	*R*	*I*	*I*	*S*	*R*	*S*	*I*
BPSWAC48	*S*	*S*	*R*	*I*	*I*	*S*	*R*	*S*	*I*
BPSWAC50	*S*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*I*
BPSWAC51	*I*	*I*	*R*	*I*	*S*	*S*	*R*	*S*	*S*

E=Erythromycin; **S**=Streptomycin; **P**=Penicillin; **N**=Norfloxin; **A**=Ampicillin; **T**=Tetracycline; **Amox**=Amoxycillin; **G**=Gentamycin; **Cif**=Ciprofloxacin.
NB: (R) No zone (Isolates having resistant to antibiotics); **(*I*)** zone ≤5mm, Isolates having some resistant to antibiotics); **(*S*)** zone between 5mm-10mm

previous studies that fresh water actinomycetes showed inhibition to gram negative bacterial isolates compared to gram positive pathogens.[25] Seven (35%) isolates showed inhibition against *Staphylococcus aureus*. A total of 6 strains, out of 20 isolates showed inhibition against at least two of the tested fungal pathogens, 65% (n=13) of the actinomycetes strain inhibited *F. oxysporum*, 50% (n=10) inhibited *F. proliferatum* and only 20% (n=4) inhibited *F. oxy. ciceri* (Table 2). This clearly shows that fresh water actinomycetes of Tuichang river is having a significant antifungal activity which is in agreement with the findings of antimycotic activity of fresh water actinomycetes in river Nile.[26] The antimicrobial activity of the isolates from fresh water systems of Pudukkottai, Tamilnadu, also supported the present study.[5]

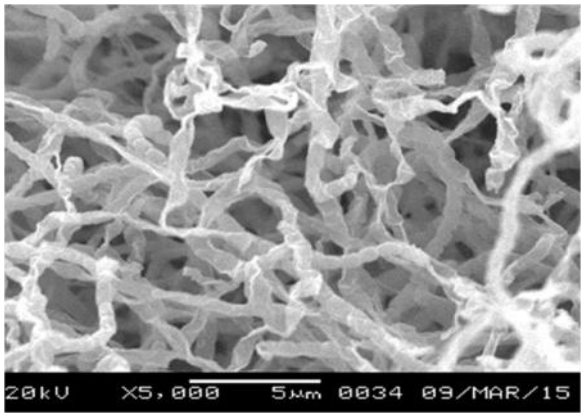

Figure 2. Scanning electron microscope showing spore chain morphology of BPSWAC23 isolate.

Antibiotics sensitivity pattern of the isolates was studied with nine different standard antibiotic discs containing erythromycin (E), streptomycin (S), penicillin (P), norfloxicin (N), ampicillin (A), tetracycline (T), amoxicillin (Amy) gentamycin (G), ciprofloxacin (Cf). Most of the 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) which indicates the potency of actinomycetes in discovering new antibiotics (Table 3).

Interestingly, of all the isolates screened, 2 strains BPSWAC23 and BPSWAC24 inhibited all the tested fungal and bacterial pathogens and showed resistance to 50% of the antibiotics used in the study (Fig 3). Biosynthetic genes PKS II and NRPS were also detected in these two isolates; which further proved the existence biosynthetic gene clusters which may be responsible for the production of antimicrobial secondary metabolites. As previously mentioned the detection of PKS II and NRPS genes have been extensively used for evaluating the biosynthetic prospective of both culturable and non-culturable microorganisms.[13]

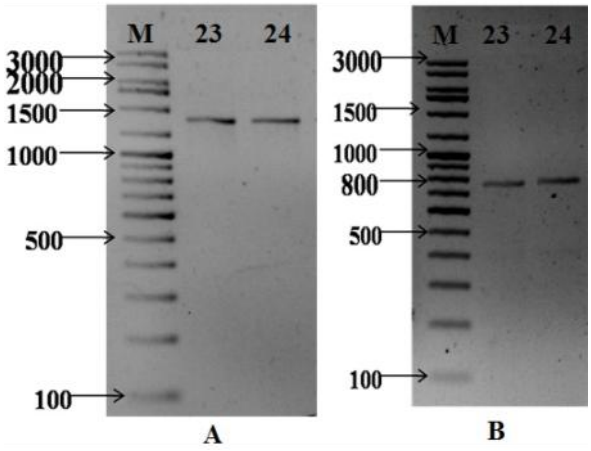

Figure 3. Amplification of biosynthetic genes (A) PKSI and (B) NRPS using degenerate primer.

The two actinomycete strains BPSWAC23 and BPSWAC24 were confirmed as *Streptomyces* sp. and *Streptomyces parvus* respectively by sequencing of 16S rRNA gene and showed 99% identity with BlastN sequences. The *Streptomyces* isolates showed a good candidate for the discovery of antibiotics which is also reported by some researchers that species of *Streptomyces*, account for more than 70% of the total antibiotic production.[28] Both the sequences were deposited in NCBI gene bank and an accession numbers has given as KM405308 for BPSWAC23 and KM405309 for BPSWAC24.

CONCLUSION

This study can be concluded that fresh water of Mizoram may harbour rich source of biologically potential actinomycetes which has the capabilities of producing secondary metabolites with antimicrobial functions. Overall findings proved the importance of actinomycetes as antimicrobial agents and suggest the possibilities of using actinomycetes as bioinoculant for biocontrol agent against major fungal phytopathogens. Hence, the investigated fresh water represented a rich source of bioactive and antagonistic actinomycetes.

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