A New Antibiotic 105- SMp from Soil Screened Streptomyces species 105 –SM
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Citation

Abstract
The present work describes, the 89 Streptomyces isolates were isolated from various soil horizons in and around Ooty (Tamilnadu, India), among those isolates the Streptomyces species 105-SM strain was selected for taxonomical, cultural characterizations, bioprocess studies along with purified antibiotic 105-SMp from Streptomyces species 105-SM isolate and its biological activities. From various soils from in and around Ooty (Tamilnadu, India), only 89 isolates were screened based on pinpoint colonies, then based on antibacterial and antifungal activities, the isolate 105-SM was selected for taxonomical, cultural characterizations and bioprocess studies. The purified antibiotic 105p was recovered from the bioprocess media by using extraction tree (column chromatography technique) and polarities of the compound 105-SMp. The purified antibiotic 105-SMp was undergone antibacterial and antifungal studies along with cytocidal studies. The purified antibiotic 105-SMp showed significant antifungal activity on Candida albicans (Ca5, Ca6, and Ca27) and found to be possessing cytotoxic activity at high concentrations. The antibiotic 105-SMp was confirmed by UV and FT IR spectral studies.

INTRODUCTION
Antibiotics have entered human life over half a century ago. They were the reason pneumonia, tuberculosis, gangrene and other infections lost their lethality for human beings. But even most powerful antibiotics cannot kill all pathogenic bacteria in the world. Bacteria have the advantage of millions of years, so humans have yet to discover many adaptations. All in all, it seems intuitive that soil bacteria which thrive in the company of organisms, from which we produce most of our antibiotics, are indeed resistant to most of these antibiotics. However, it soon became apparent during screening programmes that some microbial metabolites were discovered more frequently than others. As the number of described microbial metabolites increased, so did the probability of rediscovering known compounds. At the present time, with several thousands of described microbial metabolites, strategies must be introduced into screening programmes to increase the chances of discovering novel compounds. The filamentous actinomycetes account for a significant fraction of microbial metabolites and, among them, Streptomyces is by far the most prolific genus. The pharmaceutical industry, over several decades, has probably isolated and screened millions of Streptomyces strains. Consequently, the chances of isolating a novel Streptomyces strain have substantially diminished, and so has the probability of discovering a novel compound. Therefore it has been estimated that only a fraction of the antibiotics produced by Streptomyces strains have been discovered, identifying the undiscovered portion will require a substantial effort. Hence, based on the above said objective, an attempt was made to select Streptomyces species 105-SM strain as a suitable organism from soil for production of antibiotics 105-SMp to conform their efficacy on antibacterial, antifungal and cytotoxic activities.

METHODS AND MATERIALS
SCREENING OF SOIL ISOLATE
All the soil samples were collected from in and around Ooty (Tamil nadu, India). The screening methods were described by following the standard procedure as follows. One gram of each sample was added to 1gm of calcium carbonate (CaCO₃) in an air-dried flask which was dispersed in 100ml of sterile water with 0.1ml of Tween 80. The flasks were kept on a shaker (Remi Vortex Shaker, India) for half an hour. These flasks were considered as stock cultures. From the stock cultures, 1ml was taken and diluted with 9ml of sterile distilled water in 6 culture tubes to get 10⁻¹ to 10⁻⁵ concentrations of original sample. Then the last three
dilutions were plated on sterile petridishes for crowded plating, the dilutions were mixed with antibacterial (ampicillin 25µg ml⁻¹) and antifungal (clotrimazole 50µg ml⁻¹) agents in starch casein medium. All plates were kept at 37°C in the incubator and observed for growth every day. After five days, in each plate was observed, few Actinomycetes colonies, which were isolated, based on pin point colonies behavior, then which were preserved (Hopwood D.A.1985) in Actinomycetes agar slants for further studies.

**SCREENING FOR ANTIBIOTIC ACTIVITY**

To obtain pure cultures, the isolates were streaked into kenknight's agar plates by multiple streaking methods. Based on the preliminary morphology observation (zone of inhibition), seven isolates were selected and tested for microbial sensitivity using different stains of bacteria and fungi by cross-streak method (Bradshaw 1992). Each isolate was grown on nutrient agar medium while testing against bacteria at 37°C and in YEME agar (yeast extract –malt extract) medium in case of yeast and fungi for seven days at 28°C. Using the following test organisms such as: Gram positive bacteria: Bacillus subtilis; Staphylococcus aureus, Gram negative bacteria: Escherichia coil; Pseudomonas aeruginosa, Fungi; Aspergillus niger, Aspergillus fumigates, Yeasts: Saccharomyces cervisiae, candid albicans (Ca). The selected isolates of the Actinomycetes were streaked on their respective media in Petridishes. After 7 d, the test organisms were streaked at right angle to the original streak of Actinomycetes. For 24 h culture of bacteria and 48 h culture of fungi and yeast were used as inocula. These petridishes were kept for 24 h incubation at 37°C after inoculation for bacteria while for fungi and yeast 48 h incubation at 28°C. Based on their antibacterial and antifungal properties, 105-SM isolate was chosen for taxonomical characterization.

**TAXONOMICAL STUDIES**

Taxonomical studies of 105-SMp isolate were performed by following methods such as melanin formation, gelatin.
liquification, acid production and hydrogen sulfide production tests, along with nitrate broth technique. The cultural, physo-chemical and taxonomical characteristics of 105-SM isolate were observed on melanin formation, gelatin liquification, hydrogen sulfide production and acid production tests, along with nitrate broth technique. Microscopical studies of isolated strain 105-SM were carried out by (i) agar block method and (ii) inclined cover slip method. In the agar method, the isolated strain was prepared in thin agar block and was examined under high magnification (phase contrast microscopy, 100X) and in the inclined coverslip method the mycelia adhering to cover slips placed at an angle in growing culture was examined at high magnification. Twenty seven isolates which were taken for primary screening (Giancar'o Lancing 1993 and Bradshaw 1992) to identify antibiotic productivity, only 105-SM isolate having prominent antibiotics producing capacity was selected for the morphological and cultural characteristics studies, which were performed the method described by (ISP) International Streptomyces Project. The selected 105-SM strain was inoculated and incubated at 30 °C for 21 d in different media described as follows: ISP cultural media: ISP medium-1 Tyrosine-yeast extract broth, ISP medium-2 Yeast extract- malt extract agar, ISP medium-3 Oat meal agar, ISP medium-4 Inorganic salts starch agar, ISP medium-5 Glycerol-asparagines agar, ISP medium-6 Peptone-yeast extract iron agar, ISP medium-7 Tyrosine agar and Bennett agar. Assimilation is the utilization of carbon source (Dextrose, sucrose, lactose, maltose, D-mannitol, fructose and sorbitol) by microorganisms in the presence of oxygen. Positive assimilation of growth was identified by the change in pH of the medium.

**Figure 3**
Plate 1: shows strain filamentous using scanning electron microscope (Inclined coverslip method)

**Figure 4**
Table 3: Results of Biochemical Tests of isolate

<table>
<thead>
<tr>
<th>Biochemical Tests</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanin formation test</td>
<td>- Negative indicates no melanin formation</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>- Negative No pink color</td>
</tr>
<tr>
<td>Hydrogen sulfide production</td>
<td>- Negative No yellow color</td>
</tr>
<tr>
<td>Starch hydrolysis test</td>
<td>++ Clear zone between 10-15 mm</td>
</tr>
<tr>
<td>Gelatin liquification test</td>
<td>+ Positive Gelatin liquefied</td>
</tr>
<tr>
<td>Acid production</td>
<td>+ Positive acid production</td>
</tr>
<tr>
<td>Milk peptonozation test</td>
<td>WB, NG, WLB and pH</td>
</tr>
<tr>
<td></td>
<td>WB- White band, NG- No gas formation, WLB- Wheylike brownish medium</td>
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</tbody>
</table>

**Figure 5**
Plate 2: shows strain in Bennet’s agar slants with control
A New Antibiotic 105- SMp from Soil Screened Streptomyces species 105 –SM

Figure 6
Plate 3: shows strain in ISP-3 agar slant with control

Figure 9
Plate 6: shows strain in ISP-7 agar slants with control

Figure 7
Plate 4: shows strain in ISP-4 agar slants with control

Figure 10
Plate 5: shows strain in ISP-5 agar slant with control

BIOPROCESS FOR ANTIBIOTIC PRODUCTION FOR STRAIN

Bioprocess for Streptomyces strain 105-SM was carried out by using cultivation media with different ratios of carbon and nitrogen source in the cultivation broth in seed medium and production medium. The composition of the seed medium(100ml) consisted of glucose 2%w/v, soluble starch 1%w/v, meat extract 1%w/v, dried yeast 0.4%w/v, Soya bean meal 2.5%w/v, sodium chloride 0.2%w/v and potassium mono hydrogen phosphate (K₂HPO₄)0.005%, which was adjusted to 7.0 pH prior to sterilization , was dispersed into each of two 500 ml Erlenmeyer flasks and sterilized. A loopful of Streptomyces 105-SM (ISP medium-4 slant) was inoculated to each of the medium and cultured under shaking condition at 30° C for 3 d. The seed
culture was incubated at 28°C on a rotary shaker at 250rpm for 48 hours in 500ml conical flask containing 100ml of the seed medium. The production medium one liter (1 l) consisted of soluble starch-20g, glucose-1g, yeast extract powder-1g, calcium carbonate (CaCO₃)-3g, magnesium sulfate (MgSO₄.7H₂O)-0.25g, potassium mono hydrogen phosphate (K₂HPO₄)-5g, tryptone-2g, calcium chloride-.0004g, sodium chloride (NaCl)-0.01g, ferrous sulfate (FeSO₄.4H₂O)-0.002g, Silicone oil-0.3ml, which was adjusted pH 7.0 prior to sterilization. The resultant seed broth (20ml) was inoculated to the production medium and cultured at 30°C for 3 d, the second stage seed culture 80ml was used as an inoculum to initiate the cultivation in two liter (2 l) bioreactor containing one liter (1 l) of a cultivation medium. The cultivation was carried out at 28°C with 1200ml (1.2 l) of air per min and agitation at 200rpm. Culture growth was evaluated, using the reported method by centrifuging untreated and treated bioprocess broth at 5000rpm for 10 min.

**PRODUCT RECOVERY FOR STRAIN**

The antibiotic compound of the strain 105-SM was isolated from the fermentation broth of the strain 105-SM as outlined in schematic diagram fig-1 and activity was monitored using Staphylococcus aureus as the test organism. After 4 d of the bioprocess, the product yield showed a large zone of inhibition against the test organism . The mycelia were separated from the bioprocess broth by centrifugation. The filtrate was extracted with equal quantity of ethyl acetate and concentrated vacuum at 40 °C, obtained 1.1 gm of crude compound from extracted with 500ml methanol and concentrated in vacuum at 40 °C. On subjecting these crude extract to column chromatography (length 15cm x 3cm diameter silica gel (60-120 mesh) column) by eluting with solvents carbon tetra chloride: methanol (3:1) stepwise, a fraction containing active material (crude powder 320gm) was obtained. Further the crude powder material was subjected to column silica gel (60-120 mesh) chromatography eluted with n-Hexane: methanol (7:3) stepwise, the purified brown color powder 80mg was obtained evaporation in vacuum

**SPECTRAL ANALYSIS**

The elementary analysis to establish the chemical structure of these antibiotic 105-SMp, the spectral studies were performed by using UV and FTIR following the standard procedure .

**ANTIMICROBIAL STUDIES**

Antimicrobial study of purified antibiotic compound of 105-SMp was carried out by following two-fold serial dilution technique . The concentration of the working stock culture was 10⁶ to 10⁸ cfu ml⁻¹. The antibiotics were screened against the following organisms; Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Aspergillus Niger, Aspergillus flavus, Candida albicans Ca₅, Ca₁₀, Ca₂₅, & Ca₇.
RESULTS AND DISCUSSION

The Soil screening of Actinomycetes was done by the determinations of the Actinomycetes populations in the various soil horizon samples which were collected from in and around Ooty (Tamilnadu, India). The preliminary morphology observation of soil isolates was studied from the selected various soil samples. Only 89 isolates were isolated, which had pinpoint white colonies and twenty seven soil isolates of Actinomycetes showed zone of inhibition during the crowded-plate study. By multiple streak method in kenknight's agar media to obtained pure culture of 27 strains. Each strain was stored on Actinomycetes agar slants for preserved for further studies. The 27 isolates of Actinomycetes were then observed microscopically for the presented of filamentous, spore and fragmentation characters of Actinomycetes as shown in plate no-1, as described by Bergey's Manual of Determinative Bacteriology 23 (Cross T. 1989). These isolated strains were tested for microbial sensitivity using different strains of bacteria and fungi which were described in materials and methods by cross streak method. The results showed that only 7 isolates was found to be better antimicrobial activity. Among 7 isolates, strain 105-SM was selected based on prominent antibacterial activity and antifungal activity. The strain 105-SM was found to be significant activities against Pseudomonas aeruginosa (30mm), E.coli (25mm), Bacillus subtilis (24mm) and Staphylococcus aurues (27mm). All other isolates exhibited low to the moderate activities as shown in table no: 1. The 105-SM strain was very effective towards Aspergillus niger (20mm) but not effective towards Candida albicans (nil) while others exhibited moderate to low activity. The antifungal activity and antiyeast activity are shown in table no:2..Based on the prominent antibacterial and antifungal activities, the selected 105-SM strain was taken for the taxonomical studies, showed positive results in gelatin liquification, starch hydrolysis, and milk peptonisation tests which were carried out by procedure discussed in materials and methods, the results are showed in table no:3. It showed the positive reaction in gelatin liquification, acid production and milk peptonisation tests and negative reaction in melanin formation test, nitrate broth technique and hydrogen sulfide production. No formation of melanoid pigment was observed on peptone-yeast extract iron agar. The selected 105-SM strain was subjected to morphological, cultural and physiological characteristics (Growth characteristics, reverse color, presence of aerial mycelia with spores, soluble pigment and the microorganisms showed growth, which having a spreading
nature and having a matte surface) were observed in ISP media (ISP-2,3,4,5,7 and Bennett's agar), are summarized in Table no: 4. The aerial mycelium was yellow color on yeast malt extract agar, whitish yellow powdery on Oct meal agar, pinkish yellow color powdery on inorganic salts starch agar, yellow color leathery on tyrosine agar and yellow powdery on Bennett's agar. Reverse side of the growth culture was pale yellowish pink on yeast extract-malt extract agar, yellow color on oct meal agar, yellow color on Bennett's agar, sandal wood color on glycerol asperagine agar, whitish yellow on peptone yeast extract iron agar. Pale yellow color to dark yellow soluble color pigments was several ISP media. Positive assimilation found to be on various carbon sources with 105-SM strain in carbon utilization tests. These physico-chemical properties of 105-SM strain helped to differentiate the species and subspecies in Streptomyces. These cultural characters of these organisms are shown in Plate no: 2-8. Based on the utilization of carbohydrate sources, protein hydrolysis and peptonization properties and also the antimicrobial activity, Streptomyces 105-SM strain was selected, for parameters also include the cheaper utilization of the cultivation media. The seed medium and cultivation medium were designed based on taxonomical studies, which gave information about the nutritional requirements of the Streptomyces strain 105-SM. The bioprocess was monitored at every 12 h intervals for the packed cell volume and change in pH. The increasing acidity was neutralized by addition of 1N sodium hydroxide. The pH of broth becomes acidic (pH 6) up to 120 h after which it remained neutral. The Streptomyces strain 105-SM grew vigorously and there was a change in medium color from white cream to yellow. After 4 d of the bioprocess, the product yield showed a large zone of inhibition against the test organism. The mycelia were separated from the bioprocess broth by centrifugation. The filtrate was extracted with equal quantity of ethyl acetate and concentrated vacuum at 40 °C, followed by obtained 1.1 gm of crude compound from extracted with 500ml methanol and concentrated in vacuum at 40 °C. On subjecting these crude extract to column chromatography (length 15cm x 3cm diameter silica gel (60-120 mesh) column) by eluting with solvents carbon tetra chloride: methanol (3:1) stepwise, fraction containing active material (crude powder 320gm) was obtained. Further the crude powder material was subjected to column silica gel (60-120 mesh) chromatography eluted with n-Hexane: methanol (7:3) stepwise, the purified brown color powder 80mg was obtained evaporation in vacuum. The product recovered from the fermentation broth found to be 80mg of brown colored powder after extraction as represented in fig No.1 Schematic diagram. The antimicrobial activity was checked at each stage using test microorganism S.aureus. Inhibition of growth increased with each stage of purification. The antimicrobial activity of the purified compound 105-SMp was showed in table no5. The antibiotic showed broad spectrum of activity on gram positive and gram negative organisms. The minimum inhibition concentration (MIC) ranged between 100µg/ml to 20µg/ml respectively for 105. S.aureus was found to be susceptible to the isolated antibiotic. The antifungal activity of these compounds was also performed. The minimum fungicidal concentration (MFC) values are tabulated in table no 6. The purified antibiotic 105-SMp showed better activity against Aspergillus species and less activity against yeast. The antibiotic 105-SMp compound showed cytotoxic activity against Hep-2 vero cells only above the concentration of 20µg ml⁻¹. Since cytotoxicity is a parameter for antitumour activity, it is worthwhile to study this activity further. In an attempt to establish the chemical structure of the antibiotic produced by strain 105; spectral studies such as UV, and IR were performed and the characteristic peaks and their absorbance are shown in Table No.18. Efforts to establish the complete structure elucidation are in progress.

References

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