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Fermentation, Isolation and Purification of Antibiotic F₄₀ from *Streptomyces sulfonensis* sp.nov.

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ABSTRACT

A new *Streptomyces* F₄₀ - *Streptomyces sulfonensis* was isolated from soils of Andhra Pradesh in India by selective methods and it exhibited broad-spectrum activity against Gram-positive and Gram-negative bacteria. The antibiotic F₄₀ from culture filtrate was extracted with chloroform, ethyl acetate and n-butanol at p^H 6,7 and 8. All these extracts were concentrated to low volume (5ml) under vacuum at 45^o C on a rota-vapour apparatus and tested for their activity against *B.subtilis*. The mycelia cake portion of the strain *S.sulfonensis* was collected separately and washed 4 times with sterile distilled water and extracted with methanol (10ml) for 1 hour. The antimicrobial activity of the methanolic extract was also tested. Both mycelial and chloroform extract of broth at p^H 8 exhibited good to excellent antimicrobial activity. The broth extract after concentration in vacuo at 45^oC obtained as reddish pink solid (1.390 g). It was active against *B.subtilis* NCIM 2063. The crude antibiotic (1.390g) was subjected to various purification methods and finally obtained as pale yellow solid (15mg)-antibiotic F₄₀ and it was active against *Bacillus subtilis*.

Keywords: *Streptomyces*, *Streptomyces sulfonensis*, Fermentation, Purification, Bioautography.

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INTRODUCTION

Since the isolation of actinomycin in 1940 and streptomycin in 1944 by Waksman, the actinomycetes have received tremendous attention of the scientists. Soils, composts and fodders are common sources of actinomycetes. Waksman [1] recognized a few natural substrates as ideal sources for the isolation of actinomycetes and other streptomycetes.

The isolation of active principles of actinomycetes requires the use of methods which have found general application in the isolation of natural products. Antibiotics of actinomycetes have extremely diverse chemical structures. Most antibiotics are released by actinomycetes into the culture medium; a few of them are found in the mycelium. So, no single procedure is suitable for the isolation of all these compounds. When one is dealing with an unknown substance, it is always advisable to have an idea of the stability of the substance at various temperatures and at various pH values.

The present communication deals with the fermentation, isolation and purification of antibiotic F40 from the culture filtrate of *Streptomyces sulfonensis*, a new streptomycete isolated from soils of Andhra Pradesh.[3]

MATERIALS AND METHODS

Fermentation process studies

The slant culture of *S.sulfonensis* was grown on ATCC 172 medium[4] and it exhibited broad-spectrum activity against Gram-positive and Gram-negative bacteria(Table.1).Seed culture was prepared in a medium composed of soybean meal:10.0g,Corn steep solids:10.0g,Glucose: 5.0 g and Calcium carbonate:5.0g, in one litre distilled water, adjusted to p^H :7.0. by the addition of 0.1 N HCl with digital p^H meter 335 and distributed into 500 ml Erlenmeyer flasks and incubated for 72 hours at 28°C on a rotary shaker at 220 rpm speed and allowed to develop the seed. A 10% level of seed was used to inoculate the production medium of the following composition: Soybean meal:10.0g, Corn steep solids:5.0g,Soluble starch:10.0g, Dextrose:5.0g, Calcium carbonate:7.0g, Distilled water:1.0 litre, p^H : 7.2. The fermentation was run at 28° C for 7 days on a rotary shaker. Samples (15ml) were drawn at regular intervals. The p^H was determined with P^H meter 335& antibiotic activities were evaluated by cup-plate method [5] against *B.subtilis* NCIM 2063 as test organism. The results are presented in Table.2. All the test organisms employed in the present studies were supplied by the National Chemical Laboratory NCIM, Pune.

Extraction, isolation and purification of active compounds

Separation of culture filtrate:

The batch fermentations were conducted by shake-flask method with *S.sulfonensis* in 1000 ml conical flasks. Each flask contained 180ml of the production medium. A total of 10 flasks were run for each batch. All flasks were incubated at 28° C for 6 days on a rotary shaker. The samples were tested for the antibiotic productivity and harvested on 6th day. About 5 batches were run and the fermentation broth of each batch was collected by centrifugation at 4000 rpm for 15 minutes and subjected to extraction and purification methods.

Extraction of antibiotic F₄₀ from the culture filtrate

Selection of solvent

The centrifuged broth sample about 150ml was divided into 3 portions (each 50ml) and each portion was adjusted to p^H 6.0, 7.0 and 8.0. They were extracted thrice (3x5 ml) with selected solvents such as chloroform, ethyl acetate and n- butanol. All these extracts were concentrated to low volume (5ml) under vacuum at 45° C on a rota-vapour apparatus and tested for their activity against *B.subtilis*.

The mycelia cake portion of the strain *S.sulfonensis* was collected separately and washed 4 times with sterile distilled water and extracted with methanol (10ml) for 1 hour.The antimicrobial activity of the

methanolic extract was also tested. The results are presented in Table.3. The antibiotic F_{40} and along with active principles were almost completely extracted with chloroform at P^H 8 where as other solvent extracts gave negligible activities (Table.3).

In subsequent studies chloroform was used for extraction of antibiotic F_{40} . The crude antibiotic F_{40} thus obtained was appeared as reddish pink solid (1.390 g) active against *B.subtilis* and it was subjected to chromatographic analysis and bio-autography.

Isolation & purification of antibiotic F_{40}

Paper chromatography

Paper chromatographic studies of compounds in several systems for the purpose of their classification and identification have proved useful in systematic analysis[6]. Betina[7], Betina and Nemeč[8] determined the ionic character of unknown antibiotics and also the possibility of their isolation and purification from the PH chromatograms. In the present investigation different solvent systems have been used to analyze the crude residue. They have been chosen in order to have information on the polarity and the ionic character of the products.

Development and detection of chromatograms

Strips of the Whatman No.1 paper 6x35 cm were used. The chromatograms were developed using 500 ml glass cylinders and saturated with 25ml of the solvents. Different solvent systems were employed. The crude sample was dissolved in chloroform at a concentration of 10mg/ml and 10 μ l was spotted on the strip and allowed to dry in air. The chromatographic strips were developed by ascending chromatographic technique for 4 to 6 hours. The chromatograms were air dried and the active principles were detected bioautographically employing *B.subtilis* as test organism.

The bioautographic glass plates (32cmx23cm) were sterilized by dry heat. The sterile nutrient agar medium (250 ml) was seeded with 2 ml of 24 hours culture of *B.subtilis*, mixed thoroughly and poured into bioautographic plate and allowed to solidify. The developed paper chromatograms were placed onto the solidified agar surface. The plates were kept at 4⁰ C for 2 hours for diffusion of antibiotic into the medium. The paper strips were removed from the agar surface aseptically and the plates were incubated at 37⁰ C for 24 hours. The antibiotic position was revealed as clear inhibition zone on an opaque background.

Thin layer chromatography (TLC)

The method is applicable to samples of crude antibiotics which are themselves easily prepared and it will not identify an individual antibiotic in a crude mixture, but it will narrow the choice of identities to a small number [9]. Ikekawa, et al [10] used colour –forming reagents to locate macrolide antibiotics isolated on thin layer chromatograms. Betina and Barath[11] and Bickel ,et al[12] subsequently combined thin layer chromatography with bioautography for the detection of antibiotics. Thirty three solvent systems were used to analyze the crude product by bioautographic technique. Finally, 17% methanol in chloroform was found to be most suitable and selected for subsequent studies. The pre-coated TLC sheets supplied by E.Merck, India were used. The developed pre-coated TLC sheet was subjected to bioautographic technique as described above. Well defined zones of inhibition were observed.

Separation of the antimicrobial compound (F_{40})

Separation of the antimicrobial compound (F_{40}) into its individual components was conducted by preparative TLC using 17% methanol in chloroform as a solvent system. The preparative TLC plates (20cmx20cm) were coated uniformly with silica gel, air dried and activated at 100⁰ C for 45 minutes. The chromatographic chambers were saturated with 17% methanol in chloroform. The crude antibiotic F_{40} was applied onto the plates as a thin band. The chromatograms were run for 4 to 6 hours and they were air dried. The visible coloured spots were marked, scraped and collected separately. The other colourless portions were also scraped and collected separately. A total of 9 fractions were obtained. A total of 15 plates were used and the respective similar fractions were pooled.

Each fraction was extracted with chloroform, filtered, concentrated under vacuum at 45⁰ C and their antimicrobial activities were determined by paper disc –plate method [13]. The results are shown in Table.4. Among 9 fractions, fraction 1 showed good activity.

Purification of active fraction 1

The chloroform residue of active fraction 1 (110mg) was further purified by column chromatography employing silica gel (100-200 mesh) column. Hexane, hexane-ethylacetate, ethylacetate, ethylacetate-methanol of various combinations were employed. A total of 115 fractions (each 50ml) were collected from the column. The purity was checked by TLC studies. The spots were identified by UV fluorescence, iodine vapours and H₂SO₄ spray. Fractions with identical R_f values were pooled together which resulted in 13 fractions. Each fraction was evaporated to dryness by Büchi Rota vapour R-114 with Büchi water bath B-480 at 45⁰ C. It was further dried by ED-3 Hindhivac direct drive rotary vacuum pump.

Evaluation of antimicrobial activity

The dried residues of all 13 fractions were dissolved in dichloromethane (1mg/ml) and evaluated for their antimicrobial activities against *B.subtilis* by disc- plate method. Among 13 fractions, fraction 7 exhibited good activity.

RESULTS

Antimicrobial spectrum

The culture filtrate of *S.sulfonensis* exhibited excellent antibacterial activity as shown in Table.1.

Table 1: Antimicrobial spectrum of the culture filtrate of *S.sulfonensis*

Test organism	Inhibition zone diameter (mm)
<i>Bacillus pumilus</i> NCIM 2327	21
<i>Bacillus subtilis</i> NCIM 2063	24
<i>Staphylococcus aureus</i> NCIM 2492	19
<i>Sarcina lutea</i> NCIM 2103	30
<i>Escherichia coli</i> NCIM 2563	18
<i>Pseudomonas aeruginosa</i> NCIM 2863	24
No activity was observed against fungi and yeasts	

Fermentation process

In view of excellent antimicrobial activities, the fermentation process studies were conducted with *S.sulfonensis*. As shown in Table.2, the p^H of the culture broth was gradually increased. The antimicrobial activity was found to be optimum on 6th day and a slight decrease in activity was noticed on 7th day.

Table 2: Fermentation cycle of *S. sulfonensis*

Fermentation time in days	p ^H	* Inhibition zone diameter(mm)
0	7.4	0.0
1	7.6	0.0
2	8.0	13.0
3	8.1	16.0
4	8.6	20.0
5	8.6	22.0
6	8.7	24.0
7	9.0	21.0

**B.subtilis*

Extraction of active principles

The crude antibiotic obtained from the culture broth into chloroform extract at P^H 8 was appeared as reddish pink solid (1.390g) and exhibited excellent antimicrobial activity against test organism (*B.subtilis*).

The mycelia cake (110g) was extracted with methanol showed good antimicrobial activity against test organism.(Table.3).

Table 3: Selection of solvent for extraction of antibiotic principles

Strain	Solvent for broth Extraction	Test organism	Inhibition zone diameter (mm)	
			Broth extract	Mycelial extract
<i>S.sulfonensis</i>	Chloroform	<i>B.subtilis</i>	35.0	22.0

Purification of extracellular antibiotic principles (Broth extract)

The paper chromatography and bioautographic studies revealed as clear inhibition zones on an opaque background.

TLC studies resulted in 9 fractions. Fraction 1 showed good antimicrobial activity, fraction 2 showed moderate activity, fraction 3 to 6 exhibited poor activities and fraction 7 to 9 & the solvent CHCl₃ did not show any activities.(Table. 4).

Table 4: Antimicrobial spectra of active fractions

Test organism	Active fractions						
	1	2	3	4	5	6	
<i>Bacillus pumilus</i> NCIM 2327	+++	+	-	+		+	+
<i>Bacillus subtilis</i> NCIM 2063	+++	++	+	+	+		+
<i>Staphylococcus aureus</i> NCIM 2492	+++	+		+	-	+	-
<i>Pseudomonas aeruginosa</i> NCIM 2863	+++	+	-	-	-	-	-
<i>Escherichia coli</i> NCIM 2563	+++	+	+	+	+		-

-: No activity, +: poor activity, ++: Moderate activity,+++: Good activity

Antimicrobial activity of column fractions

Among 13 fractions, fraction 7 (F40) exhibited good activity, fraction 2,3 &5 showed poor activities and fractions 1,4,6,8,9,10,11,12 ,13 & solvent dichloromethane did not exhibit any activities.Fraction 7 was eluated with hexane-ethyl acetate (9:1).

DISCUSSION

The data of fermentation process studies of the strain *S.sulfonensis* indicates that the p^H of the culture broth was gradually increased and the antimicrobial activity was found to be maximum on 6th day and a slight decrease in activity was noticed on 7th day (Table.2) due to metabolic reactions. The antibiotic principles from broth were completely extracted with chloroform at P^H 8, which indicates that alkaline environment was favourable for extraction of active principles from culture filtrate (Table 3). The preparative TLC studies resulted in active fraction 1 which exhibited good antimicrobial activity (Table.4). Among 13 column fractions , fraction 7 showed good antimicrobial activity . The fraction 7 (F40) was appeared as pale yellow solid (15mg). It was subjected to spectral analysis for its characterization. F40 indicates the isolate number from which the active compound was isolated. In subsequent studies , F40 was identified as diphenyl sulfone antibiotic.

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