ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Preliminary studies on antifungal activity of Actinomycetes isolated from ice point

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Manuscript Info

Manuscript History:

Received: 07 September 2013
Final Accepted: 21 September 2013
Published Online: October 2013

Key words:
Cryophilic, *Planomonospora* sp. Actinomycetes, slide culture and non Streptomycetes

Abstract

The objective of the present study was to isolate antifungal metabolites from cryophilic actinobacteria isolated from ice point of manali and to evaluate their antifungal potential against *Candida sp* and *Cryptococcus* species. Totally 28 actinomycetes were isolated and 13 were found to be facultative psychrophiles. Based on spore production and mycelial morphology the isolates were identified as *Streptomyces* sp, *Micromonospora* sp, *Micropolyspora* sp, *Dactylsporangium* sp, *Intrasporangium* sp, and *Planomonospora* sp. out of 13 isolates four actinomycetes (S4, P, M1,M2 and MP) showed antifungal activity against test pathogens. The maximum inhibitory zone was 36 mm showed by *Planomonospora* sp against *Cryptococcus neoformans* and C*andida albicans* .The isolated fraction was identified as polyene in nature and it R*f* value was 1.802.

1. Introduction

Fungi are eukaryotic and have machinery for protein and nucleic acid synthesis similar to that of higher animals. It is, therefore, difficult to find out compounds that selectively inhibit fungal metabolism without affect humans. The search for new drugs against fungal infections is a major challenge to current research in mycotic diseases (Gupte *et al.*, 2002). There is lack of effective and safe antifungal antibiotics and many fungal strains are resistant to certain antimycotic drugs with resulting therapeutic failures (Macura, 1993). Therefore, there is an urgent need of non-toxic and effective antifungal antibiotics. The pioneering work of Waksman showed that actinomycetes are capable of producing medically useful antibiotics (Nolan and Cross, 1990). Actinomycetes are the main source of clinically important antibiotics producer. *Streptomyces* are prolific antibiotic producer, producing around 80% of total antibiotic products. *Micromonospora* is the runner up with less than one-tenth as many as *Streptomyces*. The antagonistic activity of *Streptomyces* to fungal pathogen is usually related to the production of antifungal compounds (El-Tarabily, 2006). The need for new, safe and more effective antifungal agents are a major challenge to the pharmaceutical industry today, especially with the obvious increase in opportunistic infections of HIV infected individual. The history of new drug discovery processes shows that novel skeletons have come, in the majority of cases, from natural sources (Bevan *et al.*, 1995). One reason for safer, broad-spectrum antifungal antibiotics with this is that when compared to antibacterial, fungi are like mammalian cells, eukaryotes and therefore agents that inhibit protein, RNA or DNA biosynthesis in fungi have greater potential for toxicity (Georgopapadakou *et al.*, 1994). Choice of natural materials like soils in researches is based on the assumption that samples from widely diverse locations are more likely to yield novel microorganisms and therefore hopefully, novel metabolites as a result of the geographical variation. Besides, the important approaches helpful in discovering new microbial species or unknown bioactive substances include isolation and characterization of microorganisms from the most extreme habitations (Lee and Hwang 2002). Manali ice point is well known cryophilic region for its huge and unexplored diversity. This diversity can be explored for isolation and characterization of antifungal activity of native actinomycetes.

2. Materials and method

Sample collection and isolation

soil sample was collected at Three different sites such as Kullu, Middle Hill and ice point of Rothang Hill, Himachalpredesh during October in a sterile container and brought up to laboratory with the help of ice bag. One ml of 10^{-5} sample was poured on Actinomycetes Isolation agar and the plates were incubated at 15° C for 45 days.

Morphological study:

The color of colony, mass of spore and pigmentation were noted on actinomycetes agar. Spore morphology was observed by simple staining and mycelial morphology was done by slide culture method followed by Sudan black stain.

Selection of actinomycets growing at 35° C:

Colonies which are Rough, Powdery colonies were selected and purified by continuous streak method on Starch casein Nitrate agar plates were incubated at 35° C for 7 days. The growing actinomycetes were subjected to Biochemical studies such as IMViC, Hydrolysis of starch, utilization of sugar were performed to differentiate the isolates.

Determination of antifungal activity:

Fermentation was carried out under 28˚C for 7 days under 150 rpm on The International Strptomyces Project-2 (ISP2) broth. The test pathogens *C.albicans, C.glabarata* and *C.neoformans* were swabbed on PDA plates and wells were made using well puncture. About 100 µl of cell free culture filtrate was loaded on the PDA plates and incubated at 28^{o} C for 24 h. To determine the polyene antifungal compound PDA plates with 1% ergosterol were prepared along with a control without ergosterol. The plates were seeded with the test organism. Wells were made with a sterile cork borer and 0.1 ml of culture filtrate was added to the well. The plates were incubated at 28°C for 24 h and observed for the zone of inhibition.

Extraction of active compound:

Cell free supernatant was collected and mixed with equal amount of Ethylacetate (1:1). The sample was shaken vigorously and the solvent phase were collected and evaporated. About 1 mg/ ml of concentrated sample was re dissolved in distilled water and separated on silica G 60 grade absorbent by chloroform: methanol: water (25:24:20) and the the Rf values was recorded. The TLC plates were exposed to Iodine Vapour and bands were collected separately and concentrated by methanol.

Bio assay:

50 µl of 1mg/ml of TLC fractions were loaded on sterile disc and used against test pathogens. The bio assay was performed by disc diffusion method.

3. Results and discussion

Isolation and Identification of actinomycetes

Nearly 28 Actinomycetes from extreme environment was isolated among which *Streptomyces* and *Micromonospora* were most abundant. Based on their spore morphology and biochemical characters the isolates and identified as, *Streptomyces sp, Micromonospora sp, Micropolyspora sp, Dactylsporangium sp, Planomonospora sp* and *Intrasporangium sp* (Table1). The frequency of isolates were 53>38>7.10>3.57>3.57>3.57 (Fig 1). Spiral chain of spore, Monospore, Sporangiospores and Cylindrical spores were observed under microscopically (Table 2). According to Kutzner (1981) for proper identification of genera and species of actinomycetes, besides morphological, various other biochemical properties such as cell wall chemo type, whole-cell sugar pattern, peptidoglycan type, phospholipids type and G+C% of DNA should be determined. Likewise, the statement of Thirumalachar and Sukapure (1964), *Micropolyspora sp* is differentiated from *Streptomyces* "only by the fragmenting nature of the vegetative mycelium" is hardly appropriate, either from a morphological or from a chemical point of view according to Lechevalier *et al.* (1961) to refer to aerobic actinomycetes forming short chains of conidia both on the substrate and the aerial mycelia by *Micropolyspora*.

Antifungal activity of secondary metabolites of isolated actinomycetes

Out of 28 actinomyctes, only 13 were grown at 35° C belongs to *Streptomyces* (5) *Micromonospora sp* (4), and each one from *Dactylsporangium, Intrasporangium, Planamonospora sp* and *Micropolyspora sp*. Cell free culture filtrate of 13 tested actinomycetes showed four were active against *C. albicans, C. glabrata* and *C. neoformans* (Table 3). In the present research the maximun zone of inhibition was 36 mm showed by *Planomonospora* sp

against *C.neoformans* and *C.albicans*. Followed by *Planomonospora* the genus *Sterptomyces* sp. (S4) showed 32 mm zone of inhibition against *C.neoformans*. The control plate without ergosterol showed an inhibition zone diameter of 36 mm, whereas the plate containing the reversal agent, ergosterol, showed a reduced inhibition zone diameter i.e. 18 and 16 mm respectively (Table 4). In this study two of non Streptomyces colonies such as *Planomonospora* sp and *Micromonospora* sp were found to be active against fungal pathogens. This is the first report that the antifungal activity of *Planomonospora* sp. *Streptomyces sp*, that are isolated from different geographical regions and show strong broad-spectrum antifungal antibiotic activities are often members of the S. *violaceusniger clade* or they are closely related strains (Getha and Vikineswary, 2002). The strains that did not exhibit antifungal activities were found to be non-clade members. Many *Streptomyces* sp showed antifungal activity against *Aspergillus sp* (Afifi *et al*., 2012) but not against *C.albicans* (Kokare *et al.*, 2004). In this present investigation, *Planomonospora* sp showed effective antifungal activity against all tested fungal pathogen and it was confirmed that the production of Polyene antifungal. Detection of Polyene antifungal compound by the addition of ergosterol was accomplished earlier by Motta and Brendeli (2002). To determine the effects ergosterol on antifngal activity the medium was supplemented with ergo sterol as reversal agent (Praaveenkumar jain and jain, 2007). The bio assay of TLC fractions reveals that the R_f value of active fraction of *Planomonospora* sp was 1.802 and *Streptomyces* sp was 1.52 (Table 5).

Table 1: Number of actinommycetes isolates

Sample site	Isolate	Number of isolate
Site 1 Kullu	*Streptomyces sp* *Micromonospora sp* *Micropolyspora sp*	7 5 2
Site 2 Mid hill (1000 M)	*Streptomyces sp* *Planomonospora sp* *Dactylosporangium sp* *Intrasporangium sp*	5 1 1 1
Site 3 Manali Ice point (2,500 M)	*Streptomyces sp* *Micromonospora sp*	3 3
Total		28

Fig 1: Frequency of Actinomycetes on Manli soil sampling site

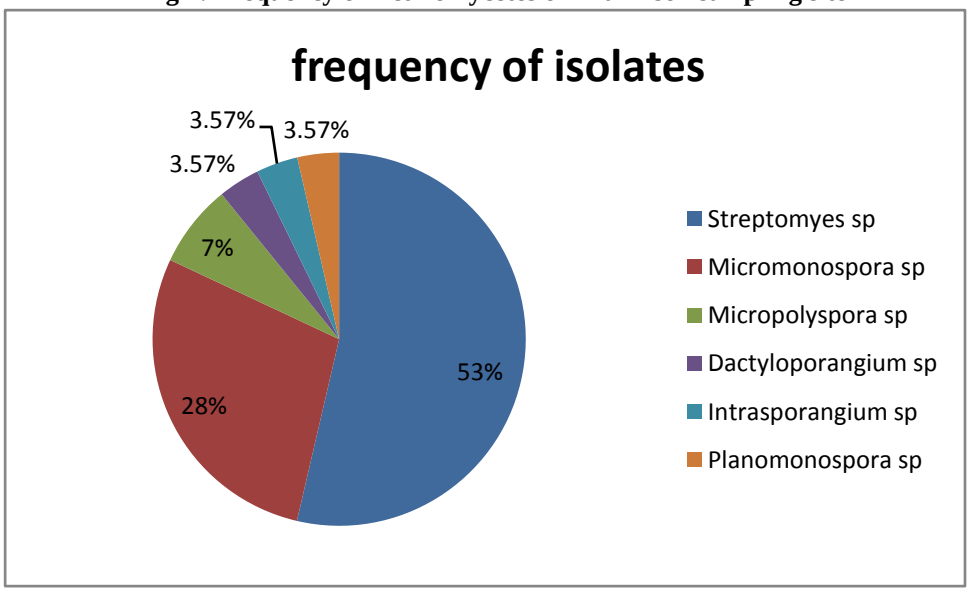

Table 2: Spore and Mycelial Morphology of Actinomycetes

S.No	GENUS	SPORE	Mycelium	Strain code
1	*Streptomyces* sp.	Long Spiral chain of spore	AM/SM	S
2	*Micromonosporaa* sp.	Monospore on substrate mycelium	AM/SM	M
3	*Datylosporangium* sp.	Short sporangiospore	SM	D
4	*Intrasporangium* sp.	Intracallaer vesicles	AM/SM	I
5	*Planomonospora* sp.	Cylindrical sporangiospore on SM	AM/SM	P
6	*Micropolyspora* sp	Short chain spore	AM	MP

Table 3: Anti fungal activity of isolted Actinomycetes

Table 4: Detection of polyene anti fungal compound of active strain

test pathogen	Diameter of Zone of Inhibition (mm)												
	S1	S2	S3	S4	S5	P	M1	M2	M3	M4	Is	Ds	MP
Candida albicans	-	-	-	-	-	36	-	-	-	-	-	-	-
Candida glabrata	-	-	-	-	-	26	20	20	-	-	-	-	-
Cryptococcus neoformans	-	-	-	32		36	22	-	-	-	-	-	-

Bioassay active $^{+}$

test pathogen	Diameter of Zone of Inhibition (mm)												
	S1	S2	S3	S4	S5	P	M1	M2	M3	M4	Is	Ds	MP
C. albicans	-	-	-	-	-	18	-	-	-	-	-	-	-
C. glabrata	-	-	-	-	-	10	20	20	-	-	-	-	-
Cryptococcus neoformans	-	-	-	32		16	22	-	-	-	-	-	-

Table 5: R_f values of compounds from chloroform extraction

Fragment	Bioassay of active compound and its R_f Value	
1	*Streptomyces* sp (S4)	*Planomonospora* sp (P)
	1.793	5.533
2	1.52^{+}	3.220
3	0.876	1.802^{+}

Conclusion

Novel psychrophilic antifungal producing Nonstreptomycetes *Planomonospora* sp was isolated and identified.. Screening of actinobacteria especially rare actinomycetes is from less explored system is very rare. For proper identification and characterization of the antimicrobial extracts it is necessary to obtain in pure form. However, a little effort was made in this preliminary screening. The Present work has resulted in selective isolation of novel soil Actinomycetes and their antifungal activity against some clinical fungal pathogens.

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