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## Stress Tolerance and Characterization of *Actinomycetes* from Salt Pan Soils of Nellore District, Andhra Pradesh.

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### ABSTRACT

*Actinomycetes* are of a great significant class of microbial resources, they are the major producers of antibiotics and other bioactive substances. Halophilic *Actinomycetes* were isolated from salt pan soil by pourplate and spread plate method using glycerol asparagine agar medium. The isolates were characterized on the basis of colony morphology, Gram's staining, phenotypic characteristics, aerial mass color and reverse side pigmentation. The antibiotic producing activity was determined by primary and secondary screening method. In this screening process NMA1, NMA2, NMA3, NMA4 and NMA5 were selected. These positive isolates were monitored for stress tolerance at various pH(5-9), temperatures(10°C-50°C) and salt concentration from 0.5%-13%.

**Keywords:** Marine *actinomycetes*, antibacterial activity, stress tolerances, phenotypic characterization

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## INTRODUCTION

*Actinomycetes* is a great significant class of microbial resources, they are great producers of antibiotics and other great value bioactive substances. So far, about two-thirds of the world's antibiotics were produced by *actinomycetes*. *Actinomycetes* population regarded as one of the great significant group of soil population [1], but after that, it is being separated from a several marine samples, including sediments obtained from deep-sea [2&3], even from greatest depth- Mariana Trench [4&5], and also in the surrounding regions of hydrothermal vents [6]. It is now favor that *actinomycetes* can be primitive to the marine environment and that this environment is likely to yield many unusual *actinomycetes* that have the full potential to produce novel antibiotics and other compounds. These marine *actinomycetes* are environmentally safe, aforesaid to their saprophytic relatives in soils, perhaps substantially impacting the advance of complex carbon principals in benthic ocean habitats [7]. However, a well-defined biodiversity and taxonomic investigation of *actinomycetes* is important to identify *actinomycetes* from the marine environment [8]. As the strains with biological action were found in large numbers, we detached active *actinomycete* strains more and more difficult from conventional environment [9]. So that the ability of the discovery of new compounds decreased. Halophiles or salt-tolerant *actinomycetes* are which are increasingly interested throughout the world as research materials of microbial physiology from adverse circumstance. Studies have shown that *Actinomycetes* segregated from the marine environment are creaturely active and have adapted to life in the sea. *Streptomyces* are especially prolific and can produce a tremendous abundant antibiotics (around 80% of the total antibiotic production) and active secondary metabolites [10].

Natural products have postscript to play a highly significant role in the drug authentication and development process; about 28 % of the new chemical entities and 42 % of the anticancer drugs preceded into the worldwide market at intervals 1981 and 2006 were natural products and their derivatives [11]. A study was done by the Santhi et al., [12]. By the collection of two marine *actinomycetes* isolated from different locations of the Manakudi Estuary of Arabian Sea in Tamilnadu, India. *Actinomycetes* are the most efficient and biotechnologically beneficial prokaryotes able to yield broad range of bioactive minor metabolites, such as antibiotics, antitumor agents, immunosuppressive promoters and enzymes. The particular metabolites are accepted to possess antibacterial, antifungal, neuritogenic, ancones, antialgal, antimalarial and anti-inflammatory action [13]. *Actinomycetes* have the scope to incorporate no end of different biologically active secondary metabolites such as vitamins, nutritional materials, herbicides, antibiotics, pesticides, anti-parasitic and enzymes like cellulose and xylanase used in waste treatment [14]. They are free living, saprophytic bacteria and a major source for the Production of antibiotics [15]. Less than one part in 1012 of the earth's soil has been screened for *Actinomycetes* [16]. Only 1-3% of *Streptomyces* antibiotics have been discovered and to find the remaining 97-99% will require modern technologies for screening, selection and enrichment of *Actinomycetes* [17&18].

## MATERIALS AND METHODS

### Sample collection

Soil samples were collected from various locations salt pans of Isukapalli village, Nellore District, India at 14°44'33.6"N-80°06'17.7"E, using Randomized block design. Soil samples were collected up to a depth of 7-8 cm by using sterile gougers. These collected soil samples were immediately placed in sterile polythene bags and stored at 4°C for future use.

### Sample pretreatment and isolation

Heat treatment was given to all the soil samples by keeping them in hot air oven at 50°C for 1hr. These Pretreated soil sample serially diluted with 0.8% NaCl and plated on to Glycerol asparagine agar (international *streptomyces* project, [19 & 20] medium. The plates were incubated at 30°C for 7 days. After 7 days of incubation the colonies were purified by repeated streaking on to fresh Glycerol Aspergine medium and preserved on 80% glycerol. Cycloheximide is used as antibiotic.

### Phenotypic characterization of the isolates

**Aerial mass colour**

For the grouping and description of *Actinomycetes* sp. the color of aerial mycelium was considered as an important character. The color of the aerial mycelium are from white, cream, redbrick and green. When the aerial mass color declines between two colors array, both the colors are recorded. In the cases where aerial mass color of a strain showed intermediate colors, then in that place both the color series were noted [21].

**Reverse side pigments**

All the isolates are grouped into two groups according to their capability to produce characteristic pigments on the reverse side of the colony, labeled as distinctive (+) and not distinctive or none (-).

**Screening**

**Antimicrobial activity**

Glycerol asparagine broth medium was used for the fermentation of *actinomycetes*. In this process isolates were subjected to fermentation and maintained in 500ml conical flask contains 50 ml of glycerol asparagine broth for 10 days at 30°C on rotary shaker at 120 rpm. After incubation the culture broth was centrifuged at 5000 rpm for 15 minutes. Supernatant was collected and pellet was scrapped. The collected supernatant was used for screening of antimicrobial activity [22]. To determine the antibacterial activity-pathogenic bacteria (*Bacillus* sp, *Staphylococcus* sp, *E.Coli* and *Klebsiella* sp.) were cultured on nutrient broth at 37°C for 24 hours. These cultures were swapped on nutrient agar medium. Four wells (2mm in diameter) were prepared in respective seeded agar plates and each well was filled with different concentrations of (10µl, 50µl, 100µl & 150µl) supernatant and 50µl of streptomycin was added in middle well of the each plate to maintain control. The plates were incubated at 37°C for 24 to 48 hours. The Zone of inhibition was recorded.

**Stress resistance**

The stress factors such as temperature, alkalinity and salinity was studied on *Actinomycete* isolates by observing their growth on glycerol asparagine medium under different stress parameters. The effect of temperature was studied by incubating the isolates at 10°C, 20°C 30°C, 40°C and 50°C. The influence of alkalinity on *Actinomycetes* growth was studied by growing the isolates at pH 5, 6, 7, 8 and 9. The *Actinomycetes* were grown on broth media with different NaCl (0.5%, 1%, 3%, 5%, 7% and 10%)

**RESULTS AND DISCUSSION**

A total of twenty two *actinomycete* isolates were isolated from salt pan soil samples. All the isolates were designated as NMA1-NMA22 (Table-1). The isolated strains were filamentous, Gram positive in nature. Results of aerial mass colour, reverse side pigmentation, anti-bacterial activity of *actinomycete* isolates NMA1, NMA2, NMA3, NMA4 and NMA5 were represented in Table 2-3 and Fig 1-5. These 5 isolates were used for further study such as stress resistance i.e. salt tolerance, tolerance to hydrogen ion concentration different temperatures (Tables 2 - 6 and figure 6-8).

**Table.1 Isolation of *Actinomycetes* from various areas in salt pans**

Area	Latitude and longitude	Nature of sample (soil/water)	No. of isolates on ISP medium
Isukapalli, Nellore dist, Andrapradesh	Latitude 14° 44' 33.6" N Longitude 80° 06' 17.7" E	Soil Sample	NMA1, NMA2, NMA3, NMA4, NMA5, NMA6, NMA7, NMA8, NMA9, NMA10, NMA11, N12, NMA13, NMA14, NMA15, NMA16, NMA17, NMA18, NMA19, NMA20, NMA21, NMA22.

**Table.2 Phenotypic characterization of *Actinomycetes* isolates**

S.NO	Name of the isolate	Color of the mycelium	Aerial mycelium	Substrate mycelium	Reverse side Pigmentation
1	NMA1	Dull white	–	+	+
2	NMA2	Dark ochre green	–	+	+
3	NMA3	White	+	–	+
4	NMA4	Dark cream	+	+	+
5	NMA5	Light brownish cream	+	+	+
6	NMA6	Dark cream	+	+	+
7	NMA7	Dull cream	–	+	+
8	NMA8	Light brick reddish cream	+	+	+
9	NMA9	Brick red	+	+	+
10	NMA10	Brick red	+	+	+
11	NMA11	Dull white	+	+	+
12	NMA12	Light brick red	+	+	+
13	NMA13	Ochre green	–	+	+
14	NMA14	Ochre green	–	+	+
15	NMA15	Reddish cream	+	+	+
16	NMA16	Reddish cream	+	+	+
17	NMA17	Dark cream	+	+	+
18	NMA18	Dull white	+	+	+
19	NMA19	Dark ochre green	+	+	+
20	NMA20	Light white	+	+	+
21	NMA21	Dark ochre green	+	+	+
22	NMA22	Light brownish orange	+	+	+

Mycelium(+)Positive, (-) Negative.Riversides pigmentation (+) distinctive,(-)non distinctive

**Table.3 Anti-bacterial activity of *Actinomycete* isolates by agar diffusion method**

S.NO	Name of the isolate	<i>Bacillus</i>	<i>Staphylococcus</i>	<i>E.coli</i>	<i>Klebshiella</i>
1	NMA1	–	+	–	–
2	NMA2	+	+	–	–
3	NMA3	+	+	–	–
4	NMA4	–	–	+	–
5	NMA5	+	+	–	–

+ positive;-negative.

**Table.4 Sodium chloride tolerance on the growth of *Actinomycete* isolates**

S.NO	Name of the isolate	0.50%	3%	5%	7%	9%	11%	13%
1	NMA1	++	++	++	+	+	+	–
2	NMA2	+++	++	+	+	+	+	–
3	NMA3	+++	++	++	+	+	+	–
4	NMA4	++	+++	++	+	+	+	–
5	NMA5	+++	++	+	+	+	+	+

–Negative;+ Poor growth; ++ moderate growth; +++good growth.

**Table.5Effect of different temperature on the growth of *Actinomycete* isolates**

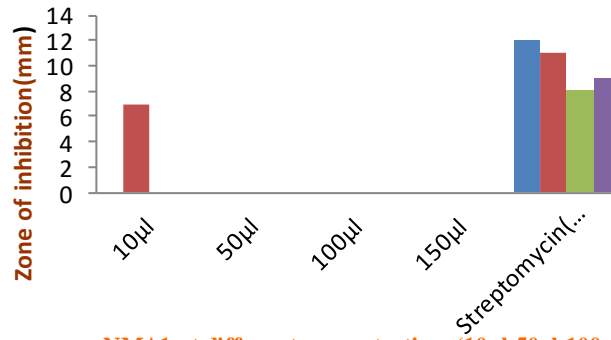
S.NO	Name of the isolate	0°c	10°c	20°c	30°c	40°c	50°c	60°c
1	NMA1	–	+	+	++	++	–	–
2	NMA2	–	+	+	++	++	+	–
3	NMA3	–	+	+	++	+	+	–
4	NMA4	–	+	+	++	++	+	–
5	NMA5	–	+	+	++	+	+	–

+ Poor growth; ++ moderate growth; +++good growth; -Negative.

**Table.6**Effect of different pH on the growth of *Actinomycete* isolates

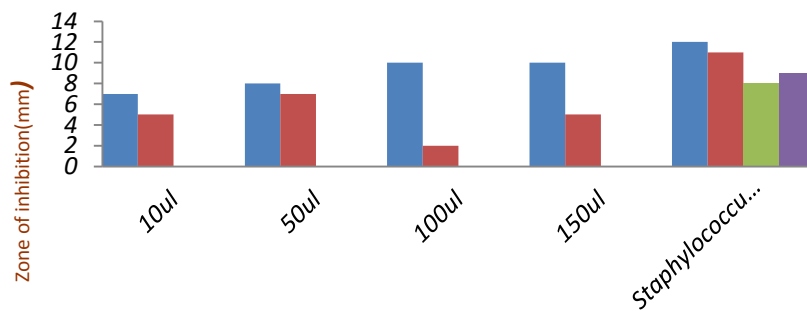
S.NO	Name of the isolate	6	7	8	9
1	NMA1	+	+	+++	++
2	NMA2	+	+	+	++
3	NMA3	+	++	+	++
4	NMA4	+	+	+	++
5	NMA5	+	+	+	++

+ Poor growth; ++ moderate growth; +++good growth; -Negative.



**NMA1 at different concentrations(10ul,50ul,100ul&150ul)**

**Fig-1: ANTIBACTERIAL ACTIVITY OF NMA1**



**NMA2 at different concentrations μl(10,50,100&150)**

**Fig -2: ANTIBACTERIAL ACTIVITY OF NMA2**

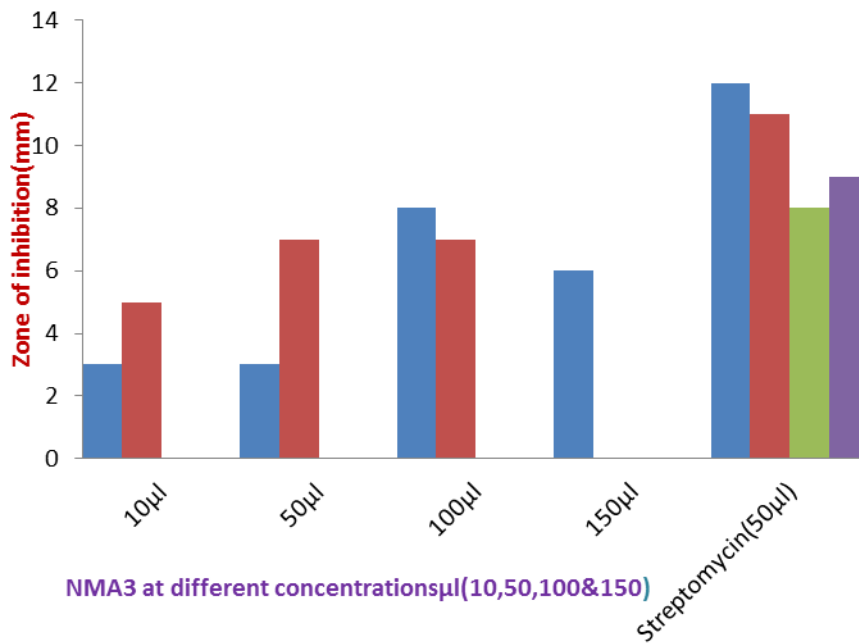
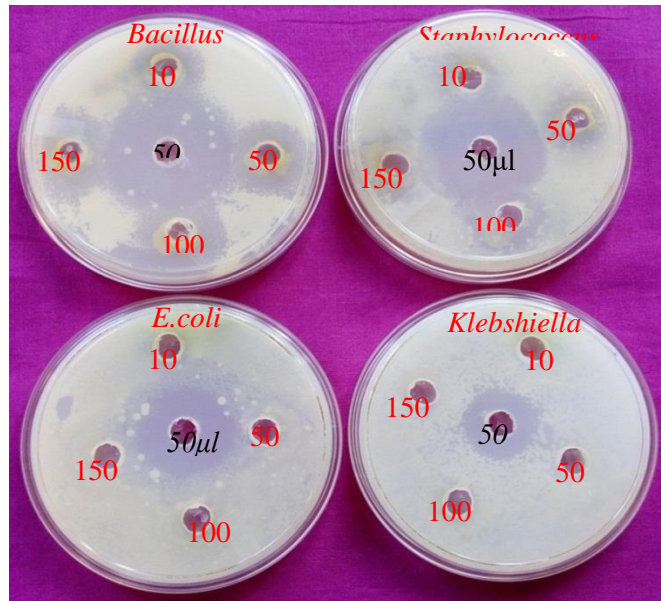
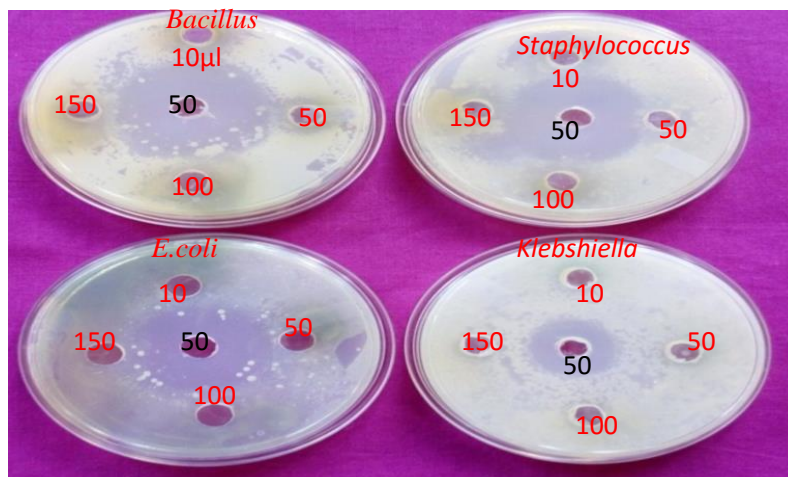
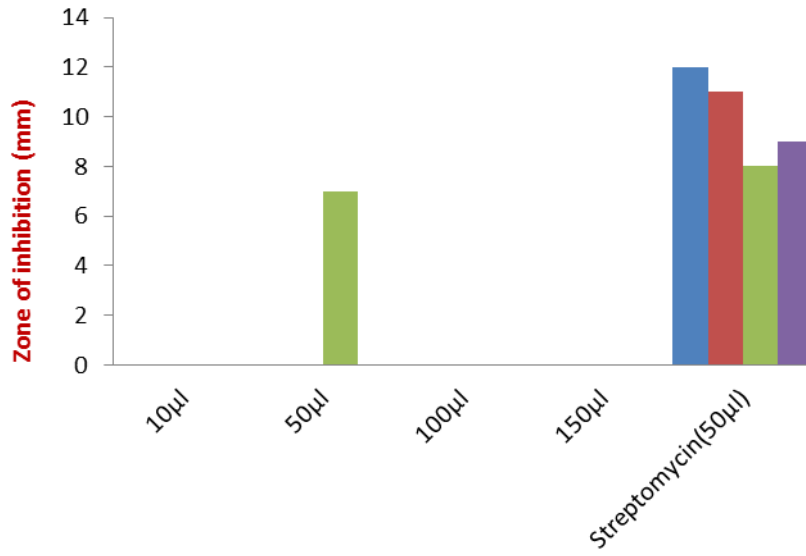


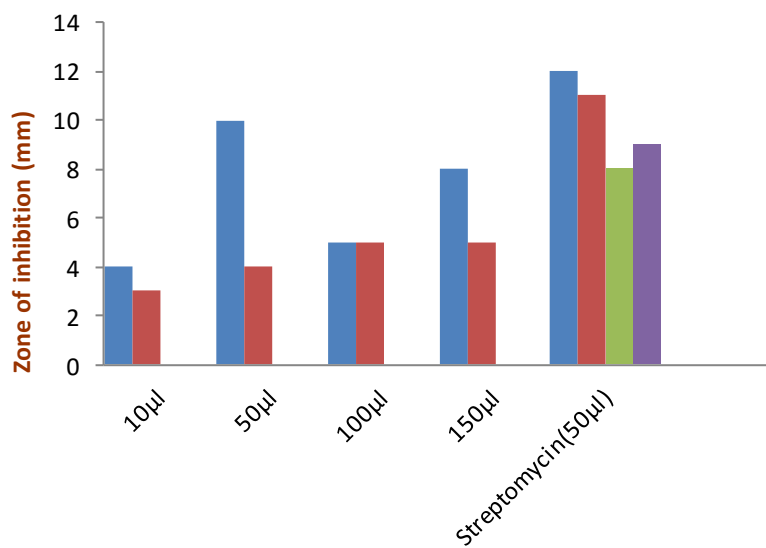
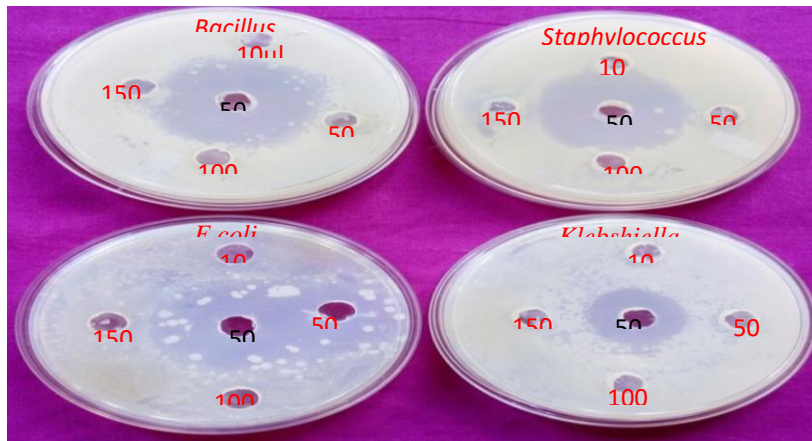
Fig-3: ANTIBACTERIAL ACTIVITY OF NMA3





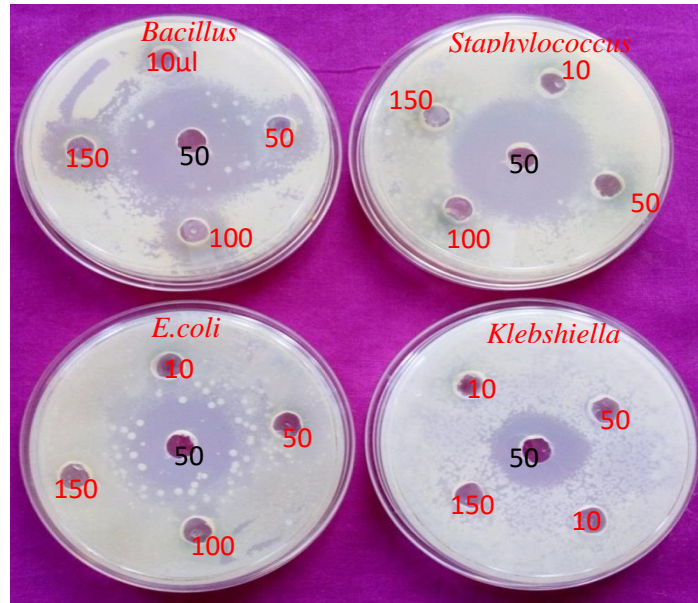
NMA4 at different concentrations µl(10,50,100,150)

Fig4:ANTIMICROBIAL ACTIVVITY OF NMA4



NMA5 at different concentrationsµl(10,50,100,&150)

Fig-5: ANTIBACTERIAL ACTIVITY OF Isolate NMA5



Different stress conditions on growth of actinomycetes isolates

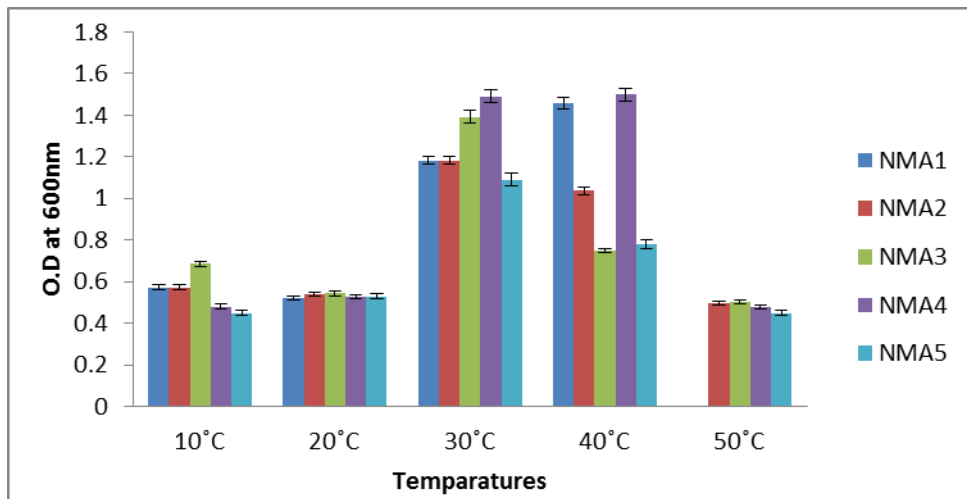


Fig-6: Effect of temperature on growth of isolates

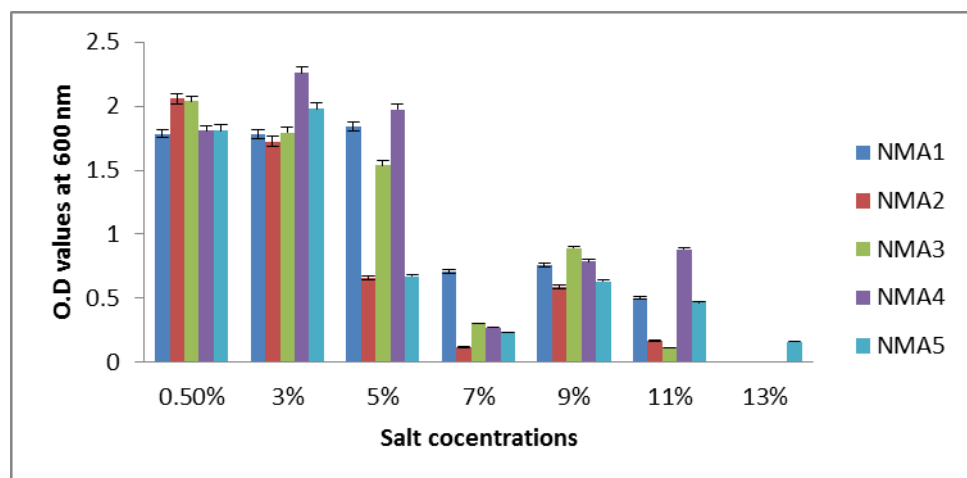


Fig-7: Effect of salt concentrations on growth of isolates



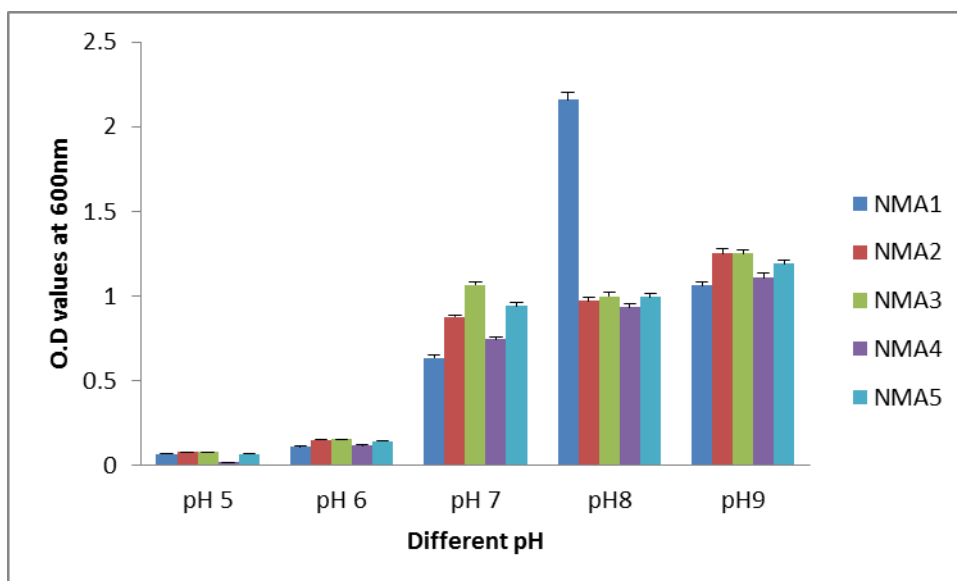


Fig-8: Effect of pH on growth of isolates

Sediment samples collected from marine salt pan soils. Serial dilution of salt pan soils was done. 22 isolates are isolated from soil sample. Marine sediment samples are good for the isolation of *actinomycetes*; [23] reviewed the literature on isolation of *actinomycetes* from marine slugs and suggested that the marine sediment may be valuable for the isolation of novel *actinomycetes*. These 22 isolates phenotypic characterization was done. Marine isolates phenotypic characterization and species relationship by physiological and biochemical characteristics described by (8). The aerial mass color of almost all strains were cream, white ochre green, brick red and only NMA22 has shown brownish orange color. [24] Have also noted that white tinct series of *actinomycetes* they were the predominate forms. Filamentous bacteria belonging to the order *Actinomycetales*, specially *Micromonospora* and *Streptomyces* strains have a rare and authenticate the capacity to produce novel antibiotics [25], hence the continued interest in screening such organisms for new bioactive compounds and it is also effectively increasingly clear that un- and under-explored environments, such as solitary biomes and marine ecosystems, are a very rich source of unique *actinomycetes* which have the capacity to produce attractive new bioactive compounds, including antibiotics [26]. Based on antibacterial activity around 22 isolates, 5 isolates (NMA1, NMA2, NMA3, NMA4 and NMA5) were selected. Stress tolerance studies have shown that, these 5 active isolates are showing growth at pH 5, 6, 7, 8 and 9. Whereas pH 7, 8 & 9 showing the good growth. Temperature like 10°C, 20°C, 30°C, 40°C and 50°C showing the growth. At 30°C and 40°C is the optimum for isolates. Salt concentration like 0.5%, 3%, 5%, 7%, 9%, 11% and 13% showing the growth. Whereas 0.5%, 3% and 5% is optimum for maximum growth of *actinomycetes* isolates.

### CONCLUSION

The investigation for novel metabolites especially from *actinomycetes* requires screening. Number of isolates in order to discover *actinomycete* population with novel compounds of antibiotics interest. The present study was an attempt to use pretreatment methods to select and isolate marine *actinomycetes*, with natural antimicrobial activity against a diversity of microbial pathogens, from the sediments of Nellore district, Andhra Pradesh.

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### REFERENCES

- [1] Kuster, E. In: Gray S, Parkinson T, editors. Liverpool University Press, Liverpool, 1968.
- [2] Walker JD, Colwell RR, Mar Biol, 1975; 30: 193-2012.

- [3] Colquhoun JA, Mexson J, Goodfellow M, Ward AC, Horikoshi K, Bull AT, Antonie van Leeuwenhoek, 1998 ;74: 27-40.
- [4] Takami H, Inoue A, Fuji F, Horikoshi K. FEMS MicrobLett,1997; 152: 279-285.
- [5] Pathom-aree W, Stach JEM, Ward AC, Horikoshi K, Bull AT, Goodfellow M, (10,898 m) Extremophiles, 2006; 10: 181-189.
- [6] Murphy P, Hill RT . Biofuture ,1998; 179: 34-37.
- [7] Mincer TJ, Jensen PR, Kauffman CA, Fenical W. Appl Environ Microbiol , 2002; 68: 5005-5011.
- [8] Das,S, Lyla,P.S and Khan,S.A, Chinese Journal of Oceanology and Limnology 2008; Vol. 26 (2), 166-177.
- [9] Jiang Ch. L., Xu L. H. *Microbial Resources*. Publisher: Science Press,1997; pp: 104-200 (in Chinese).
- [10] Thenmozhi M, Krishnan K. J Nat Environ Sci2011; 2(2).
- [11] Newman DJ, Cragg GM, SnaderKM J Nat Prod 2003; 66:1022–1037.
- [12] SatheejaSanthi.S, Jose.A, and Solomon.J, R.D, International Journal of Current Research , 2010; 3: 020-023.
- [13] Ravikumar S, Inbaneson SJ, Uthiraselvam M, Priya SR, RamuA, Banerjee MB. J Pharm Res 2011; 4(1): 294-296.
- [14] Ogunmwonyi IH, Mazomba N, Mabinya L, Ngwenya E, Green E, Akinpelu DA, et al. Afr J Microbiol Res 2010; 4(21):2223-2230.
- [15] Atta HM, Dabour SM, Desoukey SG.. Am Eurasian J Agric Environ Sci 2009; 5(3): 368-377.
- [16] Baltz RH. Back to the Future. Microbe. 2007; 2:125–31.
- [17] Clardy J, Fischbach MA, Walsh CT. Nat Biotechnol. 2006; 24:1541-50.
- [18] Goodfellow, M and Haynes, Academic Press, London.1984; 453-472.
- [19] Shirling, E.B., Gottlieb, D. *Int. J. Syst. Bacteriol.*, 1966; 16(3): 313 340.
- [20] Murray P. R., Baron J. H., Pfaller M. A., Jorgensen J. H. and Tenover F. C., (Eds.), 8th Ed., American Society for Microbiology, Washington, D.C.2003.
- [21] Shirling, E.B., Gottlieb, D. *Int. J. Syst. Bacteriol.*,1966; 16(3): 313 340.
- [22] Ruan J. Sh. Publisher: Science Press, 1992; pp: 18-109 (in Chinese).
- [23] Goodfellow, M and Haynes, Academic Press, London. 1984; 453-472.
- [24] Vanajakumar, Selvakumar.N and Natarajan.R, 1995;267-274.
- [25] Bentley, S. D., Chater, K. F., Cerdeno-Tarraga, A. M. & 40 other authors. Nature 2002; 417, 141–147.
- [26] Hong,K, Gao.A.H, Xie.Q,Y, Gao,H, Zhuang.L, Lin.H.P, Yu.H.P, Li.J, Yao.X.S, International Basel, Switzerland,2009; 7(1): 24–44.