Research Article

Production and Characterization of Actinomycin D from *Streptomyces parvulus* Isolated from *Aloe vera* (l.) Burm. F. and its Antimicrobial Activity

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ABSTRACT

Plants serve as a reservoir of large number of organisms. However, insufficient knowledge exists about the therapeutic applications of endophytic actinomycetes. The aim of this study was to screen endophytic *Streptomyces* sp. Av-R5 for antimicrobial compound for inhibitory activity against human pathogens. *Streptomyces* sp. Av-R5 was isolated from root of *Aloe vera* and identified by 16S rRNA. Structure of the antimicrobial compound was purified by HPLC and identified by FTIR, ESIMS and NMR (^{1}H and ^{13}C). *Streptomyces* sp. Av-R5produced substantial amount of actinomycin D which exhibited broad spectrum antimicrobial activity against human pathogens. Morphological, cultural, physiological and 16S rRNA sequencing studies revealed that the organism Av-R5 showed 100% similarity with *Streptomyces parvulus* NBRC 13193^T (AB184326). Two bioactive metabolites identified from the fermented broth by spectroscopic analysis were as actinomycin D and actinomycin $X_{0\beta}$. The minimum inhibitory concentration of the ethyl acetate extracted compound exhibited antimicrobial activity against Gram-positive, Gram-negative bacteria and *Candida albicans* MTCC 183 with the MIC ranging between 0.25 to 1 mg /ml. The endophytic *Streptomyces* sp. Av-R5 produced notably higher quantities 400 mg/ l of antimicrobial compound in glucose soybean meal broth medium under CCD optimized condition than previously reported actinomycins producing strains. Endophytic *Streptomyces* sp. Av-R5 may be exploited as a potential source for the commercial production of actinomycin D.

Keywords: Actinomycin D, actinomycin $X_{0\beta}$, central composite design, glucose soybean meal broth, *Streptomyces parvulus*.

INTRODUCTION

Medicinal plants are known to be rich source of bioactive compounds with therapeutic potential. Several bioactive molecules from plants have been commercially exploited. Since the endophytic actinomycetes symbiotically associate with their plant host and have genetically evolved together over a long period of time, they benefit each other by producing metabolites of biological and physiological significance complementary to each other[1]. Endophytic actinomycetes benefit host plants by means of growth promotion, stress tolerance and reduction in disease symptoms[2]. Herbaceous plants are the major host for endophytic actinomycetes followed by shrubs[3, 4]. *Streptomyces* is the dominant species followed by *Microbispora*, *Micromonospora*, *Nocardioides*, *Nocardia* and *Streptosporangium*. There are many reports in which both plant and its endophyticactinomycetes produce same metabolites such as, 6-prenylindole, kaempferol, isoscutellarin, umbelliferon and cichorin[5], fisetin, naringenin, 3'-hydroxydaidzein and xenognosin B[6]. However, reports of different antimicrobial substances produced by the endophytic actinomycetes and the host plant also exist[7, 8, 9]. *Streptomyces parvulus* are known for the production of actinomycin D. Actinomycins are a family of chromo peptide lactone antibiotics, among which actinomycin D has been widely studied. Actinomycin D can be synthesized by different species of *Streptomyces* as part of a mixture of several actinomycins[10]. It has been clinically used for the treatment against viruses, cancer and blocking cell cycle. Due to its toxicity, actinomycin D has limited clinical use as an antimicrobial agent, but is used as an important tool in molecular and cellular biology.

Endophytic actinomycetescan be a promising biological control agent of human and plant diseases and can thus be considered as a potential alternative to industrial production of antibiotics for pharmaceutical industries. Keeping this in view, *Streptomyces* sp. Av-R5 was isolated from root of *Aloe vera* and screened for their antimicrobial activity against human pathogens. The optimal conditions for production of antimicrobial compound, its thermal stability, and its proteolysis resistance to enzymes were also examined.

MATERIALSAND METHODS

Endophytic actinomycetes Av-R5

The endophytic *Streptomyces* sp. Av-R5 was isolated from root of *Aloe vera* growing in the garden of School of Studies in Life Science, Pt. Ravishankar Shukla University, Raipur, Chhattisgarh by five step surface sterilization process on starch casein agar medium (soluble starch 10 g, K_2HPO_4 2 g, KNO_3 2g, NaCl 2 g, Casein 0.3 g, $MgSO_4$ 0.05 g, $CaCO_3$ 0.02 g, $FeSO_4$ 0.01 g, Distilled water 1000 ml) supplemented with nalidixic acid (50 mg / l) and nystatin (50 mg / l) to suppress the growth of bacteria and fungi respectively. The plates were incubated at 28 ± 2°C for 21 days. After attaining visible powdery growth, colonies were transferred on starch casein agar slants for storage and preservation. *Streptomyces* sp. Av-R5 was screened for their antimicrobial activity against eight MTCC bacterial, two fungal and seven clinical human pathogens by agar well diffusion method. The inoculum of the pathogens was prepared by adding one loop full of bacterial culture in 25 ml nutrient broth and incubated at 37°C for 16-18 h. The inoculum size of each bacterial strain was standardized by adjusting the optical density of the culture broth to a turbidity corresponding to 0.08 at 620 nm using a spectrophotometer, which was equivalent to 10^8 cfu/ ml.

Cultural, morphological and molecular characterization

Cultural, biochemical and physiological characteristics of potent endophytic actinomycetes Av-R5 was examined using International *Streptomyces* Project procedures following[11]. For the microscopy the isolates were grown by cover slip culture method[12]. For morphological identification cover slips were inserted at 45° angle in inorganic salt starch agar (ISP-4) media at 28 ± 2°C for 14 days. They were observed for their mycelia structure such as presence of rectiflexibilis, retinaculiaperti and spiral chains, presence of globular sporangia, presence of flagellated spores, formation of conidia like spores on the substrate hyphae, tendency of substrate mycelium to fragment under Leica trinocular microscope at 1000x.Molecular characterization by 16S rRNA sequencing and phylogenetic tree construction of the potent isolate was carried out from Microbial Type Culture Collection and Gene Bank, IMTECH, Chandigarh. The Comparison of the nucleotide sequence of the actinomycetes isolate was done using GenBank Database through NCBI Blast (http://www.ncbi.nlm.nih.gov) andalso the 16S rRNA sequence of the endophytic actinomycetes was deposited in NCBI, EMBL and DDBJ and the sequence accession number was obtained.

Fermentation

Antibiotic production was studied in ten different medium viz., Czapek-Dox broth (CZB), glycerol asparagine broth (GAB), glucose soybean meal broth (GSB), inorganic salt starch broth (ISSB), nutrient broth (NB), potato dextrose broth (PDB), sabarouds broth (SB), soybean meal broth (SB), starch casein nitrate broth (SCNB), yeast extract malt extract broth (YEMA). Among all the media glucose soybean meal broth media (CCD optimized nutritional and physical condition) consisting of glucose 11.16 g / l; soybean meal 10.25 g/ l; $CaCO_3$ 1.32 g / l; NaCl 11.18 g / l was selected for further studies. A lot of five 500 ml Ehrlenmayer flasks containing 200 ml of the glucose soybean meal broth medium was inoculated with 6% seed inoculum and incubated at 31.42°C for 10 days at pH 7.19. After incubation, the broths were filtered through Whatman no. 1 filter paper and extracted using equal volume of six different solvent (acetone, chloroform, ethyl acetate, methanol, n-butanol, n-hexane). The extracted compound were concentrated using rota evaporator (IKA model RV 10D796) at 28°C. DMSO was used as a negative control and streptomycin (10 µg/ ml) was used as a positive control. The best solvent extract (1 mg /ml) which showed maximum antimicrobial activity was tested against all the Gram-positive, Gram-negative and fungal pathogens by agar well diffusion method.

Growth curve of *Streptomyces* sp. Av-R5

A growth curve was constructed to evaluate growth time of endophytic *Streptomyces* sp. Av-R5 and correlate it with the production phase of the active metabolite extract. Av-R5 was grown in submerged culture for 14 days under the conditions described above as optimal for the production of active metabolite extract. *B. cereus* MTCC-430 was used as an indicator organism to study the time course production of antimicrobial compound. The growth medium (CCD optimized glucose soybean meal broth)in one flask was filtered through a Whatman no. 1 filter paper, every 24 h during the 14 day growth period. Dry weight of cell mass was established on the difference between the final weight of the filter paper, after filtration & drying and the initial weight.

Purification and characterization of antimicrobial compound

The crude antimicrobial compound was tested for its purity by thin layer chromatography with n-butanol: ethyl acetate: water (9:9:1) as a mobile phase on 6×6 cm pre coated silica gel aluminium sheet (Merck). The developed spots were detected using UV light at 254 nm and their Rf values was calculated. Antimicrobial compound was further analyzed for their purity by HPLC (Shimadzu). Antimicrobial compound was dissolved in methanol (1.5 mg /ml) and was subjected to chromatography on silica gel column Hypersil BDS C18 (150 × 4.6 mm, 5µ pore size), injection volume was 20 µL. The mobile phase was acetonitrile and 5 Mm ammonium acetate in water with a flow rate of 1 ml / min and run time of 30 min. The antimicrobial compounds were characterized using spectroscopic analysis. The UV-Visible spectrum of the antimicrobial compound was determined in methanol (0.08 µg/ ml) with a JASCO UV-visible spectrophotometer (JASCO, model UV-630) at 200-800 nm. FTIR spectrum of the antimicrobial compound was recorded using JASCO FTIR model no. FTIR-4100 at 400-4000 cm^{-1} wave number range using a potassium bromide pellet technique. The spectrum was plotted as wave number along X-axis versus percentage transmission along Y-axis. The ^1H and ^{13}C NMR spectrum of the antimicrobial compound was determined in JEOL 400-MHz NMR spectrophotometer with multiple probe facility model no. AL-400 used $CDCl_3$ as a solvent system. Mass spectrum of the antimicrobial compound was recorded using Shimadzu LCMS with APCI and ESI probes (model LC-2010EV). Electron spray ionization was operated in the positive and negative ion mode and mass spectra were recorded over a range of 100-1300 m/z.

Bioactivity

Purified compound was evaluated for its MIC values against various Gram-positive and Gram-negative MTCC and clinical human pathogen cultures using nutrient broth (Standard NCCL method 2000). The inoculum for MTCC and clinical bacterial cultures to be tested were prepared by adding one loop full of culture in 25 ml nutrient broth and incubated at 37°C for 16-18 h. The inoculum size of each bacterial strain was standardizing by adjusting the optical density of the culture broth to a turbidity corresponding to 0.08 at 620 nm using a spectrophotometer which was equivalent to 10^8cfu/ ml. Dilution of the antimicrobial compound was starting from the 1, 0.50, 0.25, 0.125, 0.0625, 0.0315 mg / ml respectively. Test tubes having 1 ml of nutrient broth and different concentrations of purified antimicrobial compound was inoculated with the 100 µl of the test inoculum and incubated at 37°C for 24 h for bacteria and 28°C for 72 h for fungi then observed for the growth. Dilution in the test tube with no visible growth was streaked on nutrient agar media to confirm killing of the test organism. Concentration of the antimicrobial compound in this tube was considered as minimum inhibitory concentration (mg/ ml) of the compound against that particular test organism.

Statistical analysis

All measurements were carried out in duplicates. CCD was done with the Design Expert software package (version 9.0.4.1, State-Ease Inc., USA).

RESULTS AND DISCUSSION

The characterization of the isolate Av-R5 associated with root of *Aloe vera* on the basis of morphological, biochemical and physiological properties suggested that Av-R5 was a member of the genus *Streptomyces.* The isolate showed good growth on SCA, ISP-3, ISP-4, ISP-5, ISP-7 and poor growth on ISP-2 media.

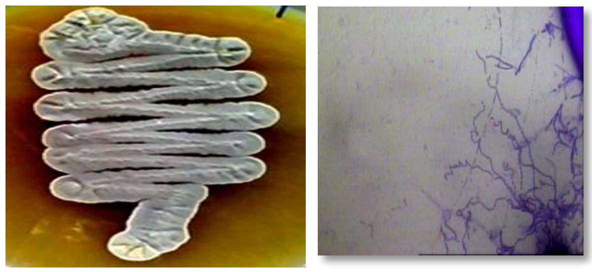
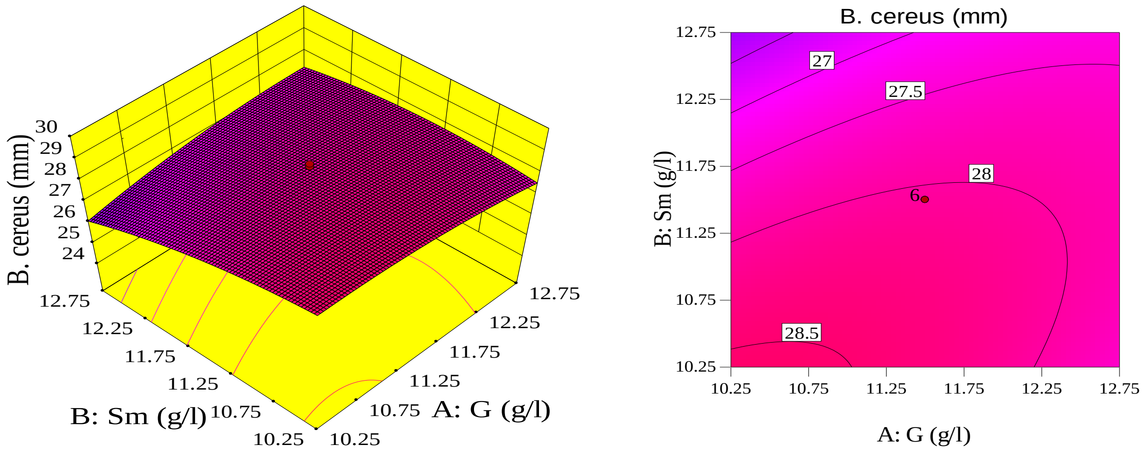


Figure1: (a) Colony characteristic of Av-R5 grown on inorganic salt starch agar medium at 28°C for 21 days **(b)** Retinaculum apertum type of spore chain (1000x).

Figure 2: Neighbor-joining phylogenetic tree for endophytic *Streptomyces* sp. Av-R5 [(*Streptomyces parvulus* NBRC 13193^T (AB184326)] from root of *Aloe vera*based on the 16S rRNA gene sequences with *Kitasatospora setae* as the out group.

It showed grey color aerial mycelium, yellow color substrate mycelium and orange yellow color pigment production in all kind of medium. Aerial mycelium showed Retinaculum Apertum type of spore chain, open loops with extended spirals of wide diameter structure (Figure1). In this study, the 16S rRNA sequence of the *Streptomyces* Av-R5 has been submitted in the Genbanks namely NCBI/EMBL/DDBJ (accession number KY771080). The gene sequence of the *Streptomyces* sp. Av-R5 and the phylogenetic analysis revealed 1476 bp sequence of the isolate 100 % similar to that of the existing species of *Streptomyces parvulus* NBRC 13193^T (AB184326)

(Figure2). *Streptomyces parvulus* was first identified by Waksman from a soil sample in 1940. Recently, *S. parvulus* has been reported as an endophyte from stem of the medicinal plant, *Dracaena cochinchinensis* and from *Codonopsislanceolata*[13, 14]. Endophytic actinomycetes have little or no host specificity and that certain species can have vast geographic distributions [15].



(A) (B)

Figure 3: (A) Response surface graph and **(B)** Contour plot shows interactive effect of glucose and soybean meal on antimicrobial activity of *Streptomyces* sp. Av-R5 against *B. cereus* MTCC-430. An elliptical nature of contour plot indicates the significant interaction between glucose and soybean meal.

The effect of different production medium on production of antimicrobial compound revealed glucose soybean meal broth better over other medium. Glucose soybean meal broth medium was monitored through CCD for enhancing the production of active antibiotic compound. The final optimized nutritional parameters which favored antimicrobial compound production were as glucose 11.16 g/ l, soybean meal 10.25 g/ l, calcium carbonate 1.32 g/ l and sodium chloride 11.25 g/ l (Figure 3). Antibiotic production was found to be directly proportional to the concentration (within a certain range) of glucose and soybean meal. A significant increase in the production of antimicrobial compound was achieved when the amount of glucose and soybean meal is between 10.25 to 11.50 g/ l. Further increased in their concentration gradually decreased the antibacterial activity. Higher concentration of glucose is generally considered as repressor of secondary metabolites; it increases the cell growth and inhibits antibiotic production due to the achievement of stationary phase [16]. Soybean meal is a complex nitrogen source and increases the production of antibiotic by *Streptomyces* sp. due to slow decomposition of these compounds in the medium[17].

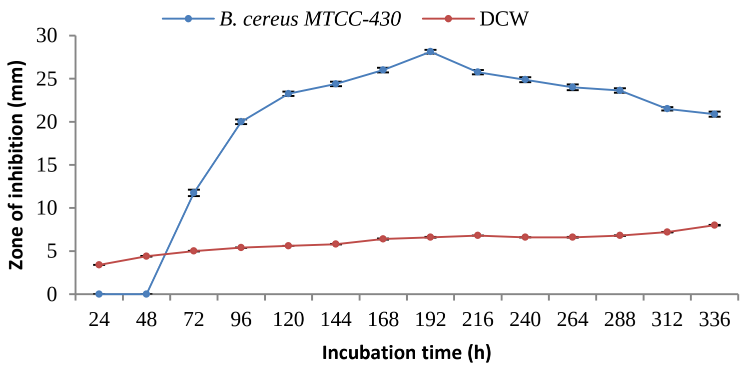
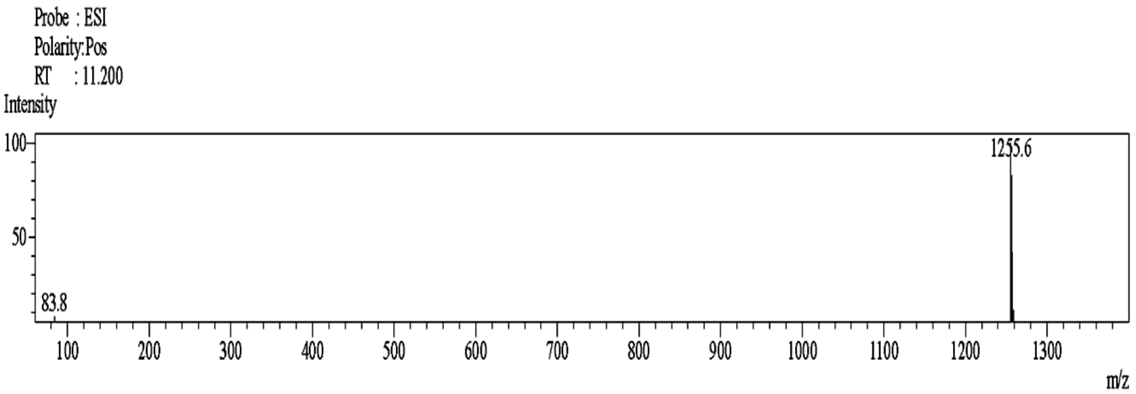


Figure 4: Growth curve of *Streptomyces sp. Av-R5* from root of *Aloe vera* cultured in glucose soybean meal broth medium. Results represent the means ± standard errors of the means of two independent experiments.

The growth curve of *Streptomyces* sp. Av-R5 revealed that antimicrobial compound production was started on 72 h of incubation with a zone of inhibition of 11.75 ± 0.37 mm against *B. cereus*. Maximum antibacterial activity was observed on 192 h of incubation with a zone of inhibition of 28.12 ± 0.23 mm against *B. cereus.* Further increase in incubation time resulted in gradual decrease in antibacterial activity although there was increase in dry cell weight (Figure 4). The antimicrobial efficacy of the solvent extracts of *S. parvulus* Av-R5 revealed that ethyl acetate extract showed maximum activity against *B. cereus* and *S. aureus*. Acetone and methanol extracted compound were next in order. However, minimum antibacterial activity was recorded with the substance extracted using chloroform. It was observe that ethyl acetate extracted antimicrobial compound showed broad spectrum antimicrobial activity against Gram-negative bacteria besides Gram-positive bacteria and also showed activity against *Candida albicans* MTCC-183 (Table 1). Ethyl acetate has been reported good extractor for actinomycin D from *S. parvulus*[18].



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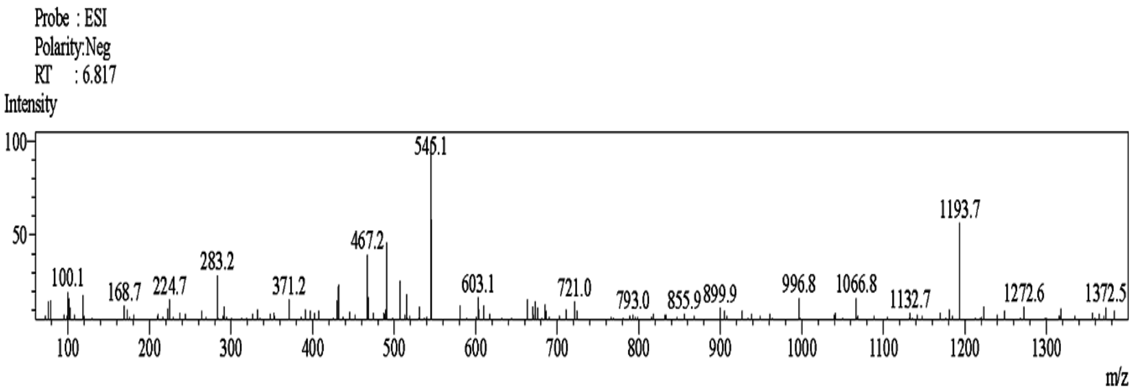

(g)

Figure 5: Mass spectral analysis of antimicrobial compound of *Streptomyces parvulus* from root of *Aloe vera.* The signal for $[M+H]^+$ was visible at m/z 1255.4 with the retention time 11.20 min. and (g) actinomycin $X_{0\beta}$. The signal for $[M-H]^-$ was visible at m/z 1272.6 with the retention time 6.81 min.

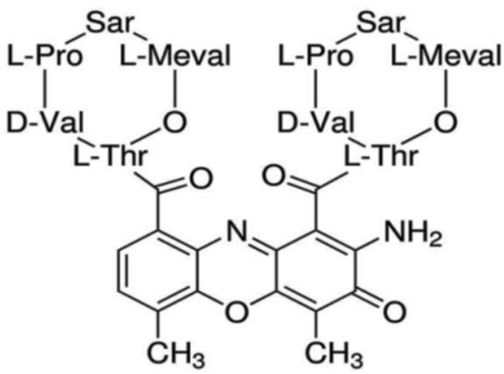

Figure 6: Chemical structure of actinomycin D from *Streptomyces parvulus* Av-R5

In the present study *Streptomyces parvulus* NBRC 13193^T (AB184326) from root of *Aloe vera* produced higher yield 400 mg / l of actinomycin D by CCD optimized condition. There are at least twenty six species of *Streptomyces* and *Micromonospora* capable of producing various forms of actinomycins[19] although in less quantity as compared from the current study. However, few strains of *Streptomyces* are reported to produce relatively large quantities of actinomycins such as *Streptomyces parvulus* DAUFPE 3124 (133 mg/ l actinomycin D under optimized medium) [20], *S. parvulus* ATCC 12434 (152 mg/ l of actinomycin D under non optimized conditions) [21], *S. sindenensis* (289 mg/ l actinomycin D) [22], *Streptomyces griseoruber* NBRC 12873 (210 mg/ l actinomycin D under non optimized fermentation conditions)[19], *S. parvulus* GQ451836 (180 mg/ l actinomycin D under non optimized condition) [23].

Table 1: Antimicrobial activity of ethyl acetate extracted compound of *Streptomyces parvulus* Av-R5 against human pathogenic organisms

S. No	Test organism	Zone of inhibition (mm)
MTCC human pathogen		
1	*Bacillus cereus* MTCC-430	19.87 ± 0.40
2	*Bacillus subtilis* MTCC-441	15.25 ± 0.36
3	*Staphylococcus aureus* MTCC-96	15.12 ± 0.29
4	*Staphylococcus epidermidis* MTCC-435	15.25 ± 0.36
5	*Escherichia coli* MTCC-1687	12.75 ± 0.25
6	*Klebsiellapneumoniae* MTCC-3384	12.87 ± 0.35
7	*Proteus vulgaris* MTCC-744	14.12 ± 0.40
8	*Pseudomonas aeruginosa* MTCC-741	15.50 ± 0.33
9	*Candida albicans* MTCC-183	15.87 ± 0.29
10	*Aspergillusniger* MTCC-872	0.00 ± 0.00
Clinical human pathogen		
1	*Bacillus cereus* JNMC-1	18.12 ± 0.35
2	*Bacillus subtilis* JNMC-2	14.12 ± 0.29
3	*Staphylococcus aureus* JNMC-3	12.00 ± 0.27
4	*Staphylococcus epidermidis* JNMC-4	9.87 ± 0.29
5	*Escherichia coli* JNMC-5	0.00 ± 0.00
6	*Klebsiellapneumoniae* JNMC-6	12.00 ± 0.27
7	*Proteus vulgaris* JNMC-7	9.00 ± 0.27

The crude antimicrobial compound was tested for the components present in it by thin layer chromatography. Single separated band was observed with the Rf value 0.64. The ethyl acetate extracted antimicrobial compound from *Streptomyces parvulus* was further analyzed for its purity by semi preparative reverse phase HPLC. Actinomycin D, actinomycin $X_{0\beta}$ were detected in crude extracts and these two major components were eluted by HPLC at 10.956 and 6.817 min respectively. The antimicrobial compound from *Streptomyces* sp. Av-R5 had characteristic odor and dark reddish orange crystalline nature. The compound was soluble in methanol, chloroform, n-butanol and ethyl acetate, less soluble in n-hexane and insoluble in water. Av-R5 showed absorbance at 433.5, 263.2 and 201.3 nm. Spectroscopic analysis of the antimicrobial compound is shown in. The IR spectrum (KBr) of the antimicrobial compound showed the presence of –OH (2926 cm^{-1}), -OH bending (1376 cm^{-1}), NH_2 stretching (3419 cm^{-1}), C-H stretch (2855 cm^{-1}), C=O stretch (1714 cm^{-1}), -C=C stretch (1644 cm^{-1}), C-C stretch in ring (1582, 1516, 1491, 1458, 1406 cm^{-1}), C=O (1316, 1300, 1264 cm^{-1}) and C-O stretching (1219, 1194, 1098, 1075, 1040 cm^{-1}) from which the presence of hydroxyls, primary amine, alkanes, carbonyl groups, alkenes, aromatic hydrocarbons, carboxylic acids and esters are inferred. The LC and ESI-MS of the isolated compounds revealed molecular ion peaks at m/z 1255.6 (M + 1) in positive mode at 11.20 RT identical to those of actinomycin D and molecular formula as $C_{62}H_{86}N_{12}O_{16}$ with different functional groups. The purity of particular peak (11.196 RT) was 50.128 %. The molecular weight 1272.6 m/z also observed in negative polarity at 6.817 RT was similar to that of actinomycin $X_{0\beta}$(Figure 5).Proton NMR in $CDCl_3$ using a JEOL-400 MHz shows aromatic protons corresponding to 6 to 8.5 ppm, -CH_3 groups present at δ 0.8 to 2.5 ppm and other protons like –OH, -NH_2 present at respective chemical shift range. ^{13}C NMR spectrum of the antimicrobial compound exhibits carbonyl resonances between 170 and 178 ppm, which indicates the carbon signals of various amino acids. The carbon signals between 115-140 ppm correspond to C=C of alkenes and the resonances of various fatty acid chains are found mainly between 10 and 40 ppm. Some of the unsaturated carbon atoms showing resonances at 122.099 ppm can be attributed to olefinic fatty acid residues. The solvent $CDCL_3$ signal was found nearer to 80 ppm. A search with these spectroscopic data indicated the compound to be actinomycin D(Figure 6).Two bioactive compounds actinomycin D and actinomycin $X_{0\beta}$ were identified by spectroscopic analysis. Structure of the antimicrobial compound was confirmed by comparison of the FTIR, ESIMS and NMR (^{1}H and ^{13}C) with the literature [18, 19]. Reports for the production of Actinomycin D by different endophytic *Streptomyces* sp. are also available such as actinomycin D from *Streptomyces* sp. Tc022 an endophyte of *Alpinia galanga*[7]; actinomycin D from *S. parvulus* KJ200636 an endophyte of *Codonopsis lanceolata*[14]. Among the related strains, *S. parvulus* are known for the production of actinomycin D. It was also reported that those polypeptide antibiotic, actinomycin D from *S. parvulus* also exhibit antimicrobial activity against Gram-positive and Gram-negative bacteria [18, 23].

The MIC of the ethyl acetate extracted antimicrobial compound of *S. parvulus* Av-R5 against Gram-positive and Gram-negative bacteria revealed the 0.25 mg/ ml against *Bacillus subtilis* MTCC 441; 0.50 mg /ml against *Bacillus cereus* MTCC 430, *Staphylococcus aureus* MTCC 96, *Pseudomonas aeruginosa* MTCC 741 and clinical human pathogens *Bacillus subtilis* JNMC 2, *Staphylococcus epidermidis* JNMC 4; 1 mg/ ml against *Staphylococcus epidermidis* MTCC 435, *Escherichia coli* MTCC 1687, *Klebsiella pneumonia* MTCC 3384, *Proteus vulgaris* MTCC 744 and clinical human pathogens *Bacillus cereus* JNMC 1, *Staphylococcus aureus* JNMC 3, *Klebsiella pneumonia* JNMC 6, *Proteus vulgaris* JNMC 7, *Candida albicans* MTCC 183.

It is concluded from the present study that *S. parvulus* Av-R5 associated with *Aloe vera* have antimicrobial potential and produced substantial amount of actinomycin D and actinomycin $X_{0\beta}$ which exhibited broad spectrum of antimicrobial activity against Gram-negative bacteria besides Gram-positive bacteria and also showed activity against *Candida albicans* MTCC-183. Thus, endophytic actinomycetes could be a potential source of not only antimicrobial compounds but also for other metabolic substances of therapeutic significance.

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