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Isolation, Characterization and Antibacterial activity of *Streptomyces torulosus* SSB

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Streptomyces **are soil dwelling, Gram positive, filamentous bacteria belonging to the phylum Actinobacteria. These are high G+C organisms noted for their characteristic 'earthy' odour which results from production of a volatile metabolite, geosmin. Streptomyces are known for their ability to produce antibiotics. In fact, two thirds of the known antibiotics are produced by actinomycetes and 80% of them are made by members of** ***Streptomyces*** **genus.**

An actinomycete strain, designated SSB, was isolated from the local soil sample. Initial studies indicated that it was a Streptomycete. It was sent to IMTECH, Chandigarh for further identification. On the basis of morphological, phenotypic and biochemical characteristics, it was identified as ***Streptomyces torulosus, SSB*****. The strain SSB was found to produce a reddish pigment on both solid as well as liquid media. The pigment production was best observed in Potato dextrose broth (Hi-media) and hence used for further studies on pigment production.** ***Streptomyces torulosus*** **is not reported earlier to produce such a pigment. The pigment produced by the strain SSB was extracted in ethyl acetate and purified by standard chromatographic techniques. The absorption spectra of the partially purified pigment were determined. It was found to be similar to Actinomycin-D. The partially purified pigment was found to have antibacterial activity against gram positive organisms.**

Key words: *Streptomyces torulosus*, Actinomycin-D, pigment.

The *Streptomycetes* are members of the bacterial order *Actinomycetales*, bacteria that resemble fungi in their branching filamentous structure. However, they are true bacteria - prokaryotic cells - unlike eukaryotic fungal cells. As they grow, they form branching filaments of cells, which become a network of strands called a mycelium, similar in appearance to the mycelium of some fungi[1].

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Actinomycetes are also unique in the way they form spores and in the production of numerous antibiotics. By far the most successful genus in this group is *Streptomyces* with over 500 species[2]. These are non-motile, filamentous, Gram-positive bacteria. *Streptomyces* species are found worldwide in soil and are important in soil ecology[3]. Much of the characteristic earthy smell of soils arises from chemicals called geosmens given off by *Streptomyces* species. *Streptomycetes* are metabolically diverse and can utilize almost anything, including sugars, alcohols, amino acids, organic acids, and aromatic compounds. This is achieved by producing extracellular hydrolytic enzymes. *Streptomycetes* are also of medical and industrial importance because they synthesize antibiotics[4]. Over 50 different antibiotics have

been isolated from *Streptomyces* species, including streptomycin, neomycin, chloramphenicol and tetracyclines. Bacteria in general and *Streptomyces* in particular, produce pigments for a variety of purposes. Some pigments are photosynthetic, others play a role in protection against UV radiations and yet others are antibacterial in nature. *S.coeilicolor* produces an indicator pigment actinorhodin[5]; *S.venezuelae* produces a sporulation pigment[6] and so on.

One such pigmented antibiotic is actinomycin. Actinomycins are chromopeptide lactone antibiotics. Numerous actinomycins have been reported to be produced by various strains of *Streptomyces*[7]. All known natural actinomycins contain the same phenoxazone chromophore and differ only in the amino acid content of the peptide side chains[8].Among the actinomycins, Actinomycin- D is the most extensively studied and widely used actinomycin in the treatment of malignant tumors[9]. The bioactivity of actinomycin-D is mainly due to its ability to intercalate into DNA duplex[10].

In the present paper, we report the isolation and taxonomy of the producer organism, its antibacterial pigment production, extraction and characterization.

MATERIAL AND METHODS

Producer organism isolation and identification

Under our screening program for antibiotic producers, an organism was isolated from the soil sample from Nagpur, Maharashtra, India by using actinomycetes isolation agar. Preliminary identification on the basis of microscopic appearance, colony characteristics, pigmentation, and liquid cultures pointed it to be a Streptomycete[11]. It was sent to MTCC, IMTECH, Chandigarh for further identification. The cultures were maintained on Potato dextrose agar (Hi-media) containing potato infusions 200g, dextrose 20g, agar 15g and distilled water 1 liter. Unless otherwise mentioned, all the chemicals and solvents were purchased from Merck and media from Hi-media laboratories, Mumbai.

On the basis of morphological, biochemical and physiological characteristics, the isolate was identified at MTCC, Chandigarh.

Characterization of isolate

To establish the identity of the isolate, it was grown on the yeast extract malt extract (ISP2) media and characteristics were assessed in accordance with the method described by Shirling and Gottlieb. Cultural characteristics were also observed on oatmeal agar (ISP3), inorganic salt starch agar (ISP4), glycerol aspargine agar (ISP5), peptone-yeast extract agar (ISP6) and tyrosine agar (ISP7). For physiological and biochemical characterization, the Gram staining properties, production of pigments, effect of different temperatures on growth, utilization of 13 different sugars (arabinose, dextrose, galactose, fructose, inositol, raffinose, rhamnose, sorbitol, salicin, sucrose, xylose, mannitol and lactose) and biochemical tests like urea, starch, casein and gelatin hydrolysis, nitrate reduction, H_2S production, oxidase production, catalase production and citrate utilization were studied following standard methods[12,13].

Growth & antibacterial pigment production

Pigment production was studied in Potato dextrose broth (PDB). The pH of the medium was adjusted to 6.8 before sterilization. Seed culture was prepared in 250ml Erlenmeyer flask containing 50ml PDB by inoculating a loopful from a slant culture and incubating at 28ºC on a shaker incubator at 150 rpm for 48 hours. Pigment production was observed in the same medium by inoculating 1 liter flask (200ml medium) with 2.5% (v/v) of seed culture and growing under same conditions for 8 days.

Pigment extraction & purification

The cells were separated from the fermented broth by centrifugation. The pigmented broth was used for successive extraction of the pigment. The solvents in the increasing order of polarity were used for successive extraction. All the successive extracts thus obtained were concentrated to 3ml and further used for spectrophotometric studies.

The UV-visible absorption spectra (190nm-1100nm) of the successive extracts were determined by using a double beam bio-spectrophotometer BL-198, Elico. The spectra were compared with the spectrum of standard actinomycin D in similar solvent conditions.

The broth was also extracted by the method detailed by Praveen *et.al.,* for standard

actinomycin-D isolation. The fermented broth was centrifuged and the supernatant separated. The pH of the supernatant was adjusted to 6.8. It was then extracted thrice with equal volume of ethyl acetate, filtered and concentrated under vacuum in a Vacuum Concentrator plus (Eppendorf AG, Germany). Crude antibiotic pigment was kept in air tight ampoules at -4ºC.

The crude antibiotic pigment was resuspended in a small volume of ethyl acetate and subjected to column chromatography in a column packed with silica gel (mesh 60-120) in ethyl acetate. The elution was carried out using ethyl acetate. Two major fractions, yellow and red in color were obtained, collected separately and stored. The first yellow fraction was designated SSB-A. All the further studies were carried out on SSB-A.

Characterization of antibacterial pigment TLC

The fraction SSB-A was subjected to Thin layer chromatography using TLC plates (Merck TLC Silica gel 60 F_{254}) with $CHCl_3$: MeOH(9:1)[14] as the mobile phase and compared to that of standard actinomycin-D.

Determination of hydrolysis products

The fraction SSB-A was subjected to acid hydrolysis in screw capped tubes containing 6N HCl for 3 hours at 100ºC. The hydrolysis products were subjected to paper chromatography with butanol : acetic acid : water (4:1:2) as the solvent system. Standard sarcosine and threonine were also loaded onto the chromatogram. Detection was effected by spraying with 0.2% ninhydrin in acetone[15]. The location of the spots was determined relative to the solvent front.

Antibacterial studies and Minimum inhibitory concentration

The antibacterial activity of the purified fraction SSB-A was assessed by agar-well diffusion assay. 0.1ml of broth cultures of test organisms was seeded by pour plate method in Mueller-Hinton agar plates. 2mm wells were dug and 10µl of fraction SSB-A was added in the wells. The diameter of the inhibition zones was determined after 24 hours of incubation at 37ºC. The test organisms used were *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 740), *Pseudomonas aeruginosa* (MTCC 647) and *Escherichia coli* (MTCC 1652).

Minimum inhibitory concentration of the antibacterial pigment was tested against *Bacillus subtilis* and *Staphylococcus aureus* by agar plate dilution method. The concentrations used were 0.05µg/ml to 25µg/ml.

RESULTS AND DISCUSSION

While screening for bioactive natural products, several microorganisms were isolated and their bioactivities were studied. One culture showing potential bioactivity was designated SSB and studied further.

Identification of the isolate

Preliminary studies showed that the organism was a *Streptomycete*. *Streptomyces* belong to the family *Streptomycetaceae* of the order *Actinomycetales*. On the basis of morphological, physiological and biochemical characteristics, the isolate was identified by IMTECH, Chandigarh as *Streptomyces torulosus*.

Table 1. Physiological characteristics of *Streptomyces torulosus* SSB

Characteristics	*S.torulosus SSB*
Temperature range of growth	25-42ºC
Urea hydrolysis	-
Starch hydrolysis	+
Caesin hydrolysis	-
Gelatin hydrolysis	+
Nitrate reduction	-
H_2S production	-
Citrate utilization	+
Catalase	+
Oxidase	-
Arabinose	+
Dextrose	+
Galactose	+
Fructose	+
Inositol	+
Raffinose	+
Rhamnose	+
Sorbitol	-
Salicin	-
Sucrose	+
Xylose	-
Mannitol	+
Lactose	-

+: Positive; -: negative

Characterization of the isolate

Cultural characteristics

Streptomyces torulosus SSB showed good growth on potato dextrose agar, ISP2, ISP3, ISP5 and starch yeast extract agar. It showed moderate growth on Sabourads agar, ISP3, ISP6, ISP7 caesin agar, starch NH_4SO_4 agar and starch glucose agar and showed less growth on nutrient agar, Krainskys agar, starch peptone agar and glucose peptone agar. The culture showed the presence of opaque, convex mycelial growth with irregular margins. The color of the aerial mycelium was grey and the substrate mycelium was pink, orange or violet. (Table 1)

Biochemical and physiological characteristics

Streptomyces torulosus SSB was found to hydrolyze starch and gelatin but not urea and casein and showed a negative result for nitrate reduction. The strain was catalase positive but oxidase negative and could utilize citrate as the sole carbon source. (Table 2) The strain showed growth at pH 5 to 9 but the growth was found to be best at pH6.8. The test strain was able to grow at a temperature range of 25ºC to 42ºC. The study on sodium chloride tolerance indicated that the strain could grow in a medium containing upto 2% NaCl concentration.

The strain utilized arabinose, dextrose, galactose, fructose, meso-inositol, raffinose, rhamnose, sucrose and mannitol as sole carbon source but failed to utilize sorbitol, salicin, xylose and lactose as sole carbon source.

Spectral studies

Spectra of the successive extracts in

Table 2. Cultural characteristics of *Streptomyces torulosus*

S.No	Media	Growth	Pigmentation	Sporulation
1	Nutrient agar	+	Violet	+
2	Sabourads dextrose agar	++	Orange	++
3	Potato dextrose agar	+++	Orange	+++
4	ISP 2	+++	Orange brown	+++
5	ISP 3	++	Yellowish brown	+
6	ISP 4	+++	Orange	+++
7	ISP 5	+++	Violet	+++
8	ISP 6	++	None	-
9	ISP 7	++	Pink	-
10	Krainskys media	+	Pink	++
11	Caesin agar	++	Orange	++
12	Starch YE agar	+++	Purple	+++
13	Starch NH_4SO_4	++	Pink	+
14	Starch glucose agar	++	Red	+
15	Starch peptone agar	+	No	+
16	Glucose peptone agar	+	Violet	++
17	Maltose peptone agar	++	Violet	++

+++Very good, ++ good, + fair, - no growth

Table 3. *In vitro* antibacterial activity

Test organisms	Zone of inhibition in mm	
	Actinomycin-D (1mg/ml)	SSB-A (1mg/ml)
Staphylococcus aureus	0.9mm	0.4mm
Bacillus subtilis	1.1mm	0.4mm
Pseudomonas aeruginosa	No zone	No zone
Escherichia coli	No zone	No zone

acetone, benzene, chloroform and methanol were found to be similar to actinomycin. The peak of 441nm was found in all the extracts. The spectra of successive extract in chloroform was found to be similar to that of standard Actinomycin D (Fig. 1).

Purification

The ethyl acetate extract of the crude pigment when subjected to column chromatography yielded two major fractions- a yellow fraction and an orange-red fraction. The yellow fraction showed potential bioactivity and was designated SSB-A and selected for further studies. The fraction SSB-A when subjected to TLC using $CHCl_3$: MeOH (9:1) as the mobile phase showed one single spot with R_f of 0.87 corresponding to that of standard actinomycin-D under similar conditions[14].

Determination of hydrolysis products

The paper chromatogram of hydrolysis products of SSB-A showed purple spots on exposure to 0.2% ninhydrin in acetone confirming the presence of amino acids in SSB-A. The R_f of two purple spots was comparable to that of sarcosine (0.26) and threonine (0.24)

Antibacterial studies

SSB-A was found to be active against gram-positive organisms like *S.aureus* and *B.subtilis* at 1mg/ml concentration but not against gram-negative organisms like *E.coli* and *P. aeruginosa.* (Table 3) The minimum inhibitory concentration of SSB-A against *B.subtilis* and *S.aureus* was found to be 15µg/ml.

In our screening program for antibiotic producers, a *Streptomyces* with potential bioactivity was isolated and designated SSB. The isolate was then sent to IMTECH, Chandigarh where it was identified as *Streptomyces torulosus*, SSB. *Streptomyces torulosus* has not been reported to produce any pigment earlier.

The orange-red pigment produced by *S. torulosus* SSB was successively extracted, concentrated and UV-visible spectra was studied. The extracts were also tested for bioactivity against *Bacillus subtilis*. The successive chloroform extract showed maximum activity and its UV-visible spectra was found to be similar to standard actinomycin-D (Fig. 1). Since the bioactive pigment is orange-yellow and produced by a *Streptomycete* and is also intercalating

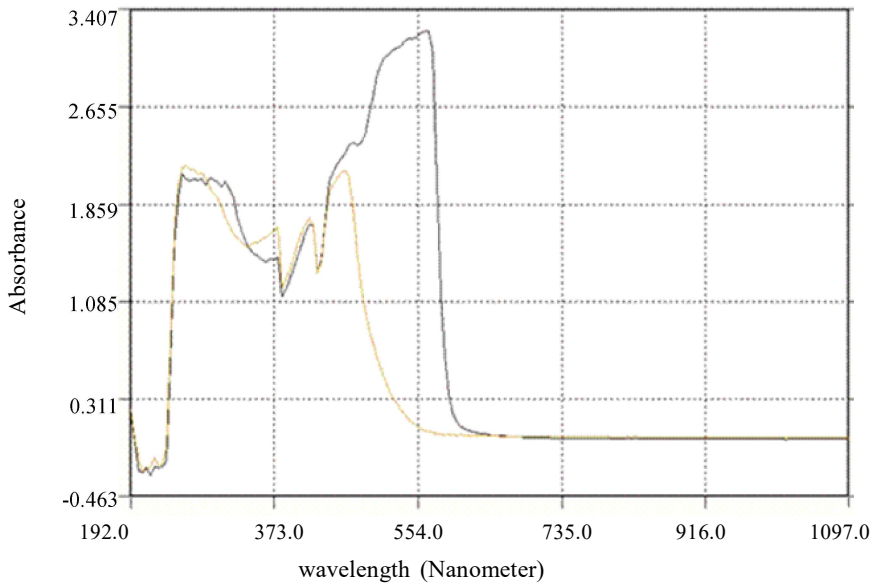

Fig. 1. Comparative spectra of successive chloroform extract and Actinomycin-D in chloroform. The coinciding spectral peaks observed were at 260nm, 378nm, 417nm and 462nm.

Black ——— : Successive chloroform extract; Yellow ——— : Actinomycin-D in chloroform.

(unpublished data), it was presumed that it might be analogous to actinomycin-D.

Hence the standard reported procedure for actinomycin extraction from the fermentation broth was followed[15]. On extracting the fermentation broth in ethyl acetate, the crude antibiotic extract which was obtained was concentrated and used for further purification. Column chromatography yielded two fractions, out of which the yellow fraction was designated SSB-A.

The TLC of the partially purified SSB-A was comparable to that of standard Actinomycin-D with R_f value of 0.87. Paper chromatography of acid hydrolysis products of SSB-A confirmed the presence of the amino acids sarcosine and threonine. Actinomycins are chromopeptide lactones. Hence, the presence of amino acids further indicated its similarity to actinomycin-D.

Antibacterial activity of SSB-A by agar-well diffusion method showed that it is active against gram-positives at a concentration of 1mg/ml but not against gram-negatives. Actinomycin-D is also reported to be more active against gram-positives as compared to that of gram-negatives at the same concentration[16]. Since the antibacterial activity was seen only against *B.subtilis* and *S.aureus*, the minimum inhibitory concentration of SSB-A against them was tested, which was found to be 15µg/ml.

From all these preliminary investigations, we conclude that the antibacterial pigment SSB-A produced by *Streptomyces torulosus* SSB belongs to the family of actinomycins. It has to be further purified and its structure determined to conclusively state that it is actinomycin-D or its derivative.

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