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Research Article

ANTIMICROBIAL ACTIVITY AND HPLC ANALYSIS OF TROPANE ALKALOIDS IN *STREPTOMYCES SPP*. ISOLATED FROM *DATURA STRAMONIUM* L.

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ABSTRACT

Aim of the present study is to isolate and identify promising antimicrobial metabolite producing *Streptomyces* strain from *Datura stramonium*. Physiological, biochemical and 16S rRNA studies strongly suggested that this isolate belonged to *Streptomyces* spp and ability to produce enzymes such as amylase, lipase and catalase. Maximum biological activity was obtained on Modified Nutrient Glucose Agar (MNGA) medium. Preliminary screening revealed that the isolate was found to be active against bacteria and fungi. Isolate showed activity against bacteria such as *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis,* and fungi such as *Aspergillus niger, Trycophyton rubrum* and *Aspergillus flavus*. The antibacterial substances were extracted using methanol from MNGA medium in which isolate had grown for five days at 28°C. The antimicrobial activity was assessed using broth microdilution technique. The lowest Minimum Inhibitory Concentration of methanol fractions of S*treptomyces spp.* Loyola UGC against *B. subtilis*, *S. aureus* was 250 mg/ml and against *Aspergillus flavus* was 61.5 mg/ml. The growth parameters such as carbon source, Nitrogen source, Inoculation period, pH and temperature were optimized. Nutritional and cultural conditions for the production of antimicrobial metabolite by this organism under shake-flask conditions have been studied. HPLC analysis of methanol extract of actinomycets showed the presence of Hyoscyamine and scopolamine.

Keywords: Endophyte, *Streptomyces*, Antimicrobial activity, Medium optimization, HPLC, Tropane alkaloids, *Datura stramonium*.

INTRODUCTION

The term 'Endophyte' was introduced by De Bary (1866) and it was assigned to all microorganisms that are inside the living tissues of the host plant asymptomatically (Glienke-Blanco *et al*. 2002). Actinomycets are noteworthy as antibiotic producers, making three quarters of all known products; the *Streptomyces* are especially prolific (Nolan and Cross, 1998). *Streptomyces* species are widely recognized as industrially important microorganisms because of their ability to produce many kinds of novel secondary metabolites including antibiotics (Bibb, 2005). The discovery of new molecules from actinomycets has marked an epoch in antibiotic research and subsequent developments in antibiotic chemotherapy (Ameriga *et al*. 2000). Searching for original sources of micro-organisms is also advisable since the environment can affect microbial metabolism and therefore, antibiotics producing micro-organisms have been isolated from the most diverse habitats such as endophytes of terrestrial plants (Strobel *et al*. 2004) and from sea organisms (Gandhimathi *et al*.2008) among other sources. The problems of drug resistance, patient's sensitivity and inability to control certain infectious diseases have given an impetus for continuous search of new antibiotics all over the world. To combat the multidrug resistant organisms, introduction of new antimicrobial compounds or antibiotics from new source is essential. *Datura stramonium* L. is a wild-growing herb known as Jimson weed belongs to the family Solanaceae. The plant distributed throughout most parts of temperate regions of the world (Berkov *et al*.2006). Whole plant is used as anti-inflammatory, central nervous system stimulant (Spring, 1989), dental and skin infections, toothache and alopecia (De Foe and Senatore, 1993). The entire plant has anti-cholinergic compounds, but the seeds contain the highest concentration (Chang *et al*. 1999). Attempt is made to isolate and identification of the endophytic actinomycets from *Datura stramonium* L. to evaluate their antimicrobial activity. The present study evaluates the isolation, identification; cultural characteristics and antimicrobial activity of *Streptomyces spp*. isolate Loyola UGC recovered from *Datura stramonium*, Tamil Nadu, India.

MATERIALS AND METHODS

Plant materials

Datura stramonium L. was collected from the Irula Tribal Women's Welfare Society (ITWWS), Chengalpattu, Kanchipuram district, Tamil Nadu, South India. The species was identified and authenticated by Dr. D. Narashiman**,** Department of Botany, Madras Christian College, Chennai, and South India.

Isolation of actinomycets

Roots and transition zones of *Datura stramonium* L. were surface sterilized by the methodology of Johannes *et al*.(2006) with some modifications. Samples were thoroughly washed with running tap water and all the visibly damaged material was excluded. Plant parts were rinsed in 0.1% Tween 20 for 30 sec, followed by bevastin for 2 to 3 min to inhibit the fungal growth, sequentially immersed in 0.1% sodium hypochlorite for 30 sec and in 75% ethanol for 3 to 5 min. After each treatment, samples were rinsed three times in sterile distilled water. Finally surface sterilized samples were aseptically dissected to expose cortex region and placed onto actinomycets isolation medium, incubated for 12 to 15 days at 28°C in dark. The isolation medium was supplemented with nalidixic acid and actidion both to a final concentration of 50µg/ml to inhibit the growth of non actinomycets microorganisms.

Physiological and biochemical characteristics

Media used were those recommended by Shirling and Gottlieb (1966) in the International Streptomyces Project (ISP) and by Waksman (1961). Mycelium was observed after incubation at 28°C for two weeks. Colors were determined according to Prauser (1964). Carbohydrate utilization was determined by growth on carbon utilization medium (ISP 9) (Shirling and Gottlieb (1966) supplemented with 1% carbon sources at 28°C. Temperature range for growth was determined on inorganic salts starch agar medium (ISP 4) using a temperature gradient incubator.

Screening of antimicrobial activity

Streptomyces spp. isolate loyola UGC was inoculated on modified nutrient glucose agar (MNGA) plates by single streak in the center. The plates were incubated at 28°C for four days. The test pathogenic bacteria (*Bacillus subtilis* MTCC 441, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* MTCC3615, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* MTCC 1688, *Klebsiela pneumonia* ATCC 15380 and *Xanthomonas spp.*) and pathogenic filamentous fungi (*Aspergillus niger* MTCC 1344, *Aspergillus flavus*, *Botyritis cinerea*, *Curvularia luenata* 46/01, *Trycophyton rubrum 57/01and*

Trycophyton mentagrophytes 66/01) were cross streaked and incubated at 28°C for 96 h. The microbial inhibitions were observed by determining the inhibition zone from the antagonist. Antimicrobial activity of the train was determined by standard cup plate method using Gram (+) and (-) bacteria and fungi Assay plates were prepared by inoculating 20 ml of Mueller Hinton agar medium with test organism. Agar-cups (6 mm diameter) were filled with 100 µl of mycelia-free culture filtrate in triplicate and the plates were incubated at 37°C for 24 h. Zone of inhibition was measured by the diameter (mm).

Media optimization for compound production

Modified Nutrient Glucose Broth (MNGB) was used as the base to determine the optimal nutritional and cultural conditions for the growth and antimicrobial compound production. Different carbon and nitrogen sources were provided to assess the growth effects and antimicrobial metabolite production. MNGB medium was supplemented with different carbon and nitrogen sources to study their effect on growth and antibiotic production. The medium (100 ml in 500 ml Erlenmeyer flask) was inoculated with 5 ml of homogenous spore suspension (0.6 O.D.), and incubated at 28°C on a rotary shaker (180 rpm) for five days. The effect of cultural conditions like different incubation temperatures (15, 20, 25, 30, 35 and 50°C), initial pH (5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9) and incubation period (0, 1, 2, 3, 4 and 8 days) on growth and antibiotic production was studied.

Extraction of antimicrobial metabolites

Streptomyces spp. isolate Loyola UGC culture broth was collected and centrifuged at 8000 rpm for 10 min. Antimicrobial compound containing supernatant was extracted using equal volume of different solvents such as hexane, ethyl acetate and Methanol. Solvents were concentrated by vacuum evaporator at 40°C.

***In vitro* antimicrobial test**

Crude extract (10 mg) was dissolved in 1 ml of dimethyl sulfoxide (DMSO): water (1:9) and used for antimicrobial study according to standard broth microdilution method (NCCLS) and the MIC was calculated. Mueller Hinton broth (Himedia, Mumbai) was prepared and sterilized by autoclaving at 121°C, 15 lbs for 15 minutes. The required concentration of the extract (1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ ml, 0.0625 mg/ml, and 0.03125 mg/ml) was added to the 96 well micro titer plate containing 0.1 ml broth. The 10 µl of log phase culture was introduced into the respective wells and the final inoculum size was 1×10^5cfu/ml. The plates were incubated at 37°C for 18 h. Positive control and solvent control (DMSO) was also included. 5 ml of the test broth was introduced on plain nutrient agar plates to observe the viability of the organism. MIC was determined as the complete growth inhibition at the lowest concentration of the extract.

High performance liquid chromatography (HPLC) analysis of extract

HPLC analysis of methanolic extract of endophytic actinomycets (MeEA) was eluting from a RP- C18 (4.6 x 150 mm, 5µm) reverse phase column with ultraviolet (UV) detector. 20µl of the sample was injected each time detected at 270 nm. The mobile phase was methanol/acetonitrile/water (25:35:40, by v/v/v) at 1.0 ml min^{-1}. The sample and mobile phase were filtered through 0.2µm PVDF filter before entering the column.

Amplification of 16s r RNA and sequencing

Genomic DNA Loyola UGC was extracted by Enticknap *et al*. (2006). The 16 S ribosomal RNA gene was amplified by using the PCR method with Taq DNA polymerase and primers 27f (5`AGT TTG ATC CTG GCT CAG 3`) and 1492 (5`ACG GCT ACC TTG TTA CGA CTT 3`). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94°C for four minutes followed by 30 cycles at 94°C for one minute, primer annealing at 52°C for one minute and primer extension at 72°C for one minute. At the end of the cycling, the reaction mixture was held at 72°C for 10 min and then cooled to 4°C. The PCR product obtained was sequenced by an automated sequencer (Genetic Analyzer 3130, Applied Biosystem, and USA). The same primers as above were used for this purpose. The sequence was compared for similarity with the reference species of *Streptomyces* contained in genomic database, using the NCBI BLAST available at http://www.ncbinlm-nih.gov/.

RESULTS AND DISCUSSION

Streptomyces spp. isolate Loyola UGC, was isolated from the transition zone of *Datura stramonium* belongs to the family Solanaceae exhibited antimicrobial activity against some Gram positive bacteria and fungi. Morphological identification of the isolate Loyola UGC was Gram-positive, substrate and circular in shape. A yellow pigment diffused in to the surrounding medium (Table1). Culture characteristics of Loyola UGC were derived on the basis of observation made after seven days of incubation on different media. These characteristic morphological properties strongly suggested that Loyola UGC belonged to the genus *Streptomyces spp*. The isolate Loyola UGC was non motile and aerobic, also showed exponential growth on medium amended with sodium chloride up to 2.5%; poor growth was absorbed at below 5% of NaCl. The preliminary screening (cross-streak method) revealed that the *Streptomyces spp*. isolate Loyola UGC was a good antibacterial and antifungal compound producer (Fig. 1).

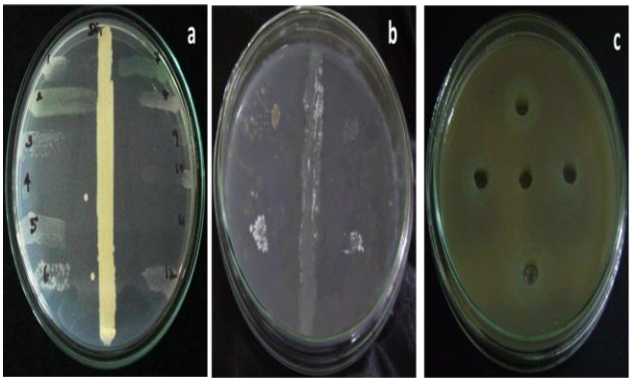

Fig 1: a & b shows prelimiminary screening of antibacterial and antifugal activity, c) Different media extract inhibited the growth of Bacillus subtilits.

Optimization of antimicrobial compound production was carried out in batch culture. The strain was able to grow in all the tested carbon sources. However maximum growth and pigment production were observed in glucose followed by maltose; and nitrogen source were observed in Soytone (Fig 2).

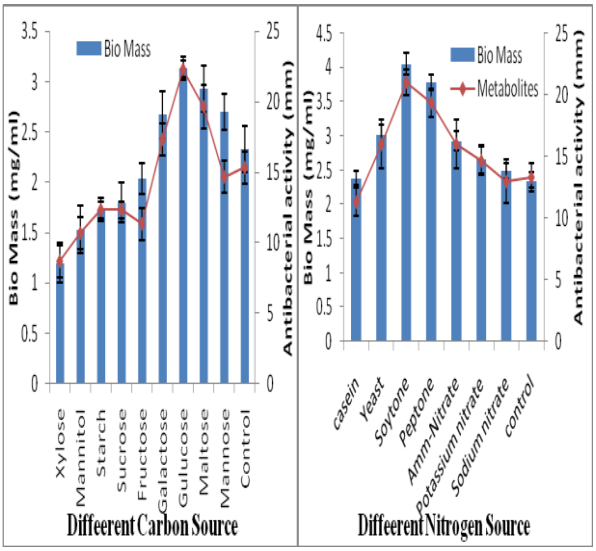

Fig. 2: Effect of different carbon and nitrogen sources for antimicrobial metabolite production.

The increased glucose level in the broth leads to the higher production of antimicrobial compound. The optimum temperature

found to be effective for growth and pigment production. Maximum antimicrobial activity was obtained at pH 7.5 and an incubation period of 5 days (Fig .3).

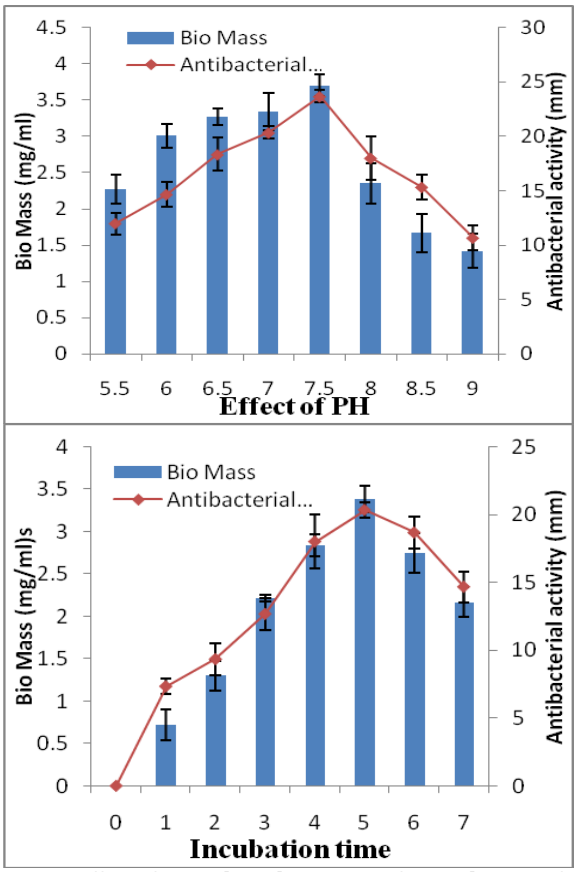

Fig. 3: Effect of pH and incubation time for production of antimicrobial compound.

The other physical parameters such as NaCl (2.5%), temperature (28°C), were optimized for the production (Table 1).

Table1 : Physiological and Biochemical characteristics of *Streptomyces spp*. isolate Loyola UGC.

Culture Character	Growth Response
Growth under anaerobic condition	-
Gram staining	+
Growth and Shape	Substrate, Circular
Motility	Non Motile
Diffusible pigments	+
Range of temperature for growth	25-30°C
Optimum temperature for growth	28°C
Range of pH for growth	6-8
NaCl tolerance	2.5%
Catalase production	+
Protease	-
Amylase	+
Lipase	+

Preliminary screening revealed that MNGA medium was very good base for the production of antibacterial compounds. The diameter of inhibition zones produced by the extracellular products of Loyola UGC was 12 mm for *B. subtilis,* 20 mm for *E. faecalis,* 19 mm *for S. aureus,* and 12 mm for *E. coli* (Table .2). Methanol extract of endophytic actinomycets showed activity against gram positive bacteria and antifungal activity (Table.3, 4). The MIC of 250 µg /ml was determined for Gram positive bacteria; *B. subtilis, E. faecalis* and *S. aureus,* whereas MIC of 500 µg /ml was observed for *E. coli, and Y. enterocolitica* Most of the gram negative bacteria showed MIC as 1000 µg /ml. The Streptomycin antibiotic used positive control (Table.3). The MIC of antifungal activity 125 µg /ml was showed against *A.niger* and *T. rubrum* (Table4). The HPLC analysis of MeEA showed the presence of hyoscyamine at 10.59 and scopolamine at 8.04 compared with authentic standard (sigma) (Fig. 4).

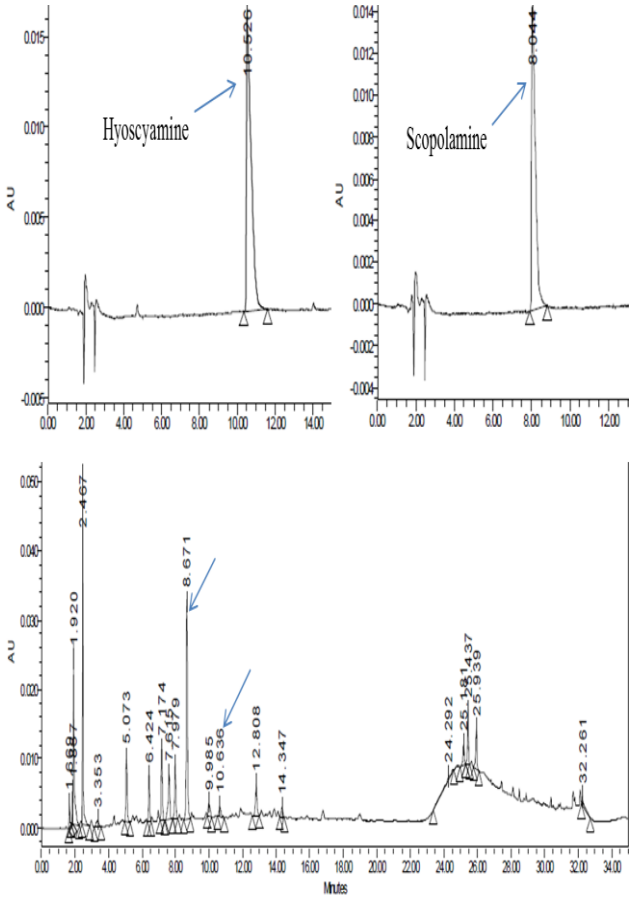

Fig4: HPLC-analysis of tropane alkaloids. a & b- Standard Hyoscyamine and Scopolamine; c- Methanol extract of *Streptomyces spp*. isolate Loyola UGC shows presence of tropane alkaloids.

The sequencing results revealed that isolate was *Streptomyces spp*. Antibiotic producing endophytic actinomycets Loyola UGC has been isolated, from *Datura stramonium* and characterized by the *Streptomyces spp*. By using a wide range of isolation media, culture characteristics combining physiological and biochemical characteristics, we identified the isolate which was found to produce pigment. Actinomycets are useful biological tools producing antimicrobials against bacteria and fungi (Okami and Hotta., 1988). Two novel antimycin antibiotics urauchimycines A and B were isolated from a fermentation of *Streptomyces spp*. Ni-80 (Imamura *et al*. 1993). The *Streptomyces spp* Loyola UGC culture showed good antimicrobial activity in solid as well as in culture broth unlike fumaradimycine, which is inactivated in fermentation broth (Maruyama *et al*. 1975). The isolate Loyola UGC was growing well in MNGA medium amended with NaCl. It can be placed in intermediate tolerance group (Tresner *et al*. 1968). The normal temperature for growth of *Streptomyces* is 28 °C. Our isolate Loyola UGC was also growing well at 28 °C. The isolated spp. exhibited antibacterial activity against Gram positive bacteria similar to another report where aerobic, filamentous bacterium TN97 isolated from soil with antimicrobial activity against Gram positive bacteria (Ben Ameur Mehdi *et al*. 2006). Our results indicated that the antimicrobial compounds were extracellular. Most of the secondary metabolites and antibiotics of microbes are extracellular in nature similarly extra cellular products of actinomycets showed potent antimicrobial activities (Hacene *et al*. 2000). The results indicated the dependence of the production of antimicrobial compound(s) on medium constituents. Similar findings have been reported by Holmalahti *et al*. (1998). It has been reported that the environmental factors like temperature, pH and incubation period have profound influence on

antibiotic production (Yoshida *et al.* 1962). Our results indicate that the synthesis of antimicrobial metabolites depends on the medium constituents. In fact, it has been shown that the nature of carbon and nitrogen sources strongly affects antibiotic production in different organisms and the antibiotic production was increased by glucose rich medium (Holmalahti *et al*. 1998). The HPLC analysis of the methanol extract showed the presence of tropane alkaloids; hyoscyamine and scopolamine. The growth of many fungi, yeasts, bacteria, and viruses were inhibited by the phytochemical compounds found in *Datura* (Youdim *et al*. 1999). Classes of alkaloids are among the major powerful poisons known (Fluck 1973). Apart from being poisonous, some alkaloids have also been proved to be useful in correcting renal disorders (Konkwara 1976); it therefore, means that the alkaloids of *D. stramonium* may be a poison that can be tried on lower or higher organisms. The secondary metabolites identified in the extract used in this study could be responsible for antimicrobial activity exhibited by these *Streptomyces spp.*

Table2: Shows medium optimization of *Streptomyces spp*. isolate Loyola UGC.

Name of the medium	Zone of Inhibition (mm)				
	B.subtilis	***E. faecalis***	***S. aureus***	***E. coli***	***E. aerogenes***
Modified Nutrient Glucose Broth	12	20	19	12	8
Actinomycets isolation broth	10	14	10	-	10
Benet Medium	-	-	-	-	-
Antibiotic production medium	*10*	8	12	8	10
Starch casein broth	*8*	10	13	-	-

Table3: Antibacterial activities of *Streptomyces spp*. isolate Loyola UGC

Test Organisms	Streptomycin (µg/ml)	Minimum Inhibitory Concentration (MIC) (µg/ml)		
		Hexane	Ethyl acetate	Methanol
Bacillus subtilis MTCC 441	6.25	-	-	250
Staphylococcus aureus ATCC25923	6.25	-	-	250
Klebsiella pneumonia ATCC 15380	2.5	-	>1000	1000
Enterococcus faecalis ATCC 29212	<50	-	-	250
Yersinia enterocolitica MTCC 840	12.5	-	>1000	1000
Xanthomonas pvoryzae MTCC 2760	25	-	-	1000
Vibrio parahaemolyticus MTCC 451	25	-	-	1000
Enterobacter aerogenes MTCC 111	25	-	>1000	1000
Escherichia coli ATCC 25922	2.5	-	-	500
Proteus vulgaris MTCC 1771	6.25	-	-	1000

Table4: Antifungal activity of *Streptomyces spp*. isolates Loyola UGC.

Test Organisms	Fluconazole (µg/ml)	Minimum inhibitory Concentrations (MIC) (µg/ml)		
		Hexane	Ethyl acetate	Methanol
Aspergillus niger MTCC 1344	100	-	-	125
Aspergillus flavus	50	-	500	61.5
Botyritis cinerea	100	-	-	250
Curvularia luenata 46/01	<12.5	-	-	500
Trycophyton rubrum 57/01	25	-	1000	125
Trycophyton mentagrophytes 66/01	25	-	500	250

CONCLUSION

From the present study, it is clear that a novel isolate of *Streptomyces* spp. Loyola UGC which produced methanol soluble extracellular product effective against pathogenic test bacteria and fungi. In view of the decline in the discovery of new lead compounds in recent years, further investigations on isolate Loyola UGC would lead to some useful anti biological products.

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