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Pageantagonistic Activity of Actinomycetes on Some Gram Negative and Gram Positive Bacteria

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Abstract: In the present research, *In vitro* study of antagonistic properties of fifty soil actinomycetes against 10 bacteria which were: *Salmonella typhi*, *Klebsiella pneumoniae*, *Serratia marsecense, E. coli, Proteus vulgaris*, *Streptococcus faecalis, Staphylococcus aureu*s, *Bacillus subtilis*, *Bacillus cereus* and *Micrococcus luteus* showed that Six of them have inhibitory effects on both gram negative and gram positive pathogenic bacteria by using agar disk diffusion method. Of all six active Actinomycetes isolates, one of them named *Streptomyces plicatus* No. 24 was selected for further study on the basis of (a) broad spectrum activity and (b) larger inhibition zone against pathogen growth, in comparison with others. Minimum Inhibitory Concentration (MIC) of the crude culture filtrate of *S. plicatus* against *Staphylococcus aureus* was determined as 0.004 mg ml^{-1}. Gram positive pathogenic bacteria were more sensitive to its metabolites than gram negative ones. A commercial product containing *S. plicatus* or its effective metabolite(s) is suggested to avoid some of diseases in human and also bacterial resistance to conventional antibiotics.

Key words: Actinomycetes · Antagonistic activity · Bacteria

INTRODUCTION

The Actinomycetes are gram positive bacteria with G+C content over that present in a wide variety of soil, water and plants [1]. They are best known for their economic importance as producers of antibiotics, vitamins, herbicides, pesticides and enzymes and they seem to have a significant role in future biotechnology [2,3]. The discovery of antibiotics had a major impact on the control of infectious disease and the development of pharmaceutical industry [2]. At present, 4000 antibiotic substances obtained from bacteria and fungi have applied in medicine [4]. The *Streptomycetes* are specially prolific and recognized as industrially important microorganisms, because of their ability to produce a greate many antibiotics including aminoglycosides, anthracyclins, glycopeptides, β- lactms, macrolides, peptides polyenes, polyethers and tetracyclines [5,6]. The *Streptomycetes* species produce about 80% of commercially and medically useful antibiotics [7]. In United State and Japan during 1953 to 1970 approximately 85% antibiotics were produced by Actinomycetes, 11% by fungi and 4% by bacteria [8].

The problem of microbial resistance to antibiotics and inability to control infectious disease has given an impetus for continuous search of new antibiotics all over the world [9]. In the course of screening for new antibiotics, several research studies are currently oriented towards isolation of new Streptomyces species from different soil and water samples [10,11]. In screening for Actinomycetes which are able to produce bioactive compounds, the exploration of new soils and habitats has been recommended [12,13]. The metabolic diversity of the Actinomycete family is due to their extremely large genome, which has hundreds of transcription factors that control gene expression, allowing them to respond to specific needs [14]. For the screening of microorganisms for the production of bioactive compounds, exploration of new soils and habitats has been recommended [15]. In the present work, Actinomycetes strain isolated from Iran soils were selected and tested for their capacity to produce compounds which are active against gram-positive and gram negative bacteria. Certainly detecting effective metabolite (s) responsible for *Streptomyces* antagonistic properties might complete our study and thus will lead to realize related genome structures.

MATERIALS AND METHODS

Isolation and Identification of Antagonistic Organisms: Soil samples were collected from grasslands and vegetable fields in different localities of Kerman province (Iran), using an open-end soil borer (20 cm in depth,

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2.5 cm in diameter) as described by Lee and Hwang [16,17]. They were taken from a depth of 8-12 cm below the soil surface. Samples were air-dried at room temperature for 7-10 days and then passed through a 0.8 mm mesh sieve and were preserved in polyethylene bags at room temperature before use. 10 g of air-dried soil samples were mixed with sterile distilled water (100 ml). Mixtures were shaken vigorously for 15 minute and then allowed to settle for 15 minute. Portions (1ml) of soil suspensions (diluted 10^{-1}) were transferred to 9 ml of sterile distilled water and subsequently diluted to 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Inoculate consisted of adding aliquots of 10^{-3} to 10^{-6} soil dilution to autoclaved CGA (1 ml in 25 ml CGA) at 50°C before pouring the plates and solidifications. Three replicates were considered for each dilution. Plates were incubated at 28°C for up to 7 days and thus dry colonies of Actinomycetes were isolated by pour plate technique [18,19].

Test Organism: Ten human pathogenic bacteria (clinical isolates) including: *S. aureus*, *B. cereus*, *B. subtilis, M. luteus, S. faecalis, E. coli, S. typhi, S. marsecense, K. pneumoniae* and *P. vulgaris* were cultured overnight at 30°C in nutrient broth. Suspensions of them with an optical density of McFarland 0.5 were made in sodium chloride solution [20].

Determination of Antibacterial Activity: Antibacterial activity of pure actinomycetes cultures performed by using disk diffusion method [21,22]. From each actinomycetes isolated, surface culture was prepared by using sterile swab on CGA medium. After 4-6 days incubation at 28°C, 6mm agar disks were prepared by using sterile cork borers. Disks were then aseptically transferred to muller hinton agar (oxoid) plates having fresh lawn culture of the test bacterial. Plates were incubated at 28°C for 2 days and bioactivity was evaluated by measuring the diameter of inhibition zones [23,24].

Antibacterial activity was indicative as growth of test bacteria isolates was prohibited in the direction of active *Streptomyces* isolate. Antibacterial activity around the Actinomycetes agar disk was evaluated as follows and the ratings used were modified from those of Lee and Hwang and El-Tarabily *et al.* [21] no inhibition (-); [25] weak inhibition = partial inhibition of growth, measured as a diameter of 5-9 mm (+); [2] moderate inhibition = almost complete inhibition of growth, measured as a diameter of 10-19 mm (++); [4] strong inhibition = complete inhibition, measured as a diameter of > 20 mm (+++) [26,16].

Submerged Cultures and Preparation of Crude Extract: The most active isolate was grown in submerged cultures of CG broth medium on rotary shakers under 130 rpm at 28°C. To monitor the activity versus post seeding time, aseptically small aliquots of culture media were taken every 24 hr for 20 days and the activity was evaluated against four optional pathogens: *S. aureus, M. luteus, K. pneumonia* and *P. vulgaris* by well diffusion-method. To prepare crude extract, after 14 days of post seeding which the activity reached its maximum, the cultures were harvested; spores and mycelia were excluded by filtration through two layers of cheese cloth. The clarified sap was then dried to dark crude under reduced air at 50°C [19].

Determination of Minimum Inhibitory Concentration (MIC): To measure the MIC value of crude extract of the most active isolate, two-fold serial dilutions of 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0/019, 0/009, 0/004. 0/002 and 0/001 mg ml^{-1} were prepared in Dimethyl sulfoxide: Methanol (1:1 v/v) and assayed against one of the test human pathogens, S. *aureus*, by well-diffusion method as described by Shahidi Bonjar [19]. The MIC was defined as the lowest concentration that is able to inhibit any visible growth.

Scanning Electron Microscope Studies: Ornamentation of spore chains and mycelial morphology of the *S. plicatus* were examined with use of a scanning electron microscope model CAMSCANMV2300. Preparation for the scanning electron microscope consisted of using the culture of 21-28 day old growing on CGA and depositing the specimen onto specimen aluminum stubs which held by a piece of double stick scotch tape. The stubs were coated in a sputter coater for 2 min. Afterwards the specimens were viewed and digital electron micrographs were prepared at magnification of 6000-20000 X with an accelerating voltage of 20 kv accordingly.

RESULTS

Screening and Bioassays: In screening for Actinomycetes with antibacterial activity, fifty isolates were screened. The potent actinomycetes were characterized by morphological and biochemical methods. The observed structure was copared with Bergeys manual of systematic Bacteriology [11,3]. Six isolates showed activities against the test bacteria. *S. plicatus* (isolate No. 24) had maximum activity against the tested bacteria in comparison with others based on (a) broad spectrum activity and (b) larger zone of inhibition and was selected for further evaluations.

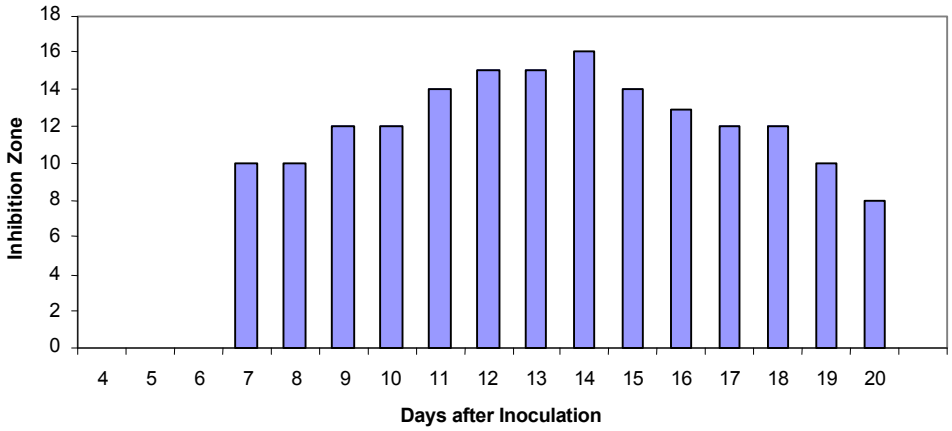

Fig. 1: *In vitro* bioassay results of *Streptomyces plicatus* No. 24 against *S. aureus* in rotary cultures indicative of production time versus inhibition zones

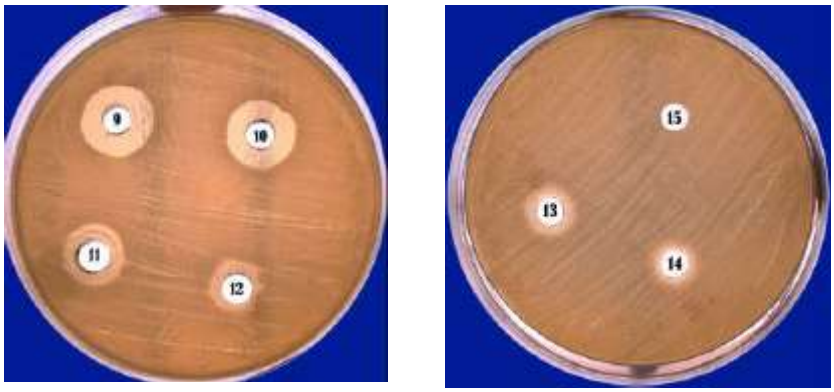

Fig. 2: MIC of *Streptomyces plicatus* No. 24 against *S. aureus* by well diffusion method

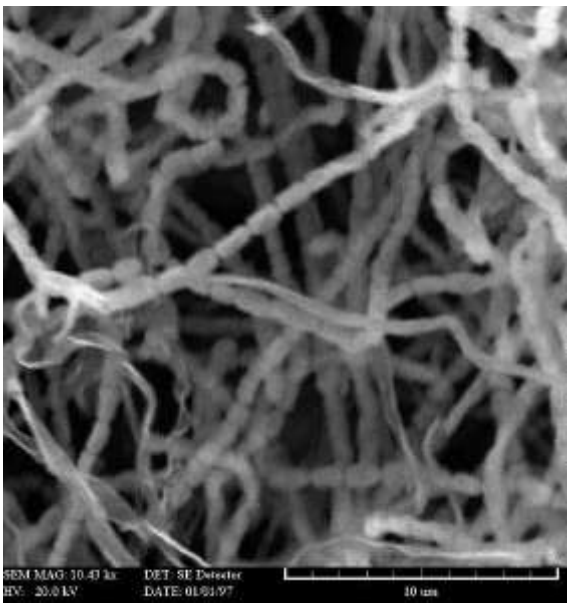

Fig. 3: Scanning electron micrograph of spore chains of *Streptomyces plicatus* No. 24.

Table 1: Bioassay result of MIC of *Streptomyce plicatus* No. 24 against *S. aureus* by well diffusion method

Concentration (mg/ml)	Inhibition Zone	Concentration (mg/ml)	Inhibition Zone
40	+	0/15	+
20	+	0/078	+
10	+	0/039	+
5	+	0/019	+
2/5	+	0/009	+
1/25	+	0/004	+
0/625	+	0/002	-
0/31	+	0/001	-

Monitoring Activity in Shaked Culture: In rotary submerged culture, activity of *Streptomyces* isolate No. 24 against *S. aureus, M. luteus, K. pneumonia* and *P. vulgaris* reached the maximum at 14th post inoculation day (1). It means 14th days after inoculation is the best time for showing maximum inhibition zone and is suitable to harvest cultures for preparation of crude extract for future investigations (Figure 1).

Determination of MIC: In well diffusion-method, MIC of the crude of *S. plicatus* against *S. aureus* was determined as 0/004 mg ml $^{-1}$ (Figure 2 and Table 1).

Scanning Electron Microscope Studies: Scanning electron micrograph of spore chains of *S. plicatus* is shown in Figure 3.

DISCUSSION

The antibacterial resistance is presently an urgent focus of research and new antibiotics are necessary to combat pathogens. The emergence and dissemination of antibacterial resistance is well documented as a serious problem worldwide [27]. The emergence of bacterial resistance threatens to return us to the era before the development of antibiotics [28]. This situation recommends the need for the investigation of new, safe and effective antimicrobials for replacement of invalidated antimicrobials [29]. Actinomycetes have been recognized as source of several secondary metabolites, antibiotics and lytic enzymes among which *Streptomyces* spp. have been shown to have characteristics which make them useful as antagonistic agents against pathogens. A total of 50 actinomycetes isolates were screened from different location in Iran. 6 samples of them showed antibacterial activity against the pathogenic bacteria . The best isolate was *Steptomyces plicatus* with MIC = 0.004 mg ml^{-1}. All actinomycetes were isolated at mesophilic temperature (25-35°C). These findings were in agreement with other authors [25, 2, 15, 24] who found that, most actinomycetes were isolated at mesophihc temperature . Gram positive bacteria were more sensitive than gram negative bacteria. Although the nature and type of active antibacterial principles involved in the present study are not clear, but the prominent antibacterial activity of *S. plicatus* highlights it as a candidate for further investigation of antagonistic activity on human bacterial pathogens.

Our results are similar to the findings of Singh and Gurusiddaiah and Manjula, C. et al in which growth of *B. subtilis, E.coli* and *S. aureus* could be inhibited by *Streptomyces* spp. [24,30]. The crude extract of *S. plicatus* showed antibacterial activity against several species of human pathogens including both Gram-positive and Gram-negative bacteria. These findings indicated that our produced substance might be the alternative antimicrobial substance as a tool for controlling human diseases. A commercial product containing *S. plicatus* or its effective metabolite (s) is suggested to avoid some of diseases in human. Identification the effective metabolites and search genes responsible for its function can be the topic for future and perfecting researches which also consider actinomycetes (like *S. plicatus*) as antagonistic agents.

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