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Short Communication

Isolation, characterization and chromatography based purification of antibacterial compound isolated from rare endophytic actinomycetes *Micrococcus yunnanensis*

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ABSTRACT

Endophytic actinomycetes are considered as one of the relatively unexplored potential sources in search of antibiotic producer against antibiotic resistant pathogens. A potent strain isolated from *Catharanthus roseus* that displays antibacterial potential against antibiotic resistant human pathogen *Staphylococcus aureus* was characterized and designated as *Micrococcus yunnanensis* strain rsk5. Rsk5 is capable of producing optimum antibacterial metabolites on starch casein medium at 30 °C, pH 5 and 2% NaCl condition. The crude antibacterial agent was extracted from fermentation broth by ethyl acetate and separated by TLC using chloroform-methanol (24:1, v/v) solvent system with R_f value of 0.26. It was partially purified by flash chromatography, followed by HPLC and analyzed by ultraviolet visible spectrophotometer to get absorption maxima at 208.4 nm. The ESI-MS spectra showed molecular ion peaks at *m/z* 472.4 [M-H], which does not match with any known antibacterial compound.

1. Introduction

The emerging problem of antibiotic resistance pathogens promotes resurgence in search of new and useful biologically active compounds from potentially important organisms. One of the significant and relatively overlooked organisms is endophytic actinomycetes that reside in the inner tissues of higher plants and have attracted more attention in recent years by production of several antibiotics such as alnumycin, munumbicins A to D, coronamycins [1,2], and novel antitumor agent anthraquinones, lupinacidins A and B [3]. Thus, endophytic actinobacteria are considered as potential sources in search of novel antimicrobial compounds which act as alternatives to combat multidrug-resistant human pathogens [4].

The *Catharanthus roseus* is an important medicinal plant used in folk and modern medication. Potent endophytic actinomycetes belongs to genus *Micrococcus* and was isolated from *C. roseus* during our studies on novel antibiotic sources. Natural antibacterial substance was partially purified by chromatography-based purification approaches. The present work was focused on characterization and partial purification of antibacterial substance of endophyte *Micrococcus yunnanensis* strain rsk5 isolated from *C. roseus* from Rajkot, India.

2. Materials and methods

2.1. Selective isolation of endophytic actinomycetes

Endophytic actinomycetes was selectively isolated by modified protocol of Johannes et al. [5] from root, stem and leaf samples of *C. roseus* plant collected from Rajkot, India. The surface-sterilized samples were transferred on humic acid vitamin agar (HV) containing nystatin (50 μg/mL) and nalidixic acid (10 μg/mL) to avoid fungal and gram-negative bacterial contamination in the medium and incubated at 28 °C for 21 days [6]. Success of surface disinfection process was confirmed by inoculation of distilled water from the final rinse and surface-sterilized tissue on the HV agar plate to check microbial growth.

2.2. Preliminary screening for antibacterial activities

The primary screening for antibacterial activities of isolated actinomycetes was performed by cross-streak method [7]. The bacterial pathogens such as *Staphylococcus aureus* MTCC 96, *Bacillus subtilis* MTCC 441, *Bacillus megaterium* MTCC 2444, *Enterococcus faecalis* MTCC 439, *Proteus vulgaris* MTCC 1771, *Salmonella typhimurium*

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MTCC 1251, *Pseudomonas aeruginosa* MTCC 2453 and *Escherichia coli* MTCC 739 were used in the study. Antagonistic effect of isolates against test organisms was recorded. One of the potent strains, rsk5, produces antibacterial compound against test cultures and was selected for further studies.

2.3. Biochemical and 16S rRNA gene sequence based characterization

Characterization of potential endophytic actinomycetes strain rsk5 was carried out up to genus level by physiological and biochemical characterization. Genomic DNA was extracted by modified protocol of Kieser et al. [8]. 16S rDNA gene was amplified by PCR using primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGHTACCTTGTTACGACTT-3′) in a thermal cycler (Bio-Rad, USA). PCR product was purified, sequenced, compared with similar 16S rDNA gene sequences using the BLAST search program, aligned using ClustalW program and submitted to the GenBank database. Phylogenetic tree was constructed using MEGA5 software by neighbor-joining method with bootstrap testing.

2.4. Optimization for growth and metabolite production

The inhibitory effect of the 30 µL ethyl acetate extract along with standard antibiotic penicillin (HiMedia, India) was tested by agar well diffusion method as described by Gebreyohannes et al. [9]. The effects of media, incubation period, temperature (20, 25, 30, 35, 40, 45 and 50 °C), pH (5–10) and NaCl concentration (0%–15%, m/v) on strain rsk5 were studied to get optimum growth and antibacterial activity.

2.5. Fermentation and extraction

Repeated fermentations were carried out to obtain a total volume of 8 L starch casein culture broth. The biomass was removed from culture broth by centrifugation at 10,000 rpm for 10 min followed by filtration. The supernatant was concentrated and extracted five times with one volume of ethyl acetate and one volume of culture filtrate. The ethyl acetate extract was dried under vacuum using rotary evaporator, desorbed in methanol and bioassayed for the presence of antibacterial substance.

2.6. Chromatography based purification of the antibacterial compound

The crude extract was purified by analytical thin layer chromatography (TLC) on TLC sheet using ascending method and elution was optimized with chloroform-methanol (24:1, v/v), chloroform-methanol (7.5:17.5, v/v), methanol-dichloromethane-water (1:1:1, v/v/v), benzene-acetic acid-water (4:1:5, v/v/v) and acetonitrile-water (92.5:7.5, v/v) successively. Bands of TLC sheets were scraped off carefully, dissolved in methanol, and centrifuged at 10,000 rpm for 10 min to remove silica, and antibacterial assay was performed. Optimized solvent system was used to separate antibacterial compound on bulk scale using preparative TLC on 1 mm thick silica gel 60 (Merck Ltd., India). Active bands of preparative TLC were merged, concentrated (120 mg/mL) and further purified by automated flash chromatography system (Yamazen Co., Ltd., Japan).

The light-yellow TLC extract was mixed with silica gel 60, packed in the silica gel column (30 µm), fractionated by different solvent gradient systems at 10 bar pressure and monitored by UV detector at 254 nm. The eluate was collected using fraction collector, evaporated and then each fraction was analyzed by bioassay. Fractions containing antibacterial activity were merged, concentrated and further purified. The final purification of active fraction was carried out by reverse phase high performance liquid chromatography (HPLC) (Agilent 1220 Infinity, USA) using a C_{18} column (Agilent Eclipse Plus C_{18}, 4.6 mm×100 mm, 3.5 µm) at a constant temperature of 40 °C. The flow rate was maintained at the rate of 1 mL/min and the eluate was detected using UV detector. The prominent peak compounds were collected separately and concentrated, and 30 µL extract was bioassayed against the indicator organism *S. aureus*. Penicillin was considered as standard antibiotic for comparison of potency. Analytical HPLC was repeated several times to get sufficient volume of antibacterial compound for further analysis. The absorption spectrum of the active molecules in methanol was scanned in UV–visible region between 200 and 700 nm by Shimadzu UV 1800 spectrophotometer. The electro-spray ionization mass spectra (ESI-MS) of HPLC purified active compound were recorded with Shimadzu LCMS-8030 triple quadrupole liquid chromatograph mass spectrometer equipped with electrospray ionization interface and coupled to the UPLC system (Shimadzu, Japan) at negative ion detection mode in the scan range from m/z 100 to 500. The mass spectrometers were operated under following optimized conditions: probe voltage, −3.5 kV; desolvation line temperature, 250 °C; heat block temperature, 400 °C; nebulizing gas flow rate, 1.5 L/min; and drying gas flow rate, 10 L/min. HPLC separation was accomplished using reverse phase C_{18} column (150 mm × 4.6 mm, 4.6 µm) using a linear gradient at the flow rate of 0.4 mL/min. The HPLC gradient system consists of 10 mM acetonitrile in water (solvent A) and 10 mM acetic acid (solvent B). The gradient elution program was 0–10 min 10%–90% B, 10–11 min 90%–100% B and 11–20 min 100% B. Injection volume was 20 µL and column temperature was maintained at 40 °C.

3. Results and discussion

3.1. Isolation of endophytic actinomycetes

Association of endophytic actinomycetes with medicinal plants may lead to participation in metabolic pathways, enhancement of its own natural bioactivity and gain of some genetic information to produce biologically active compound [10]. Hence, the present study exploited antibacterial potential of endophytic actinomycetes isolated from a medicinal plant *C. roseus* of Rajkot, Gujarat, India. Till date no endophytic actinomycetes has been reported from root of *C. roseus*. A total of 11 morphologically distinct endophytic actinomycetes were isolated from internal leaf, stem and root tissues of *C. roseus*. Among the isolated organisms, 6 (54.55%) isolates were obtained from root and 5 (45.45%) were isolated from leaf whereas no actinomycetes was obtained from stem tissues. Our result suggests that endophytic actinomycetes residing in root possesses more antimicrobial potentials than the other parts of the plant. It can be anticipated that endophytic actinomycetes presenting inside the root tissues may play an important role in plant protection by development of antibacterial potentials against pathogens [11].

Effective surface sterilization is a crucial step for the study of endophytes. Alkaline environment in surface sterilization favors growth of actinomycetes but not endophytic fungi. Furthermore, 10% $NaHCO_3$ inhibits the growth of fungal endophytes, so that endophytic actinomycetes could grow out of tissues earlier than fungi. No microbial growth was observed on HV agar plate containing sterile distilled water from the final rinse and surface-sterilized tissue. These results confirm that sterilization technique was efficient, no epiphytes were grown and all the isolates were endophytic actinomycetes.

3.2. Preliminary screening for antibacterial activity

All isolates were screened for their antibacterial activities, among which three organisms from root tissues exhibited their antibacterial potentials against several gram-positive and gram-negative bacteria (Table 1). Among these three organisms, strain rsk5 was found to be more potent which possesses broad spectrum antibacterial activity and hence it was chosen for further study.

Table 1
Antimicrobial activity of endophytic actinomycetes isolated from *Catharanthus roseus* against test pathogens.

Extract	*S. aureus*	*B. subtilis*	*B. megaterium*	*E. faecalis*	*P. vulgaris*	*S. typhimurium*	*P. aeruginosa*	*E. coli*
EA1	+	+++	++	+++	++	++	+++	+
EA2	–	–	+	+	–	–	–	–
EA3	–	–	–	–	–	+	–	–
EA4	–	–	–	–	–	–	–	–
EA5	+++	+++	+++	+++	+++	+++	+++	+++
EA6	–	++	++	++	+	++	+	++
EA7	–	–	+	–	–	+	–	–
EA8	–	–	–	–	–	–	–	+
EA9	–	+	–	+	–	–	–	–
EA10	–	–	–	–	–	+	–	–
EA11	–	–	–	–	–	–	–	–
Penicillin	–	+++	+++	+++	+++	+++	+++	+++

+++: Good activity; ++: Moderate activity; +: Weak activity; _: No activity.

3.3. Characterization of isolate rsk5

Morphology of strain rsk5 on tryptone soya agar showed yellow-colored, smooth, and circular colony with entire margin. Rsk5 is aerobic, gram-positive, coccus-shaped bacteria which do not produce endospore. Rsk5 could grow at temperature range of 4–45 °C, pH 5–8 and NaCl concentration of 0%–12% (m/v). Physiological and biochemical characteristics of rsk5 are shown in Table 2.

The 16S rDNA sequence (1463 bp) of the strain rsk5 has got the accession number KU991822 and confirmed the identification of the strain at species level. Phylogenetic tree of rsk5 showed 99% similarity with *Micrococcus yunnanensis* strain N1-7 (GenBank Accession Number: JX094178.1 based on nucleotide homology and phylogenetic analysis (Fig. 1)). The DNA G+C content was derived as 56.86%, which is different from other known *Micrococcus* sp. having range between 66.3% and 73.3%. Rsk5 has been characterized and identified as *Micrococcus yunnanensis* strain rsk5 based on morphological, physiological, biochemical features and 16S rDNA sequence based characters. Significant variation in characteristics and DNA G+C content from other known *Micrococcus yunnanensis* strongly suggests that rsk5 is a novel strain. To date, no report is available on antibacterial property of *Micrococcus yunnanensis*; therefore, this is the first report of antibacterial potentials of this species.

Table 2
Physiological and biochemical characteristics of strain rsk5.

S. no.	Characteristic	Strain rsk5
1	Sporulation	–
2	Pigmentation	–
3	Starch hydrolysis	–
4	Gelatin hydrolysis	–
5	Lipid hydrolysis	–
6	Casein hydrolysis	+
7	Anaerobic growth	–
8	Indole production	+
9	Methyl red	–
10	Voges proskauer	+
11	Nitrate reduction	+
12	Oxidase	–
13	Urease	–
14	Alkaline phosphatase	+
15	β-galactosidase	–
16	Catalase	+
17	Coagulase	–
18	Arabinose	+
19	Galactose	–
20	Rhamnose	–
21	Glucose	+
22	lactose	–
23	Sucrose	–

"+" positive, "-" negative.

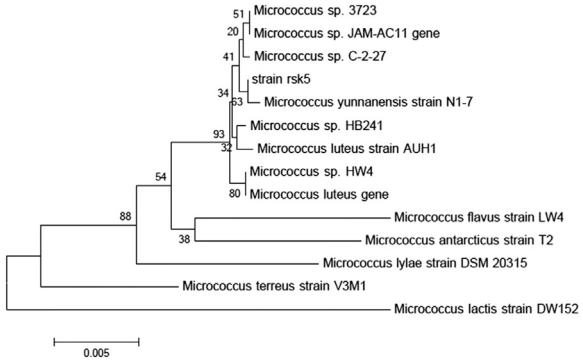

Fig. 1. Rooted neighbor joining phylogenetic tree of strain rsk5 based on 16S rRNA gene sequences, showing the relationship between strain rsk5 and related representative species of the genus *Micrococcus*. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. The scale bar indicates 0.005 substitutions per nucleotide position.

3.4. Optimization for growth and metabolite production

The strain rsk5 showed broad spectrum antibacterial activity against all test organisms including antibiotic resistant *S. aureus*. *S. aureus* was insensitive to penicillin but was found to be most sensitive to the rsk5 extract; hence further study was carried out with it [12]. Among the seven-selected media, strain rsk5 grown on starch casein medium showed maximum growth as well as antibacterial activity against *S. aureus* (Fig. 2A). SCM posseses three specific nitrogen sources which directly influence the production of antibacterial metabolites [13].

Rsk5 had the highest growth as well as antibacterial activity at 30 °C and there was no activity at 45–50 °C (Fig. 2B). Rsk5 could grow up to 45 °C. This result was consistent with that put forwarded by Iwai and Omura [14] where they concluded that the temperature range responsible for increased production of secondary metabolites is narrow. Highest growth of strain was obtained at pH 7 but optimum antibacterial activity was observed at pH 5 (Fig. 2C). The present study revealed that slight change in acidic condition elevated protective mechanisms of endophytic actinomycetes rsk5 by induced production of antibacterial agents [15,16]. Rsk5 can tolerate wide range of salinity up to 12% NaCl and grow in absence of NaCl but optimum growth as well as antibacterial metabolite production and was observed at 2% NaCl (Fig. 2D).

Antibacterial activity of strain rsk5 grown in starch casein medium was started from ninth day of incubation and then it was continuously increased and reached a maximum on twelfth day. Growth and product kinetics of rsk5 showed that production of antibacterial metabolites started during stationary phase and hence the product must be a secondary metabolite.

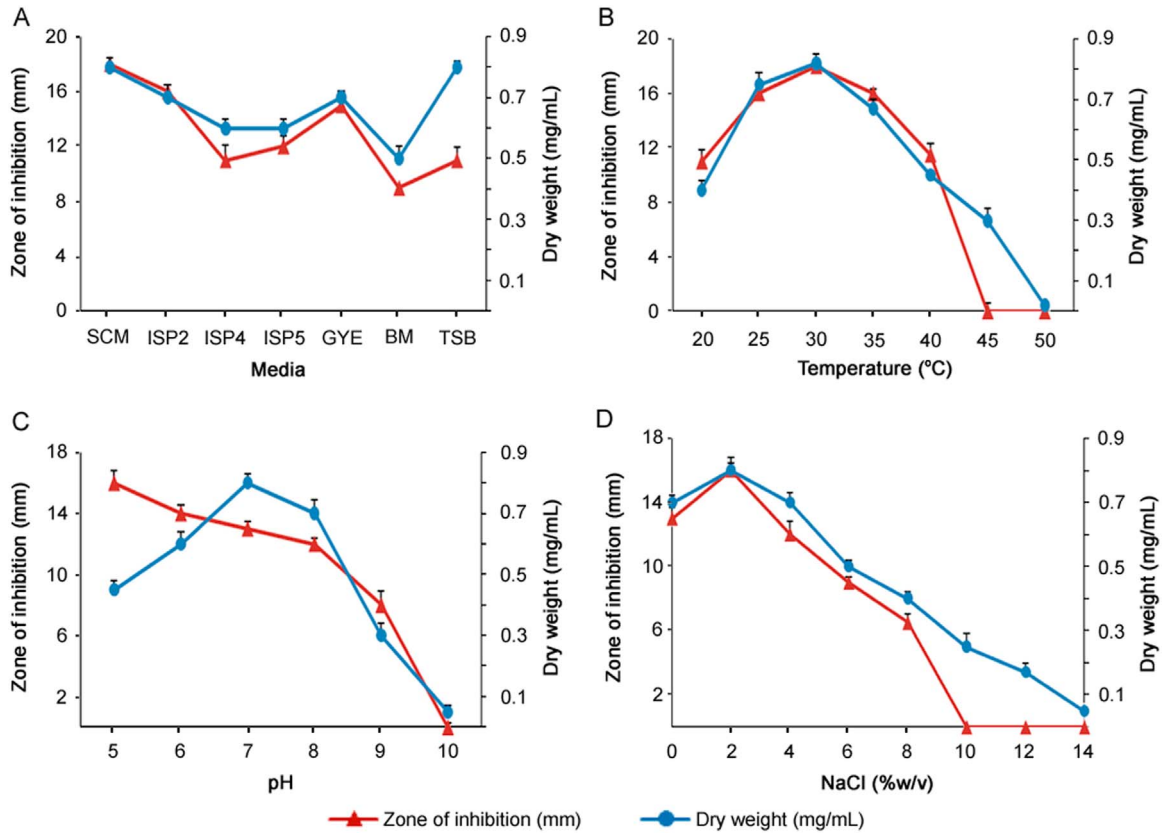

Fig. 2. Effects of (A) media, (B) temperature, (C) pH and (D) NaCl on growth and antibiotic production.

3.5. Fermentation and extraction of the antibacterial agent

Antibacterial secondary metabolites were secreted in extracellular medium by actinomycetes [17]. Selection of solvent system was based on clear separated bands with sharp zone of inhibition against test culture. Ethyl acetate extract of rsk5 was dried to get yellow crude extract which was further assayed against *S. aureus* to confirm the heat stability of metabolites [18].

3.6. Chromatography based purification of the antibacterial compound

Among the five solvent systems chosen for TLC, chloroform-methanol (24:1, v/v) was found to be the best solvent system for the separation of antibacterial metabolites (data not shown) [19]. Bioassay of TLC extract reveals that the compound showing antibacterial activity migrated through the plate with R_f value of 0.26. Analytical TLC followed by preparative TLC was found to be a suitable method to get enough antibacterial agents. Multiple preparative TLC was performed to get sufficient amount of active metabolites.

Flash chromatography was found to be an efficient technique for separation of antibiotic because large quantity of compound can be separated at a time by applying medium pressure [20,21]. TLC extract was further purified by flash chromatography with isocratic elution with 350 mL hexane/ethyl acetate (gradient from 100% hexane/0% ethyl acetate to 0% hexane/100% ethyl acetate) at the flow rate of 3 mL/min. Forty-eight tubes of 7 mL fraction volume were collected. Antibacterial assay of fractions 21–25 showed presence of antibacterial metabolite that was further mixed, concentrated and further recuperated in methanol.

Partial purification was performed with analytical HPLC where optimization of mobile phase reveals that acetonitrile-0.1% acetic acid (60:40, v/v) was the most suitable solvent system and active component was detected by UV at 210 nm. HPLC chromatogram of metabolic extract showed seven peaks; fraction of each peak was tested for antibacterial activity. Fraction 4 (retention time: 1.24 min) presented a single sharp isolated peak and showed potential antibacterial activity (Fig. 3). HPLC with optimized conditions was found to be very effective for final purification of antibacterial compound [22]. The result of bioassay against *S. aureus* showed 1.4-fold increases in antibacterial activity. The UV absorption maxima of purified antibacterial compound produced by strain rsk5 were recorded at 208 nm. The ESI-MS spectra showed one major and three minor peaks which seem to be irrelevant. The prominent molecular ion peaks at m/z 319.3 [M-H] lead to a molecular weight 320 g/mol (Fig. 4). This compound seems to be novel as it differs from other known antibacterial secondary metabolites.

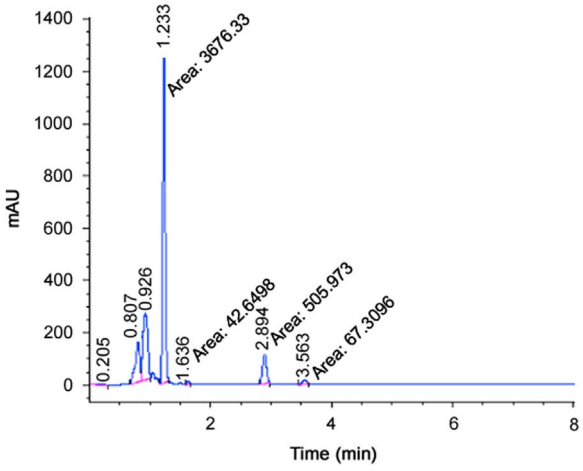

Fig. 3. Chromatogram of separated antibacterial compound by preparative HPLC (retention time=1.24 min).

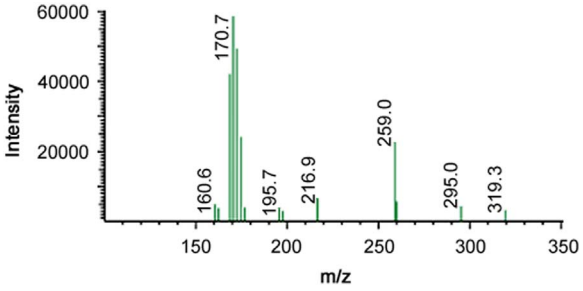

Fig. 4. ESI–MS spectra of antibacterial compound.

4. Conclusion

The search of antibiotic producer from unexplored sources against drug-resistant bacteria is still in demand. The present study indicates that medicinal plants are important source for exploration of antibiotic producing actinomycetes. Endophytic actinomycetes *Micrococcus yunnanensis* strain rsk5 has proved its capabilities of being a potential candidate in the search for an antibacterial compound against antibiotic resistant human pathogenic bacteria *S. aureus*. Chromatographic separation was an efficient technique for separation of antibacterial compound. Further purification and structure elucidation of active compound and investigation of its molecular mechanisms can be a promising approach for future antimicrobial drug development programs.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpha.2017.05.001.

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