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Study on Biological Significance of Actinomycetes Isolates from Industrial Area

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ABSTRACT

The main focus of this study was to isolate some antibiotic producing actinomycetes strains from soil. Soilsamples were taken from various places of the effluent drainage of Malanpur industrial area, Bhind M.P. Eleven soil samples were collected, serially diluted and spread on actinomycetes isolation agar supplemented with amoxicillin and cycloheximide for inhibition of bacteria and fungi, respectively and incubated at 28 ± 2°C for 7-14 days. Twenty-five potential colonies were further purified and stored in agar slants and glycerol stocks. Morphological and biochemical characterization was done and purified isolates were test against pathogenic microorganisms for screening. Selected isolates with antagonistic properties were inoculated in production media for the extraction of secondary metabolites or antimicrobial products. Agar well diffusion was used for antimicrobial activity of crude extracts against Escherichia coli (E. coli), Methicillin-Resistant Staphylococcus aureus (S. aureus) and Vancomycin-Resistant Enterococci (VRE). Extract of selected 25 isolates were shown to be active against most of the selective microorganism. Zone of inhibition were recorded in the range of 15-19 mm. Isolates AGU 25 has been found most effective against all selective microorganisms. It shows strong inhibition activity against Vancomycin-Resistant Enterococci (VRE). All the isolates of this study were gram positive and their colony morphology was studied microscopically. Potent antibiotics from these actinomycetes could contribute a lot to fight against antibiotic resistant pathogens. Selected isolates have been shown strong antimicrobial activity against resistant pathogens.

Keywords: Actinomycetes; Isolation; Antimicrobial activity; Antibiotics; Antimicrobial product

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INTRODUCTION

Actinomycetes are classified as a group of gram-positive bacteria that are unique for their spore forming abilities and formation of mycelia structures [1]. The population of actinomycetes have been differing with the soil type, soil pH, geographical location and climatic condition [2]. A*ctinobacteria* are widely distributed in both terrestrial and aquatic ecosystems, mainly in soil, where they play an essential role in recycling refractory biomaterials by decomposing complex mixtures of polymers in dead plants, animals and fungal materials. They are also important in soil biodegradation and humus formation as they recycle the nutrients associated with recalcitrant polymers, such as chitin, keratin, and lignocelluloses, this produces several volatile substances like geosmin responsible of the characteristic “wet earth odour” and exhibit diverse physiological and metabolic properties, for example the manufacture of extracellular enzymes [3]. Actinomycetes play a vital role in the soil such as mineralization of organic matters, immobilization of nutrients, antibiosis and production of plant promoters [4]. Actinomycetes are of special interest, since they are known to produce chemically diverse compounds with a wide range of biological activities. The demand for new antibiotics continues to grow due to the rapid emerging of multiple antibiotic resistant pathogens causing life threatening infection. Nature is considered to be the richest and versatile source for new antibiotics in respect of engineered biosynthetic antimicrobial compound [5]. These bacteria have been noted to serve as rich reservoirs of medicinal antibiotics and are therefore extremely relevant to scientists, pharmaceutical industries and agricultural industries [6]. Out of 22,500 bioactive compounds obtained from microbes, greater than 50% of the known natural antibiotics are obtained from actinomycetes. Among actinomycetes, about 7,600 compounds are produced by *Streptomyces*

species [7]. The isolated compounds from actinomycetes has a broad spectrum of biological activities such as antibiotic, antifungal, toxic, cytotoxic, neurotoxic, anti-mitotic, antiviral and anti-neoplastic activities [8]. New targets for active components from actinomycetes have been screened for anti- HIV, immuno-suppression, anti-inflammation, Alzheimer disease, ageing processes, some tropical diseases [9]. Bioactive compounds are characterized by using the techniques of chromatography and spectrophotometry. Chromatographic techniques followed by bioautography are very useful in the identification of bioactive fractions from the metabolite mixture [10].

Novelty of bioactive molecules can be determined by comparing their Rf values with those of known antibiotics from databases to eliminate the well characterized compounds during the screening programs. Nowadays, analytical HPLC is a well-established standard technique for the characterization of bioactive compounds. High resolving power of HPLC serves as important method for isolation and purification of antibiotics. Since, the purified molecule is routinely used in antimicrobial assays and antibiotic preparations, HPLC techniques provide a valuable tool for generating highly pure preparations for characterizing the antimicrobial activities. HPLC has the ability to analyse both volatile and non-volatile compounds; hence it is employed in clinical laboratories [11].

EXPERIMENTAL SECTION

Soil Sampling and Pre-treatment

Soil samples were collected from different niche habitats of Malanpur industrial area, Bhind, M.P. Soil samples were taken from various places of effluent drain of Malanpur industrial area. Samples were collected by inserting a sterilized polyvinyl corer into the sediments. The corer is sterilized with alcohol before sampling at each location. Each collection was made from 6-12 inches depth of the surface of ground. These samples were placed in sterile poly bags and sealed tightly and transported immediately to the laboratory. Soil samples were pretreated with heat at 45˚C in hot plate 3-4 hours, for the removal of moisture from the soil samples. Soil pretreatment was required for inhibiting or eliminating unwanted microorganisms [12].

Isolation of Pure Culture of Actinomycetes

Actinomycetes strains were isolated as pure culture by using standard microbiological method. Soil sample were serially diluted in sterile water up to 10^{-7}. An aliquot of 0.1 ml of each dilution was taken and spread evenly up to over the surface of actinomycete isolation agar (AIA) medium [13] supplemented with nystatin (50 µg/ml). Plates were incubated at 30˚C for 7 days. After 7 days observation, whitish pin-point colonies, characteristic of actinomycetes with a clear zone of inhibition were seen. These pinpoint colonies with inhibitory or clear zone of inhibition were selected and purified.

Morphological Characterization

Actinomycetes isolates was inoculated into seven different International Streptomyces Project media (ISP 1 and ISP 7) and incubated for 5 days at room temperature. The colonies were observed microscopically (100X) and morphology was observed with respect to colour, aerial mycelium, size, nature of colony, reverse side colour and feeling the consistency with a sterile loop [14].

Gram Staining

Smear was prepared by spreading the culture on a glass slide followed by heat drying. The smear was covered with crystal violet for 30-60 second and washed off with water. The smear was covered with Gram's iodine for 30-60 sec followed decolourization with alcohol and washed with water. Finally, the smear was stained with safranin for two minute. After washing and drying the slides were viewed under 100X microscope [15].

Preservation of Actinomycetes Isolates

For the short time storage, purified isolates were inoculated into ISP 1 incubated at 28 ± 2˚C for 2-3 days and was stored at 4˚C. For long storage, purified actinomycetes had been transferred in 40% glycerol stock then preserved in a refrigerator at -20˚C.

Fermentation and Extraction of Secondary Metabolites

Solvent extraction method is generally used to extract the secondary metabolites from terrestrial actinomycetes. Actinomycetes isolates were inoculated in 100 ml Starch Casein Broth and kept for incubation at 30°C for 7 days at 150 rpm rotation [16]. After fermentation, the turbidity of medium was changed and centrifugation was done at 10,000 rpm for 10 minutes to remove cells and debris, now resultant broth was added with equal volume of ethyl acetate to extract secondary metabolites. The ethyl acetate phase was separated from aqueous phase using separating funnel. It was then evaporated to dryness at 45-50˚C.The crude extract was collected, weighed (mg/ml) and store for further use.

Preparation of the Inoculums

The test organisms were procured from IMTECH, Chandigarh. For the preparation of the inoculum, test bacteria were taken from the stock culture and were grown separately in their respective broth and incubated at 37ºC for 24 hours. This culture was used for the antibacterial assays. The inoculum was standardized at 1×10^8 CFU/ml comparing with turbidity standard (0.5 McFarland tube) (Table 1).

Table 1: List of test organisms

S.No	Test organism	Code No
1	*Staphylococcus aureus*	MTCC-25923
2	*Methicillin-resistant Staphylococcus aureus*	ATCC-700789
3	*Salmonella typhi*	ATCC-19430
4	*Shigella flexneri*	ATCC-29903

Antimicrobial Activity

The antimicrobial activity of the compounds was tested by agar well diffusion method. For the analysis of antimicrobial activity, the compound obtained from solvent extraction was dissolved in DMSO (20 mg/ml). The compound was screened for antibacterial activity against multidrug resistant bacteria (*Staphylococcus aureus, Methicillin-Resistant Staphylococcus aureus, Salmonella typhi, Shigella flexneri*). After 24 hrs incubation, the diameter of zone of inhibition was measured and recorded in mm.

Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration of bioactive compound against test organisms was determined by broth two-fold serialdilution method [17]. MIC values of test tubes were observed by taking the optical density (OD) at 600 nm by using spectrophotometer. The tube with least concentration of extract shows no growth has been taken as the MIC value for the respective organism.

Thin Layer Chromatography and Bioautography

Fractionation and purification of extracted secondary metabolites was carried out by TLC. Further detection of antimicrobial activity of purified compounds has done on same TLC plate by bioautography. Mobile solvent Ethyl acetate: Methanol (60:40) was used for TLC. 10 µl of solvent extract was spotted 2cm from the bottom of TLC plate which then travels up the plate by capillary action [18]. When the solvent was travelled almost to the top of the plate, the plate was removed; marked the solvent front with a pencil, and then was allowed to evaporate. The antibiotic compound separated by TLC was dried and put in sterile Petri plate, in which 15 ml of sterile molten nutrient agar seeded with 2% test organism was poured and incubated at 37°C for 24 hrs [19]. After incubation zone of inhibition around the spot of antibiotic was observed. Zone of inhibition was visualized by spraying the TLC plates with 0.2% MTT aqueous solution with 0.1% Triton X-100 followed by incubation in water vapour chamber at 37˚C for half an hour. The sterile zone on the media proved the presence of active antibacterial compounds was determined.

RESULTS AND DISCUSSION

Isolation and Purification of Actinomycetes from Soil Sample

Eleven soil samples were collected at the depth of 6-12 inch from different niche habitats of the effluent drainage of Malanpur industrial area, Bhind, Madhya Pradesh, India. Soil samples were serially diluted and spread over actinomycetes isolation agar plates. Mixed culture was seen (Figure 1), and transferred into another AIA medium plates at 28 ± 2°C for 7-14 days. Pure colonies of actinomycetes were obtained after repeated streaking (Figure 2). On the basis of different morphology, appearance and colour, total 39 colonies are selected and purified on AIA.

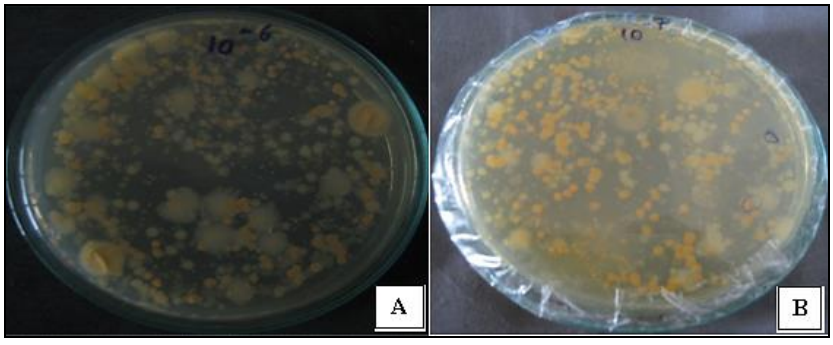


Figure 1: Microbial colonies on AIA plate after 7-14 days of incubation (crowd plate method)

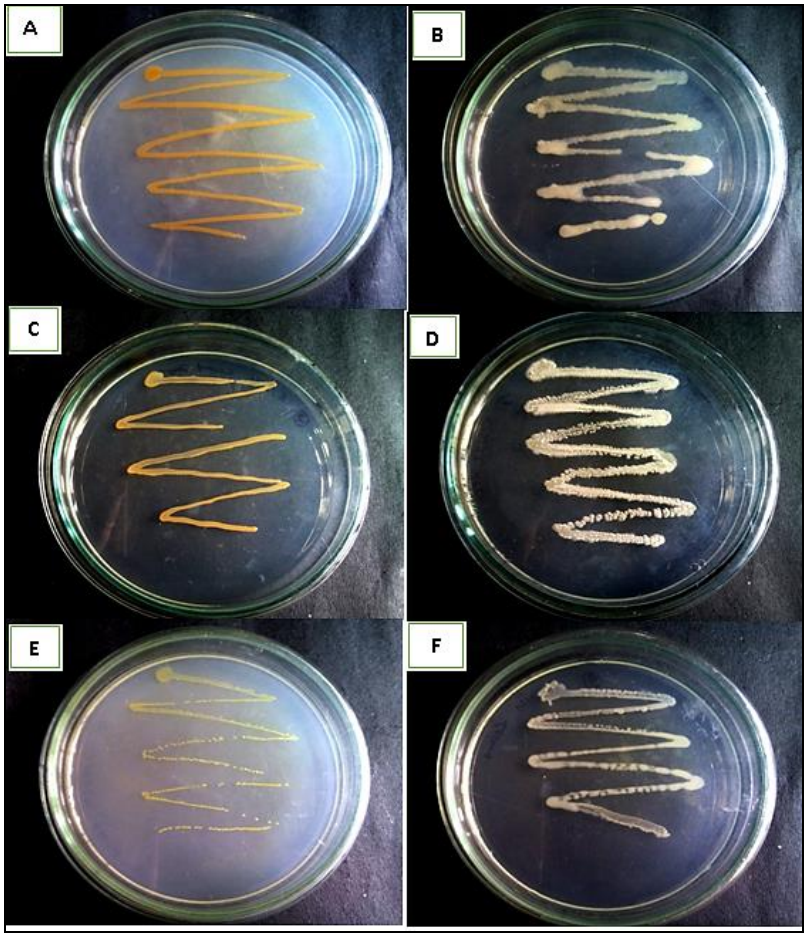


Figure 2: Purified isolates by repeated streaking method

Morphological Characterization

Microscopic characterization:

All 39 isolates were found to be gram positive after staining. Microscopic view of isolates under microscope 100X was found to be filamentous, highly branched, rods, cocci and streptococci in shape (Figure 3).

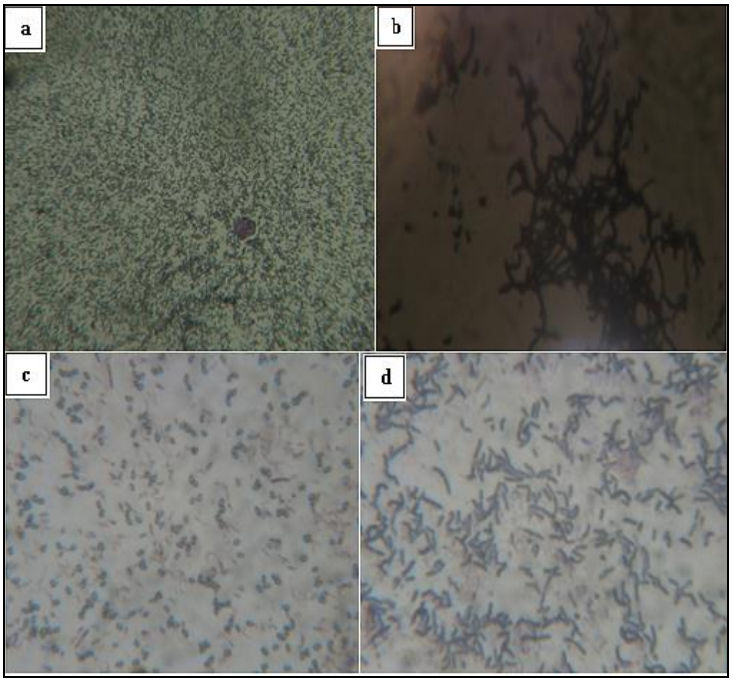


Figure 3: Results of gram staining of purified isolates observed at 100X

Macroscopic characterization:

Morphological characterization of isolates includes the colour of aerial and substrate mycelium, pigment production and branching pattern of isolates. Aerial mycelium of isolates was observed in white, cream, orange, pale yellow, brown and purple in colour. The colour of substrate mycelium of isolates was white, yellow, orange, pale yellow, brown and purple. Results for morphological study were shown in Table 2.

Table 2: Microscopic and morphological characterization (aerial and substrate)

Isolates	Gram Staining	Shape	Mycelium Colour		Pigment Production
			Aerial	Substrate	
MLN1	+ive	Cocci	Cream	Cream	No
MLN 2	+ive	Filamentous	White	White	No
MLN 3	+ive	Cocci	Purple	Purple	Purple
MLN 4	+ive	Diplococci	Orange	Orange	Light Orange
MLN 5	+ive	Cocci	White	White	No
MLN 6	+ive	Rod	White	Green	No
MLN 7	+ive	Diplococci	Cream	Cream	No
MLN 8	+ive	Cocci	Off White	Off White	No
MLN 9	+ive	Cocci	Rust Brown	Rust Brown	Brown
MLN10	+ive	Cocci	Fluorescence	Fluorescence	Fluorescence
MLN 11	+ive	Cocci	Yellow	Yellow	No
MLN 12	+ive	Diplococci	Off White	Off White	No
MLN 13	+ive	Rod	Fluorescence	Fluorescence	Fluorescence
MLN14	+ive	Cocci	Off White	Off White	No
MLN15	+ive	Rod	White	White	No
MLN16	+ive	Diplococci	Off White	Off White	No
MLN17	+ive	Diplococci	White	Pale Yellow	No
MLN18	+ive	Filamentous	White	Off White	No
MLN19	+ive	Cocci	Cream	Fluorescence	Fluorescence
MLN20	+ive	Rod	Cream	Cream	No
MLN21	+ive	Rod	Orange	Orange	Light orange
MLN22	+ive	Diplococci	Off White	Cream	No
MLN23	+ive	Diplococci	Pale yellow	Pale Yellow	Light Yellow
MLN24	+ive	Cocci	White	White	No
MLN25	+ive	Cocci	Light Orange	Orange	Orange
MLN26	+ive	Cocci	Yellow	Yellow	No
MLN27	+ive	Cocci	Off White	Off White	No
MLN28	+ive	Cocci	Off White	Cream	No
MLN29	+ive	Cocci	Light Brown	Light Brown	Brown
MLN30	+ive	Rod	White	White	No
MLN31	+ive	Diplococci	Pale Yellow	Pale Yellow	Light Yellow
MLN32	+ive	Rod	White	White	No
MLN33	+ive	Filamentous	Yellow	Yellow	Light Yellow
MLN 34	+ive	Cocci	Pale Yellow	Pale Yellow	No
MLN 35	+ive	Cocci	Off White	Cream	No
MLN 36	+ive	Diplococci	Cream	Cream	No
MLN 37	+ive	Filamentous	White	White	No
MLN38	+ive	Cocci	White	White	No
MLN 39	+ive	Cocci	White	White	No

Antibacterial Activity of Solvent Extract

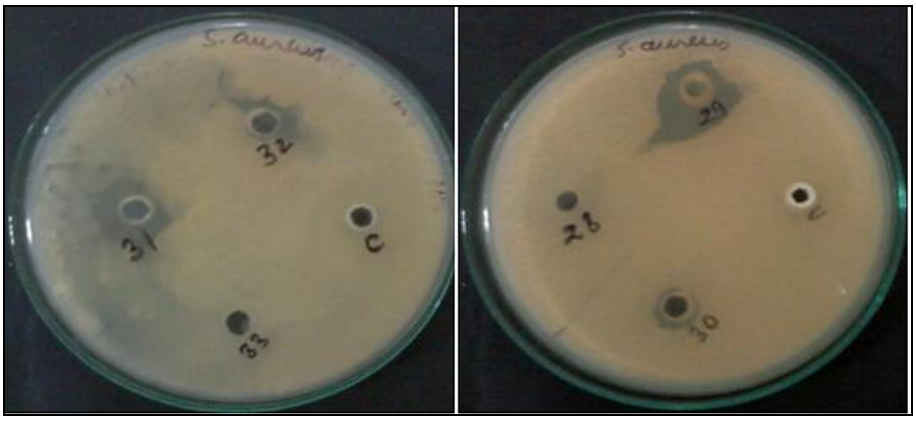
Antibacterial activity against human pathogenic microorganisms was evaluated by agar well diffusion method. Out of thirty-nine isolates, thirty-seven isolates displayed broad spectrum of antibacterial activity against test organisms in the range of ZOI 10- 25 mm. Most of the Crude extract from actinomycetes isolates showed antimicrobial activity against *S. aureus.* Bioactive compounds exhibit moderate activity against *Shigella flexneri* and *Salmonella typhi* and less active against MRSA (Table 3 and Figure 4).

Table 3: Antibacterial activity (ZOI in mm) results against selective pathogenic strains

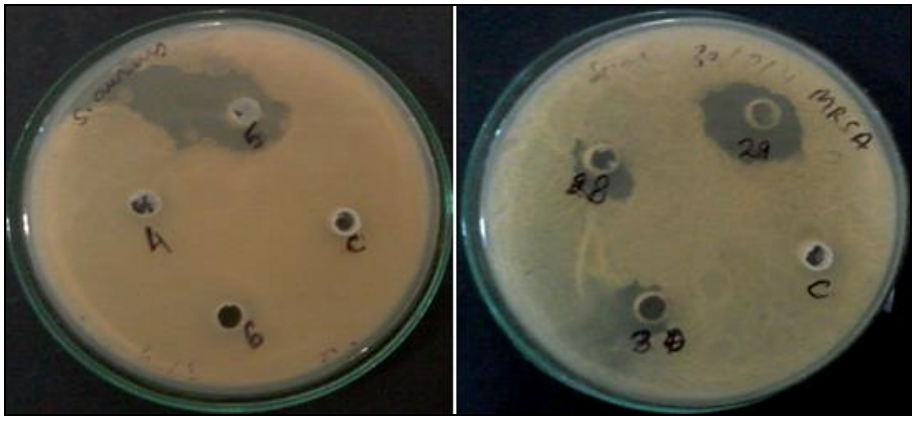
Actinomycetes Isolates	ZOI against Test Organisms (mm)			
	PS1	PS2	PS3	PS4
MLN 1	10	-	-	12
MLN 2	10	11	10	17
MLN 3	12	15	14	14
MLN 4	-	11	10	12
MLN 5	17	20	14	16
MLN 6	10	11	-	12
MLN 7	14	14	12	15
MLN 8	-	17	18	17
MLN 9	15	16	11	15
MLN 10	-	12	-	-
MLN 11	-	10	11	-
MLN 12	-	15	16	15
MLN 13	12	13	11	11
MLN 14	11	-	-	-
MLN 15	10	10	-	-
MLN 16	13	16	15	17
MLN 17	15	17	16	14
MLN 18	-	14	12	14
MLN 19	-	12	12	-
MLN 20	-	-	-	-
MLN 21	-	11	12	-
MLN 22	15	14	-	-
MLN 23	14	16	15	-
MLN 24	20	17	15	16
MLN 25	17	18	17	19
MLN 26	16	18	15	18
MLN 27	16	15	17	25
MLN 28	-	13	12	13
MLN 29	-	17	20	17
MLN 30	-	-	13	-
MLN 31	12	17	16	-
MLN 32	-	14	-	-
MLN 33	-	-	-	11

MLN 34	-	-	-	-
MLN 35	14	15	12	12
MLN 36	12	11	-	-
MLN 37	13	11	-	12
MLN 38	11	15	-	13
MLN 39	16	16	-	15

Note: PS1: *Shigella flexneri;* **PS2:** *Staphylococcus aureus*; **PS3:** *Methicillin resistant Staphylococcu aureus*; **PS4:** *Salmonella typhi*

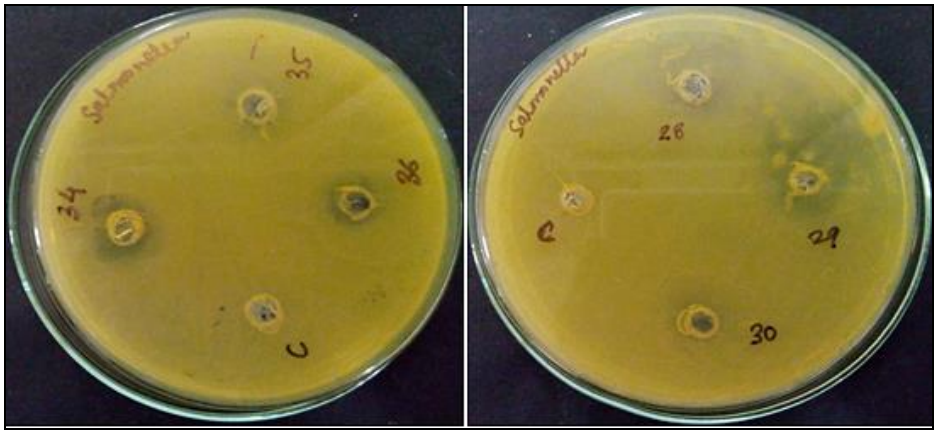


S. aureus ***S. aureus***

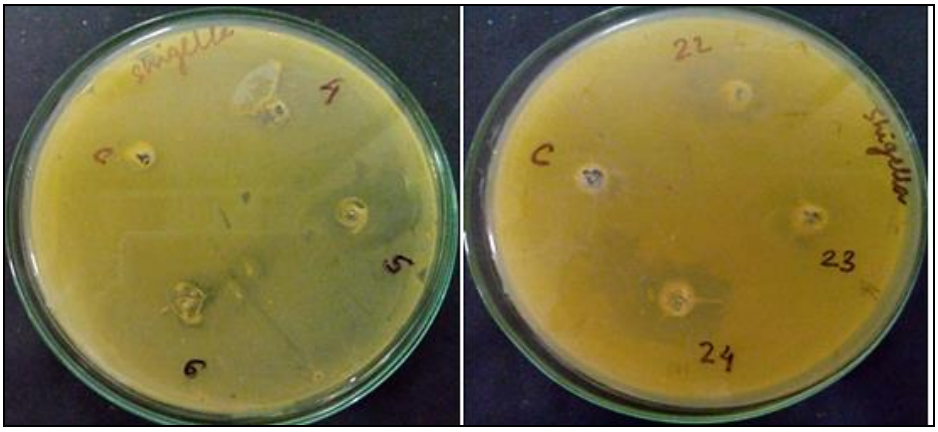


S. aureus ***Methicillin resistant S. aureus***

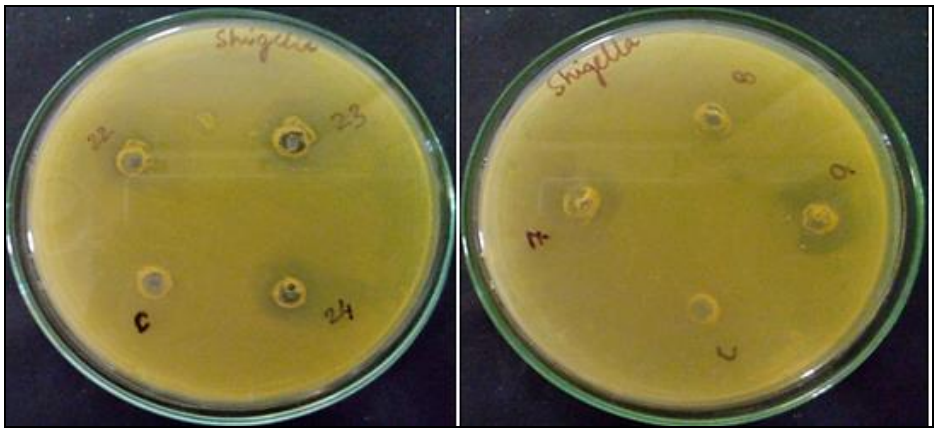
Methicillin resistant S. aureus ***Salmonella typhi***



Salmonella typhi ***Salmonella typhi***



Shigella flexneri ***Shigella flexneri***



Shigella flexneri ***Shigella flexneri***

Figure 4: ZOI of bioactive compounds against pathogenic strains

Minimum Inhibitory Concentration (MIC)

Bioactive compounds were found to be more effective against *S. aureus*. Minimum inhibitory concentration (MIC) of selected bioactive compound that exhibit potent activity against *S. aureus* was determined. The Minimum inhibitory concentration test (MIC) showed gradual decrease in optical density with addition of supernatant in increasing volume; resulting in inhibition of growth of *S. aureus*. Optical density of control was found to be 1.52. The optical density and MIC value of bioactive compounds is depicted below in Table 4.

Table 4: MIC of the bioactive compounds from actinomycetes

Bioactive compounds	Optical Density at 600nm against *S. aureus*				MIC of bioactive compounds (mg/ml)
	20 mg/ml	10 mg/ml	5 mg/ml	2.5 mg/ml	
MLN 5	0.245	0.85	1.373	1.44	10
MLN 8	0.28	0.417	0.853	0.927	5
MLN 16	0.285	0.335	1.07	1.295	10
MLN 17	0.187	0.195	0.521	1.416	5
MLN 24	0.181	0.363	0.78	0.952	5
MLN 25	0.118	0.269	0.307	0.875	5
MLN 26	0.061	0.083	0.091	0.126	5
MLN 27	0.095	0.115	0.13	0.793	2.5
MLN 29	0.345	0.529	0.705	1.02	5
MLN 31	0.293	0.384	0.601	0.983	5
MLN 39	0.194	0.274	0.593	1.2	5

Thin Layer Chromatography and Bioautography

Characterization of selected bioactive compounds were done by using chromatographic technique; TLC. Different solvent system (Ethyl acetate: Methanol – 60:40, Butanol: acetic acid: water- 4:1:5, chloroform: methanol- 10:90) were used to fractionate the bioactive compounds. Ethyl acetate: Methanol- 60:40 were found to be best solvent system for TLC. Chromatographic techniques followed by bioautography are very useful in the identification of bioactive fractions from the metabolite mixture. The Rf value of fractions were estimated. Rf values of some antibiotics determined by means of thin layer chromatography are depicted in Table 5 (Figure 5).

Table 5: Rf values of bioactive compounds produced by actinomycetes

Bioactive compounds	Rf values
MLN 5	0.85
MLN 8	0.62
MLN 16	0.74
MLN 17	0.68
MLN 24	0.66
MLN 25	0.6
MLN 26	0.77
MLN 27	0.72
MLN 29	0.76
MLN 31	0.69
MLN 39	0.83

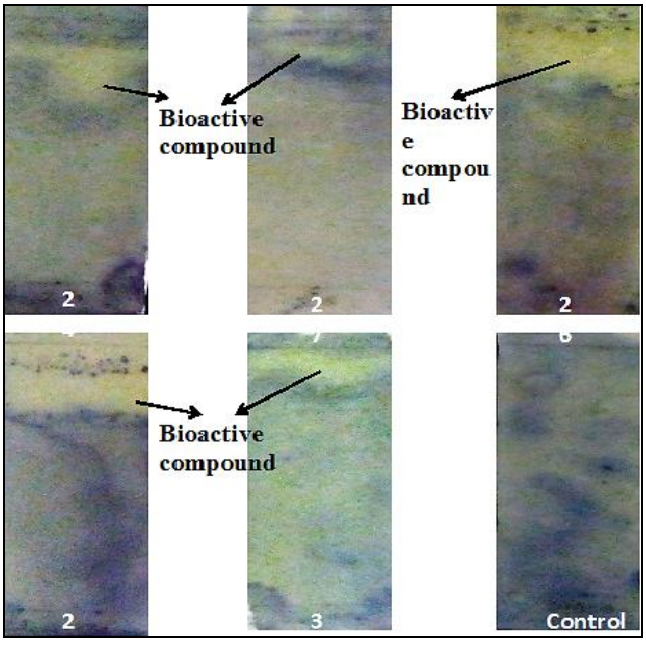


Figure 5: Bioautography of bioactive compounds produced by actinomycetes

CONCLUSION

All the isolates of this study were gram positive and their colony morphology was studied microscopically. Potent antibiotics from these actinomycetes could contribute a lot to fight against antibiotic resistant pathogens. Selected isolates have been shown strong antimicrobial activity against resistant pathogens.

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