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Research Article

ISOLATION AND CHARACTERIZATION OF ANTI-MRSA PRODUCING ACTINOMYCETES FROM SOIL

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ABSTRACT

The aim of the present study was to isolation and production of antibiotics from *Actinomycetes* spp. from the soil sample. The antibiotic produced by *Actinomycetes* spp. were tested against MRSA strains isolated from clinical sample. 20 soil samples were collected from different areas or localities of Nagpur city in different sterilized plastic locking bags. The soil samples were serially diluted on Actinomycete Isolation Agar (AIA) and different strains was identified on the basis of morphological, biochemical and cultural characteristics. The antibiotic susceptibility pattern was performed of well diffusion method. In this study, 28 *Actinomycete* isolates were obtained from 20 soil samples. The isolates were grown in starch casein broth and after incubation, broths were centrifuged and the secondary metabolites were extracted in ethyl acetate and used as test sample for antimicrobial assay against MRSA by agar well diffusion method in MHA medium. Among all the isolates, only 6 had shown the antagonistic effect against MRSA and the zones of inhibition obtained were in the range of 13-26mm. This study showed that the drug obtained from *Actinomycetes*, which is a group of organisms resembling both characteristics of bacteria and fungi showed antibacterial activity against MRSA.

INTRODUCTION

The WHO defines antimicrobial resistance as, "a microorganism's resistance to an antimicrobial drug that was once able to treat an infection by that microorganism. A person cannot become resistant to antibiotics". Resistance is the property of the microbe, not the person or other organism infected by the microbe. (*Francois P et. al. 2008)* Antimicrobial resistance (AMR) is when microbes are less treatable with one or more medication used to treat or prevent infection. This makes these medications less effective in both treating and preventing infection. Resistant microbes may require other medications or higher doses – often with more side effects, some of which may be life-threatening on their own. Some infections become completely untreatable due to resistance. All classes of microbes develop resistance: fungi –antifungal resistance, viruses – antiviral resistance, protozoans – antiprotozoal resistance, and bacteria – antibiotic resistance. Microbes which are resistant to multiple antimicrobials are termed *multidrug resistant* (MDR) (or, sometimes in the lay press, *superbugs*). (*Jensen SO et al. 2009).* Antimicrobial resistance is a growing problem in the world and causes millions of deaths every year. (Pantosti A *et. al.* 2007)

Antibiotics should only be used when needed and only when prescribed. Health care providers should try to minimize the spread of resistant infections by using proper sanitations techniques including hand washing or disinfecting between each patient. Prescribing the correct antibiotic is important and doses should not be skipped. The shortest duration needed should be used. Narrow-spectrum antibiotics should be used rather than broad-spectrum antibiotics when possible. Cultures should be taken before treatment when indicated and treatment potentially changed based on the susceptibility report.(Leekha, Surbhi *et. al.* 2011)

Call for new antibiotic therapies have been issued, but there is continuing decline in the number of approved drugs. (Woodford N *et. al.* 2007) Infection by resistant microbes may occur outside of a healthcare institution or within a healthcare institution. (Cassir N *et. al.* 2014) Common types of drug-resistant bacteria include methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA), extended spectrum beta-lactamase (ESBL), vancomycin-resistant *Enterococcus* (VRE), multidrug-resistant *A. baumannii* (MRAB). (Michael CA *et. al.* 2014).

Staphylococcus aureus is a Gram-positive coccal bacterium that is a member of the Firmicutes and is frequently found in the nose, respiratory tract, and on the skin. It is often positive for catalase and nitrate reduction. Although *S. aureus* is not always pathogenic, it is a common

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cause of skin infections such as abscesses, respiratory infections such as sinusitis, and food poisoning. Pathogenic strains often promote infections by producing potent protein toxins, and expressing cell-surface proteins that bind and inactivate antibodies. The emergence of antibiotic-resistant strains of *S. aureus* such as MRSA is a worldwide problem in clinical medicine. (Ogston A 1984)

Methicillin – resistant *Staphylococcus aureus* (MRSA) is a bacterium responsible for several difficult to treat infections in humans. It is also called oxacillin - resistant *Staphylococcus aureus* (ORSA). In the 1950's, many infections became resistant to penicillin and methicillin [16]. MRSA is any strain of *Staphylococcus aureus* that has developed, through the process of natural selection, resistance to beta – lactam antibiotics, which include the penicillins (methicillin, dicloxacillin, nafcillin, oxacillin etc.). MRSA is especially troublesome in hospitals, prisons, and nursing homes, where patients with open wounds, invasive devices, and weakened immune systems are at greater risk of nosocomial infection than the general public. MRSA began as a hospital-acquired infection, but has developed limited endemic status and is now sometimes community-acquired and livestock-acquired. The terms HA-MRSA (healthcare-associated MRSA), CA-MRSA (community-associated MRSA) and LA-MRSA (livestock-associated) reflect this distinction. (Moran GJ *et. al*. 2006)

Depending on the severity of "MRSA" infections, improper preventive measures and also long term use of drugs causing various side effects used for "MRSA" treatment, there is a need to isolate or find a new drug having least or no side effects with high efficiency of treating the infection. This study approaches new drug can be obtained from *Actinomycetes*, which is a group of organisms resembling both characteristics of bacteria and fungi.

The *Actinomycetes* is used to indicate organisms belonging to the *Actinomycetales*, a major subdivision of the Prokaryotae, the kingdom that comprises all organisms with a prokaryotic cell. Sometimes the names "*Actinomycetes*" are used restrictively for the members of the genus *Actinomyces* only. *Actinomycetes* were long regarded as fungi, as is reflected in their Greek name ("action"= ray, "mykes" = mushroom or fungus). The name refers to the radial arrangement of filaments in *Actinomyces bovis* sulphur granules. (Kluytmans J *et. al.*1997) The organisms with characteristics common to both bacteria and fungi, but yet possessing distinctive features to delimit them in to a distinct category are the *Actinomycetes*. In the strict taxonomic sense, *Actinomycetes* are clubbed with bacteria the same class of *Schizomycetes* and confined to the order *Actinomycetales*. (Agriinfo 2015) It is any member of a heterogeneous group of gram-positive, generally, anaerobic bacteria noted for a filamentous and branching.

Actinomycetes are numerous and widely distributed in soil and are next to bacteria in abundance. They are widely distributed in the soil, compost etc. They are sensitive to acidity/low pH (optimum pH range 6.5 to 8.0) and waterlogged soil conditions. The population of *Actinomycetes* increase with depth of soil even up to horizon 'C' of a soil profiler. They are heterotrophic aerobic and mesophilic (25-30^{0}C) organisms and some species are commonly present in compost and manures are thermophilic growing at 55-65^{0}C temperature (eg: *Thermoactinomycetes*, *Streptomyces*) [104]. A number of *Actinomycetes* form a close association with plants and act as growth – promoting and biocontrol agents. Some *Actinomycetes* form lichen-like associations with green algae called actinolinchens. Though most *Actinomycetes* are saprophytes (feeding on decaying organic material), some caused diseases in plants (e.g. potatoscab, gall and wilt) and animals. Most of the *Actinomycetes* are aerobic, but a few can grow under anaerobic conditions. *Actinomycetes* belonging to the order of *Actinomycetales* are grouped under four families viz *Micobacteriaceae*, *Actinomycetaceae*, *Streptomycetaceae* and *Actinoplanaceae*. Actinomycetous genera which are agriculturally and industrially important are present in only two families of *Actinomycetaceae* and *Streptomycetaceae*. In order of abundance of soils, the common genera of *Actinomycetes* are *Streptomyces* (nearly 70%). *Nocardia* and *Micromonospora* although *Actinomycetes*, *Actinoplanes*, *Micromonospora* and *Streptosporangium* are also encountered. (Hotam Singh Chaudhary 2013)

Actinomycetes are also the source of numerous antibiotics. Most of actinomycital antibiotics used to combat plant diseases belonging to the genus *Streptomyces* and are used as direct pesticides. Other genera also demonstrate promising biocontrol properties. Among the antibiotics obtained from *Actinomycetes* which are used in human and veterinary medicine are amphotericin, cycloheximide, nystain and streptomycin. (Hotam Singh Chaudhary 2013)

MATERIAL AND METHOD

Isolation of Actinomycetes from different soil samples

Soil samples were collected from different areas or localities of Nagpur in different sterilized plastic locking bags. Serial dilutions were prepared up to 10^{-7} using sterile distilled water and agitated by simply shaking and then allowed to settle. Actinomycete Isolation Agar (AIA) was prepared as per the composition containing cycloheximide (anti-fungal agent) at concentration of 50µg/ml, sterilized, poured into sterile petri plates and were allowed to solidify.0.1ml of each dilution from 10^{-4} to 10^{-6} was inoculated in Actinomycete Isolation Agar plates previously solidified by spread plate technique using sterile glass spreader. The plates were incubated at 35^0-37^{0}C for two to three days until good growth was observed. (Pine L, Waksman SA, Hopwood DA 1985) Isolated colonies were maintained on different Actinomycete Isolation Agar slant for further study.

Identification of Actinomycetes from different soil samples:

Actinomycete was characterized by various morphological and biochemical methods. Morphological characterization consisted of macroscopic and microscopic methods including ~ Gram Staining, motility, appearance, shape, margin and elevation of the colony, the colour of spores, aerial mycelium and substrate mycelium, gram staining, diffusible pigment, motility of spores, spore chain, and spore surface. (Hemashenpagam N 2011, C. manjula 2009) Biochemical characterization consisted of various biochemical tests including sugar utilization, urease test, catalase production, H_2S production, nitrate reduction test, catalase test, oxidase test, IMViC test and antibiotic susceptibility testing.

Screening of Actinomycete cultures for Antibiotic production against MRSA

Starch Casein Broth was prepared as per the composition, sterilized and cooled. Spore suspension of all *Actinomycete* isolate was inoculated in 20ml of starch casein broth containing cycloheximide (anti-fungal agent) at a concentration of 50µg/ml in 100ml conical flasks. Flasks were incubated at 35^0-37^0C for seven days. After incubation, a slight colour change was observed in the broth from white to dark yellow. Broth was centrifuged at 10,000 rpm for 10 minutes in a cooling centrifuge. Mueller Hinton Agar (MHA) was prepared as per the composition, sterilized and poured into sterile petri plates and allowed to solidify. 6mm wells were bored in MHA plates (2 wells per plate). Centrifuged broth was mixed with equal amount of ethyl acetate and vigorously shaken in separating funnel and allowed to settle for few minutes to obtain two separate layers. The bottom layer was removed and discarded. The upper layer liquid present in the separating funnel was collected in clean 100ml conical flasks. (This upper layer is believed to contain added ethyl acetate along with the bioactive/antimicrobial compound/secondary metabolites produced by *Actinomycetes* during incubation of seven days). 100-150µl of this collected liquid was loaded into one of the wells bored in MHA plates swabbed with MRSA and an equal amount of fresh ethyl acetate was loaded into another well of the same plate as a control. Plates were incubated at 37^0C for 24 hours and observed for the zone of inhibition. (Kirby-Bauer, 2002)

RESULTS AND DISCUSSION

Table 1 Antibiotic susceptibility pattern of MRSA.

Sr.No.	Antibiotic	Disc Content (µg)	Zone Size (mm)	Inference
1.	Linezolid (Lz^{30})	30	35	Sensitive
2.	Vancomycin(VA^{30})	30	19	Sensitive
3.	Amikacin(Ak^{30})	30	NZ	Resistant
4.	Ofloxacin(Of^{5})	5	12	Resistant
5.	Gentamicin(G^{10})	10	NZ	Resistant
6.	Azithromycin(At^{15})	15	NZ	Resistant
7.	Teicoplanin(TE^{30})	30	18	Sensitive
8.	Lomefloxacin(Lm^{10})	10	NZ	Resistant

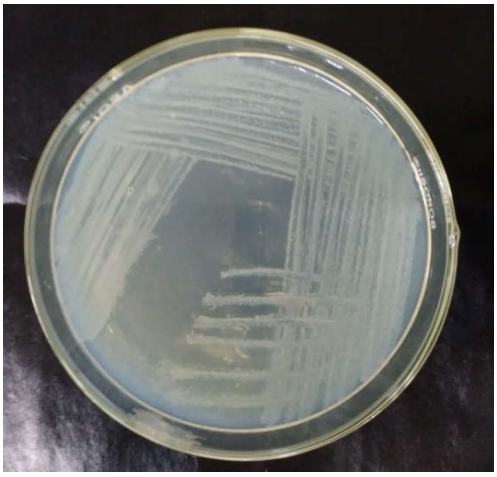

Fig 1 Colonies of *Actinomycete on* Actinomycete Isolation Agar (AIA)

Antibiotic production by Actinomycete and their antibacterial activity against MRSA

The technique of using ethyl acetate was also followed by Smriti Singh et. al. (2012). Out of total 28 isolates including *Streptomycete*, only 6 were found to show an antibacterial effect against MRSA by showing a distinct zone of inhibition.

Sr. No.	Isolate No.	Zone of Inhibition (mm)
1.	12	18
2.	19	26
3.	23	14
4.	25	15
5.	27	13
6.	28 (*Streptomycete)*	19

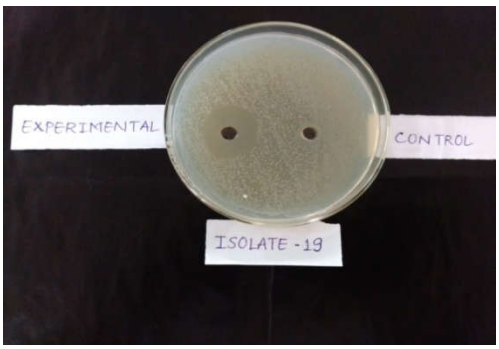

Fig 2 Antibacterial activity of antibiotic produced by *Actinomycete* against MRSA

In the work of Li Xu *et. al.* (2015), they had isolated 61 *Actinomycete* isolates from soil. They had grown the cultures in 2A and soybean meal media and after incubation broth was centrifuged to obtain a supernatant as a test sample for antimicrobial assays by agar well diffusion method against *S. aureus, E. coli, C. albicans* and MRSA. Among all the isolates, only 13 had shown the antagonistic activity against the used organisms. The zones of inhibition obtained for about 50% of isolates of 2A medium against MRSA were in the range of 15-33mm.Whereas in this present study, 28 *Actinomycete* isolates were obtained from 16 soil samples. The isolates were grown in starch casein broth and after incubation, broths were centrifuged and the secondary metabolites were extracted in ethyl acetate and used as a test sample for antimicrobial assay against MRSA by the same method as of Li Xu *et. al* in MHA medium. Among all the isolates, only 6 had shown the antagonistic effect of MRSA and the zones of inhibition obtained were in the range of 13-26mm which were in the line of zones obtained by Li Xu *et. al*., for some isolates and were lesser for the other ones.

Ahmad Siti Junaidah *et.al*. (2014) had obtained the 4 *Streptomyces* isolates and 4 MRSA isolates from Strain of University Kebangsaan Malaysia. The sources of these *Streptomyces* isolates were medicinal plants. These 4 *Streptomyces* isolates were preceded for fermentation in nutrient broth, centrifuged after incubation followed by extraction in methanol. These extracts were tested against MRSA through disc assay method on MHA medium. One of the 4 *Streptomyces* isolate was found to be most potent anti-MRSA and zones of inhibition obtained against the 4 MRSA isolates were in the range of 16-30mm. As compared to their work, 28 *Actinomycete* isolates were obtained from 16 soil samples in this present investigation. The isolates were grown

in starch casein broth and after incubation, broths were centrifuged and the secondary metabolites were extracted with ethyl acetate and used as a test sample for antimicrobial assay against MRSA by agar well diffusion method in MHA medium. Among all the isolates, only 6 had shown the antagonistic effect of MRSA and the zones of inhibition obtained were in the range of 13-26mm which were in the line of zones obtained by Ahmad Siti Junaidah *et.al*.

A total of 230 *Actinomycetes* were obtained by Hongjian Zhu *et.al*. (2013) from the soil of three-river headwater national nature reserve in Qinghai province in China. After the primary screening, they obtained only a single isolate which produced an inhibition zone of 21mm when screened by cylinder and plate assay. After the secondary screening, the secondary metabolites of the same isolate were extracted with ethyl acetate, the inorganic phase of culture medium had no inhibitory effect on MRSA while the organic phase produced a zone of 23mm. They proved that this isolate showed typical characteristics of *Streptomyces sp.* In this present study, 28 *Actinomycete* isolates were obtained from 16 soil samples. The isolates were grown in starch casein broth and after incubation, broths were centrifuged and the secondary metabolites were extracted in ethyl acetate and used as test sample for antimicrobial assay against MRSA by agar well diffusion method in MHA medium. Among all the isolates, only 6 had shown the antagonistic effect against MRSA which were much more in number as compared to Hongjian Zhu *et.al*. and the zones of inhibition obtained were in the range of 13-26mm which were greater as compared to zones obtained by Hongjian Zhu *et.al*. Here we can say that this study was much more satisfactory as compared to the total isolates taken, isolates that showed antagonistic activity and zones on inhibition obtained

In the work of Hotam S. Chaudhary *et. al*. (2013), a total of 31 *Actinomycete* isolates were obtained from soil samples. They had grown the cultures in tryptone-yeast extract broth and after incubation were centrifuged followed extraction of secondary metabolites in ethyl acetate from the supernatant obtained. These extracts were then used as a test sample for antimicrobial assays by agar well diffusion method against 12 human pathogens including MRSA. Among all the isolates, 26 had shown the antagonistic activity against MRSA. The zones of inhibition obtained for these isolates were in the range of 11-21mm. In this present investigation, 28 *Actinomycete* isolates were obtained from 16 soil samples. The isolates were grown in starch casein broth and after incubation, broths were centrifuged and the secondary metabolites were extracted in ethyl acetate and used as a test sample for antimicrobial assay against MRSA by the same method as of Hotam S. Chaudhary *et. al*. in MHA medium. Among all the isolates, only 6 had shown the antagonistic effect of MRSA and the zones of inhibition obtained were in the range of 13-26mm which were in the line of zones obtained by Hotam S. Chaudhary *et. al*., for some isolates and were greater for the other ones, proving this work to be much satisfactory as compared to their work.

Seven *Actinomycete* isolates were obtained from soil samples of Gwalior City in India by Smriti Singh *et. al*. (2012). The isolates were suspended in starch casein broth for submerged fermentation and after incubation, the broths were centrifuged followed by extraction in ethyl acetate. These extracts were used as a test sample for antimicrobial assays by agar well diffusion method against *S.aureus, E.coli,* MRSA and VRE. Among all the isolates, only one had shown the antagonistic activity against the used organisms. The zones of inhibition obtained for these isolates were in the range of 13-15mm. 28 *Actinomycete* isolates were obtained from 16 soil samples in this present study. The isolates were grown in starch casein broth and after incubation, broths were centrifuged and the secondary metabolites were extracted in ethyl acetate and used as a test sample for antimicrobial assay against MRSA by the same method as of Smriti Singh *et. al.* in MHA medium. Among all the isolates, only 6 had shown the antagonistic effect of MRSA and the zones of inhibition obtained were in the range of 13-26mm which were much greater than the zones obtained by Smriti Singh *et. al*. From this we can conclude this study to be much satisfactory and successful.

A total of 64 different *Actinomycetes* were isolated from soil of various regions of Nakhon Si Thammarat by Sumeth Naorungrote *et.al*. (2011). For primary screening, the isolates were grown on yeast extract-malt extract (YM) agar plates. The colonies were transferred on MHA plates swabbed with MRSA isolates and antibacterial activity was observed by agar plug method. A total of 10 MRSA isolates were used. Among the 64 isolates, only 19 showed activity against MRSA. The zones obtained were in the range of 3-21mm. For secondary screening, 5 most potent anti-MRSA isolates were screened by agar well diffusion method. Briefly, the culture broth was collected from 6 days *Actinomycetes* culture YM and was loaded into wells bored in previously MRSA swabbed MHA plates. The zones of inhibition were in the range of 15-25mm. As compared to their work, 28 *Actinomycete* isolates were obtained from 16 soil samples in this present investigation. The isolates were grown in starch casein broth and after incubation, broths were centrifuged and the secondary metabolites were extracted in ethyl acetate and used as a test sample for antimicrobial assay against MRSA by agar well diffusion method in MHA medium. Among all the isolates, only 6 had shown the antagonistic effect of MRSA and the zones of inhibition obtained were in the range of 13-26mm which was a little greater for one isolate and was in the line of zones obtained for other isolates as in comparison to Sumeth Naorungrote *et.al*. 75 dissimilar desert *Actinomycete* strains were isolated by Wael N. Hozzein *et.al*. (2011) from the soil samples. The isolates were grown in 100ml ISP2 medium and after incubation, 10% of the *Actinomycete* strain were transferred to starch nitrate production medium and again incubated. Later, the fermentation broth was collected and examined activity by using agar well diffusion method in nutrient agar plate seeded with test organism. A total of 11 test organism were used including MRSA. The zones of inhibition obtained for these isolates were in the range of 15-26mm against MRSA. As compared their work, 28 *Actinomycete* isolates were obtained from 16 soil samples in this present study. The isolates were grown in starch casein broth and after incubation, broths were centrifuged and the secondary metabolites were extracted in ethyl acetate and used as test sample for antimicrobial assay against MRSA by agar well diffusion method in MHA medium. Among all the isolates, only 6 had shown the antagonistic effect of MRSA and the zones of inhibition obtained were in the range of 13-26mm which were in the line of zones obtained by Wael N. Hozzein *et.al*.

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