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RESEARCH ARTICLE

ISOLATION AND IDENTIFICATION OF ACTINOMYCETES ISOLATED FROM KARACHI SOIL AND SCREENING OF ANTIMICROBIAL COMPOUNDS

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ABSTRACT

Twenty strains of actinomycetes were isolated from a soil sample in Karachi and screened for antimicrobial activity both by primary screening method (perpendicular streak and spot inoculation method) and secondary screening method (agar well diffusion method) against several test organisms including bacteria and fungi. Out of twenty strains eight showed broad spectrum activity in primary screening. The secondary screening was done with those strains which gave activity against test microorganisms in primary screening. The strains were identified using various biochemical tests and their optimum growth conditions (temperature, pH and at different concentrations of NaCl) were also determined. The most promising strain amongst all was SZ020 which was identified as *Pseudonocardia* sp.

INTRODUCTION

Actinomycetes are slow growing, gram positive bacteria with high G+C ratio. They resemble to fungi because of their filamentous appearance and spore production property (**Waksman, 1940**) and they resemble to bacteria because of presence of peptidoglycan in their cell wall and possession of flagella (**Mythili and Das, 2011**). Their habitats include soil, sea sediments, invertebrates, near hydrothermal vents, sea floor, sea plants, ocean surface, in human and cattle bodies (**Rosebury, 1944**). The genome size of actinomycetes varies from too large to too small. Actinomycetes play variety of roles like degradation of recalcitrant xenobiotic compounds, organic matter present in soil and waste release from agriculture field and urban community (**Heur *et al*., 1997**), fix nitrogen symbiotically in non-leguminous plants (**Benson and Silvester, 1993**), produce agro active compounds which are used to control phytopathogens (**Doumbou *et al*., 2001**) etc. Most actinomycetes species have capability to synthesize biologically active secondary metabolites such as antibiotics, herbicides, antitumor, anticancer, pesticides, antiparasitic, immunomodulator, vitamins and enzyme inhibitors (**Oskay *et al*., 2004; Naikpatil and Rathod, 2011**). Among these compounds, the compounds which are important therapeutically and also commercially are the antibiotics.

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About two-thirds of known antibiotics are produced by actinomycetes (**Hozzein *et al*., 2011**). About 80% of these antibiotics are produced by *Streptomyces* (**Kim and Garson, 2005**). The *Micromonospora* is the runner up in the antibiotic production and produces one tenth of antibiotics (**Lam, 2006**). As organisms are getting resistance against preexisting drugs we continuously need to search for the new compounds to which bacteria are not resistant for this purpose scientists are exploring different habitats where novel actinomycetes can be found. The goal of this research was to isolate actinomycetes from soil, which was cultivated but dry and less granulated due to over use of synthetic fertilizers and screen them for antibacterial and especially antifungal activity.

MATERIALS AND METHODS

Sampling: soil sample was collected near rhizospheric region of an ornamental plant at the depth of 10-12 cm this sample was collected in a dry and clean polythene bag and was air dried at ambient temperature for 9 days.

Culture media: Isolation of actinomycetes was done on oat meal agar (Oat meal 20gm, Agar 15gm, Trace salt solution ($FeSO_4.7H_2O$ 0.1gm, $MnCl_2.4H_2O$ 0.1gm, $ZnSO_4.7H_2O$ 0.1gm, distilled water 100ml) 1.0ml, distilled water 1000 ml, pH 7.2. Primary and secondary screening for the strains was done using nutrient agar. For the characterization purpose

yeast extract glucose agar, yeast extract malt extract agar, oat meal agar, inorganic salts starch agar, Jensen's agar, nutrient agar, starch casein agar and Sabroud's dextrose agar were used.

Test organisms: The bacteria test strains include *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Micrococcus luteus*, *Klebsiellapneumoniae*, *Salmonella typhi*, *Streptococcus faecalis*, *Streptococcus pyogenes, Corynebacteriumdiphtheriae*, *Corynebacterium Xerosis*, *Corynebacteriumhofmannii*, *Methicillin-resistant Staphylococcus aureus*, *Vancomycin resistant Enterococcus*, *Shigella dysenteriae* and *Shigella flexneri,* and the fungi test strains include *Candida albicans*, *Saccharomyces cerevisiae, Aspergillus niger* and *Aspergillus terreus* were used. The bacterial strains were stored in the form of glycerol stock and fungal strains were store on the slants of potato dextrose agar. They were sub cultured as needed.

Isolation of Actinomycetes: One gram of air dried soil sample was taken and added in the flask containing 100ml of sterile distilled water. The flask was shaken vigorously to release microorganisms from the soil particles then kept at smooth horizontal surface in still state for 15 minutes to allow the soil particles to get settle down. This suspension was 1:100 times diluted. Further dilutions were made up to $1:10^6$. 100 µl from the last three dilutions ($1:10^4$, $1:10^5$ and $1:10^6$) was transferred onto the oat meal agar plate and spread with sterile spreader. The plates were incubated for 7 days at ambient temperature. The strains of actinomycetes were selected on the basis of colonial morphology. The microscopy of the isolates was performed and characteristics of mycelium and spores types and numbers per sporangium were noted. The actinomycetes strains were preserved on slants and as glycerol stock and screened for antimicrobial potential.

Primary screening methods

Perpendicular streak method: The actinomycetes strains were streaked perpendicularly in the middle of the nutrient agar plate; the streaked plates were incubated at 37 ˚C for 3-4 days. After getting sufficient growth test organisms were streaked perpendicular to the producer actinomycetes strains and the plates were incubated at 37 ˚C for 24 hrs for bacteria and at ambient temperature for 4-5 days for unicellular fungi.

Spot inoculation method: The spots of actinomycetes strains were inoculated on the nutrient agar. The plates were incubated at 37 ˚C for 3-4 days. When the spots of actinomycetes grown they were layered with soft agar of nutrient agar (for bacteria) and potato dextrose agar (for fungi) which was pre seeded with test organism, Plates were incubated at 37 ˚C for 24 hrs for bacteria and at ambient temperature for 4-5 days for single cell fungi. To check the activity of actinomycetes against filamentous fungi the actinomycetes strains were spot inoculated on potato dextrose agar and grown for 4-5 days at ambient temperature after that a disk of actively growing fungal mycelium was inoculated at some distance from actinomycetes spot and then plates were incubated for 4 days at room temperature. The results were recorded by measuring zone of inhibition.

Secondary screening of actinomycetes

Crude extract: The producer strains which gave broad spectrum activity in primary screening methods were further evaluated by secondary screening method. In doing this the producers were inoculated in 50ml of nutrient broth and incubated at 37 ˚C in shaking incubator for 10 days. After 10 days inoculated broth was centrifuged at 10000 rpm for 10 min. Supernatant was taken and again centrifuged twice at 5000 rpm for 15 min. The collected crude extract was kept at refrigerator temperature. Then crude extract was tested against test organisms by agar well diffusion.

Solvent extraction method for extracellular metabolites: Solvents used for the extraction of extracellular metabolite were ethanol, methanol, ethyl acetate and chloroform. 5ml of crude extract was taken in sterile falcon tube and added with 5ml of solvent separately. Gently mix for 1 hr. Then it was centrifuged at 10000 rpm for 10 min. After centrifugation two phases were obtained the upper phase containing antimicrobial metabolites was collected in an Eppendorf and the activity of the extract was tested by agar well method.

Agar well diffusion method: The nutrient agar plate was layered with the soft nutrient agar pre seeded with the test organism when soft agar solidified the wells were dug with the help of sterile borer. The 100 µl of extract was added in the well. Sterile nutrient broth was also added in one well as a negative control. Then plates were kept in refrigerator for 15 min to allow the diffusion of extract and later on incubated at 37 °C in inverted position for 24 hrs.

Table 1. Biochemical and physiological characterization of strain *Pseudonocardia* sp. SZ020

Tests	*Pseudonocardia*sp. SZ020
Indole	-
Methyl Red test	+
Vogues Proskauer test	-
Citrate utilization	-
Catalase test	-
Gelatinase test	+
Urease test	-
H_2S production	-
Glucose utilization	-
Mannitol utilization	-
Lactose utilization	-
Xylose utilization	-
Fructose utilization	-
Sucrose utilization	-
Growth at 15˚C	+
Growth at 25˚C	+
Growth at 35˚C	-
Growth at 45˚C	-
Growth at 55˚C	-
Growth in the presence of NaCl 3%	+
NaCl 5%	+
NaCl 7%	+
NaCl 9%	+
NaCl 11%	+
pH 3	+
pH 5	+
pH 7	-
pH 9	-
pH 11	-

Key: +/ growth and -/no growth

Characterization of actinomycetes

Active strains were grown on different media for morphological characterization and biochemical characterization was performed using various tests. The active strains were also checked for their optimum growth conditions by growing on different temperatures (15˚C, 25˚C, 35˚C, 45˚C and 55˚C), pH (3, 5, 7, 9, and 11) and NaCl concentration (3%, 5%, 7%, 9% and 11%). the growth was measured in terms of optical density.

RESULTS AND DISCUSSION

Isolation of actinomycetes: The soil sample was collected from depth as it has been found out that number of actinomycetes at depth is relatively higher than the soil surface due to appropriate pH and water content in depth (Basavaraj *et al*., 2010). The sample was air dried for one week at 30 ^{0}C as this reduces the population of gram negative bacteria and other microorganisms (**Jeffrey, 2010**). The dry, pigmented, chalky, small to medium sized colonies which showed characteristic features of actinomycetes were selected and purified by streaking on nutrient agar. Total 20 strains were isolated. The culture of purified strains were maintained on slants and preserved in the glycerol stock also.

Screening of strains: The strains were assessed for their antimicrobial potential. The primary screenings was performed by perpendicular streak method (**Egorov, 1985**) and spot inoculation method (**Kumar *et al*., 2010**). The actinomycetes strains were first tested by perpendicular streak method and it was found that six out of twenty strains showed activity against gram positive test organisms and only four strains showed activity against gram negative organisms. The results of spot inoculation method showed that eight isolates had potential to inhibit gram positive microorganisms and five isolates inhibited gram negative organisms. The difference between the sensitivity of gram negative and gram positive bacteria is due to their cell wall morphology. The gram negative organisms have outer layer which consists of lipopolysaccharide which decreases the cell wall permeability and in this way interrupts the entrance of lipophilic molecules in cell. Cell wall of gram negative organisms also contains porins which serve as selective barrier for the passage of hydrophilic molecules. The gram positive organisms are more sensitive to antimicrobial compounds because they do not have outer layer and their cell wall is more permeable to these compounds (**Scherrer and Gerhardt, 1971**). Strains *Faenia* sp.SZ014 and *Pseudonocardia* sp. SZ020 showed broad spectrum activity. The broad spectrum activity is may be due to the production of more than one antimicrobial compound that make actinomycetes effective against both gram negative and gram positive organisms (**Gurung *et al.*, 2009**), these five isolates were selected for secondary screening. Secondary screening of active isolates was performed by agar well method (**Pandey *et al*., 2004**).

The secondary screening results were conclusive. Those strains which gave activity in primary screening did not exhibit antimicrobial potential in secondary method except *Pseudonocardia* sp. SZ020 (only against some microorganisms). The reason behind this is may be the difference in the morphology of actinomycetes when grow on solid medium (filamentous mycelia) and in liquid broth (fragementing mycelia). Also many of the actinomycetes are poor fermenter (**Pandey *et al*., 2004**). It may also be possible that the active compounds release by actinomycetes become inactive or chemically modified in broth or bind to the component of liquid medium (**Gurung *et al.*, 2009**).

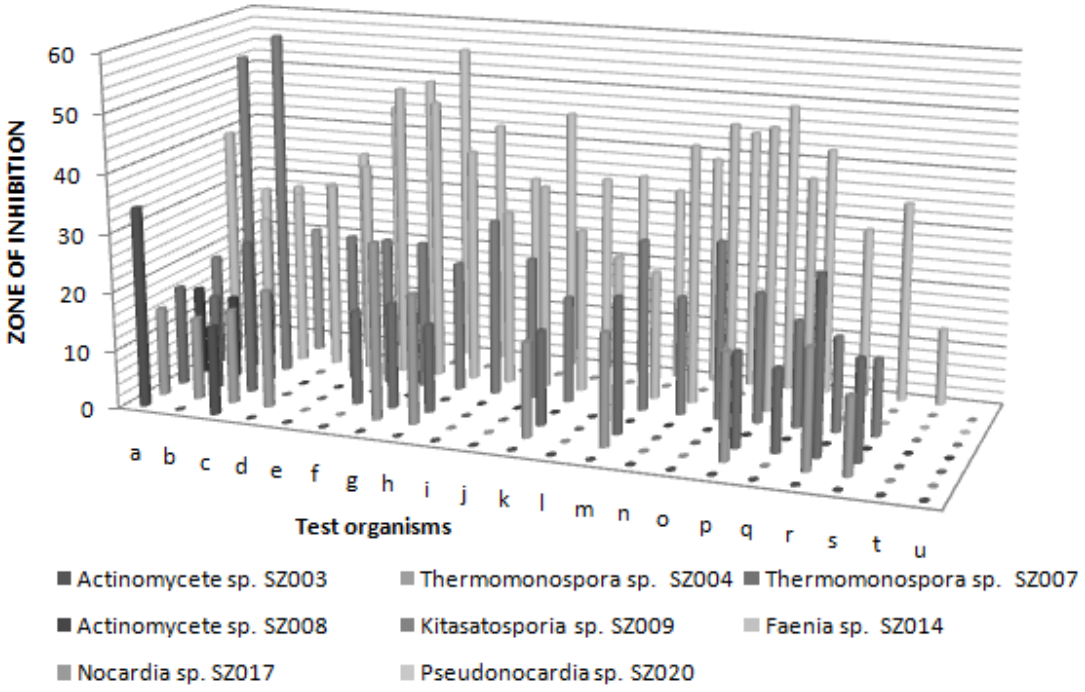
One of the reasons is may be the failure of diffusion of inhibitory compound in aqueous environment. This observation was reported by **Rosenfield and Zobell, 1947**. They reported that antimicrobial compounds are closely bound to the outer surface of cell and release into solid medium slowly. **Lemos *et al.* (1985)** reported that bound antibiotics excrete in the environment slowly and continually and prevent the colonization of competitors in its surrounding (**Hosny*et al*., 2011).** For the isolation of antimicrobial metabolites from broth different solvents including methanol, ethanol, chloroform and ethyl acetate were used. This procedure is referred as solvent extraction method (**Khan and Patel, 2011**). Only the chloroform and ethyl acetate extract of strain *Pseudonocardia* (SZ020) showed activity against MRSA and *M. lutues*. Other solvent did not extract the antimicrobial metabolite from fermented broth. The reasons for the failure of metabolite extraction by using solvents could be the presence of polar functional group in metabolite which make metabolite insoluble in solvent and soluble in water, inadequate shaking of the mixture, and the use of inappropriate solvents (**Gurung *et al.*, 2009**). The strains showing activity against bacteria were also tested against *Candida albicans, Saccharomyces cerevisea, Aspergillus niger* and *Aspergillus tereus*. Spot inoculation and perpendicular streak methods were used to check activity against *Candida albicans*and *Saccharomyces cerevisae.* The activity against *Aspergillus niger* and *Aspergillus tereus* was tested by the method used by **Prapagdee *et al.* (2008)**. Only three isolates exhibited antifungal activity against *C. albicans* and *S. cerevisea* and one isolate exhibited activity against *Aspergillus niger*. The potential of inhibiting fungi is attributed to the production of secondary antifungal compounds and extracellular hydrolytic enzymes (**Prapagdee *et al*., 2008**). The growth kinetics of the most active and broad spectrum activity showing strain *Pseudonocardia* sp. SZ020 was also determined by following the method mentioned by **Khan and Patel (2011)**. The strain started to produce antibiotic at day 3 but the activity of the extract was not too strong because of various reasons as described above. This strain completed its life cycle in 6 days.

At present we need to find out such isolates which secret novel antimicrobial compounds as the preexisting drugs have been failed due to the development of resistance among the microorganisms. The prevalence of increasing resistance is mainly due to the dissemination of existing resistant pathogenic strains or due to emergence of peculiar resistive pathogens (**World Health Organization, 2001**). The present study is also a little contribution towards this need, the isolate *Pseudonocardia* sp. SZ020 showed broad spectrum activity against many microorganisms and can be a considered as candidate in regard of searching potential antimicrobial compounds.

Table 2. Antimicrobial activity of actinomycetes strains by spot inoculation method (zone of inhibition in mm)

Actinomycete isolates	a	b	c	d	e	f	g	h	i	J		l	m	n	o	p	q	r	s	t
Actinomycete sp. SZ003	34	0	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Thermomonospora sp. SZ004	15	14	16	20	0	0	30	22	0	0	16	0	19	0	0	18	0	20	13	0
Thermomonospora sp. SZ007	17	16	26	0	0	16	18	15	0	0	16	0	23	0	0	16	14	30	17	0
Actinomycete sp. SZ008	15	14	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kitasatosporia sp. SZ009	19	55	59	0	25	25	25	22	30	24	18	0	29	20	30	22	18	16	13	0
Faenia sp. SZ014	40	25	30	32	36	50	48	40	30	35	28	24	22	44	48	48	40	0	0	0
Nocardia sp. SZ017	0	0	22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudonocardia sp. SZ020	27	28	29	35	44	49	55	42	33	45	34	35	33	39	44	49	42	29	34	13

Test organisms
a: *B. subtilis*; b: *S. aureus*; c: *M. luteus*; d: *S. faecalis*; e: *S. pyogenes*; f: *C. diphtheriae*; g: *C. xerosis*;
h: *C. hofmannii;* i: MRSA; j: VRE; k: *E. coli*; l: *P. aeruginosa;* m: *P. mirabilis*; n: *K. pneumoniae*; o: *S. typhi*;
p: *S. dysenteriae*; q: *S. flexneri*; r: *C. albicans*; s: *S. cerevisiae*; t: *A. niger*



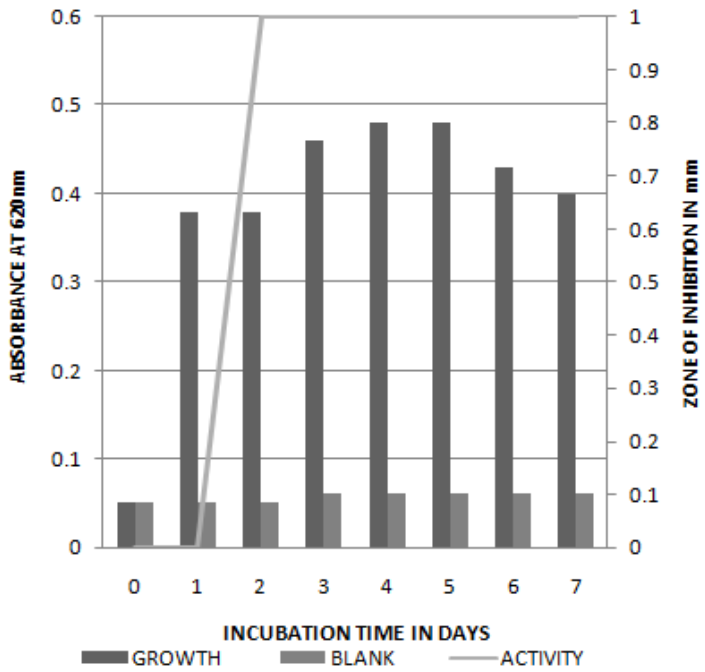


Fig. 1. Growth kinetics of *Pseudonocardia* sp. SZ020

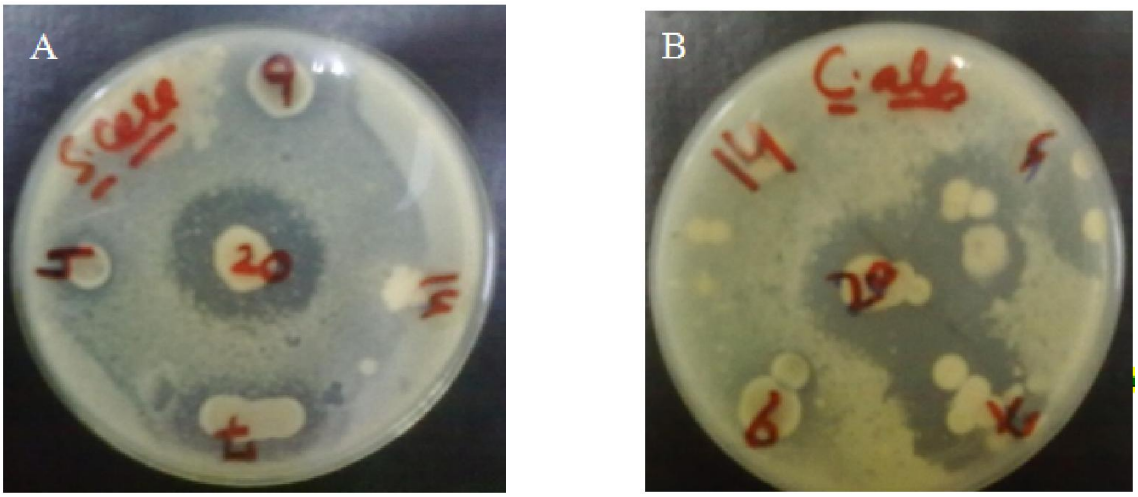


Figure 2. Antifungal activity of *Thermomonospora* sp. SZ004, *Thermomonospora* sp. SZ007, *Kitasatosporia* sp. SZ009 and *Pseudonocardia* sp. SZ020 against (A) *S. cerevisiae* (B) *C. albicans*

The further studies related to the determination of structure of the antimicrobial compounds released by *Pseudonocardia* sp. SZ020 is currently going on.

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