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Isolation and Screening of Antimicrobial Actinomycetes from the Soil Surrounding Different Medicinal Plants of Saurashtra with Future Scope to Produce Antimicrobial Compounds therefrom

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ABSTRACT

Background: *The need for new and useful compounds to provide assistance and relief in all aspects of the human condition is ever growing. The screening approach has been employed extensively in the search for microorganisms capable of producing useful antibiotics. Actinomycetes have the capability to synthesize those substances.*
Objective: *The object behind research is isolation and screening of Actinomycetes producing new useful antimicrobial compound which may cause death of microbes which has been got resistance.*
Methods*: In our study we are selected 11 medicinal plants from 3 regions of saurashtra. Soil sample collected from microflora part up to 20 cm depth of soil, diluted up to 10^6. Different media used for isolation and make pure colony of actinomycetes. Colonies of actinomycets were selected then color of Aerial and vegetative mycelium was studied. Cultures of isolates were prepared on ISP media and morphological charecteristics studied.*
Results: *Out of 66 isolates 58 were identified up to generic level. For primary screening for antimicrobial activity we used cross streak method. It was found that out of 66 isolates 45 (68.2%) isolates were showing activity against test microbes and 21 (31.8%) isolates not showing any activity.*
Conclusion*: it was concluded that actinomycetes isolates from different soil samples around medicinal plant area of saurashtra region are good source to produce antimicrobial compounds. Too many ecological niches still remain unexplored, needs to be studied for novel actinomycetes.*

Keywords: Actinomycetes; Isolation; Microflora, Mycelium; Antimicrobial

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INTRODUCTION

Natural products are naturally derived metabolites and/or by-products from microorganisms, plants, or animals. These products have been exploited for human use for thousands of years, and plants have been the chief source of compounds used for medicine. These plant products, in general, enhanced the quality of life, reduced pain, suffering, and provided relief, even though an understanding of the chemical nature of bioactive compounds in these complex mixtures and how they functioned remained a mystery. It was not until Pasteur discovered that fermentation is caused by living cells that people seriously began to investigate microbes as a source for bioactive natural products [1].
Soil Microbiology is the branch of science which deals with study of soil microorganisms and their activities in the soil. From the microbiologist view point, soil is one of the most dynamic sites of biological interactions in the nature. It is the region where most of the physical, biological and biochemical reactions related to decomposition of organic weathering of parent rock take place. Soil is an admixture *of* five major components viz. organic matter, mineral matter, soil-air, soil water and soil microorganisms/living organisms. The amount/proposition of these components varies with locality and climate. Soil is an excellent culture media for the growth and development of various microorganisms. Soil is not an inert static material but a medium pulsating with life. Soil is now believed to be dynamic or living system. Soil contains several

distinct groups of microorganisms and amongst them bacteria, fungi, actinomycetes, algae, protozoa and viruses are the most important. But bacteria are more numerous than any other kinds of microorganisms [2].

The microbial population is very high which decreases with depth of soil. Living organisms present in the soil are grouped into two categories.

1. Soil flora (micro flora) e.g. Bacteria, fungi, Actinomycetes, Algae and
2. Soil fauna (micro fauna) animal like eg. Protozoa, Nematodes, earthworms, moles, ants, rodents.

Relative percentage of soil microorganisms are: Bacteria-aerobic (70%), anaerobic (13%), Actinomycetes (13%), Fungi/molds (03%) and others (Algae Protozoa viruses) 0.2-0.8%.

Microbes in the soil are important to us in maintaining soil fertility/productivity, cycling of nutrient elements in the biosphere and sources of industrial products such as enzymes, antibiotics, vitamins, hormones, organic acids etc.

Scope and importance of soil microbiology [3], can be understood in better way by studying aspects like 1. Soil as a living system 2. Soil microbes and plant growth 3. Soil microorganisms and soil structure, 4. Organic matter decomposition 5. Humus formation 6. Biogeochemical cycling of elements 7. Soil microorganisms a bio-control agents 8. Soil microbes and seed germination 9. Biological nitrogen fixation 10. Degradation of pesticides in soil.

Actinomycetes

These are the organisms with characteristics common to both bacteria fungi but yet possessing distinctive features to delimit them into a distinct category. In the strict taxonomic sense, actinomycetes are clubbed with bacteria the same class of Schizomycetes and confined belonging to the important order of actinomycetales. They are unicellular like bacteria, but produce a mycelium which is non-septate and more slender, tike true bacteria they do not have distinct cell-wall and their cell wall is without chitin and cellulose (commonly found in the cell wall of fungi) [4] (Figure 1 and Figure 2).

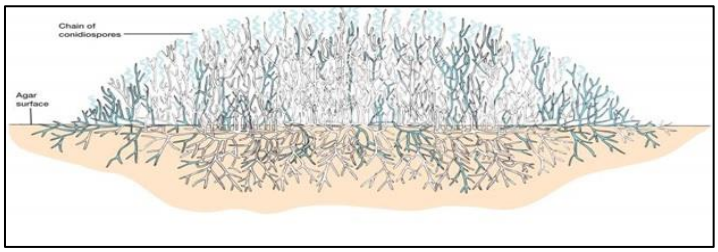

Figure 1: Show powdery consistency and stick firmly to agar surface [5]

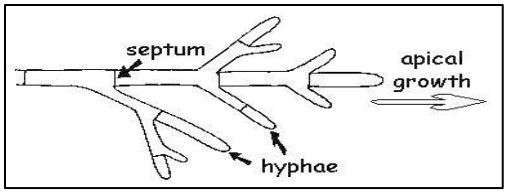

Figure 2: Hyphae and conidia/sporangia like fungi [5]

On culture media unlike slimy distinct colonies of true bacteria which grow quickly, actinomycetes colonies grow slowly, show powdery consistency and stick firmly to agar surface. They produce hyphae and conidia/sporangia like fungi. Certain actinomycetes whose hyphae undergo segmentation resemble bacteria, both morphologically and physiologically. Actinomycetes are numerous and widely distributed in soil and are next to bacteria in abundance.

They are heterotrophic, aerobic and mesophilic (25-30°C) organisms and some species are commonly present in compost and manures are thermophilic growing at 55-65°C temperature (eg. Thermoatinomycetes, Streptomyces). Actinomycetes belonging to the order of Actinomycetales are grouped four families viz Mycobacteriaceae, Actinomycetaceae, Streptomycetaceae and Actinoplanaceae. Actinomycetous genera which are agriculturally and industrially important are present in only two families of Actinomycetaceae and Strepotmycetaceae [6] (Table 1).

Table 1: Number of antibiotics produced by major group of microorganisms [7]

Taxonomic groups	Number of antibiotics
Bacteria other than actinomycetes	950
Actinomycetes	4600
Fungi	1600

Figure 3 represents that most of the drugs mainly produced by Actinomycetes.

Figure 3: Applications of Actinomycete [6]

The greatest variety of antibiotics is produced by Actinomycetes among all microbes. More than 50% of the known natural antibiotics are produced by Actinomycetes. Two thirds of the present day antibiotics are of Actinomycetes origin. The discovery of antimicrobials like penicillin and tetracycline paved the way for better health for millions around the world. However, with the development of antimicrobials, microorganisms have adapted and become resistant to previous antimicrobial agents.

MATERIAL AND METHODS

Selection of Area

In our study we are selected randomly three regions of saurashtra such as Rajkot, Junagadh and Gir somnath.

Selection of Places

Places are selected randomly for collection of samples like Agriculture university gardens, Junagadh, Girnar forest, junagadh, Herbal garden of RK University, Rajkot, Plant Nursary in talala gir forest, Plant nursery in Mangrol.

Selection of Plants

Plants selected on the basis of probability to getting actinomycets which having antimicrobial activity. Plants are *Aloe vera, Azadirachta indica, Syzygium cumini, Datura stramonium, Rosa indica, Pongamia pinnata, Oscimum sanctum, Allium sativum, Allium cepa, Trigonella foenum-graecum and Psoralea corylifolia.*

Samples were collected by inserting a sterilized polyvinyl corer into the sediments with help of stainless sterile scoops and spatula. These articles were sterilized with alcohol before sampling at each location. All the samples were collected up to 20 cm depth of ground surface. These samples collected on sterile tubes and air tight immediately after collection of samples. Care must be taken while collection and transportation of the samples to reduce contamination of the samples. Soil samples air dried at room temperature in laminar air flow for one day and stored at 4°C until further process.

Bacteria commonly grow up to densities around 10^9 CFU/ml, although the maximum densities vary tremendously depending on the species of bacteria and the media they are growing in. Therefore, to get readily countable numbers of bacteria, we make a wide range of dilutions with the goal of having one or two dilutions with countable numbers and clear colony formations.

For dilution of the sample each glassware and articles are previously sterile by autoclave. One gram of dried soil was suspended in 99 ml sterile water in conical flask and shake flask for 1-2 hrs in rotary shaker there after take 1 ml from the flask by sterile pipette and added in sterile test tube for with 9 ml sterile water and vortex for 10 minute and then serially take 1 ml from each test tube follow same procedure and diluted samples up to 10^6 and make aliquots of 0.1 ml from each dilutions.

After sterilization of the media antifungal Nystatin was added (50 μg/ml) then media were poured into Petri dishes under sterile condition (laminar air flow) and allow cooling for sufficient time for solidification of media. The diluted soil samples were taken 0.1 ml quantity and spread on the three of the media with help of sterile bent glass road and kept at 28°C for 1 week, growth of microbes were observed each day and produced actinomycetes colony were purified on the Petri dishes using streak methods on the same media. Three types of media are used for isolation of soil samples Starch Casein Agar Media, Yeast Mannitol Agar Media and Nutrient Agar media.

Based on the growth of the colony and microbes on the all three different types of media mainly Starch casein agar media and yeast mannitol agar media are selected for the further process in study. Identify the colony of the actinomycetes and based on their growth on the media select same media for the preparation of the pure colony of the actinomycetes on that media by streak plate method.

All the pure colony of actinomycetes then picked by inoculationg loop and for further use of the culture we require to storage of the culture for long time duration so that we use glycerol broth method for the preservation of our culture.

Actinomycetes Isolates their Colony and Morphological Characteristics

After incubation typical colonies of actinomycets were selected then color of Aerial mycelium and color of vegetative mycelium (reverse side) was studied. As per colony appearance Actinomycete colonies can easily be distinguished on the plate from those of fungi and true bacteria. They are compact, often leathery giving a conical appearance, and have a dry surface by using 11 plant soil and three regions of saurashtra area we got total 66 actinomycets isolates [8,9].

Colony Characters were Studied as Following Aspects

(a) Colony texture and morphology
(b) Shades of colony pigmentation were observed for various shades such as blue, red, rose yellow, green and brown
(c) Color of colony reverse was observed such as yellow, brown, blue, green, red, orange and violet [8,9].

Morphological Charecters

The cover slip cultures of actinomycets isolates were prepared and morphological charecteristics were studied. The isolates were grown on ISP agar as simple cover slip cultures [10,11].

A sterilized cover slip was carefully inserted at an angle of about 45° in media plate until about an half of the cover slip was in the medium. An actinomycetes isolates were then inoculated along with line where the upper surface of the cover slip meets media. The plates were then incubated at 30°C for 7 days. After incubation, the cover slip was carefully removed with respect to its orientation and placed in upward on a slide and used for microscopic observations.

Microscopic Examination

The following microscopic observations were done using cover slip culture

(a) Presence or absence of substrate mycelium
(b) Fragmentation of substrate mycelium
(c) Presence of sclerotia or sponrangia
(d) Sporulation on substrate mycelium
(e) Spore chain morphology: rectiflexibilities, retinaculiaperti or spirals [9-11].

Primary Screening for Antimicrobial Activity

There are many techniques for detecting antimicrobial activity; most of them are based on methods involving diffusion through solid or semi-solid culture media to inhibit the growth of sensitive microorganisms. The cross-streaking is an easy and relatively rapid method for screening cultures in search for new antibiotics and thus establish a "spectrum" of inhibiting properties of any bacterium, mold, or actinomycetes which will grow discretely on an agar plate [12-15].

Studies have revealed that the ‘cross streak method’ resulted in higher inhibition zones on indicator bacteria than those obtained by agar well diffusion method but the major drawback of the ‘cross streak method’ was difficulty in obtaining quantitative data, since the margins of the zone of inhibition were usually very fuzzy and indistinct but for initial analysis this method is choice of interest.

Hence, in the present study, we reported the isolation as well as the inhibitory effects of actinomycetes isolates on various human pathogenic bacteria using a cross streak method.

The bacteria strains were maintained on slants of Nutrient Agar determination of antimicrobial activities of 66 pure actinomycetes cultures were performed by cross-streak method (CSM). Muller-Hinton agar plates were prepared and inoculated with actinomycetes cultures by a single streak in the centre of the petridish and incubated at 20 ^{o}C for 7 days.

This was done to provide enough time for the active organism to produce the antibiotic substance which will diffuse into the agar medium.

RESULTS AND DISCUSSION

Actinomycetes Isolates

We found total 66 actinomycets isolates by using 11 plant soil and three regions of saurashtra area.

Aerial Mycelium Colors of Actinomycetes Isolates

As shown in Table 2 it was found that actinomycts isolates from different samples were showing different colors of aerial mycelium like white (57.58%), cream (18.18%), yellow (9.09%), gray (6.06%), brown (4.55%), pinkish (1.52%) and bluish (3.03%).

Vegitative Mycelium Colors of Actinomycetes Isolates

As shown in Table 3 it was found that actinomycts isolates from different samples were showing different colors of vegitative mycelium like yellow (48.48%), white (18.18%), cream (12.12%), brown (10.61%), gray (9.09%) and redish (1.52%).

Table 2: Aerial mycelium colony color

Sr. No	Soil sample		Aerial Mycelium Colony Color							Total
			White	Cream	Yellow	Brown	gray	Pinkish	Bluish	
1	AV	No	4	4	1	-	-	-	-	9
		%	44.4	44.4	11.2	-	-	-	-	100
2	AI	No	6	1	1	-	-	-	-	8
		%	75	12.5	12.5	-	-	-	-	100
3	SC	No	4	1	1	-	-	-	-	6
		%	66.67	16.67	16.67	-	-	-	-	100
4	DS	No	3	-	1	-	-	-	-	4
		%	75	-	25	-	-	-	-	100
5	RI	No	5	1	-	-	2	-	-	8
		%	62.5	12.5	-	-	25	-	-	100
6	PP	No	2	1	-	-	-	1	-	4
		%	50	25	-	-	-	25	-	100
7	OS	No	5	1	1	-	1	-	-	8
		%	62.5	12.5	12.5	-	12.5	-	-	100
8	AS	No	3	-	-	2	-	-	-	5
		%	60	-	-	40	-	-	-	100
9	AC	No	1	-	-	1	-	-	2	4
		%	25	-	-	25	-	-	50	100
10	TF	No	3	2	-	-	-	-	-	5
		%	60	40	-	-	-	-	-	100
11	PC	No	2	1	1	1	-	-	-	5
		%	40	20	20	20	-	-	-	100
No. of Colonies			**38**	**12**	**6**	**4**	**3**	**1**	**2**	**66**
Percentage			**57.58**	**18.18**	**9.09**	**6.06**	**4.55**	**1.52**	**3.03**	**100**

Table 3: Vegitative mycelium colony color

Sr. No	Soil sample		Vegitative Mycelium Colony Color						Total
			Yellow	White	Cream	Brown	Gray	Reddish	
1	AV	No	4	2	2	1	-	-	9
		%	44.44	22.22	22.22	11.12	-	-	100
2	AI	No	3	1	1	2	1	-	8
		%	37.5	12.5	12.5	25	12.5	-	100
3	SC	No	3	1	1	1	-	-	6
		%	50	16.67	16.67	16.67	-	-	100
4	DS	No	2	1	1	-	-	-	4
		%	50	25	25	-	-	-	100
5	RI	No	4	1	1	-	2	-	8
		%	50	12.5	12.5	-	25	-	100
6	PP	No	2	1	-	-	-	1	4
		%	50	25	-	-	-	25	100
7	OS	No	4	2	-	-	2	-	8
		%	50	25	-	-	25	-	100
8	AS	No	3	1	-	1	-	-	5
		%	60	20	-	20	-	-	100
9	AC	No	1	-	1	1	1	-	4
		%	25	-	25	25	25	-	100
10	TF	No	2	2	1	-	-	-	5
		%	40	40	20	-	-	-	100
11	PC	No	4	-	-	1	-	-	5
		%	80	-	-	20	-	-	100
No. of Colony			**32**	**12**	**8**	**7**	**6**	**1**	**66**
Percentage			**48.48**	**18.18**	**12.12**	**10.61**	**9.09**	**1.52**	**100**

Microscopic Examination

Out of 66 actinomycetes isolates 58 isolates were identified up to generic level. As shown in Figures 4-10. It was found that out of 58 identified actinomycetes isolates and Figure 11 shows Actinomycetes isolates genus percentage [8,16].

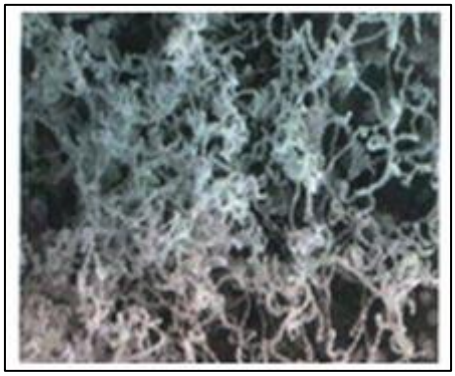

Figure 4: Streptomyces

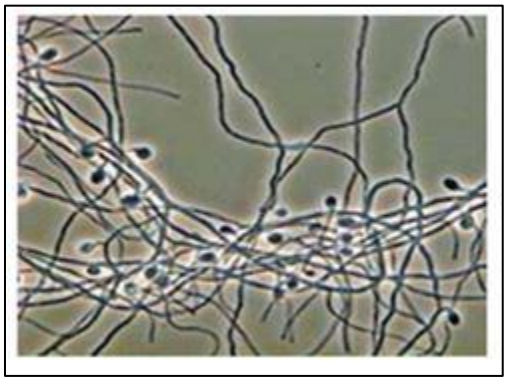

Figure 5: Micromomospora

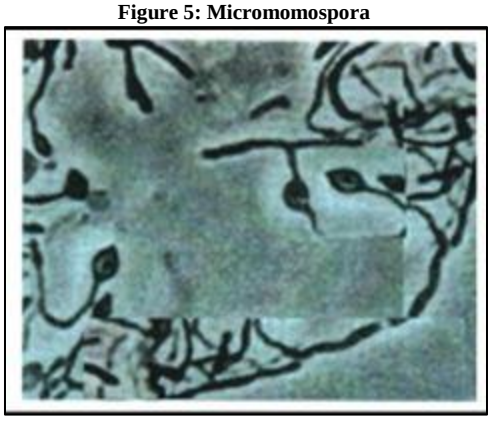

Figure 6: Intrasporangium

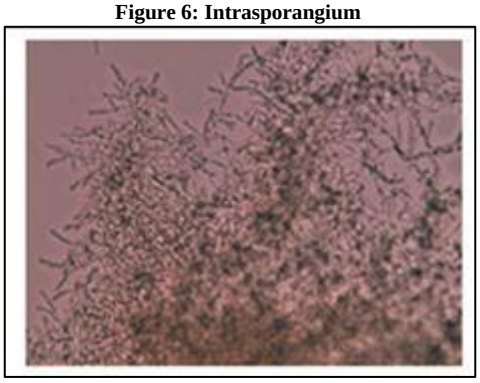

Figure 7: Saccharopolyspora

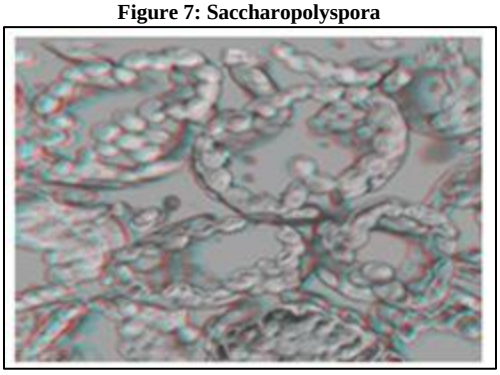

Figure 8: Streptosporangium

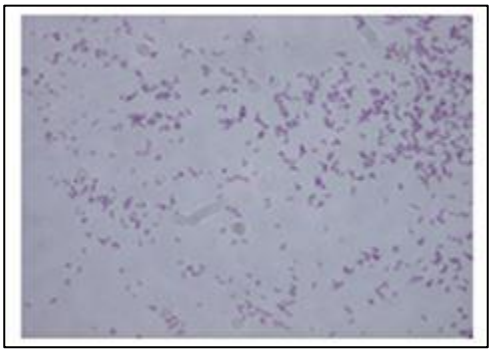

Figure 9: Rhodococcus

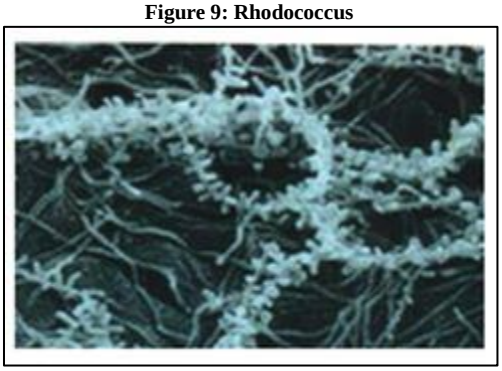

Figure 10: Saccharomonospora

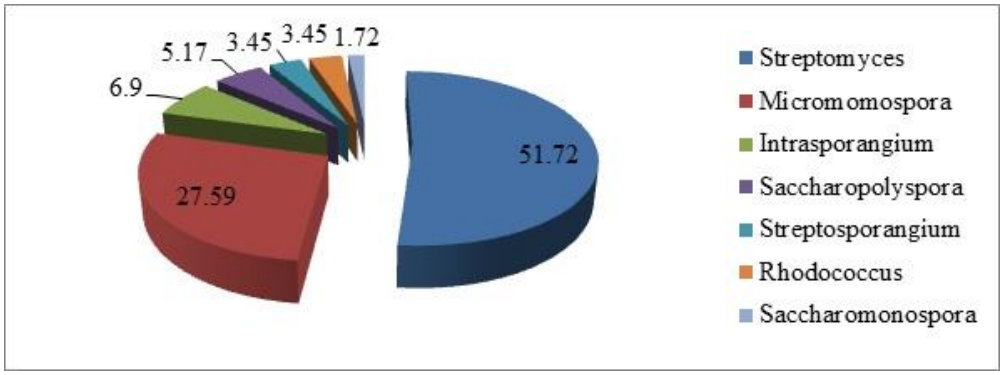

Figure 11: Actinomycetes isolates genus percentage

Screening for Antimicrobial Activity

During primary screening it was found that out of 66 actinomycetes isolates by using *Bacillus subtilis* microbial culture 43 (65%) observed active amongst them 8 (12.12%) shows more than 20 mm zone of inhibition, 21 (31.82%) shows 15 to 20 mm zone of inhibition, 6 (9.09%) shows 10 to 15 mm zone of inhibition, 8 (12.12%) shows less than 10 mm zone of inhibition and 23 (35%) have not shown any zone of inhibition hence inactive against this test pathogen [17-20].

During primary screening it was found that out of 66 actinomycetes isolates by using *Staphylococcus aureus* microbial culture 35 (53%) observed active amongst them 10 (15.15%) shows more than 20 mm zone of inhibition, 16 (24.24%) shows 15 to 20 mm zone of inhibition, 3 (4.55%) shows 10 to 15 mm zone of inhibition, 6 (9.09%) shows less than 10 mm zone of inhibition and 31 (47%) have not shown any zone of inhibition hence inactive against this test pathogen.

During primary screening it was found that out of 66 actinomycetes isolates by using *Proteus vulgaris* microbial culture 29 (44%) observed active amongst them 6 (9.09%) shows more than 20 mm zone of inhibition, 8 (12.12%) shows 15 to 20 mm zone of inhibition, 7 (10.61%) shows 10 to 15 mm zone of inhibition, 8 (12.12%) shows less than 10 mm zone of inhibition and 37 (56%) have not shown any zone of inhibition hence inactive against this test pathogen.

During primary screening it was found that out of 66 actinomycetes isolates by using *Escherichia coli* microbial culture 40 (61%) observed active amongst them 6 (9.09%) shows more than 20 mm zone of inhibition, 21 (31.82%) shows 15 to 20

mm zone of inhibition, 2 (3.03%) shows 10 to 15 mm zone of inhibition, 11 (16.67%) shows less than 10 mm zone of inhibition and 26 (39%) have not shown any zone of inhibition hence inactive against this test pathogen.

During primary screening it was found that out of 66 actinomycetes isolates by using *Klebsiella aerogenes* microbial culture 39 (59%) observed active amongst them 8 (12.12%) shows more than 20 mm zone of inhibition, 19 (28.79%) shows 15 to 20 mm zone of inhibition, 3 (4.55%) shows 10 to 15 mm zone of inhibition, 9 (13.64%) shows less than 10 mm zone of inhibition and 27 (41%) have not shown any zone of inhibition hence inactive against this test pathogen.

During primary screening it was found that out of 66 actinomycetes isolates by using *Pseudomonas aeruginosa* microbial culture 25 (38%) observed active amongst them 3 (4.55%) shows more than 20 mm zone of inhibition, 11 (16.67%) shows 15 to 20 mm zone of inhibition, 2 (3.03%) shows 10 to 15 mm zone of inhibition, 9 (13.64%) shows less than 10 mm zone of inhibition and 41 (62%) have not shown any zone of inhibition hence inactive against this test pathogen.

During primary screening it was found that out of 66 actinomycetes isolates by using *Salmonella typhi* microbial culture 20 (30%) observed active amongst them 0 (0%) shows more than 20 mm zone of inhibition, 10 (15.15%) shows 15 to 20 mm zone of inhibition, 1 (1.52%) shows 10 to 15 mm zone of inhibition, 9 (13.64%) shows less than 10 mm zone of inhibition and 46 (70%) have not shown any zone of inhibition hence inactive against this test pathogen.

During primary screening it was found that out of 66 actinomycetes isolates by using *Enterobacter aerogenes* microbial culture 22 (33%) observed active amongst them 0 (0%) shows more than 20 mm zone of inhibition, 8 (12.12%) shows 15 to 20 mm zone of inhibition, 4 (6.06%) shows 10 to 15 mm zone of inhibition, 10 (15.15%) shows less than 10 mm zone of inhibition and 44 (67%) have not shown any zone of inhibition hence inactive against this test pathogen (Table 4 and Figure 12).

Table 4: Distribution of antagonistic activity of actinomycetes zone of inhibition (mm)

Sr. no.	Test Organism	No active		>20		15 to 20		10 to 15		<10		Total	
		No	%	No	%	No	%	No	%	No	%	No	%
1	*Bs*	23	34.85	8	12.12	21	31.82	6	9.09	8	12.12	66	100
2	*Sa*	31	46.97	10	15.15	16	24.24	3	4.55	6	9.09	66	100
3	*Pv*	37	56.06	6	9.09	8	12.12	7	10.61	8	12.12	66	100
4	*Ec*	26	39.39	6	9.09	21	31.82	2	3.03	11	16.67	66	100
5	*Ka*	27	40.91	8	12.12	19	28.79	3	4.55	9	13.64	66	100
6	*Pa*	41	62.12	3	4.55	11	16.67	2	3.03	9	13.64	66	100
7	*St*	46	69.70	0	0.00	10	15.15	1	1.52	9	13.64	66	100
8	*Ea*	44	66.67	0	0.00	8	12.12	4	6.06	10	15.15	66	100

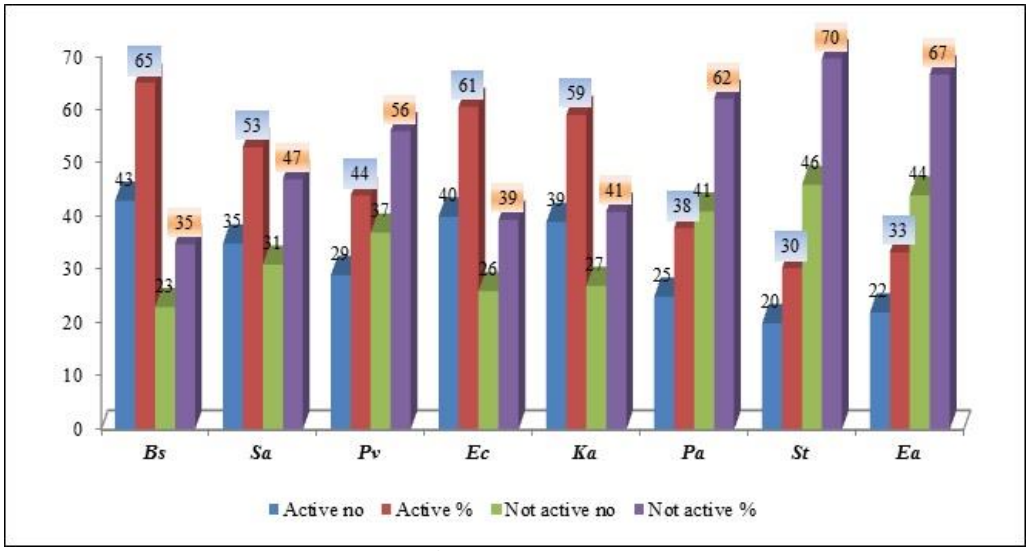

Figure 12: Distribution of antagonistic activity against test microbes

CONCLUSION

It was found that out of 66 actinomycetes isolates 45 (68.2%) actinomycetes isolates were showing activity against test microbes and 21 (31.8%) actinomycetes isolates not showing any activity against test microbes. It was also found that out of 66 actinomycetes isolates 43 (65%), 35 (53%), 29 (44%), 40 (61%), 39 (59%), 25 (38%), 20 (30%) and 22 (33%) were showing antagonistic activity against *Bacillus subtilis, Staphylococcus aureus, Proteus vulgaris, Escherichia coli, Klebsiella aerogenes, Pseudomonas aeruginosa, Salmonella typhi* and *Enterobacter aerogenes* respectively. Thus it was concluded that actinomycetes isolates from different soil samples around medicinal plat area of saurashtra region are good source of antagonistic actinomycetes.

Future Work Plan and Scope

Perform secondary screening, purification and characterization of antimicrobial compounds using different chromatographic techniques followed by *in vitro* microbial assay. Too many ecological niches still remain unexplored, needs to be studied for novel actinomycetes.

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CONFLICT OF INTERESTS

All authors have none to declare.

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