Isolation and Detection of polyene antifungal producing Actinomycetes from Kodaikanal Hill

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ABSTRACT

The present study reveals that screening of polyene antifungal producing actinomycetes against *Candida sp* and *Cryptococcus* species. Nearly twelve different actinomycetes strains were isolated and identified as *Streptomyces* sp, *Micromonospora* sp, *Planopolyspora* sp. *Microployspora* sp and *Intrasporangium* sp. The frequency of isolates were 5>4>1>1>1. Out of 12, four isolates were found to be active against fungal pathogen C*andida albicans, C.glabrata* and *Cryptococcus neoformans.* Among the four active antifungal producing actinomycetes *Planopolyspora* sp. was detected as polyene antifungal producer and the active fraction (KP1 F2) showed 4.882 R_f value.

Keywords: Polyene, ISP Medium, antifungal, actinomycetes, non-Streptomycetes

Introduction

Important systemic pathogenic fungi on human are *Candida albicans*, *Candida glabrata* and *Cryptococcus neoformans* those causing candidiasis and cryptococcosis. It is life threatening disease and opportunistic infection for AIDS. The antagonistic activity of Streptomyces to fungal pathogen is usually related to the production of antifungal compounds[1]. Among the different types of drugs prevailing in the market, antifungal antibiotics are very few but vital group of drugs whereas, it has important role in the control of mycotic diseases. The need for new, safe and more effective antifungal agents are a major challenge to the pharmaceutical industry today. The history of new drug discovery processes shows that novel skeletons have come, in the majority of cases, from natural sources[2]. The search for greater potency has been progressing for safer, broad-spectrum antifungal antibiotics that inhibit protein, RNA or DNA biosynthesis in fungi have greater potential for toxicity[3]. Until the 1970s, fungal infections were considered largely treatable and the demand for new medicines to treat them was very small. Before this period, antifungal chemotherapy included only two kinds of compounds: potassium iodide, effective in the treatment of sporotrichosis; and two useful polyenes, nystatin and amphotericin B, which were introduced in the 1950s. Therefore, only a limited number of antifungal agents (polyenes and azoles plus the recently introduced Cancidas) are currently available for the treatment of life-threatening fungal infections. These antifungal agents show some limitations, such as the

significant nephrotoxicity of amphotericin B and emerging resistance to the azoles[4-5], despite several recent improvements, such as lipid formulations of polyenes with lower toxicity and new triazoles (voriconazole, rovuconazole and pasaconazole) with a wider spectrum of action, including activity against some azole-resistant isolates[6].

Actinomycetes are group of gram positive filamentous bacteria from diverse ecological niches is known to produce chemically diverse compounds with wide range of biological activities[7]. Among of soil microbes, actinomycetes are source of important therapeutically products[8]. It has been found that some endophytic microbes can produce valuable pharmaceutical substances of biotechnological interest[9]. Therefore, screening of actinomycetes from this habitat is important for identification of useful strains that produce novel bioactive compounds[10]. Fungicidin, later designated as nystatin, is a tetraene. It was the first polyenic antibiotic isolated from a culture of *Streptomyces noursei*. The polyene macrolide antibiotic nystatin produced by *Streptomyces noursei* ATCC 11455 is an important antifungal agent contains a polyketide moiety with 38 macrolactone ring[11]. Other tetraene antibiotics were later isolated include rimocidin, chrominute, antimycoin, sistomycosin, pimaricin, etruscomycin, unamycin, protocidine, and akitamycin. None of these has found, so far as is known, any practical application. Actinomycetes are the most economical, biotechnologically valuable industrially important group of prokaryotes produced vast array of bioactive metabolites notably chemically diverse antibiotics[12]. Actinomycetes are the main source of clinically important antibiotics, most of which are too complex to be synthesized by combinatorial chemistry, making three quarters of all known products; the Streptomyces are especially prolific, producing around 80% of total antibiotic products. Micromospora is the runner up with less than one-tenth as many as *Streptomyces*[13] used for antibiotic production.

Materials and method

Sample Collection

The soil sample was collected from the coffee garden, Kodaikanal during October 2017 in a sterile container and brought up to laboratory with the help of ice bag.

Isolation of actinomycetes [14]

The serial dilution set up was prepared and sterilized and the soil samples were brought up to the laboratory condition. One gram of soil sample was serially diluted up to 10^{-8} dilution. The dilution such as 10^{-3}, 10^{-4} and 10^{-5} were taken and pour plated over the starch casein agar medium. The plate is then incubated at 15˚c for 7-15 days until the colonies were developed.

Identification of isolated actinomycetes

The colour of colony, mass of spore and pigmentation were noted on actinomycetes isolation agar(AIA). The genus has been identified through spore morphological by sudan black staining followed by slide culture method.

Fermentation for metabolite production

The International Streptomyces project (ISP 2) medium broth were prepared and sterilized. The isolated organisms were inoculated in ISP 2 medium broth and then incubated at 28˚C for 5-10 days under 150 rpm.

Solvent extraction

The fermentation medium was centrifuged at 10,000 rpm for 10 minutes and the cell free supernatant was collected and filtered with Nitro Cellulose Acetate filter paper then it was mixed with equal amount of ethylacetate (1:1). The sample was shaken vigorously and the solvent phase were collected and evaporated.

Determination of Antifungal activity

The test pathogens *Candida glabrata, C.albicans, Cryptococcus neoformans* were swabbed on PDA agar plates. About 100 µl of purified fermented culture filtrate extracted with ethylacetate and concentrated at 5mg/ml was loaded on sterile disc and placed over agar plates. All the plates are incubated at 35^{o} C for 24-48 h. To determine the polyene antifungal the medium was supplemented with Ergo sterol and assayed separately.

Separation of active compound by TLC

The concentrated compound of active strain was separated on silica G 60 grade absorbent by chloroform: methanol: ethylacetate (25:24:20). The TLC plates were UV radiated and then exposed to Iodine vapour and the R_f values were calculated.

R_f= Distance of solvent / Distance of solute

RESULTS

Isolation and Identification of Actinomycetes

Totally 12 actinomycetes strains were isolated and identified as *Planopolyspora sp, Streptomyces sp, Micromonospora sp, Micropolyspora sp,* and *Intrasporangium sp*. Among these 12 Actinomycetes, five comes under the genus *Streptomyces* sp, 4 comes under the genus *Micromonospora*, and the remaining isolates are each one under *Intrasporangium*, *Planopolyspora* and *Micropolyspora* (table 1). Isolates in single genus was differentiated based on their spore and mycelium. The differences in characteristics of aerial

mycelia color and soluble pigments they produce, is one of an indication of the diversity of actinomycetes isolates in the sampling sites. Isolates belongs to *Stereptomyces* sp produced both aerial and substrate mycelium with reticuli spiral spores. Genera of Micropolyspora produced fragmented aerial mycelium with short chain of spore. Isolates comes under *Micromonospora* sp showed monospore on aerial mycelium. *Intrasporangium* sp showed powdery, substrate mycelium with zygospore and the *Planopolyspora* sp with short cylindrical spore. It is clear that cultures that were collected from sampling site were potential for screening of active substances.

Antifungal activity of isolated actinomycetes

Out of 12 tested actinomycetes, four were active against Candida and Cryptococcus (table 2). Among them, *Streptomyces* shows moderate antifungal activity against Candida. In the present research the maximun zone of inhibition was 39 mm recorded in *Planopolyspora* sp (KP1) against *C.neoformans*. It is very rare to identify the non Sterptomycetes as novel antifungal products. Followed by *Planopolyspora* the genus Sterptomyces designated as KS2 showed 34 mm zone of inhibition against *C.neoformans*. The genus *Planopolyspora sp* (KP1) only active against 3 test pathogens and it was found to produce polyene antifungal agent. The zone of inhibition was reduced when the medium is supplemented with ergosterol. Other three active strains were comes under non polyene antifungal producers. Antifungal activity of *Planaopolyspora* sp without ergosterol showed 39 mm zone of inhibition where as the plate contains reversal agent showed a reduced inhibition zone to 23 mm respectively for *Cryptococcus neoformans*. It confirms the production of polyene antifungal agent against *Candida sp* and *Cryptococcus sp*. The concentrated active compound dissolved in double distilled water separated by TLC showed different fluorescent band under UV at 365 nm. Active fraction of KP1 was identified with the help of iodine vapour and bio assayed against *Candida sp* (image 1). The activity of collected spot given by the extract of active strain was linear and differ in their R_f values (table 3). The R_f value of active strain were 7.090 (KS2), 4.882 (KP1) and 5.533 (KS4).

Discussion

Morphologically different colonies were isolated and identified. All the actinomycetes were identified based on mycelium and spore production. Proper identification of genera and species of actinomycetes, besides morphological and physiological properties, 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. Streptomyces differentiated from *Micropolyspora* sp by forming short chains of conidia on fragmented mycelium on the substrate and the aerial mycelia[15-16]. Many author reported that the highest occurrence of streptomycete like strain of the grey aerial mycelium[17]. *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[18]. The strains that did not exhibit

antifungal activities were found to be non-clade members. Many *Streptomyces* showed antifungal activity against *Aspergillus* sp but not against *C.albicans*[19]. Detection of Polyene antifungal compound by the addition of ergosterol was accomplished on *Planopolyspora* sp and reported earlier by Motta and Brendeli[20]. To determine the effects ergosterol on antifungal activity the medium was supplemented with ergosterol as reversal agent [21]. For complete characterization of an antibiotic it should be isolated in pure form. However, a little effort was made in this approach to purify the compound and the antimicrobial agents obtained in this study can't be declared as new antibiotics, there is the probability of finding new antibiotics in soil because of its wide biodiversity. For proper identification of the antimicrobial extracts it is necessary to obtain in pure form. The present work has resulted in selective isolation of novel soil actinomycetes and their antifungal activity against some clinical yeast pathogens.

Conclusion

Searching for drugs against fungal infections is a major challenge to current research in mycotic infection. A rare actinomycetes such as *Planopolyspora* sp and *Streptomyces* sp were isolated and found to be antifungal producer.

Table 1: Study of spore and morphology of isolated Actinomycetes

Strain code	Name of the isolate	Aerial mycelium and spore morphology	Reverse side
KM1	*Micromonospora sp*	Light ash, powdery, monospore	Yellowish green
KI1	*Intrasporangium sp*	Yellow to White, powdery, moist, no pigmentation, zygospore	Dull yellow
KS1	*Streptomyces sp*	Chalky white, powdery, diffusible pigment, refractile spiral spore	Yellowish white
KS2	*Streptomyces sp*	white to ash, rough powdery, no pigmentation, long spiral chain spore	Dull white
KP1	*Planopolyspora sp*	Dark Grey, powdery, slightly rough, Pigmentation, short cylindrical spore	Reddish yellow
KS3	*Streptomyces sp*	Sandal white, powdery, no pigmentation, long spiral chain spore	Dull white
KS4	*Streptomyces sp*	Ash, powdery, orange brown pigmentation, spiral chain spore	Light yellow
KM2	*Micromonospora sp*	light grey , non diffusible pale yellow pigment	Green
KM3	*Micromonospora sp*	White fine powdery, pinkish red monospore	Pale yellow
KMP1	*Micropolyspora sp*	Fine powdery, ash, no pigmentation, short chain spiral spore, Fragmented mycelium	Dull white
KS5	*Streptomyces sp*	White powdery, rough , non diffusible pale yellow pigment, sporangia spore	brown
KM4	*Micromonospora sp*	Chalky white, no pigmentation, monospore	Fluorescent yellow

Table 2: Anti fungal activity of isolated actinomycetes

Strain code		*C.albicans*	*C.glabrata*	*Cryptococcus neoformans*
KM1		Nil	Nil	Nil
KI1		Nil	Nil	Nil
KS1		Nil	Nil	Nil
KS2		Nil	Nil	34 mm
KP1	Without ergo sterol	32 mm	26 mm	39 mm
	With ergo sterol	24 mm	18 mm	23 mm
KS3		Nil	Nil	Nil
KS4		Nil	20 mm	18 mm
KM2		Nil	20 mm	Nil
KM3		Nil	Nil	Nil
KMP1		Nil	Nil	Nil
KS5		Nil	Nil	Nil
KM4		Nil	Nil	Nil

Table 3: R_f values of compounds from ethylacetate extraction

Active fraction	TLC active compound		
Isolate	KS2	KP1	KS4
R_f	7.090	4.882	5.533

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