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# Isolation of Antibiotic Producing Actinomycetes from Saltpans

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**ABSTRACT**

Actinobacteria was an important origin of novel bioactive metabolites with significant pharmaceutical applications. A total of 22 isolates were isolated from the salt pan soils and studied their cultural, physiological characteristics. Finally, they were subjected to the screening for antibiotic producing actinomycetes by agar well diffusion assay against two gram positive (*Bacillus subtilis*, *Staphylococcus aureus*) and two gram negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*). Among the tested, two groups of gram positive bacteria were more sensitive and inhibited by 17 isolates (81.8%) and gram negative bacteria were less sensitive and inhibited by 11 isolates (50%). However, both gram+ve and gram-ve bacteria were inhibited by 6 isolates (27%).

## Introduction

Actinomycetes are the most extensively assigned group of microorganisms in nature which mainly inhabit the soil (Oskay *et al.*, 2004). Approximately 80% of the world's antibiotics are well-known to come from Actinomycetes, mostly from the genera *Streptomyces* and *Micromonospora* (Pandey *et al.*, 2004). Actinobacteria are a best source of various bioactive compounds they are antibiotics, pesticides, enzymes, immune modulators, herbicides, anti-infective and anticancer agents(Newman and Cragg2007, Takahashi andomura, 2003). *Streptomyces* are the largest genus of Actinobacteria proposed by Waksman and Henrici in 1943. They are Gram positive, aerobic, spore forming filamentous bacteria (Abussaud *et al.*, 2013). They produce an extensive branching substrate mycelium that are tough, leathery and produce diffusible pigments (Euzeby, 2008). Their aerial mycelium bears chains of arthrospores. Development of antibiotic resistance microbes to the commonly available antibiotics and antifungal agents has necessitated the requirement of new compounds and the members of the genus

*Streptomyces* offers promising lead compounds (Jayapradha Ramakrishnan, 2009).Traditional screening methods have led to the isolation of common microorganisms capable of producing metabolites, which have already been extensively studied and established (Okami and Hotta 1988; Kurtboke *et al.,* 1992). Among the current strategies of natural-product screening, improved methodologies for isolating the uncommon and less studied rare actinomycetes are required to avoid the repeated isolation of the strains that produce known bioactive metabolites, and to improve the quality of the screened natural products (Takahashi and Omura 2003;Berdy 2005;singh*et al.,* 2009). The metabolites produced by halophiles like ectoine, betaine, carotenoid pigments, enzymes, anticancer and antibacterial compounds have immense applications in pharmacy and biomedicine (Thombre and Oke, 2016). Marine sediment, soil, water, contaminated regions on the seashore, salt lakes, saline soils, alkaline–saline habitats, brines, and other regions are good sources for the selection of novel halophilicactinomycetes. The present investigation was undertaken to isolate, characterize and screen the actinomycetes from salt pan sediment soils.

## Materials and Methods

### Collection of soil samples

The soil samples were collected using randomised block design at a depth of 6-10cm saltpan sediments situated at Nellore district of Andhra Pradesh (14˚44ꞌ33.6˚N-80˚06ꞌ17.7˚E). The collected samples were stored at $4^0$C in sterile polythene bags.

### Isolation of actinomycetes

Actinomycetes were isolated using standard dilution plate technique(Rahman *et al.,* 2010). Using Glycerol asparagine agar, yeast extract and malt extract Agar media (Downes andIto 2001), starch casein agar medium's (Kuster and Williams 1964) containing nystatin (50µg/ml).The Prepared plates were incubated at $28^0$C for 7 days. Pure cultures were obtained after repeated subcultures and maintained as spore suspension in 20% (v/v) glycerol at -$20^0$C or further use.

### Cultural and physiological characteristics of actinomycetes

Cultural charcteristics of the isolates were studied according to the Shirling and Gottlieb(1966) based on their growth of the colony, colour of the aerial mycelium and substrate mycelium, pigmentation (Macroscopically) and also Physiological conditions like Temperature, pH and NaCl concentrations were observed for halophilic actinomycete isolates (SVNMA1, SVNMA2, SVNMA3, SVNMA4, SVNMA5, SVNMA6, SVNMA7, SVNMA8, SVNMA9, SVNMA10, SVNMA11, SVNMA12, SVNMA13, SVNMA14, SVNMA15, SVNMA16, SVNMA17, SVNMA18, SVNMA19, SVNMA20, SVNMA21 and SVNMA22).

### Screening of actinomycetes for antibiotics

The clinical isolates namely *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Klebsiella pneumoniae* were procured from Sri Venkateswara Ramnarain Ruia Government General Hospital, (Ruia Hospital) Tirupati. Nutrient agar and nutrient broth were used for routine culturing. A total of 22 actinomycete isolates were subjected to submerged fermentation technique (Atta and Ahmad 2009). They were inoculated in 1.0 litre flask containing 600ml yeast extract and malt extract broth and incubated on rotary shaker (120rpm) at 30ºC for 7 days. Fermeted broth was filtered through Whatman no.1 filter paper and then centrifuged at 5000 rpm for 20 min and supernatants were tested for antibacterial activity by agar well diffusion method (Baker *et al.*,1991). Different concentrations of supernatants (50 µl, 100µl, 150 µl and 200 µl) were placed into the wells they were previously seeded with bacterial pathogens; they are gram positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*). The plates were incubated at 30ºC for 48 hours and the zone of inhibition was measured. All the tests were done in triplicates.

## Results and Discussion

### Isolation of antibiotic producing actinomycetes

Various media and methods were employed for the isolation of actinomycetes from solar saltpan soils. In order to standardize the isolation procedure few preliminary investigations such of media type, pour plate and spread plate techniques were compared.

Three different types of media namely Glycerol asparagine (ISP-5), Yeast extract and malt extract agar medium (ISP-2) and Starch casein agar medium were tested to support the growth of large number of colonies from marine sediments. Among the three media tested, ISP-2 consistent in all dilutions supported higher number of colonies (Table-1). Therefore ISP-2 media was selected for preliminary routine work.

Both pour plate method and spread plate methods were evaluated for this suitability for isolation of antibiotics. Among the two tested methods distinct colonies were appeared in pour plate method. In spread plate method due to high colony number distinct colonies are less. Therefore, pour plate method was employed for isolation of actinomycetes (Table-2). Isolation of actinomycetes is the first and the most important step of actinomycetes resource development. The primary aim of such investigations has been to develop the most rapid and accurate method for proving the novelty of newly isolated strains (Hain *et al.*, 2007). Isolation and Screening of actinomycetes be going to choose a useful medium in extension tochoice of major ecological environment as the division of outside sources. Various media and methods were employed for the isolation of actinomycetes, in order to standardize the isolation procedure few preliminary investigations such of media type, pour plate and spread plate techniques were compared. Three different types of media namely Glycerol asparagine (ISP-5), Yeast extract and Malt extract agar medium (ISP-2) and Starch casein agar medium.

### Pure Cultures of Actinomycetes

The colony growth in most of the isolates was scanty (SVNMA1, SVNMA3-4, SVNMA6, SVNMA9-11, SVNMA13-19). Four isolates exhibited moderate growth (SVNMA2, SVNMA7, and SVNMA8) and a single isolate (SVNMA12) exhibited abundant growth.

All pure cultures were maintained on ISP-2 medium (Plate-1,2,3&4). In case of aerial mass of the colonies ranged from whitish ash to dark ash, light brown to dark brown, light pink to dark pink. Riverside pigmentation and soluble pigments were not detected in most of the isolates except SVNMA2, SVNMA3 and SVNMA13.

Similarly, melanoid pigmentation was absent in all the isolates except SVNMA13.The cultural characteristics of antibiotic producing actinomycetes were presented in Table-3.In all 22 isolates, some isolates exhibited the optimum growth at 30°C and some isolates exhibited the optimum growth at 40°C. When compared to 10°C, 20°C and 50°C(Fig-1).

**Table.1** Colony Forming Units (CFU) of actinomycetes on various media

| Name of the media | Colony Forming Units (CFU) ml/g sediment | | | |
|---|---|---|---|---|
| Dilutions | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ |
| Glycerol asparagine agar medium (ISP-5) | $1.4 \times 10^{1}$ | $1.2 \times 10^{2}$ | $1.0 \times 10^{3}$ | $0.7 \times 10^{4}$ |
| Yeast extract and malt extract agar medium (ISP-2) | $1.9 \times 10^{1}$ | $1.4 \times 10^{2}$ | $1.4 \times 10^{3}$ | $1.2 \times 10^{4}$ |
| Starch Casein Agar | $1.0 \times 10^{1}$ | $0.9 \times 10^{2}$ | $0.5 \times 10^{3}$ | $0.4 \times 10^{4}$ |

**Table.2** Pour Vs Spread plate methods in Yeast extract and malt extract agar media

| Dilutions | Pour plate | Spread plate |
|---|---|---|
| **$10^{-1}$** | $1.9 \times 10^{1}$ | $6.8 \times 10^{1}$ |
| **$10^{-2}$** | $1.4 \times 10^{2}$ | $5.1 \times 10^{2}$ |
| **$10^{-3}$** | $1.4 \times 10^{3}$ | $2.6 \times 10^{3}$ |
| **$10^{-4}$** | $1.2 \times 10^{4}$ | $1.1 \times 10^{4}$ |

**Table.3** Cultural and physiological characteristics ofactinomycete isolates

| Name of the isolate | Growth of the colony | Aerial mass/Colour of the colony | Substrate mycelium colour | Reverse side pigmentation* | Melanoid pigmentation* | Soluble pigment* |
|---|---|---|---|---|---|---|
| **SVNMA 1** | Scanty | Ash | Ash | ND | ND | ND |
| **SVNMA 2** | Moderate | Brownish yellow | Whitish ash | Light brown | ND | Light brown |
| **SVNMA 3** | Scanty | Ash | Light ash yellow | Yellow | ND | Light yellow |
| **SVNMA 4** | Scanty | Creamy | Creamy | ND | ND | ND |
| **SVNMA 5** | Abundant | Dark green | Green | ND | ND | ND |
| **SVNMA 6** | Scanty | Ash | Whitish ash | ND | ND | ND |
| **SVNMA 7** | Moderate | Yellowish | Pale yellow | ND | ND | ND |
| **SVNMA 8** | Moderate | Light white | Pale yellow | ND | ND | ND |
| **SVNMA 9** | Scanty | Creamy | Light pink | ND | ND | ND |
| **SVNMA 10** | Scanty | Pale ash | Ash | ND | ND | ND |
| **SVNMA 11** | Scanty | Whitish | Whitish | ND | ND | ND |
| **SVNMA 12** | Moderate | Pink | Light brown | ND | ND | ND |
| **SVNMA 13** | Scanty | Ash | Dark brown | Brown | Brown | Light black |
| **SVNMA 14** | Scanty | Brownish cream | Light pink | ND | ND | ND |
| **SVNMA 15** | Scanty | Dark ash | Dark ash | ND | ND | ND |
| **SVNMA 16** | Scanty | Creamy | Creamy | ND | ND | ND |
| **SVNMA 17** | Scanty | Dark creamy | Dark creamy | ND | ND | ND |
| **SVNMA 18** | Scanty | Ash | Ash | ND | ND | ND |
| **SVNMA 19** | Scanty | Creamy | Dark pink | ND | ND | ND |
| **SVNMA 20** | Scanty | Pink | Cream | ND | ND | ND |
| **SVNMA 21** | Scanty | Light yellow | Whitish | ND | ND | ND |
| **SVNMA 22** | Scanty | Dark brown | Brownish | Dark green | ND | ND |

*ND Not detected

**Table.4** Screening of selected antibiotic producing actinomycetes (SVNMA1-SVNMA22) for antibacterial activity

| Name of the isolate | Zone of inhibition(mm) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ***Bacillus subtilis**** | | | | ***Staphylococcus aureus**** | | | | ***Escherichia coli**** | | | | ***Klebsiella pneumoniae**** | | | |
| | **Fermented broth** | | | | | | | | | | | | | | | |
| | **50µl** | **100µl** | **150µl** | **200µl** | **50µl** | **100µl** | **150µl** | **200µl** | **50µl** | **100µl** | **150µl** | **200µl** | **50µl** | **100µl** | **150µl** | **200µl** |
| **SVNMA 1** | - | - | - | - | - | 1.0±0.0 | 3.0±0.0 | 3.0±0.0 | - | 2.0±0.0 | 2.0±0.0 | 4.0±0.0 | - | - | - | - |
| **SVNMA 2** | - | - | - | 4.0±0.0 | - | - | - | - | - | - | - | 3.0±0.0 | - | - | - | - |
| **SVNMA 3** | 1.0±0.0 | 3.0±0.0 | 2.0±0.0 | 3.0±0.0 | - | - | - | - | - | 2.0±0.0 | 3.0±0.0 | 4.0±0.0 | - | - | - | - |
| **SVNMA 4** | 4.0±0.0 | 6±0.1 | 7±0.1 | 8±0.1 | - | - | - | - | - | - | - | - | - | - | - | - |
| **SVNMA 5** | - | - | - | - | - | - | - | - | 2.0±0.0 | 3.0±0.0 | 4.0±0.0 | 5.0±0.1 | - | - | 4.0±0.0 | 5.0±0.1 |
| **SVNMA 6** | - | - | 2.0±0.0 | 3.0±0.0 | - | - | - | - | - | - | - | 4.0±0.0 | - | - | - | - |
| **SVNMA 7** | - | - | - | - | - | - | - | - | - | - | - | - | - | 1.0±0.0 | 2.0±0.0 | 3.0±0.0 |
| **SVNMA 8** | 10±0.2 | 12±0.2 | 13±0.2 | 15±0.3 | - | 3±0.0 | 4±0.0 | 6±0.1 | - | - | - | - | - | - | - | - |
| **SVNMA 9** | - | - | 2.0±0.0 | 3.0±0.0 | - | - | - | 2.0±0.0 | - | - | - | - | - | - | - | - |
| **SVNMA 10** | - | - | - | 2.0±0.0 | - | - | - | 4.0±0.0 | - | - | - | - | - | - | - | - |
| **SVNMA 11** | 5.0±0.1 | 8±0.1 | 9±0.1 | 11±0.2 | - | - | - | - | 4±0.0 | 5±0.1 | 5±0.1 | 11±0.2 | 2±0.0 | 3±0.0 | 2±0.0 | 9±0.1 |
| **SVNMA 12** | - | - | 2.0±0.0 | 3.0±0.0 | - | - | - | 2.0±0.0 | - | - | - | - | - | - | - | - |
| **SVNMA 13** | 2.0±0.0 | 3±0.0 | 4±0.0 | 4±0.0 | - | - | - | - | 2±0.0 | 5±0.1 | 3±0.0 | 10±0.2 | 1±0.0 | 2±0.0 | 2±0.0 | 4±0.0 |
| **SVNMA 14** | - | 2.0±0.0 | 3.0±0.0 | 3.0±0.0 | - | - | - | - | - | - | - | - | - | - | - | - |
| **SVNMA 15** | - | - | - | 2.0±0.0 | - | - | 2.0±0.0 | 3.0±0.0 | - | - | - | - | - | - | - | - |
| **SVNMA 16** | - | - | - | 2.0±0.0 | - | - | - | - | - | - | - | - | - | - | - | - |
| **SVNMA 17** | - | - | - | - | - | - | - | - | - | 1.0±0.0 | 2.0±0.0 | 3.0±0.0 | - | - | - | - |
| **SVNMA 18** | - | - | - | - | - | - | - | - | - | - | 1.0±0.0 | 2.0±0.0 | - | - | - | - |
| **SVNMA 19** | - | - | 2.0±0.0 | 2.0±0.0 | - | - | - | - | - | - | - | - | - | - | - | - |
| **SVNMA 20** | - | 2.0±0.0 | 3.0±0.0 | 3.0±0.0 | - | 3.0±0.0 | 2.0±0.0 | 3.0±0.0 | - | - | - | - | - | - | - | - |
| **SVNMA 21** | - | - | - | - | - | - | - | - | - | - | - | 10±0.2 | - | - | - | - |
| **SVNMA 22** | | 1.0±0.0 | 1.0±0.0 | 2.0±0.0 | - | - | 1.0±0.0 | 2.0±0.0 | - | - | - | - | - | - | - | - |

The values are the Means ± S.E for the experiments, *- no zone of inhibition

**Plate.1** Pure cultures of actinomycete isolates (SVNMA1-SVNMA6)

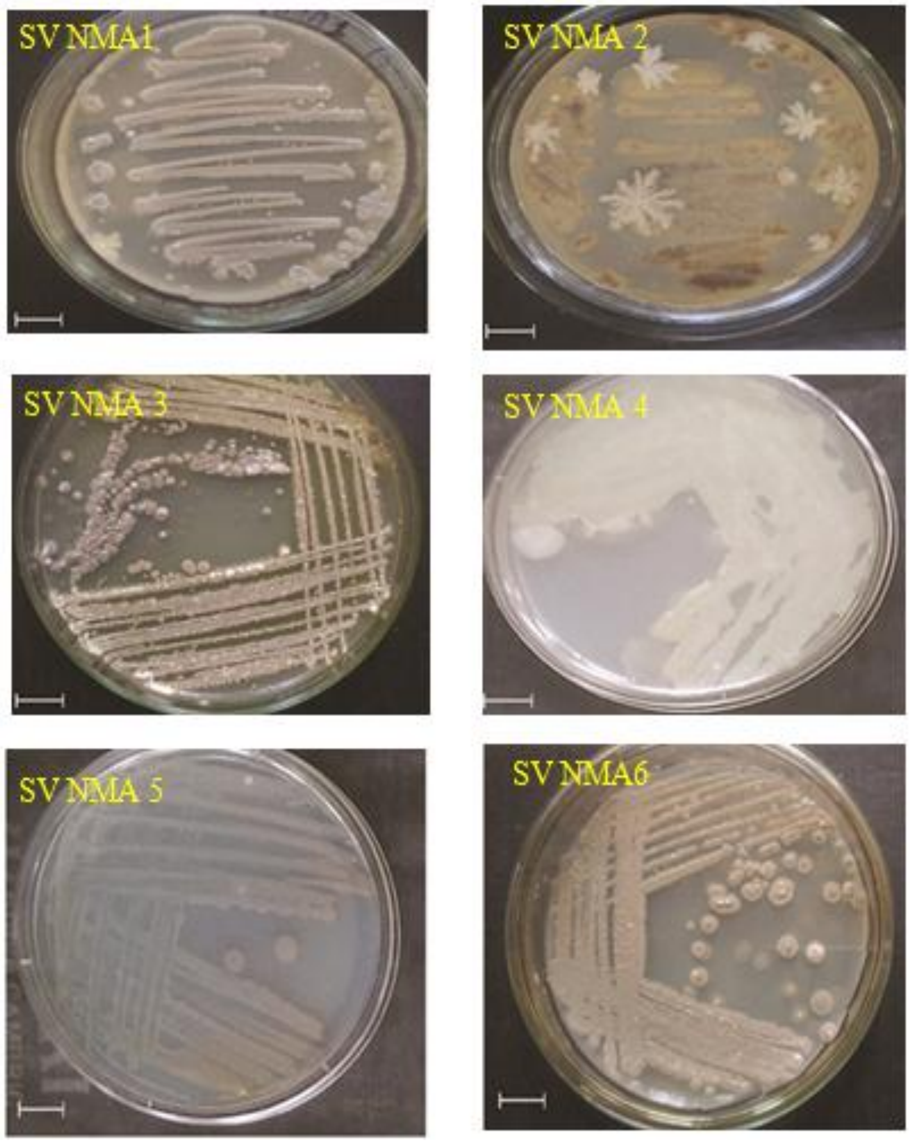


**Plate.2** Pure cultures of actinomycete isolates (SVNMA7-SVNMA12)

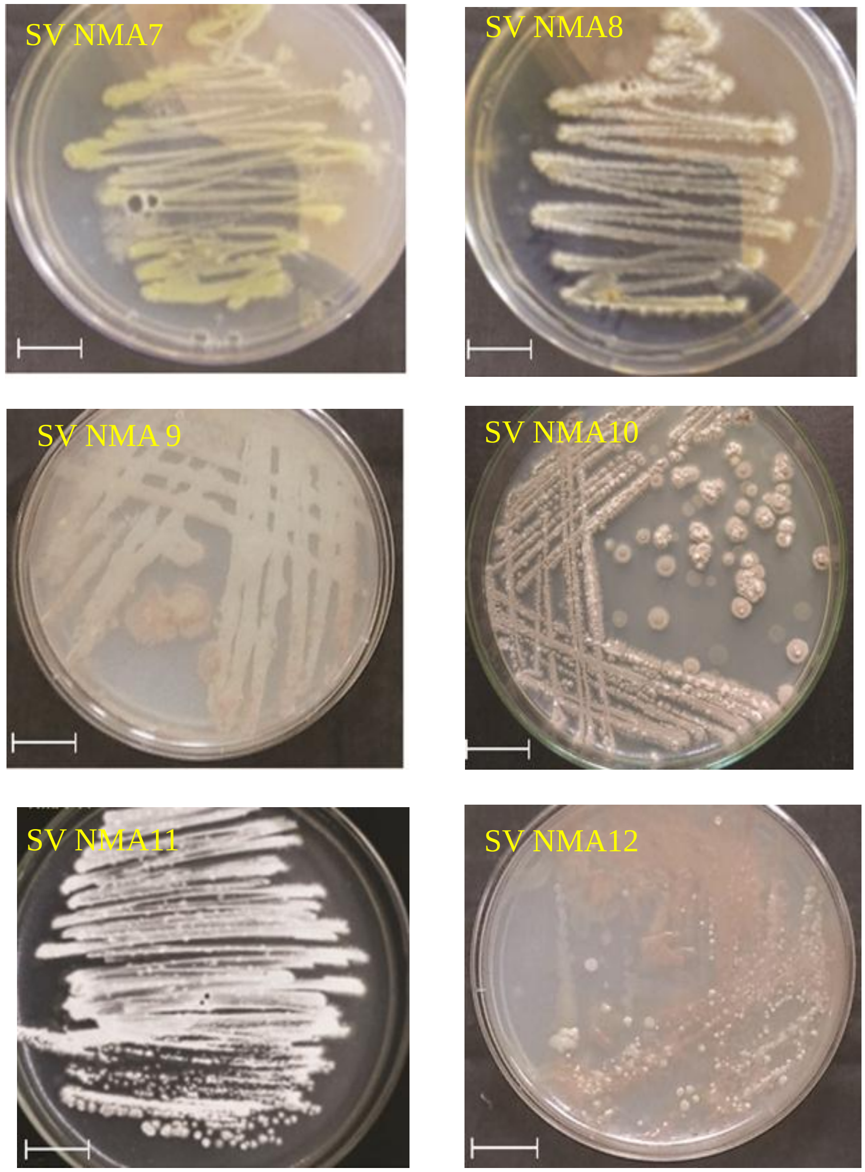


**Plate.3** Pure cultures of actinomycete isolates (SV NMA13-SVNMA18)

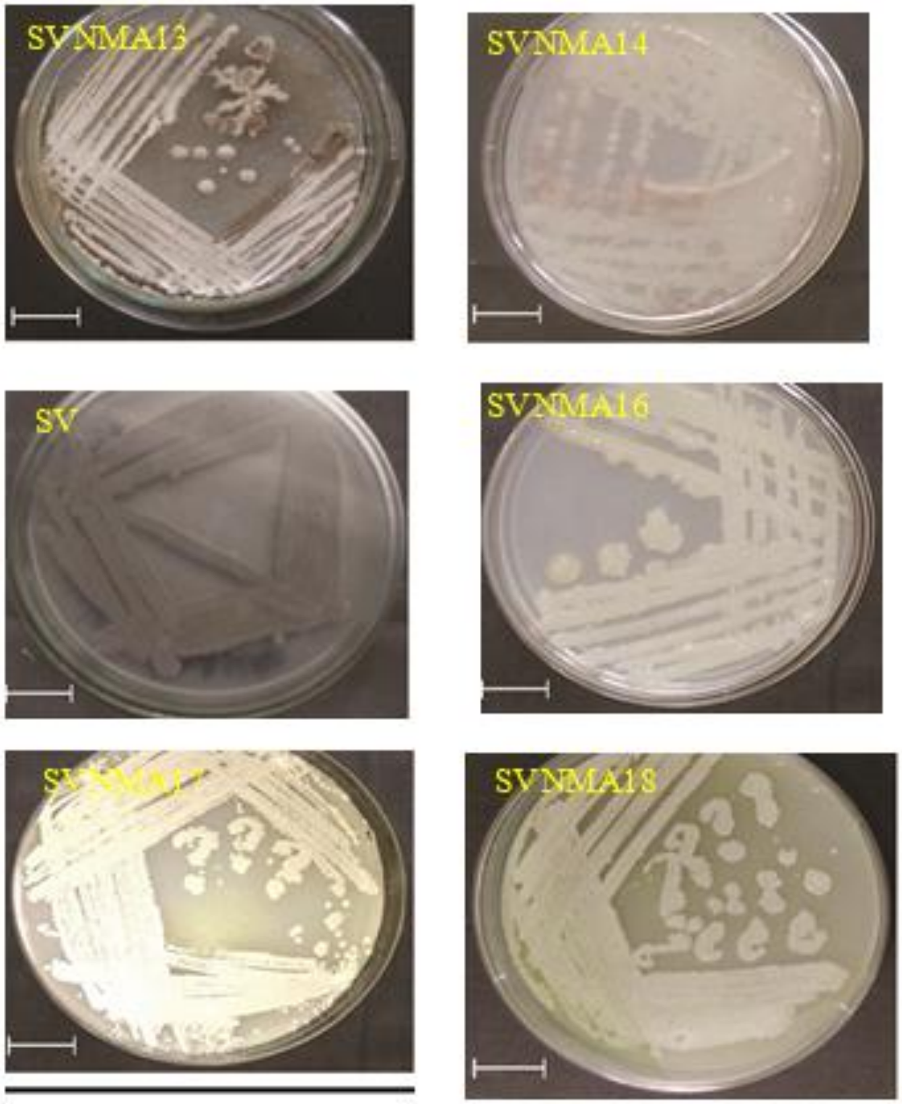


**Plate.4** Pure cultures of actinomycete isolates (SV NMA19-SVNMA22)

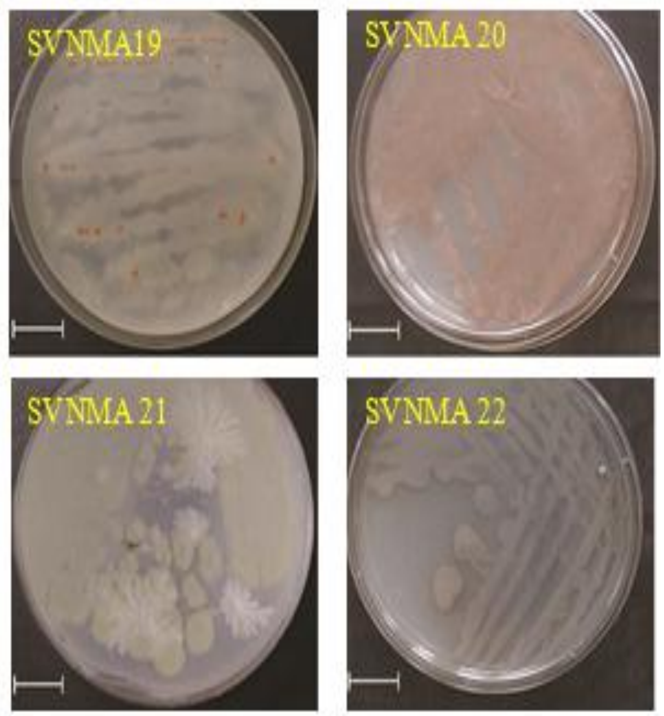



**Fig.1** Effect of temperature on growth of actinomycete isolates

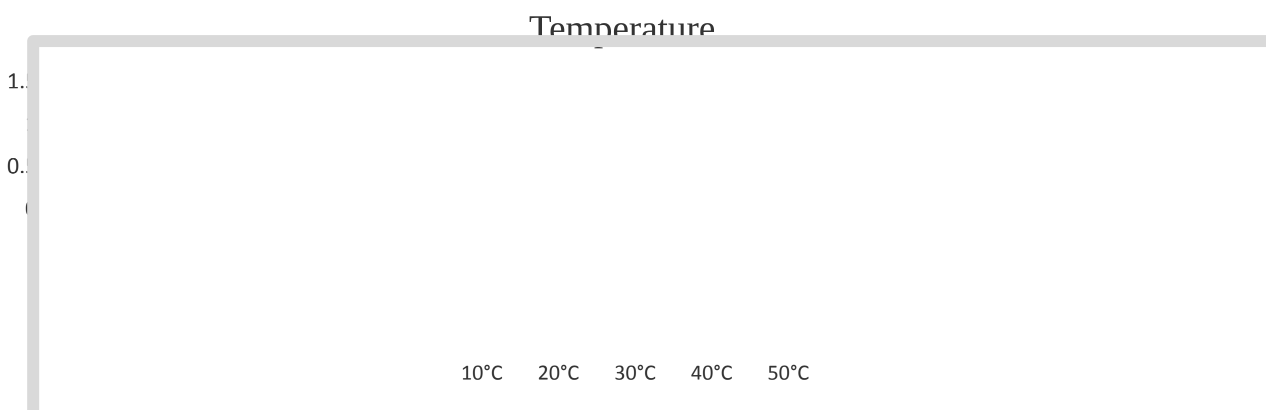


**Fig.2** Effect of pH on growth of actinomycete isolates

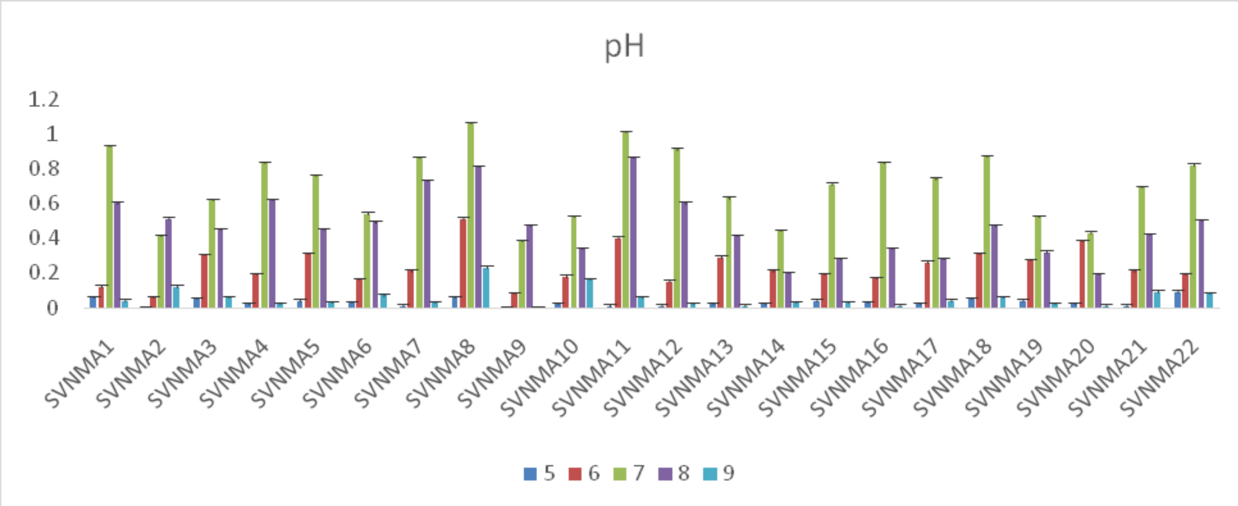
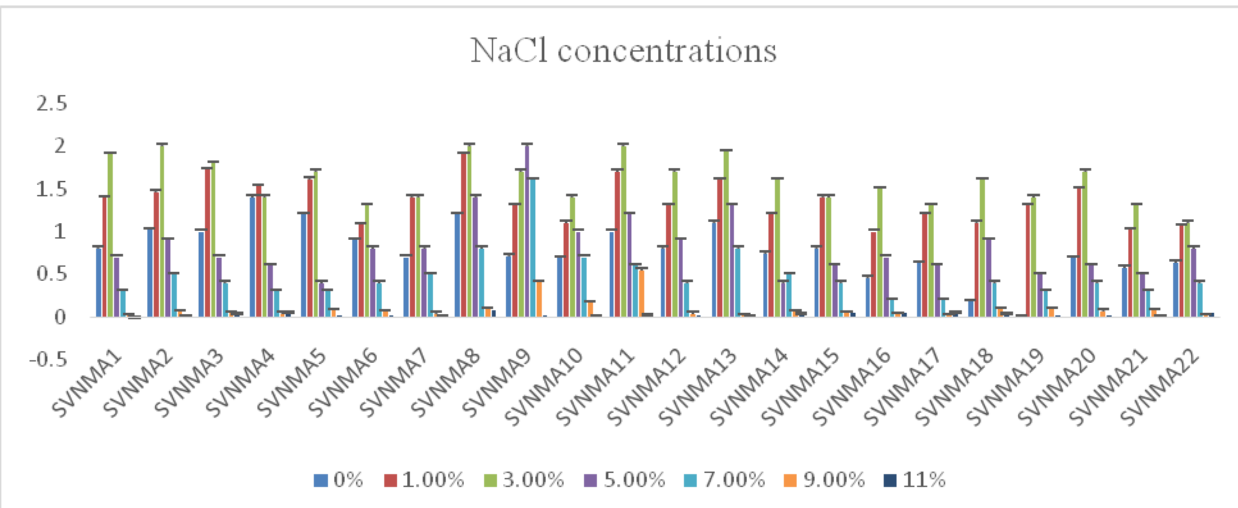



**Fig.3** Effect of NaCl concentrations on growth of actinomycete isolates




In case of pH some isolates exhibited the optimum growth at pH 7.0 and some isolates exhibited the optimum growth at pH 8.0 also (Fig-2).In case of NaCl concentrations some isolates exhibited the optimum growth at 3.0% and some isolates exhibited the optimum growth at 1.0% also (Fig-3). *Streptomyces spp. VITSVK9* showed the maximum growth with highest biomass at 30°C and at 5% of NaCl concentration in the medium and at pH -7.0; and the growth of the strain was inhibited in the absence of NaCl in the medium (Sauravand Kannabiran, 2010).A strain of *Streptomyces grancidicus* with optimum activity at 40°C, pH-7.0 and 1.5% NaCl has been reported in 2015 from Indian soil (Krishnan and Kumar, 2015).

## Screening of selected antibiotic producing halophilic actinomycetes for antibacterial activity

All the 22 isolates SVNMA1-SVNMA22 were screened for antibacterial activity using agar well diffusion assay against two gram positive (*Bacillus subtilis, Staphylococcus aureus*) and two gram negative bacteria (*Escherichia coli*, *Klebsiella pneumonia*).

The screening results showed 27.5% isolates are active against single bacteria, 59.09% were active against two bacteria and 4.5% isolates were active against three bacteria. Among the tested two groups of bacteria gram positive were more sensitive and inhibited by 17 isolates (81.8%) and gram negative bacteria were less sensitive and inhibited by 11 isolates (50%). However, both gram+ve and gram-ve bacteria were inhibited by 6 isolates (27%). The zone of inhibition in case of gram+ve bacteria ranges from 1.0 mm to 15mm and in case of gram–ve bacteria ranges from 1.0 mm to 11 mm. Among the tested gram+ve bacteria *Bacillus subtilis* is more sensitive than *Staphylococcus aureus*. 72% isolates exhibited sensitivity against *Bacillus subtilis* and 36% isolates exhibited sensitive against *Staphylococcus aureus*, 22% isolates were not active against any two gram+ ve bacteria. *Escherichia coli*is more sensitive than *Klebsiella pneumonia*. *Escherichia coli* is sensitive to 45% isolates whereas *Klebsiella pneumonia* is sensitive to 22 isolates. Remaining 50% isolates were not active against any two gram-ve bacteria(Table-4). Three potential isolates with broad spectrum activity or strong antibacterial activity were selected for further studies. They are SVNMA8, SVNMA11 and SVNMA13. While the screening of the novel secondary metabolites, isolated actinomycetes exhibiting more activity against gram positive bacteria than gram negative bacteria were much encountered. This was similar to the findings of another study (Kokare *et al.*, 2004). Actinomycetes have been confirmed as a potential origin of bioactive compounds and the affluent source of secondary metabolites (Suthindhiran and Kannabiran, 2009). Various media and plating methods were employed for the isolation of actinomycetes, the best one is ISP-2 medium and pour plate method. A total of 22 actinomycetes isolates were isolated and cultural characteristics were studied. Aerialmass of the colonies ranged from whitish ash to dark ash, light brown to dark brown, light pink to dark pink. Riverside pigmentation and soluble pigments were not detected in most of the isolates except SVNMA 2, SVNMA 3 and SVNMA 13. Similarly, melanoid pigmentation was absent in all the isolates except SVNMA 13.All the 22 isolates (SVNMA1– SVNMA 22) were screened for antibacterial activity. Among that, SVNMA8, SVNMA 11 and SVNMA 13 were more potent.

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