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Screening of Local Actinomycete Isolates in Manipur for Anticandidal Activity

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Abstract: The present study was aimed at screening of local actinomycete isolates for anticandidal activity, selection and characterization of potent strain (s) and initial optimization of fermentation conditions for anticandidal production. Of 35 actinomycete isolates in our collection from various biotopes in Manipur that were screened by cross-streak method, 2 strains (5.7% of isolates screened) NRP1-14 and NRP1-26, obtained from Nambul river in Imphal, Manipur, India, showed anticandidal activity. Based on relative potency, NRP1-14 was selected for further studies. It has been identified as *Streptomyces mutabilis* NRP1-14 based on phenotypic and genotypic characteristics. Among various media tested for submerged fermentation of the bioactive strain, Starch Casein Nitrate (SCN) broth was found to be the best. The strain produced significant activity against *Candida albicans* MTCC 227 in SCN broth after 3 days of incubation in SCN broth of pH 7.2 at 28°C under shaking speed of 150 rpm. The NRP1-14, interestingly, also exhibited significant antagonistic activity against several rice fungal pathogens-*Bipolaris oryzae*, *Curvularia oryzae*, *Fusarium oxysporum* and *Pyricularia oryzae*. Anticandidal activity is being optimized by improving cultural and environmental conditions for fermentation. This study shows that biotopes in Manipur can yield promising actinomycetes for use in medicine and agriculture. To our knowledge, this is the first report of a *Streptomyces mutabilis* strain with potent anticandidal activity. The NRP1-14, therefore, warrants further investigation for possible application as anticandidal and biocontrol agents (BCAs).

Key words: Antifungal, *Streptomyces mutabilis*, *Candida albicans*, actinomycetes, Manipur

INTRODUCTION

Infectious diseases rank next to cardiovascular diseases (CVDs) as the leading cause of death worldwide. Emergence of new and resistant fungal pathogens, increasing incidence in immunocompromised patients and environmental and health problems of chemical fungicides dictate the search for novel antifungal agents for human diseases and plant protection (Sharma and Kumar, 2009; Guo *et al*., 2008; Augustine *et al*., 2005). *Candida* sp. cause 6.2% of human infections, ranking as the 4th most prevalent infectious agent (Cowen *et al*., 2009).

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The search for new antifungal agents (AFAs) has been rather slow (Gupte *et al*., 2002). This is because molds and fungi being eukaryotic, it is difficult to find selective agents targeting fungal metabolism without significant toxicity to humans (Georgopapadakou and Walsh, 1994) and also, till recently, the antifungal market was considered too small to warrant development of new AFAs (Georgopapadakou and Walsh, 1996). Currently used AFAs are either highly toxic (amphotericin B) or pathogens have acquired resistance (azoles). Hence, there is an urgent demand for new antifungal agents and drugs.

Novel lead compounds usually come from natural sources especially plants and microorganisms (Bevan *et al*., 1995; Cragg *et al*., 1997; Nolte *et al*., 1997; Shadomy, 1987). Actinomycetes are the most prolific producer of antibiotics, accounting for about 80% of known antibiotics (Berdy, 2005) As terrestrial sources are getting exhausted, survey is increasingly directed towards underexplored habitats, niche ecosystems, or extreme biotopes supplemented by novel selective media and/or pretreatment methods for isolation of rare actinomycetes or novel strains of *Streptomyces*. Manipur holds great promise for such bioprospecting studies as it is largely unexplored for actinomycetes though it is rich in biodiversity, being part of Indo-Burma Hotspot (Myers *et al*., 2000; Ningthoujam *et al*., 2009). In our continuing search for bioactive actinomycetes from various biotopes in Manipur, we now have a substantial collection of 172 lake, 75 forest, 50 river, 35 cave, 25 endophytic and 10 salt spring isolates.

The present study reports the results of screening randomly selected 35 isolates from our collection for anticandidal activity, identification of the bioactive strains and initial data on fermentation conditions for antifungal production.

MATERIALS AND METHODS

Isolation

Soil and sediment samples were obtained from various niche habitats in Manipur such as Loktak lake, Bishnupur district and Nambul river, Imphal West district, Shirui jungle, Shirui hills and other forests in Ukhrul district, Salt spring at Shikhong and other sites, Thoubal district, Kangkhui cave, Ukhrul district, Manipur, India. Most of these samplings were done in the period Jan-Oct, 2008. Short-term preservation, pretreatment of the samples and subsequent isolation and preservation of actinomycetes were performed at MBRL, Department of Biochemistry, Manipur University Canchipur, India.

Actinomycetes were isolated from soil and sediment samples collected from various biotopes in Manipur by dilution plating on Starch Casein Nitrate Agar (SCNA) (Kuster and Williams, 1964) and Chitin Agar (Hsu and Lockwood, 1975). After incubation at 28°C for up to 3 weeks, actinomycete colonies were purified by subculturing. Pure colonies were preserved in Bennett's agar slants at 4°C.

Test Pathogens

Candida albicans (MTCC 227), *Curvularia oryzae* (MTCC 2605), *Fusarium oxysporum* (MTCC 287) and *Pyricularia oryzae* (MTCC 1477) were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. *Bipolaris oryzae* was procured from Life Science Department, Manipur University, Imphal, India.

Screening

Primary screening was done by cross-streak method (Madigan *et al*., 1997) and secondary screening by Kirby-Bauer method (Bauer *et al*. 1966). For primary screening actinomycete isolates are grown on SCNA and indicator fungi are grown on Sabouraud Dextrose Agar (SDA). Actinomycetes were streaked in the middle of the plate and allowed to grow for about 3 days and then test fungi are streaked perpendicular to the actinomycetes and further incubated for 48-72 h at 30°C and inhibition profiles were then measured.

For secondary screening, actinomycete strains were grown in SCN broth and inoculated in wells (6 mm diameter) punched in SDA plates containing lawns of test fungi and inhibition zones were measured after 48-72 h of incubation at 30°C.

Antagonism against phytopathogenic fungi was assayed by dual culture (Yuan and Crawford, 1995). Percent inhibition was measured by the formula:

$$\text{Inhibition (\%)} = [(A-B)/A]\times 100$$

where, A is diameter of fungal growth in control plate and B is diameter of fungal growth in experimental plate.

Characterization of Bioactive Strains

Phenotypic characterization of bioactive strain was performed as per ISP protocols and standard procedures (Shirling and Gottlieb, 1966; Holt *et al*., 1994). Genotypic characterization was done according to standard procedures with the help of CCMB, Hyderabad, India.

Fermentation

Several standard microbiological media such as Nutrient Broth (NB), *Streptomyces* Broth (SB), Tryptic Soy Broth (TSB), SCN broth and LB broth were tested to find out the most suitable medium for shake flask fermentation of the bioactive strains and inhibition zones tested at different times of incubation by Kirby-Bauer method (Bauer *et al*., 1966).

RESULTS AND DISCUSSION

Isolation

Several actinomycete isolates were obtained from various biotopes in Manipur by selective isolation (data not shown).

Screening

Out of 35 randomly chosen isolates, 2 strains obtained from Nambul river in Imphal, Manipur, India, NRP1-14 (Fig. 1) and NRP1-26 were found to be antagonistic against *C. albicans* in cross-streak test. This was also confirmed by disc diffusion assay (Kirby-Bauer method) after shake-flask fermentation in SCN broth. Based on relative potency (Fig. 2), NRP1-14 was selected for further studies (Table 1).

Characterization of Bioactive Strain

NRP1-14 was subjected to phenotypic characterization (Table 2, 3). Based on the morphological, physiological and biochemical characteristics and genotypic and phylogenetic analysis with the help of Centre for Cellular and Molecular Biology (CCMB),

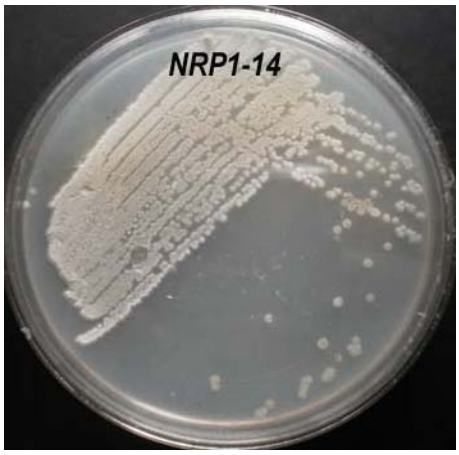

Fig. 1: NRP1-14

Fig. 2: Anticandidal activity of NRP1-14

Table 1: Time course of bioactivity of *Streptomyces mutabilis* NRP1-14

Days of incubation	Inhibition zone (mm)[#]
3	17
5	20
7	19

[#]Results are averages of 3 replicates

Hyderabad, India, the strain was identified as *Streptomyces mutabilis* NRP1-14 (Fig. 3) based on analysis of the phylogenetic tree with related strains obtained from BLAST search, using neighbor joining method.

Fermentation

Among various media tested for bioactive metabolite production by NRP1-14 in submerged fermentation, SCN broth (pH 7.2, 28^{0}C, 150 rpm) was found to be the best.

Table 2: Major phenotypic characteristics of *Streptomyces mutabilis* NRP1-14

Characteristics	Results
Gram's staining	+
Optimum pH for growth	5-6 (grows at pH 5-10; no growth at pH 11)
Optimum temp. of growth (°C)	25-30 (range 20-42, no growth at 55)
NaCl tolerance (%, w/v)	0-2 optimal (range: 0-10)
Degradation of (%, w/v)	
Tyrosine (0.5)	+
Xanthine (0.4)	+
Growth in presence of (%, w/v)	
Phenol (0.1)	+
Phenol (0.5)	+
Hydrolysis of	
Casein	w
Gelatin	+
Starch	+
Urea	+
Nitrate reduction	+
MR test	-
VP test	-

+: Positive, w: Weak positive, -: Negative

Table 3: Growth morphology of NRP1-14 on different media

Medium	Aerial mycelium	Substrate mycelium
SA	Pearl cream	Off white
ISP 1	Classic white	Off white
ISP 2	Colorless	Colorless
ISP 3	Regency grey	Chamois
ISP 4	Greyish	Classic white
ISP 5	Suede	Sandalwood
ISP 6	Colorless	Colorless
ISP 7	Suede	Classic white

SA: Streptomyces agar, ISP: International streptomyces project

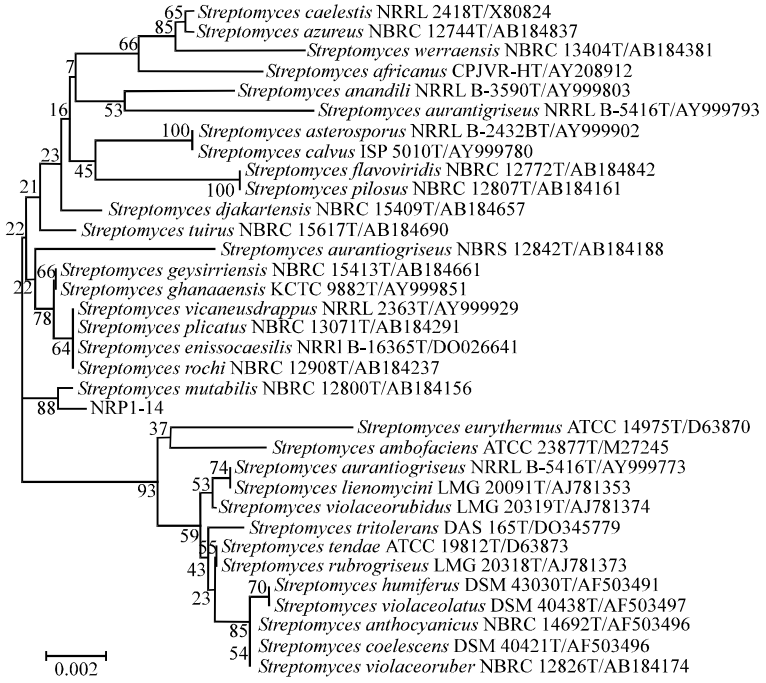

Fig. 3: Phylogenetic tree of NRP1-14

Table 4: Biocontrol activities of *Streptomyces mutabilis* NRP1-14

Pathogens	Inhibition (%)[b]
Bipolaris oryzae	51.2
Curvularia oryzae	42.0
Fusarium oxysporum	46.6
Pyricularia oryzae	60.5

[b]Results are averages of 3 replicates

Biocontrol Activities of NRP1-14

Streptomyces mutabilis NRP1-14, interestingly, also shows promising antagonistic activities against several important fungal pathogens of rice (Table 4) besides inhibiting *Candida albicans*.

The strain *Streptomyces mutabilis* NRP1-14 has been shown to exhibit promising bioactivities against *C. albicans* and several plant pathogenic fungi. It was isolated from a river soil sample. Cross (1981) reported that freshwater habitats are fruitful for isolation of antibiotic-producing actinomycetes. Manipur abounds in lakes, streams, rivers and other wetlands and freshwater habitats. Reports on freshwater actinomycetes are however, scanty compared to that of marine actinomycetes except for some publications on lake actinomycetes (Terkina *et al*., 2006).

Recent reports on antifungal *Streptomyces* species are predominantly of biocontrol strains for agricultural use as compared to anticandidal strains for potential medical applications (Han *et al*., 2008). To our knowledge, this is the first report of a *Streptomyces mutabilis* strain antagonistic to *C. albicans*. The few reports on bioactive strains of *Streptomyces mutabilis* are about antibacterial or anticoccidal activities (Fehr *et al*., 1977).

CONCLUSION

Streptomyces mutabilis NRP1-14 reported in this study shows significant bioactivities against both *Candida albicans* and several plant pathogenic fungi. Further studies are needed to explore its potential for possible medical and agricultural applications. Characterization of the bioactive metabolite (s) of this strain and optimization of its production must now be done. This will be the focus of our further investigations.

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REFERENCES

Augustine, S.K., S.P. Bhavsar and B.P. Kapadnis, 2005. A non-polyene antifungal antibiotic from *Streptomyces albidoflavus* PU 23. J. Biosci., 30: 201-211.

Bauer, A.W., W.M. Kirby, J.C. Sherris and M. Turck, 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol., 45: 493-496.

Berdy, J., 2005. Bioactive microbial metabolites. J. Antibiot., 58: 1-26.

Bevan, P., H. Ryder and I. Shaw, 1995. Identifying small-molecule lead compounds: The screening approach to drug discovery. Trends Biotechnol., 113: 115-121.

Cowen L.E., S.D. Singh, J.R. Kohler, A.K. Zaas and W.A. Schell *et al*., 2009. Harnessing Hsp90 function as a powerful, broadly effective therapeutic strategy for fungal infectious disease. Proc. Natl. Acad. Sci. USA., 106: 2818-2823.

Cragg, G.M., D.J. Newman and K.M. Snader, 1997. Natural products in drug discovery and development. J. Nat. Prod., 60: 52-60.

Cross, T., 1981. Aquatic actinomycetes: A critical survey of the occurrence, growth and role of actinomycetes in aquatic habitats. J. Applied Bacteriol., 50: 397-423.

Fehr, T., H.D. King and M. Kuhn, 1977. Mutalomycin, a new polyether antibiotic taxonomy, fermentation, isolation and characterization. J. Antibiot., 30: 903-907.

Georgopapadakou, N.H. and T.J. Walsh, 1994. Human mycoses: Drugs and targets for emerging pathogens. Science, 264: 371-373.

Georgopapadakou, N.H. and T.J. Walsh, 1996. Antifungal agents: Chemotherapeutic targets and immunogenic strategies. Antimicrob. Agents Chemother., 40: 279-291.

Guo, B., Y. Wang, X. Sun and K. Tang, 2008. Bioactive natural products from endophytes: A review. Applied Biochem. Microbiol., 44: 136-142.

Gupte, M., P. Kulkarni and B.N. Ganguli, 2002. Antifungal antibiotics. Applied Microbiol. Biotechnol., 58: 46-57.

Han, J.H., I.C. Hwang, S.H. Cho, C. Jang and N.G. Kim *et al*., 2008. Description of *Streptomyces neopeptinus* sp. nov., an actinobacterium with broad spectrum antifungal activities. J. Microbiol., 46: 295-299.

Holt, J.G., N.R. Kreig, P.H.A. Sneath, J.T. Staley and S.T. Williams, 1994. Bergey's Manual of Determinative Bacteriology. 9th Edn., Williams and Wilkins, Baltimore.

Hsu, S.C. and J.L. Lockwood, 1975. Powdered chitin agar as a selective medium for enumeration of Actinomycetes in water and soil3. Applied Microbiol., 29: 422-426.

Kuster, E. and S. Williams, 1964. Selection of media for isolation of streptomycetes. Nature, 202: 928-929.

Madigan, M.T., J.M. Martinko and J. Parker, 1997. Brock Biology Microrganisms. 8th Edn., Prentice Hall International Inc., New Jersey, pp: 440-442.

Myers, N., R.A. Mittermeier, C.G. Mittermeier, G.A.B. Da Fonseca and J. Kent, 2000. Biodiversity hotsopts for conservation priorities. Nature, 403: 853-858.

Ningthoujam, D.S., S. Sanasam, S. Nimaichand and P. Sanjenbam, 2009. Screening and optimization studies of native anticandidal actinomycetes from Manipur (Indo-Burma Biodiversity Hotspot), India. Proceedings of the 15th International Symposium on the Biology of Actinomycetes (ISBA15), Shanghai Jiaotong University, Shanghai, Aug. 20-25,2009.

Nolte, F.S., T. Parkinson and D.J. Falconer, 1997. Isolation and characterization of fluconazole-and amphotericin B-resistant *Candida albicans* from blood of two patients with leukemia. Antimicrob. Agents Chemother., 41: 196-199.

Shadomy, S., 1987. Preclinical Evaluation of Antifungal Agent. In: Recent Trends in the Discovery, Developments and Evaluation of Antifungal Agents. Fromtling, R.A. (Ed.). Prous Science, New Jersey, pp. 8-14.

Sharma, S. and P. Kumar, 2009. *In vitro* antifungal potency of some plant extracts against *Fusarium oxysporum*. Int. J. Green Pharm., 3: 63-65.

Shirling, E.B. and D. Gottlieb, 1966. Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol., 16: 313-340.

Terkina, I.A., V.V. Parfenova and T.S. Ahn, 2006. Antagonistic activity of actinomycetes of Lake Baikal. Applied Biochem. Microbiol., 42: 173-176.

Yuan, W.M. and D.L. Crawford, 1995. Characterization of *Streptomyces lydicus* WYEC 108 as a potential biocontrol agent against fungal root and seed rots. Applied Environ. Microbiol., 61: 3119-3128.