Actinomycetes screening for bioactive potential isolated from the moist forest soils of Pakistan

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ABSTRACT

Five moist soil zones in temperate forests of Pakistan were investigated for the bioactive potential of actinomycetes. The identification of the isolates was based on their cultural and morphological characteristics among which 60 isolates were screened and recognised out of 208 isolates. The isolates identification falls under three genera including Actinomyces, Streptomyces and Nocardia spp. each with the total number of 31, 17 and 12 isolates identified respectively. The identified isolates were further screened for bioactive potential among which 15 isolates produced bioactive substances against one or more indicator strains of gram positive and gram negative bacteria and fungi. The results clearly demonstrated that temperate forest ecosystems are providing a key habitat to bioactive actinomycetes, which have an important medical, scientific and economic significance globally in general and in particular for developing countries like Pakistan.

Introduction

Actinomycetes are gram-positive bacteria that form filamentous mycelia with high G+C (guanine +cytosine) content and are widely distributed in a variety of natural and manmade environments, particularly constituting a significant component of the microbial population in temperate forest soils (Debananda *et al.*, 2009; Lam, 2006 and Ndonde & Semu, 2000 and Watve *et al.*, 2001). About 100 genera of *actinomycetes* exist in natural habitats including diverse forest zones (Yokota, 1997). Some genera such as *Streptomyces* and *Actinoadura* are widely distributed, which can be isolated from different temperate soil habitats (Williams *et al*., 1989). Among the gram-positive bacteria, *Actinomycetes* exhibit the greatest morphological differentiation with branching hyphae and specialised spore-bearing structures (Kim & Garson, 2005; Prescott *et al.,* 1993).

These bacteria are globally significant and have been extensively studied (Cragg & Newman, 2005; Bull *et al.,* 2000 and Debananda *et al.*, 2009), due to their ability to produce novel antibiotics (Goodfellow *et al.*, 1989; Williams *et al*., 1983; Crandall & Hamil, 1986; Williams *et al*., 1989 and Korn & Kutzner, 1992). Of one thousand different antibiotics known today, more than 70 % are produced by *Actinomycetes* (Bull & Stach, 2005; Edward, 1980; Imada & Okami, 1998; Kim & Garson, 2005). In addition to antibacterial activity, *actinomycetes* also produce commercially important bioactive compounds such as avermectin (Prescott *et al.,* 1993) and other secondary metabolites with biological activities (Blunt & Prinsep, 2006 and Debananda *et al.,* 2009) hence *actinomycetes* strains have many gene clusters involving the biosynthesis of melanin, carotenoid, siderophore, polyketide and peptide compounds (Omura *et al.,* 2001). To our knowledge, *actinomycetes* isolation from temperate forest soils in Pakistan have not been undertaken by researchers and this geographic region may have important bioactive *actinomycetes* traits, which could be of medical and economic benefits to Pakistan.

The current study was conducted to isolate potential soil *actinomycetes* strains from five diverse geographic regions, which fall in the temperate forests of Pakistan. The isolates were further screened for bioactive potential against selected strains of gram positive and gram negative bacteria and fungi.

Material and Methods

Study Area

Five temperate forest zones from diverse geographic locations: Miadam Forest Swat (MFS), Kalam Forest Swat (KFS) and Osherai Dara Forest Dir (OFD), Ayubia Forest Hazara (AFH), Nathiagali Forest Hazara (NFH) of Malakand and Hazara Divisions in north western Pakistan, respectively (Fig. 1) were selected for soil sampling and subsequent isolation and screening of bioactive *actinomycetes*.

These temperate forests zones having suitable rich soil conditions (~40 to 60 % volumetric water content and 5 to 8 % organic carbon on dry soil mass basis), acting as a growing media for bioactive *actinomycetes*, and these conditions provided the preliminary basis for selection of the study area for soil sampling.

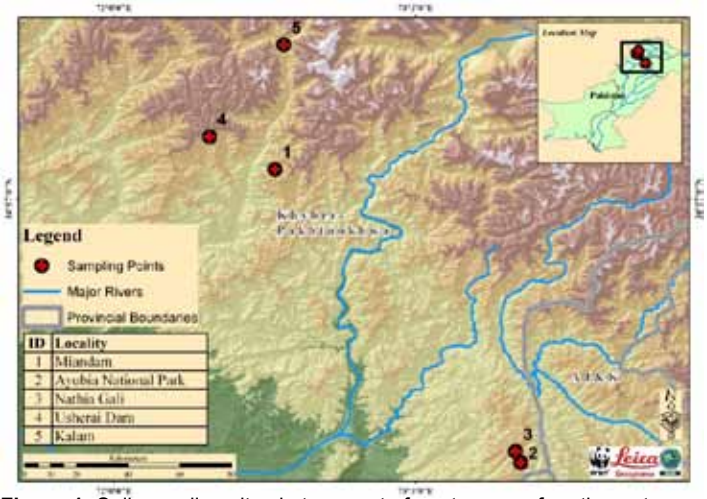

Figure 1: Soil sampling sites in temperate forest zones of north-western Pakistan

Data Collection

Soil Sample collection and pre-treatment

The soil samples were collected during May, 2009 to February,

2010. A total of 15 plots were randomly located in each forest zone (5 zones). A total of 75 soil samples were collected at a depth of 15 to 20 cm below the surface (in the mineral soil layer) to avoid sampling the organic layer acting as a nutrient source for other organisms, where most of the soil microbial activities occur. The samples were packed in sterile polyethylene bags and aseptically transported to the laboratory for further analysis. The collected samples were air dried for seven days, mixed and homogenised manually by removing roots. One gram of each soil sample was grounded, pulverised and passed through a 60 um mesh sieve for the isolation of *actinomycetes*. Each soil sample was pre-treated with 0.1 gm of calcium carbonate ($CaCO_3$) and incubated at 25^{0}C for two weeks. The soil samples were suspended in 99.0 ml sterile distilled water (Laidi *et al.*, 2006), placed in incubator shaking it at 150 rotations per minute for 30 minutes (Laidi *et al.*, 2006 and Saadoun & Gharaibeh, 2003).

Isolation of actinomycetes

Several media types were used for the selective isolation of *actinomycetes* from the soils slurries. The plate dilution method was used for the isolation of *actinomycetes* following the method of Seong *et al.,* (2001). Serially diluted suspensions were spread uniformly on a selective media of hair hydrolysate vitamin Agar (HHVA) and incubated for 2-7 days at 25^{0}C. Plates were checked for the growth of the desired *actinomycetes* colonies after incubation.

Characterisation of the isolates

Morphological characters of the isolates were investigated by gram staining method according to Hucker and Conn (1923). Common morphological characters were observed by incubating the Oatmeal Agar plates in the dark at 28^{0}C for three weeks. Cover slip culture method (Kawato and Shinobu, 1959) was carried out for the microscopic examination of the cross-hatched cultures of the isolates. Oil immersion microscope was used for determining the mycelium colour, structure and arrangements on mycelia as described by Prauser (1964). Bergey's Manual of Systematic Bacteriology (Williams *et al.,* 1983a, 1983b) was followed for the structure resemblance and comparison while the identified colour and colony-morphology were recognised according to the method of Shirling and Gottlieb (1966). The isolates were then cultured on starch-casein agar media according to the method of Pridham (1964) for the determination of colour of the arial mycelia of *actinomycetes*.

Screening of the isolates for bioactive potential

Pure isolates were screened for bioactive potential against pathogenic test organisms following the method of Kirby-Bauer as detailed in Debananda *et al.*, (2009). Isolates were inculcated and grown on GS medium (Antibiotic producing medium) through shaking the isolates in a shaker (150 rpm) at ambient temperature (~22^{o}C). Test pathogens of Gram positive bacteria *Staphylococcus aureus* (MTCC 96), *Micrococcus luteus* (MTCC 106), *Bacillus subtilis* (MTCC 121) and *Gram negative bacteria Escherichia coli* (MTCC 739) were cultured on Nutrient Agar (NA) plates while yeast/fungus *Candida albicans* (MTCC 227) and *Aspergillus Niger* (MTCC 1344) were cultured on Sabouraud Agar (SA) by following the standard procedure according to Debananda *et al.*, (2009). Antibiotic discs were saturated with the crude antibiotic extract and were applied against test-pathogens cultures on NA and SA plates. After 48 hour incubation, the zones of inhibition of the pure isolates against the test pathogens were analysed systematically.

Results and Discussion

Culture and morphology of the isolates

Numerous types of bacterial and fungal colonies of *actinomycetes* were identified among which 30 to 45 colonies were observed on each plate. Colonies selection was made, based on their colony appearance. A total of 208 isolates were subjected to microscopic analysis among which 63 isolates were obtained from the soil samples of Miadam Forest (MFS) followed by 47 from Kalam Forest (KFS), 39 from Ayubia Forest (AFH), 30 from Nathiagali Forest (NFH) and 29 from Osherai Forest (OFD), respectively. Further identification was done based on their cultural and morphological characteristics among which 60 isolates were screened. The isolates identification falls under three genera including *Actinomyces, Streptomyces, and Nocardia* as shown in Table 1.

Bioactive potential of the isolates

In a total of 60 isolates screened, 15 produced bioactive substances against one or more of the test-pathogens while 8 isolates exhibited broad spectrum bioactive potential (Fig. 2) and the results are comparable to the studies conducted by Debananda *et al.*, (2009) and Slavica *et al.,* (2005). These isolates showed bioactive potential against gram positive, gram negative bacteria and yeast/ fungi. The results of this study were similar to the studies conducted by Edwards (1980) and Egorov (1985). The isolate NFH8 (Fig. 2) showed broad spectrum bioactive potential against gram positive bacteria, S*taphylococcus aureus* (MTCC 96) and *Micrococcusluteus* (MTCC 106). Isolate KFS8 also exhibited broad spectrum bioactive potential against *Bacillus subtilis* (MTCC 121); Isolates KFS3 and AFH2 also showed broad spectrum bioactive potential against gram negative bacteria. Isolate NFH8 (Nathiagali Forest Hazara) exhibited broad spectrum antimycotic activity against *Candida albicans* (MTCC 227) while KFS8 against *Aspergillus niger* (MTCC 1344), respectively (Fig. 2). Similar and comparable results were obtained by Atta (2009) and Saadoun & Gharaibeh (2003), which show the fidelity of the experimental protocols adopted.

Table 1: Recognition and classification of actinomycetes into genera's based on cultural and morphological characteristics

No of Isolates	Actinomycetal isolate	Colony description	Microscopic assessment
31	*Actinomyces*	Branched and filamentous and micro colonies	Non acid fast and Gram positive pleomorphic cells, Y and V shaped on filament
17	*Streptomyces*	White, Gray and occasional pinkish color colony with powdery appearance as concave, convex and/or flat surface	Multiple long branching, non fragmenting, long chains , spirali and/or coils
12	*Nocardia*	Shiny colonies with aerial filaments on blurry surface	Non acid fast and Gram positive pleomorphic cells, bacillary and coccoid structure; rarely partial mycelium which fragments readily and hence produce rod shape or coccoid cell

Antibacterial potential against Gram-positive bacteria

The results revealed that six isolates exhibited antimicrobial potential against gram positive bacteria *Staphylococcus aureus,* seven against *Micrococcus luteus* and eight against *Bacillus subtilis.* The results also indicated that isolates MFS3, MFS4, KFS7, AFH2, NFH4 and NFH8 have antimicrobial potential against *Staphylococcus aureus,* while AFH2 produced minimum inhibition zone of 10 mm and NFH8 produced a maximum zone of 30 mm in diameter. The isolates MFS3, MFS4, KFS3, AFH2, AFH9, NFH4, NFH8, NFH10 and OFD3 produced antimicrobial substances

against *Micrococcus luteus;* OFD3 showed a minimum inhibition zone of 16 mm in diameter while NFH8 showed a maximum zone of inhibition of 30 mm. The antimicrobial potential of isolates were also analysed against *Bacillus subtilis*. Results indicated that MFS8, KFS8, AFS2, AFH3, AFH9, NFH4, NFH8 and OFD4 isolates produced antimicrobial metabolites. MFS8 isolate showed a minimum zone of 9 mm in diameter as compared to KFS8 which produced 30 mm zone of inhibition (Fig. 2). These results suggests that antimicrobial activity is diverse in *actinomycetes* collected from temperate forest soils and can be of significance for further insight and synthesis of these identified bioactive materials.

Antibacterial potential against Gram-negative bacteria

The isolates also showed a potential of antibacterial activity against gram negative bacteria. Six isolates: KFS3, AFH2, AFH3, AFH7,

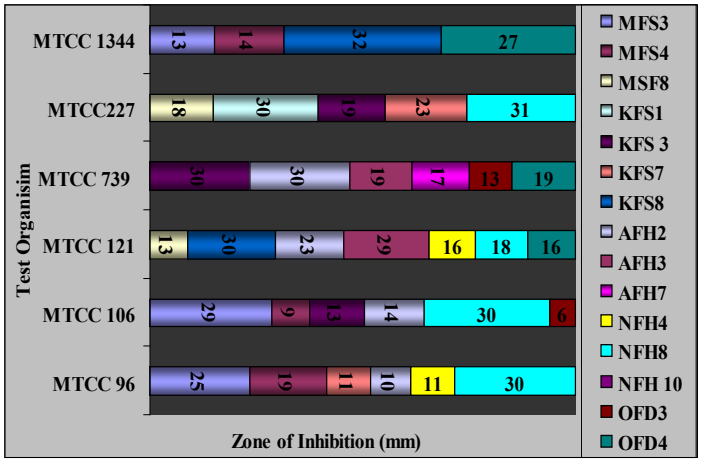

Figure 2: Antimicrobial and antimycotic activity of the isolates against *Staphylococcus aureus* (MTCC 96)*, Micrococcus luteus (MTCC* 106), *Bacillus subtilis* (MTCC 121), Escherichia *coli* (MTCC 739), *Candida albicans (*MTCC 227) and *Aspergillus Niger* (MTCC 1344)

OFD3 and OFD4 clearly inhibited the growth of *Escherichia coli*. The isolate OFD3 produced a minimum zone of 13 mm against *Escherichia coli* as compared to maximum zones of 30 mm diameter produced both by KFS3 and AFH2 (Fig. 3) demonstrating clearly that both have the capacity to completely restrict the growth of *Escherichia coli*. Similar results were obtained by Lo and Ho (2001) and Slavica *et al.,* (2005).

Antimycotic potential against fungi/yeast

For antimycotic potential against fungi, 11 isolates were used, of which 5 isolates were effective against *Candida albicans* while 6 isolates were effective against *Aspergillus niger*. MFS8, KFS1, KFS3, AFH9 and NFH8 showed inhibition zones against *Candida albicans*. MFS8 introverted to the minimum zone of 18 mm in diameter while the maximum inhibition zones of 30 and 31 mm was exhibited both by KFS1 and NFH8 respectively, showing their strong action against the *species*. The test-pathogen *Aspergillus niger* was subjected to all isolates among which MFS3, MFS4, KFS7, KFS8, AFH2 and OFD4 produced antimycotic substances against the *species*. The low inhibition zone of 13 mm in diameter was produced by MFS3 isolate while maximum antimycotic activity by producing an inhibition zone of 32 mm in diameter was shown by KFS8 (Figure 3), being the maximum inhibition zone produced in the current investigations compared to the results observed by Taechowisan *et al.,* (2003). It is probable that the isolates from these forests may have an added advantage of expressing a strong action by KFS8 and further studies are recommended to elucidate its mode of strong action.

Conclusion

The results demonstrate that the temperate soils of north-western Pakistan under temperate forests constitute a significant component of the *actinomycetes* population. Majority of the isolates showed broad spectrum bioactive potential and antimycotic and antibacterial activity against one or more test pathogens. It is also evident from the current study that these forest ecosystems in Pakistan have great potential for the discovery of bioactive actinomycetes, which could be used in future for significant bioactive strains isolation.

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