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RESEARCH ARTICLE

SCREENING OF ACTINOMYCETES FROM INDIGENOUS SOIL FOR PRODUCTION OF EXTRACELLULAR METABOLITES

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ABSTRACT

Actinomycetes are abundantly present in soil and they produce a variety of antimicrobial compounds that can be used as chemotherapeutic agent in order to limit the infection. In present study, actinomycetes were isolated from different ground soils. Primarily these isolates were screened for extracellular metabolites production by conventional methods. Cross streak method and double agar overlay methods were used in this screening. Initially we have isolated 33 actinomycetes strains from different soil samples and screened them for antimicrobial potential. About 51.51 % of isolated strains showed the antagonistic properties against one or two tested gram positive bacteria. The best strain IAS 1, IAS 7, IAS 10, IAS 11, showed maximum zone of inhibition against *M.luteus*. The chemical nature of ISA 10 was assessed by simply heating the supernatant and we found that the extra cellular metabolite activity was absent in heated sample suggesting the protein nature of it

INTRODUCTION

Actinomycetes are Gram positive bacteria and an important source of antibiotics (Valli *et al*., 2012). They have high G+C content 55-77% (Lo *et al.,* 2002; Ningthoujam *et al.,* 2009; Lam *et al.,* 2006 and Ndonde *et al.,* 2000). They belong to order actinomycetales and composed of around eighty genera (Stackebrandt *et al.,* 1997 and Goodfellow *et al.,* 1983) .They are distributed ubiquitously including water, soil, and marine (Gebreyohannes *et al.,* 2013). Actinomycetes are economically and biotechnologically feasible prokaryotes. They produced a variety of bioactive compounds that can be used to treat infections, they include antitumor, antifungal, antibacterial agents (Bizuye *et al.,* 2013) and enzymes (Jeyadharshan *et al.,* 2013). Among all genus, *Streptomyces* are the best secondary metabolites producers (Valli *et al.,* 2012). First antibiotic, streptomycin has been isolated from *Streptomyces* in 1945 by A. Waksman (Atta *et al.,* 2010). Up till now, a number of antibiotics have been isolated from actinomycetes including anthracyclines, peptides, macrolides β-lactams, actinomycins and tetracyclines etc. Variability among genus of actinomycetes is of great significance in many areas of science especially in antibiotics (Magarvey *et al.,* 2004). Now a days multi-drug resistant pathogenic bacteria is an issue of extreme concern in the world whose numbers are continuously increasing day by day and resulting in rapid spread of infectious diseases, leading to high morbidity and mortality (Hong *et al.,* 2009 and Alanis *et al.,* 2005). New antibiotics are also frequently in use for pathogens that seems to be responsible for emergence of resistant pathogens in clinical cases (Lewis, 2013). However, there are some microbes which are easily destroyed by selective antibiotics are not frequently available, furthermore, antibiotics that are discovered yet are expensive and have more side effects (Bizuye *et al.,* 2013). Most of the Actinomycetes do not cultivate in lab condition that are the important source of most of the antimicrobial drugs, that's why we are unable to examine their potential of producing novel antibiotics (Schatz *et al.,* 1945).

Over the past few years, actinomycetes that have been isolated and screened for antibiotics were found to be previously reported or are found to be re-isolated strains, however, unexplored ecosystem or less explored ecosystem, like marine, desert, forest, caves and hills has been found to be a more promising source for isolating new bioactive novel compounds from Actinomycetes (B´erdy *et al.,* 2012 and Nachtigall *et al.,* 2011). Because of the increasing resistance of microorganisms towards discovered antibiotics there is a need to explore the potential of novel strains of actinomycetes for their new secondary metabolites and to study its role in the field of antibiotics.

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MATERIALS AND METHODS

Sample collection

Soil samples were collected from different grounds of University Of Karachi at a depth of 10-15 cm.

Chemicals

Chemicals include ethanol, NaCl, Na_2HPO_4, KH_2PO_4, H_2SO_4 and $BaCl_2$. Different microbiological media including Nutrient agar, Nutrient broth, Agar technical, and Heart infusion agar procured from Oxoid.

Sample processing and isolation of Actinomycetes

Samples were placed in an empty petri dish for two days (Jeffrey, 2008). Dilution series were begin by adding 1 gm of soil sample in 100 mL of saline and then serially diluted till 10^{-5} according to the protocol used by Rahman *et al.* (2011) with slight modification. 100µl from 10^{-3}, $10^{-4}$and 10^{-5} were transferred onto half strength nutrient agar (Taha *et al.*, 2007) and allowed to dry. Plates were incubated at room temperature for 3 days.

Colonial and morphological characterization

Actinomycetes were identified by their morphological and colonial characteristics. (Gurung *et al.,* 2009). Their morphologies were identified through Gram staining and were differentiated according to their shape, size and color of colonies.

Tested cultures

Clinical isolates were selected to evaluate antibacterial activity of Actinomycetes strains. These include: *Staphylococcus aureus, Staphylococcus epidermidis, Bacillus subtilis, Enterococcus fecalis, Micrococcus luteus, Salmonella typhi, E.coli, Pseudomonas aeruginosa, Acinetobacter* and *Proteus mirabilus.*

Primary screening of metabolites

Primary screening of metabolites were performed by two methods**:**

1. Cross streaking

Actinomycetes strains IAS 26 to IAS 33 were analyzed by cross streak method (Mohseni *et al.,* 2013). These strains were streaked at the corner of plate and incubated for 3 days at room temperature. Overnight fresh cultures of test organism were selected and their suspension was prepared in PBS until the turbidity was matched to 0.5 McFarland standards. These test organisms were streaked perpendicular to the Actinomycetes strains by dipping the sterile cotton swab in suspension of test cultures. Plates were incubated at 37 °C for 24 hours.

2. Double agar overlay method

For preliminary screening of antibacterial activity of Actinomycetes, double agar overlay method was used (Shetty *et al*., 2014). Actinomycetes strains were stabbed on half strength nutrient agar and incubated at room temperature for 2-3 days. After appearance of growth 5mL 1% soft agar containing 100 µL of *M.luteus* (which was matched with 0.5 McFarland standards) were overlayed over half strength nutrient agar and incubated at 37°C for 24 hours. After a day, zone of inhibition was measured. Strains that showed antibacterial activity against *M.luteus* were further analyzed with other test organisms. Concentration of isolates and test culture was maintained by matching the turbidity with 0.5 McFarland standards. For evaluation of antibacterial activity 8 µL from isolate suspension was inoculated on half strength nutrient agar. Plates were incubated at room temperature for 2 days. When growth appeared on plate, 5mL 1% soft agar containing 100 µL of test culture (which was matched with 0.5 McFarland standards) was poured over it. Plates were incubated for 24 hours at 37°C.

Determination of nature of bioactive compound

Actinomycetes strains that showed activity against test cultures were inoculated in 50 mL of half strength nutrient broth and incubated for 2 days at room temperature; adapted and modified Mohseni *et al.* (2013). To extract the bioactive compounds, inoculated broth was centrifuged at 5000 rpm for 10 min at 4°C modified from Valli *et al.* (2012). Supernatant were passed through membrane filter of 0.45 µm size. The filtered supernatants were transferred into two aliquots. 1 was heated at 100ºC for 1 min and other remained unheated. To determine the nature of bioactive compound, Agar well diffusion method was used. *M.luteus* was used as the test culture. 100 µL from both aliquots were transferred into wells. Plates were incubated at 37 ºC for 24 hours.

RESULTS AND DISCUSSION

The purpose of this study was to evaluate antimicrobial activity of Actinomycetes. Due to emergence of multidrug resistant human pathogens there is a need to discover new antibiotics which are effective against these pathogens. (Mohseni *et al.,* 2013), to overcome this problem we can use the potential of Actinomycetes, that are able to produce bioactive compounds and an important source of secondary metabolites (Suthindhiran *et al.,* 2009)

Isolation of Actinomycetes from ground soil

We have isolated 33 Actinomycetes strains from various ground soils of University of Karachi as shown in Table 1. Half strength nutrient agar (Taha *et al.*, 2007) was used for their isolation. They showed their optimum growth at room temperature after incubation of 3 days.

Table 1. Actinomycetes strains isolated from ground soil from University of Karachi

S. No.	Places at University of Karachi	No. of isolates
1	Valika ground	6
2	N.B.P ground	14
3	HBL ground	6
4	Silver jubilee gate ground	7

Table 2. Antibacterial activity of Actinomycetes against *M.luteus*

Isolate No.	Activity against *M.luteus* (mm)	Isolate No	Activity against *M.luteus* (mm)	Isolate No	Activity against *M.luteus* (mm)
IAS 1	30	IAS 11	40	IAS 21	-
IAS 2	28	IAS 12	-	IAS 22	-
IAS 3	23	IAS 13	-	IAS 23	-
IAS 4	-	IAS14	-	IAS 24	-
IAS 5	24	IAS 15	-	IAS 25	-
IAS 6	20	IAS 16	-		
IAS 7	30	IAS 17	-		
IAS 8	20	IAS 18	-		
IAS 9	-	IAS 19	-		
IAS 10	45	IAS 20	-		

Table 3. Antibacterial activity of Actinomycetes strains against gram positive bacteria by cross streak method

Isolate No.	Growth pattern			
	S.epidermidis	*S.fecalis*	*S.aureus*	*M.luteus*
IAS 26	+	+	+	+
IAS 27	GI	+	+	+
IAS 28	+	GI	+	+
IAS 29	+	GI	+	+
IAS 30	+	GI	+	+
IAS 31	+	GI	+	+
IAS 32	+	GI	+	+
IAS 33	+	+	+	+

Keys: + show no inhibition of growth GI shows growth inhibition

Table 2. Antibacterial activity of IAS 10 against clinical isolates – shows no zone of inhibition

Test organisms	IAS 10		
	Average diameter of zone of inhibition (mm)		
	Exp 1	Exp 2	Exp 3
Staphylococcus aureus	-	-	-
Staphylococcus epidermidis	19	19	20
Bacillus subtilis	14	15	14
Enterococcus .fecalis	-	-	-
Micrococcus luteus	20	19	19
Salmonella typhi	-	-	-
E.coli	-	-	-
Pseudomonas aeruginosa	-	-	-
Acinetobacter	-	-	-
Proteus mirabilus	-	-	-

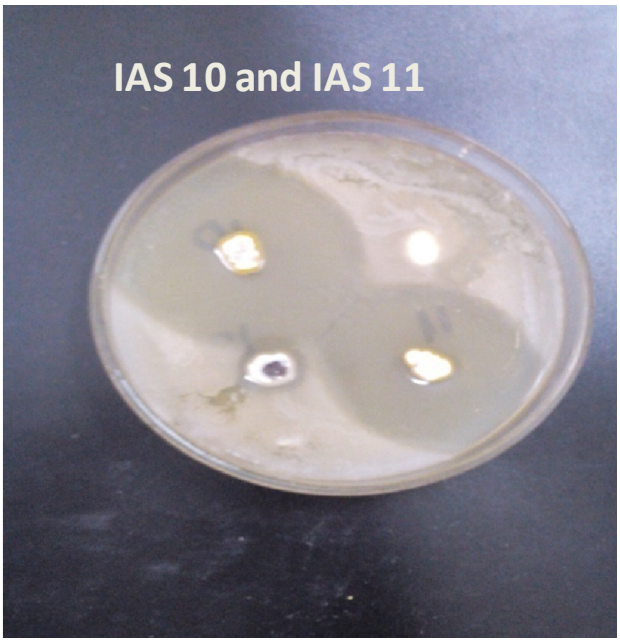


Fig. 1. Antibacterial activity of IAS 10 and IAS 11 against *M.luteus*

Fig. 2. Antibacterial activity of Actinomycetes strains against gram positive bacteria by cross streak method

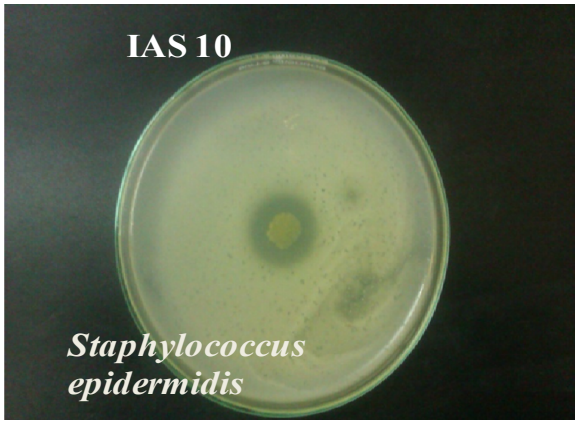


Fig. 3. Antibacterial activity of IAS 10 against *S.epidermidis*

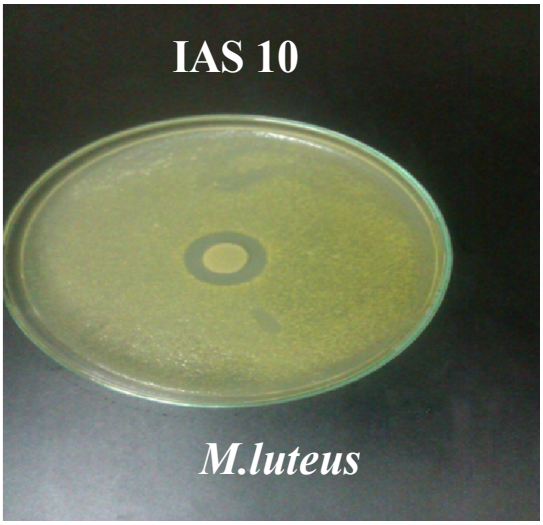


Fig. 4. Antibacterial activity of IAS 10 against *M.luteus*

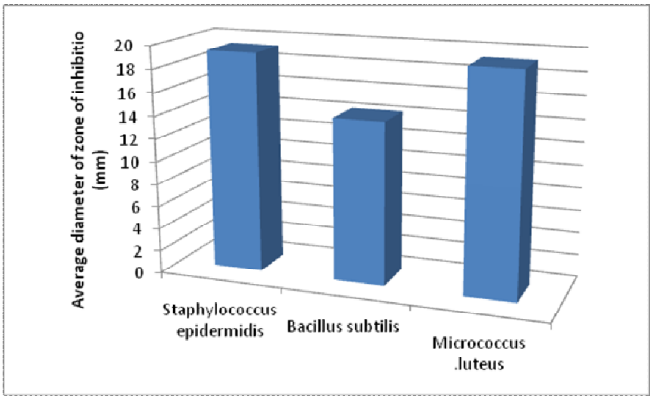


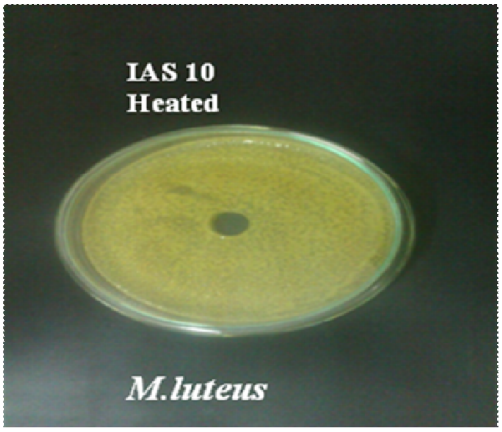
Fig. 5. Antibacterial activity of IAS10 against test cultures

Colonial and morphological characterization

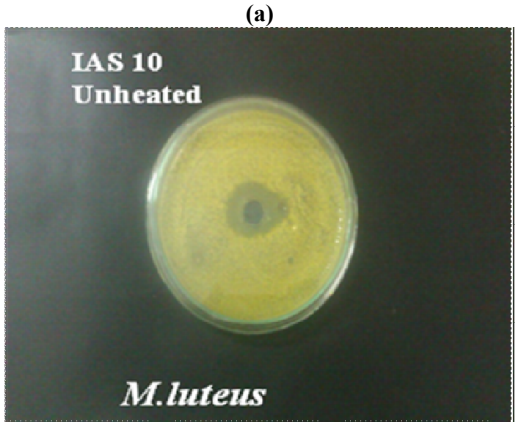
All of the isolated strains were found to be gram positive, having fine thread like morphology along with spores (Gurung *et al.,* 2009). Their colonies on half strength nutrient agar were chalky white, dry, nodular and sticky to agar.

Primary screening of metabolites

We have isolated 33 Actinomycetes strains from different soil samples. Out of 33 Actinomycetes strains 17 showed antibacterial activity against tested organisms. IAS 10 showed maximum activity against test cultures while least activity was shown by IAS 6. In this research two different screening methods were used including cross streak method (Mohseni *et al.,* 2013) and double layer overlay method (Shetty *et al*., 2014). Antibacterial activity of isolated strains was screened against different clinical isolates among which, *M luteus* was found to be the most sensitive organism



(a)



(b)

Fig. 6. IAS 10 filtrate heated (a) and unheated (b)

1. Cross streaking method

Cross streaking method was performed by using four bacterial cultures i.e., *S.epidermidis*, *S.fecalis*, *S.aureus*, *M.luteus*. Out of 8 strains five of them were found to have antimicrobial properties.

2. Double layer overlay method

For preliminary screening, all of the isolated Actinomycetes strains were tested for their antibacterial activity against *M.luteus.* Out of 25 isolates, 9 isolates showed antagonistic activity and it was concluded by measuring zone of inhibition. IAS 10 showed maximum zone of inhibition of 45 mm and it was selected to test with different clinical isolates other than *M.luteus*. Isolates showed more activity against gram positive bacteria than gram negative bacteria, this was similar to the finding of Das *et al.,* 2014, resistant showed by gram negative bacteria may be due to the presence of outer membrane that contain lipopolysaccharide **(**Parunago *et al*., 2007). All experiments were performed in triplets in order to increase the

reliability of the results. After maintaining inoculum size of isolate, double agar overlay method was re-performed and we saw IAS 10 showed activity against different clinical isolates as well.

Determination of nature of metabolites

In order to determine the nature of metabolites produced by Actinomycetes, an experiment was performed by using IAS 10. Extraction of metabolites was done by using the method as performed by Valli *et al.* (2012) with slight modifications. The well onto which unheated filtrate was added showed inhibition, while the well onto which heated filtrate was added, did not give any activity, this shows that the nature of metabolites produced by IAS 10 is most likely to be protein, as proteins gets denatured when heated at a high temperature.

Conclusion

The present research highlights the importance of soil actinomycetes which are quite active in producing antagonistic metabolites. In this study we found 17 out of 33 isolates (51.5%) are efficient in producing antimicrobial substances which are effective against *S.epidermidis, S.fecalis*, *S.aureus* and *M.luteus*. Further, one of the strain IAS 10, was found to produce extra cellular metabolites that are easily inactivated by heating, suggesting the protein nature of metabolite. Future research will be carried out in determining the molecular nature of this bioactive metabolite, produced from soil actinomycetes.

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