

# Isolation, Characterization and Antimicrobial Activity of Endophytic Actinobacteria from Medicinal Plants

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## Sreenivasan *et al.*: Bioactive Actinobacteria from Medicinal Plants

The current study is aimed to isolate endophytic actinobacteria from various medicinal plants for exploring its antimicrobial activity against clinical pathogens. Out of 50 actinobacterial cultures isolated from 11 medicinal plants, 58 % of the cultures showed antimicrobial activity against at least two out of nine pathogens tested. Strain KCA1 isolated from *Phyllanthus niruri* leaves showed maximum zone of inhibition against all of the nine pathogens tested. Strain KCA1 exhibited maximum level of antimicrobial metabolite production in solid state fermentation during 9<sup>th</sup> d of incubation. Three well separated spots were observed when the ethyl acetate extract of strain KCA1 was analyzed through thin layer chromatography. One spot with retention factor value 0.82 was found to inhibit *Staphylococcus aureus* in thin layer chromatography bio autographic assay. The active fraction eluted from thin layer chromatography was further characterized by gas chromatography-mass spectroscopy analysis. The mass spectral data revealed the presence of three compounds *viz.* 2, 4-di-tert-butylphenol, 1-hexadecanol and 1-nonadecene present in major proportions which are responsible for the antimicrobial activity of the strain KCA1. In fermentation experiment, variables such as glucose, yeast extract and sodium chloride were found to influence the antimicrobial compound production. The potential strain KCA1 was identified as *Streptomyces* sp. on the basis of microscopic, cultural, physiological and 16S ribosomal ribonucleic acid analysis.

**Key words:** Endophytic actinobacteria, *Streptomyces*, antimicrobial, bioautography, gas chromatography-mass spectroscopy

Antimicrobial Resistance (AMR) is one of the most concerning issues of human health<sup>[1,2]</sup>. It is reported that more than 700 000 deaths occur every year because of AMR in global level. It is statistically estimated that about 10 million people may possibly die every year due to AMR by 2050 without global intervention which would actually be more compared with cancer deaths by 2050. In India, it is estimated that there would be two million AMR-related deaths by 2050 without global intervention<sup>[3]</sup>. The management of AMR is difficult and requires a multinational approach. Indeed, no single initiative in the prevention and containment of AMR has proven entirely successful and hence development of a series of necessary mechanisms has been suggested<sup>[4]</sup>. The reduction in the number of novel antibiotics that have been found and produced in recent years is indeed a challenge to the researchers<sup>[5]</sup>. This has called for increased search for novel antibiotics to be discovered.

Microorganisms are producing a wide variety of nanoparticles, which can perform wide range of bioactivities<sup>[6,7]</sup>. This is especially notable for the phylum

actinobacteria, which have been reported to produce more than 10 000 different bioactive molecules. Among them almost 45 % of the bioactive metabolites are currently known and most of which have been isolated from the *Streptomyces*<sup>[8]</sup>. Endophytic microorganisms live in healthy plant tissues and develop a symbiotic relationship with the host plant without causing any disease. The biodiversity of endophytic actinobacteria is known to be a valuable resource. A variety of studies have explored a wide spectrum of taxonomic status of medicinal plant-associated endophytic actinobacteria<sup>[7,9-11]</sup>.

To our knowledge, to date, there are very limited reports on the diversity and antimicrobial potential of endophytic actinobacteria associated with *Phyllanthus niruri* (*P. niruri*), *Ocimum tenuiflorum* (*O. tenuiflorum*),

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*Azadirachta indica* (*A. indica*) and *Mentha arvensis* (*M. arvensis*) and also no reports were found with *Cassia fistula* (*C. fistula*), *Eclipta prostrate* (*E. prostrate*), *Lawsonia inermis* (*L. inermis*), *Senna auriculata* (*S. auriculata*), *Rhizophora apiculata* (*R. apiculata*), *Murraya koenigii* (*M. koenigii*) and *Coriandrum sativum* (*C. sativum*). Thus, the objectives of our study are as follows: To isolate and identify the endophytic actinobacteria from selected medicinal plants; to screen the antimicrobial potential of endophytic actinobacteria against various clinical pathogens; to separate and analyses the bioactive compounds from potential endophytic actinobacteria KCA1 using Thin Layer Chromatography (TLC) based bio autography and Gas Chromatography-Mass Spectroscopy (GC-MS) analysis.

## MATERIALS AND METHODS

### Sample collection:

Medicinal plants were collected from regions of Trivandrum, Kerala and vicinity of Sathyabama Institute of Science and Technology, Chennai, Tamil Nadu. A total of 33 samples including root, stem and leaf of 11 different medicinal plants such as *P. niruri*, *C. fistula*, *A. indica*, *O. tenuiflorum*, *M. arvensis*, *E. prostrate*, *L. inermis*, *S. auriculata*, *R. apiculata*, *M. koenigii* and *C. sativum* were collected for this study. The plant parts were rinsed thoroughly in running tap water to remove sand and dust particles and placed inside a zip-lock cover and were brought to the laboratory.

### Isolation and characterization of endophytic actinobacteria:

The plant parts were cut into small pieces and were subjected to surface sterilization so as to ensure the removal of surface microbes. The most commonly used surface sterility includes ethanol (70 % for 1 min) and a strong oxidant or general disinfectant like household bleach (Sodium hypochlorite (NaOCl)) with 2 %-5 % (w/v), available chlorine (for 2-4 min). Then the surface sterilized samples were crushed in a mortar and pestle. 1 ml of crushed sample was serially diluted from  $10^{-1}$  to  $10^{-5}$  using sterile 9 ml water blank. 100  $\mu$ l of aliquot from  $10^3$ - $10^5$  dilutions were plated on Starch Casein Agar (SCA) supplemented with nalidixic acid (20  $\mu$ g/ml) to inhibit the growth of fast-growing eubacteria colonies and nystatin (50  $\mu$ g/ml) to suppress the fungal growth. After incubating the plates for 20 d-28 d at 28°, all the endophytic actinobacterial

colonies were recovered and sub-cultured in individual International *Streptomyces* Project-2 (ISP2) agar plates. After confirming the purity, morphologically dissimilar actinobacterial colonies were sub-cultured on ISP2 agar slants as well as in 20 % glycerol broth and stored at 4° and -20°, respectively. Cultural characteristics such as growth rate, consistency, aerial mass color, reverse side pigment and soluble pigment production and microscopic characteristics such as presence of aerial and substrate mycelium, mycelia fragmentation and spore chain morphology were recorded.

### Preliminary determination of antimicrobial activity:

Antimicrobial activity of all the endophytic actinobacterial cultures were tested by agar plug method against clinical pathogens such as *Staphylococcus aureus* (*S. aureus*)-ATCC 29213, *Bacillus cereus*, *Micrococcus* sp., *Escherichia coli* (*E. coli*)-ATCC 25922, *Providencia* sp., *Vibrio* sp., *Aeromonas hydrophila* (*A. hydrophila*), *Klebsiella pneumonia* (*K. pneumoniae*)-ATCC 13882 and *Candida albicans* (*C. albicans*). Inoculum with 0.5 McFarland's standard was prepared using sterile nutrient broth and inoculated onto Muller Hinton Agar (MHA) plates using sterile cotton swabs. Agar plugs of 10 d old endophytic actinobacterial strains with 5 mm diameter were taken from ISP2 agar plates and placed over the MHA agar plates seeded with test pathogens. Zone of inhibition was measured after 24 h of incubation at 37° and the results were expressed in millimetre in diameter. Actinobacterial strain which showed promising antimicrobial activity was selected as potential strain for further studies.

### Effect of fermentation method on bioactive metabolites production by the strain KCA1:

Effect of solid-state and submerged fermentation on bioactive metabolite production by the strain KCA1 was investigated. Spores of the endophytic actinobacterial strain KCA1 was inoculated into 5 ISP2 agar plates (90 mm) and 100 ml of ISP2 broth. ISP2 agar plates were kept in the incubator at 28° for 15 d whereas the ISP2 broth containing flasks were incubated in rotary shaker with 120 rpm at 28° for 15 d. After 3 d, for every 24 h, agar plug from ISP2 agar plates were taken and tested for antimicrobial activity against *S. aureus* by agar plug method. Similarly, each 2 ml of endophytic actinobacterial strain KCA1 inoculated in ISP2 broth was taken and centrifuged at 10 000 rpm for 10 min. Further, the cell free supernatant was collected and antimicrobial activity was tested against *S. aureus* by adopting agar well diffusion method.

### Extraction of bioactive metabolites:

The spores of the endophytic actinobacteria KCA1 was inoculated into ISP2 agar plates for its growth and bioactive metabolite production. After incubating the plates for 7 d, the spores were scrapped out and the agar blocks were collected and mixed with hexane, ethyl acetate, chloroform and methanol (1:2 ratio) for the extraction of metabolites. After 24 h, the agar blocks were removed and the solvents were collected and evaporated using rotary evaporator so as to get the crude extract. The antimicrobial activity against *S. aureus* and *E. coli* was tested for four different extracts of the strain KCA1 by agar well diffusion method.

### Secondary determination of antimicrobial activity:

The crude ethyl acetate extract was then checked for antimicrobial activity against various clinical pathogens using disc diffusion method. Sterile empty filter paper disc (HiMedia) with 5 mm diameter was impregnated with 20 µl of endophytic actinobacteria KCA1 ethyl acetate crude extract (100 µg/ml) and kept for drying. The panel of clinical pathogens, as mentioned above, was grown in nutrient broth. Inoculum equivalent to 0.5 McFarland standards was spread evenly over the surface of MHA plate using sterile cotton swab. Once the discs were dried, they were placed over the pathogens inoculated MHA plates and incubated at 37°. After 24 h of incubation the diameter of the zone of inhibition was measured and expressed in millimeter.

### Purification of bioactive compounds:

The crude ethyl acetate extract of strain KCA1, which showed promising antimicrobial activity, was subjected to separation through TLC. 3 µl of crude extract was spotted onto silica gel plates (pore size 60Å, mesh size: 230-400, particle size 40-63 µm, Merck) using capillary tube and then developed with ethyl acetate:dichloromethane (8:2, v/v) gradient as an eluting solvent mixture. The separated components were visualized under Ultraviolet (UV) light at 254 and 365 nm. The antimicrobial active fraction was detected by bio autography approach. The developed TLC plates were overlaid on MHA agar medium supplemented with 0.1 % (w/v) 2, 3, 5-triphenyltetrazolium chloride (tetrazolium red) and *S. aureus*-ATCC 29213 used as the test pathogen at a final concentration of 10<sup>7</sup> CFU/ml. The plate was incubated at 37° for 24 h. Clear zone of inhibition indicated the position of antimicrobial compounds on the TLC plates and the Retention factor (R<sub>f</sub>) value was calculated. The active fraction was eluted

by preparative TLC and purified using ethyl acetate.

### Identification of bioactive metabolites by GC-MS analysis

The partially purified TLC fraction was further subjected to identify the bioactive metabolites by using GC-MS analysis. Agilent technologies 6890–5973N with capillary column TG-5 MS Phenyl Methyl Siloxane (30 m×250 µm×0.25 µm) system was used. Mass detector used in split mode and helium gas with flow rate of 1.0 ml/min was used as a carrier. Injector was operated at 230° and oven temperature for initial setup was 60° for 2 min, ramp 10 min to 280° for 8 min.

### Characterization, optimization and taxonomy of potential actinobacterial strain:

The potential endophytic actinobacterial strain KCA1 was characterized based on their phenotypic<sup>[12]</sup> 16S ribosomal Ribonucleic Acid (16S rRNA) analysis. Morphological characteristics were observed in different culture media such as tryptone agar (ISP1), yeast extract-malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salts-starch agar (ISP4), glycerol-asparagine agar (ISP5), peptone-yeast extract-iron agar (ISP6) and tyrosine agar (ISP7). The results were noted after incubation at 30° for 7 d-10 d. The strain KCA 1 was studied for the utilization of carbon, nitrogen, minerals sources and enzyme production. Also the effect of different pH levels and Sodium chloride (NaCl) concentration on its growth were studied<sup>[13]</sup>.

Strain KCA1 grown at all the above conditions was tested for its antimicrobial activity against *S. aureus* and *E. coli* by agar plug method as described above.

### Molecular characterization:

The genomic Deoxyribonucleic acid (DNA) of potential endophytic actinobacteria KCA1 was isolated using solute ready genomic DNA kit (HiMedia-HiPurA *Streptomyces* DNA purification kit), according to the manufacturer's guidelines. The polymerase chain reaction amplification of 16S rRNA gene of strain KCA1 was performed by using the primers 27F 5'-AGAGTTTGATCMTGGCTCAG-3' (forward) and 1492R 5'-TACGGYTACCTTGTTACGACTT-3' (reverse)<sup>[14]</sup>. Amplified product was carried out for sequencing at Euro fins Genomics India pvt. Ltd., Bangalore. The identification of phylogenetic 9 h and calculation of pairwise 16S rRNA gene sequence similarities were achieved using the Molecular Evolutionary Genetics Analysis (MEGA) version 7

and basic local alignment search tool analysis (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The phylogenetic tree was constructed using the aligned sequences by the neighbor-joining method<sup>[15]</sup> using Kimura-2-parameter distances in the MEGA 7 software. To determine the support of each clade, bootstrap analysis was performed with 1000 replications. The obtained 16S rRNA sequence was submitted to GenBank to get the accession number.

## RESULTS AND DISCUSSION

Totally 50 endophytic actinobacterial cultures were isolated from eleven medicinal plant species based on different colony morphology. All the cultures showed good growth on ISP2 agar medium while most of them have the typical morphology of *Streptomyces* (Table 1). Among the 50 cultures, 37 of them appear to be powdery in nature while 10 others showed a leathery consistency and 3 strains appeared to be smooth. The aerial mass colour of the actinobacterial cultures were found to be grey (28), white (9), cream (5), brown (3), orange (2), pink (2) and yellow (1) with a diverse range of reverse side pigments like pale yellow (31), brown (10), pale brown (5), pink (2) and orange (2). Some of the strains had soluble pigments like pale yellow (9), pink (1) and brown (3) which were dispersed all over the plates. All the endophytic actinobacteria showed the presence of aerial and a branched substrate mycelium which differentiate into short, straight to flexuous chains of smooth-surfaced spores. Passari *et al.*<sup>[16]</sup> isolated 42 endophytic actinobacteria from seven medicinal plants and most of them are identified as *Streptomyces*. Similar to this present study surface sterilization is one of the important processes to study endophytic actinobacterial isolation, whereas Qin *et al.*<sup>[17]</sup> conducted similar work and isolated 2174 actinobacteria from 90 plant samples.

They reported most of the strains as *Streptomyces* (87%) according to the preliminary morphology identification. Bhatia *et al.*<sup>[18]</sup> isolated most of the actinobacteria in SCA media from fresh and dried root and shoot materials of *P. niruri*. Gangwar *et al.*<sup>[19]</sup> isolated forty endophytic actinobacteria from root, leaves and stem of medicinal plants including *O. sanctum* and most of them are identified as *Streptomyces*. According to literature survey, this study might be the first report of endophytic actinobacterial isolation from medicinal plants such as *C. fistula*, *E. prostrata*, *L. inermis*, *S. auriculata*, *R. apiculata*, *M. koenigii* and *C. sativum*.

Out of 50 endophytic actinobacterial isolates, 29 isolates showed activity against minimum of 2 out of 9 pathogens tested whereas two isolates namely KCA1 and KCA2 showed positive activity against all of the 9 pathogens tested (Table 2). The isolate KCA1, isolated from the leaves of *P. niruri* showed maximum inhibition against all the pathogens with the zone of inhibition ranging 13.2±0.6 mm to 22.8±0.4 mm in diameter. Endophytic actinobacteria isolated from medicinal plants *P. niruri*, *O. sanctum*, *A. indica* and *M. arvensis* showed strong antimicrobial activity against various pathogens<sup>[18-20]</sup>. Bhatia *et al.*<sup>[18]</sup> reported that endophytic actinobacteria isolated from *P. niruri* showed maximum zone of inhibition against gram negative pathogen *P. fluorescens*, which is correlated with our present study, KCA1 isolated from *P. niruri* also showing highest zone inhibition against the gram negative pathogens *E. coli* (14.8±0.4 mm), *Vibrio* sp. (13.2±0.6 mm), *Providencia* sp. (13.2±0.2), *A. hydrophila* (20.5±0.28) and *K. pneumoniae* (22.5±0.3). Similar to our study, endophytic actinobacteria isolated from *A. indica* showed antimicrobial activity against *P. fluorescens*, *E. coli* and *Bacillus subtilis*<sup>[21]</sup>.

The strain KCA1 showed a good bioactive metabolite

**TABLE 1: BIOACTIVE METABOLITES IDENTIFIED FROM THE *Streptomyces* STRAIN KCA1 THROUGH GC-MS ANALYSIS**

Compound name	RT	MM	Area (%)	MF	Activity	References
2,4-Di-tert-butylphenol	22.579	206	12.58	C <sub>14</sub> H <sub>22</sub> O	Antibacterial; antiviral; antifungal; antioxidant; anti-inflammatory; cytotoxicities; phytotoxicity; allelopathy and autotoxicity; insecticidal and nematocidal activities	[30-33]
1-Hexadecanol	25.207	242	11.8	C <sub>16</sub> H <sub>34</sub> O	Antimicrobial; antifungal activity	[34,35]
1-Nonadecene	30.797	266	19.45	C <sub>19</sub> H <sub>38</sub>	antituberculosis, anticancer, antioxidant, antimicrobial activity	[36-39]

**TABLE 2: EFFECT OF CRITICAL MEDIUM COMPONENTS ON ANTIMICROBIAL COMPOUNDS PRODUCTION BY *Streptomyces* STRAIN KCA1**

Characteristics	Variables	Growth	Antimicrobial activity <i>S. aureus</i> (mm)
Cultural Characteristics	ISP1 (Tryptone agar)	+++	15.2±0.2
	ISP2 (Yeast extract malt extract)	+++	22.2±0.2
	ISP3 (Oatmeal agar)	+++	0.0
	ISP4 (Inorganic salts- starch)	++	19.8±0.4
	ISP5 (Glycerol asparagine)	+	0.0
	ISP6 (Peptone Yeast extract iron )	++	0.0
	ISP7 (Tyrosine agar)	+++	16.2±0.2
Utilization of Carbon source	Glucose	++	20.2±0.2
	Arabinose	+	0.0
	Sucrose	++	14.5±0.3
	Xylose	+	13.2±0.2
	Inositol	+	0.0
	Mannitol	+	17.8±0.2
	Fructose	++	17.5±0.3
	Rhamnose	-	0.0
	Raffinose	-	0.0
	Cellulose	-	0.0
	Peptone	++	14.8±0.2
	Yeast Extract	+++	20.5±0.3
Utilization of nitrogen source	Malt Extract	+++	0.0
	KNO <sub>3</sub>	-	0.0
	Soybean (NaNO <sub>3</sub> )	+++	12.5±0.3
	Asparaginase	+++	0.0
Enzyme production	Glutaminase	+++	0.0
	Amylase	++	0.0
	Protease	++	0.0
	Lipase	-	0.0
	5	-	0.0
pH	7	+++	22.2±0.2
	9	++	0.0
	11	-	0.0
	0	++	11.8±0.2
	1	+++	21.5±0.3
NaCl tolerance (%)	2.5	+	0.0
	5	-	0.0
	7.5	-	0.0
	10	-	0.0

production in both solid as well as in submerged state fermentation. From 3 d to 15 d, it was noted that the metabolites produced by the strain KCA1 showed maximum activity against *S. aureus* in solid state fermentation compared to that of submerged fermentation (fig. 1). The metabolite production was thus confirmed to be faster and stable in solid state having an inhibition zone of  $15.2 \pm 0.2$  mm in diameter on 3 d to  $21.5 \pm 0.9$  mm, which was the maximum, on 9 d when compared to that of submerged fermentation where the zone of inhibition ( $12.5 \pm 0.8$  mm) showed on 7 d and it reaches the maximum level on 9 d with the inhibition zone of  $14.8 \pm 0.7$  mm. However, a gradual reduction in the inhibition zone was observed from 8<sup>th</sup> and 10<sup>th</sup> d of its growth in solid and submerged state fermentation respectively. Our present study correlated with Radhika *et al.*<sup>[22]</sup> who reported that the bioactive metabolite production from *Streptomyces* showed antimicrobial activity against *S. aureus* in both solid and submerged state fermentation. But most of the *Streptomyces* strains were reported to show bioactive metabolite production in solid state and failed to show activity when produced in liquid culture. Gebreyohannes *et al.*<sup>[23]</sup> studied using eleven potential actinobacteria for solid and submerged state fermentation and bioactivity was also checked against various pathogens. Similar to our study, they reported that both the fermentation methods were suitable for bioactive metabolite production and showed provident antimicrobial activity against various pathogens.

The ethyl acetate extract of KCA1 showed activity against all the test pathogens the maximum activity was observed against *S. aureus* ( $20.0 \pm 0.3$  mm) followed by *Bacillus cereus* ( $19.83 \pm 0.4$  mm), *K. pneumoniae* ( $19.0 \pm 0.3$  mm) and *C. albicans* ( $16.83 \pm 1.2$  mm). Also,

the crude extract of KCA1 showed least antimicrobial activity against *Vibrio* sp. ( $11.17 \pm 1.1$  mm) followed by *A. hydrophila* ( $11.83 \pm 0.4$  mm), *Providencia* ( $12.33 \pm 0.9$  mm) and *E. coli* ( $13.5 \pm 0.8$  mm) (fig. 2). Our present finding of secondary screening also correlated with that of other reports. This evidenced that the crude extract of actinobacterial strains produced by solid state fermentation showed antimicrobial activity against wide range of pathogens such as *S. aureus*, *E. coli*, *P. aeruginosa*, *K. pneumoniae* and *S. typhi* in secondary screening using disc diffusion method<sup>[23]</sup>. They also reported that the promising antimicrobial inhibition against various pathogens suggested that endophytic actinobacteria may be the crucial candidates for the production of bioactive metabolites<sup>[17]</sup>.

Among the different solvents used for extraction of secondary metabolites from the potential strain KCA 1, ethyl acetate extract showed maximum zone of inhibition against *S. aureus* ( $20.5 \pm 0.7$  mm), while no results were obtained for hexane and methanol extracts. However, chloroform extract showed  $9.5 \pm 0.3$  mm zone of inhibition against *S. aureus*. Similarly, Das *et al.*<sup>[24]</sup> also reported that ethyl acetate extract of *Streptomyces* showed maximum activity against *S. aureus*. The ethyl acetate extract of *Streptomyces* sp. VITPSA showed highest range zones of inhibition against *P. aeruginosa* and *S. aureus*, which was also higher than the standard chloramphenicol<sup>[25]</sup>. Our present study exactly correlates with other reports showing the antimicrobial activity of different solvent extracts of endophytic actinobacteria against *E. coli*, *P. mirabilis*, *P. aeruginosa*, *K. pneumoniae* and *Enterobacter* and proving that ethyl acetate extract was the most predominate than other solvents<sup>[26]</sup>.

A total of six bioactive compounds were detected by

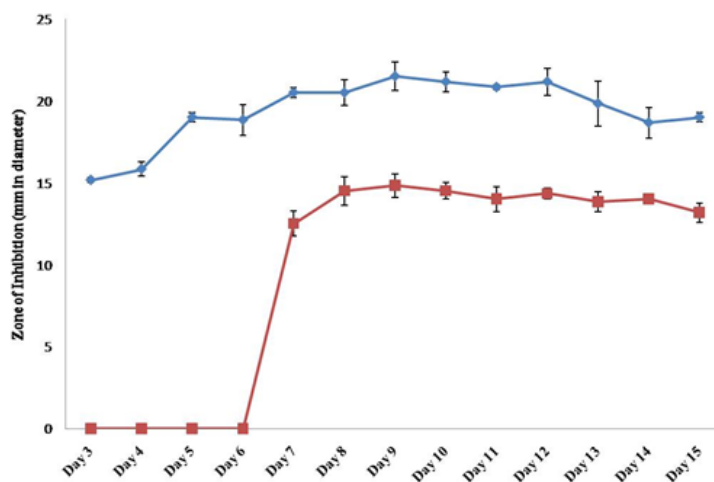
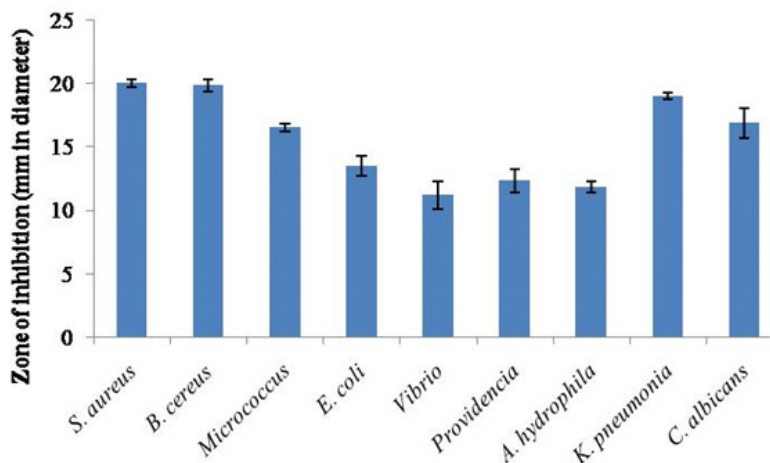


Fig. 1: Effect of solid and submerged fermentation for antimicrobial metabolite production from the strain KCA1

Note: (—●—) Solid and (—■—) Submerged



**Fig. 2: Antimicrobial activity of potential strain KCA1 against different pathogens**

TLC using the solvent system of dichloromethane and chloroform with the ratio of 8:2. In which, one bioactive compound showed a clear zone of inhibition against *S. aureus* and had an  $R_f$  value of 0.82. The antimicrobial activity of the bioactive compound was found to be  $21.8 \pm 0.3$  mm zone of inhibition against *S. aureus* which was collected by preparative TLC. Our research finding exactly correlated with Vaishnavi *et al.*<sup>[27]</sup> reported that partially purified ethyl acetate extract from *Streptomyces* showed inhibition against *S. aureus*, which was purified and detected by TLC and bio autography techniques. TLC based bioautography is a high throughput method which can be used for screening of bioactive molecules and to study their activity<sup>[28]</sup>. Our present study also correlated with other report stated that antibacterial compound Actinomycin D produced by *Streptomyces* TBRC 8912 was purified and detected by TLC based bioautography<sup>[29]</sup>.

In this present study GC-MS analysis of partially purified active TLC fraction of *Streptomyces* sp. KCA1 confirmed the presence of major three compounds with different retention times as illustrated in fig. 3. The identified compounds and their retention time, molecular mass, peak area (%), molecular formula and their potential activities are shown in Table 1. The most prevailing major compounds such as 2, 4-di-tert-butylphenol ( $C_{14}H_{22}O$ ), 1-Hexadecanol ( $C_{16}H_{34}O$ ) and 1-Nonadecene ( $C_{19}H_{38}$ ) were found to be the derivatives of aromatic compounds. Similarly,  $C_{14}H_{22}O$  by TLC based approach and showed strong antimicrobial activity against *Aspergillus*<sup>[30,31]</sup>. Also, *Streptomyces mutabilis* G61 isolated from Sahara soil sample which produced  $C_{14}H_{22}O$  having strong antimicrobial activity<sup>[32]</sup>. The endophytic *Streptomyces globosus* JQ926176 produced  $C_{14}H_{22}O$  which has strong antioxidant activity<sup>[33]</sup>.

Another remaining two bioactive compounds  $C_{16}H_{34}O$  and  $C_{19}H_{38}$  were also isolated from *Streptomyces* strains showed antimicrobial, anticancer and anti-tubercular activity<sup>[34-39]</sup>. Thus, the present study is clearly revealed that the three major compounds identified through GC-MS could be the key contributing factors to the antagonistic potential of *Streptomyces* strain KCA1.

The cultural characteristics of the strain KCA 1 is shown in Table 1. It showed good growth on ISP1, ISP2, ISP3 and ISP7 and moderate growth on ISP4, ISP5 and ISP6. The potential strain KCA1 was found to utilize wide range of carbon (Glucose, Arabinose, Sucrose, Xylose, Inositol, Fructose, Mannitol, Raffinose, Rhamnose, and cellulose), nitrogen (Peptone, Yeast extract, Malt extract, Potassium nitrate ( $KNO_3$ ) and Soybean) and mineral sources (NaCl, Magnesium chloride, Manganese chloride, Potassium chloride, Calcium carbonate) for their growth and secondary metabolite production. The strain KCA1 was also found to have asparaginase, glutaminase, amylase and protease production. Other factors like pH and NaCl were also found to regulate the growth and secondary metabolite production of the strain KCA1 (Table 1). Among all the parameters tested, the potential strain KCA1 was found to show maximum growth and antimicrobial activity against *S. aureus* and *E. coli* on utilization of glucose as carbon source, yeast extract as nitrogen source, NaCl as mineral source and pH 7 (Table 2). Similarly, Manikkam *et al.*<sup>[40]</sup> also studied the influence of different carbon sources for the growth and metabolite production against *S. aureus*. It showed glucose as the most predominant carbon source followed by fructose, xylose and sucrose which were also correlated with our present study which showed highest growth of inhibition of *S. aureus* ( $20.2 \pm 0.2$  mm) followed by

mannitol ( $17.8 \pm 0.2$  mm), fructose ( $17.5 \pm 0.3$  mm), sucrose ( $14.5 \pm 0.3$ ) and xylose ( $13.2 \pm 0.2$ ). Al-ghazali *et al.*<sup>[41]</sup> reported that *Streptomyces* sp. LH9 used dextrose as a carbon source and produced maximum activity against *E. coli*, *P. aeruginosa*, *S. aureus* and *S. agalactiae*. They also studied different nitrogen sources for metabolite production and reported peptone as the excellent nitrogen source against *E. coli*, *P. aeruginosa*, *S. aureus* and *S. agalactiae*, whereas our present study showed yeast extract having maximum activity followed by peptone and soybean against *S. aureus* and *E. coli*<sup>[41]</sup>. Manikkam *et al.*<sup>[40]</sup> reported that malt extract used as nitrogen source showed maximum activity against *S. aureus*. Our results correlates with Abdelwahed *et al.*<sup>[42]</sup> who reported soybean meal and yeast extract as the most favorable nitrogen source for antimicrobial compound production for *Streptomyces cyaneus* DN.37 and *Streptomyces lavendulae* DN.7, respectively. Al-Farraj *et al.*<sup>[43]</sup> also reported that glucose and yeast extract used as the carbon and nitrogen sources influenced maximum antibiotic productions than others. Actinobacteria can tolerate wide range of pH and are able to grow in higher saline condition<sup>[44]</sup>. Our present study reported that the strain KCA1 showed good growth in pH 7 and was able to tolerate NaCl up to 1 %. This investigation also

coincides with other's reports<sup>[40,45]</sup>.

The strain KCA1 showed powdery type of colony with gray aerial mass color in ISP2 agar medium (fig. 4a). Scanning electron microscope images of this strain showed substrate mycelia and extensively branched aerial hyphae that are further differentiated into smoothly surfaced spores (fig. 4b). The 16S rRNA gene sequence of the strain KCA1 showed 99.45 % similarity with *Streptomyces* sp. and had a query length of 1452 bp. The accession number MW470667 was received from Genbank for the strain KCA1. A phylogenetic tree was constructed based on 16S rRNA gene sequences to show the comparative relationship between strain KCA1 and other related *Streptomyces* species (fig. 5). The comparative analysis of 16S rRNA gene sequence and phylogenetic relationship showed that strain KCA1 lies in clade close with *Streptomyces* sp. strain KBR3 and *Streptomyces* sp., RAN54. This microscopic and molecular identification is also agreed with other reports<sup>[27,46]</sup>.

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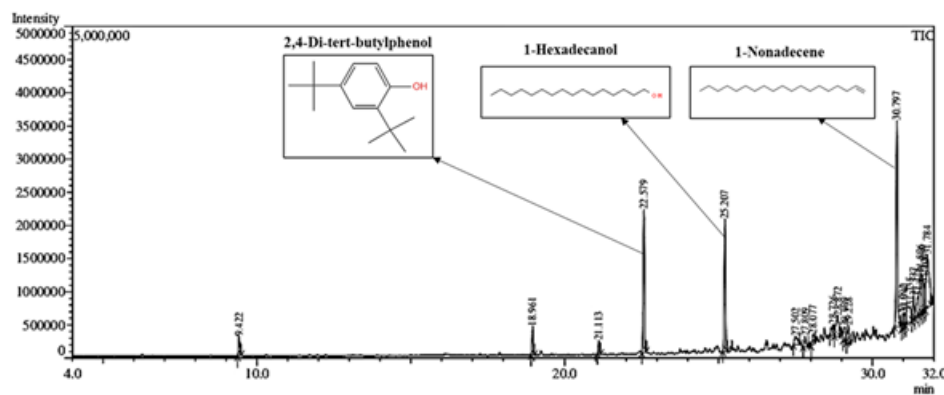


Fig. 3: GC-MS chromatogram of TLC based partially purified *Streptomyces* sp. KCA1

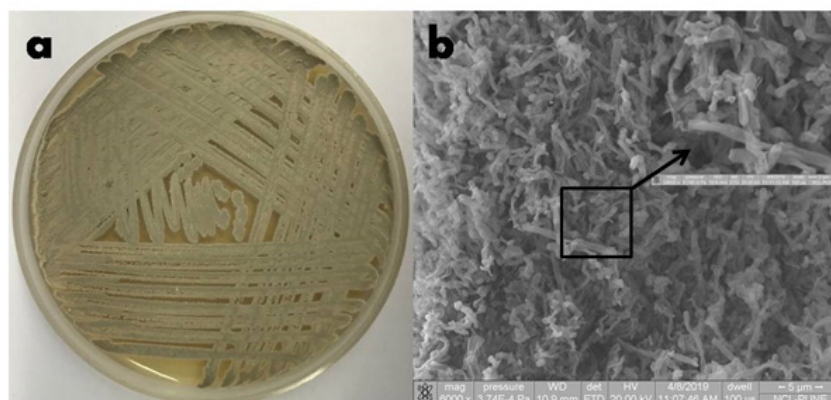
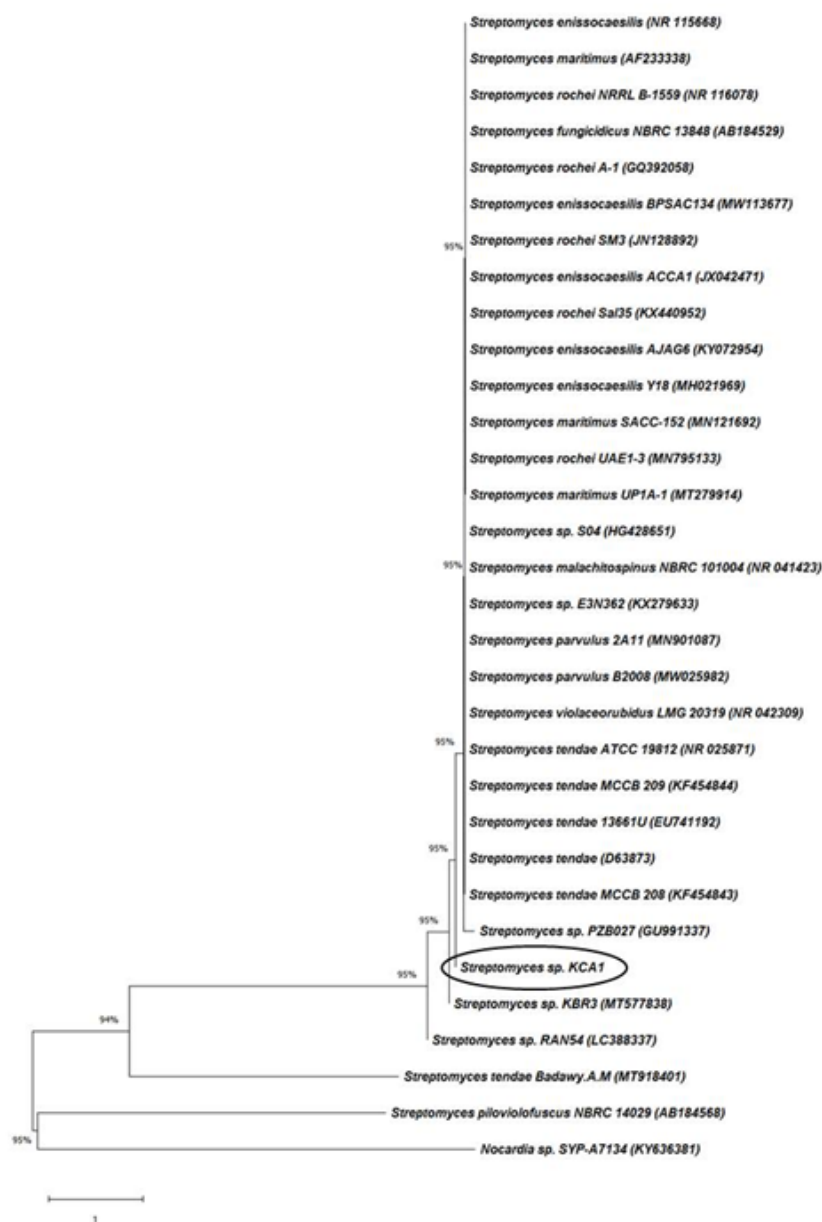


Fig. 4: Morphological view of potential strain KCA1. (a): Cultural morphology and (b): Microscopic (SEM) morphology





**Fig. 5:** Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences shows the strain KCA1 to be a sister taxa of *Streptomyces* sp. within the family Streptomycetaceae. *Nocardia* sp. SYP.A7134 was used as out-group

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