Impact of modified conventional methods on isolation of actinomycetes population

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ABSTRACT

Actinomycetes from diverse environment are well known antibacterial producers. Isolation of undiscovered actinomycetes from marine environment switched over by recovering non Streptomyces colonies. Rare genera of actinomycetes were isolated from marine sediments by selective isolation procedures. Totally 95 actinomycetes colonies were isolated and 11 of them were isolated by conventional dilution method. Among the isolates, 72.7 % of them belong to *Streptomyces* sp and 27.2% of them non Streptomyces in nature. Maximum recovery (87.5%) of genus *Streptomyces* sp was isolated from the sample treated 0.1% phenol. Isolation of nonstreptomyces colonies were also recovered by different adopted isolation methods such as, 0.5M sucrose gradient centrifugation (80%) followed by dry heat treatment (64%). The non Streptomyces colonies were identified as *Nocardia sp*, *Planomonospora sp, Micropolyspora sp, Streptosporangium sp* and *Micromonospora sp.* The research confirms application of heat treatment and sucrose gradient centrifugation promotes the selective isolation of non Streptomyces from environmental samples.

Key words: nonstrptomyces; Planomonospora; Sucrose solution; SDS; Phenol

Introduction

Actinomycetes are widely distributed in natural environment and play an important role in antibiotics production[1]. Marine-derived actinomycetes are rich sources of novel secondary metabolites which harbour unique structures and have diverse antimicrobial activity. Marine microorganisms widely distributed on our oceans of the earth and emerging as the great source for antibacterial antifungal, anti infective, enyme inhibitors and antiviral. The marine ecosystem need the use of reliable selective isolation procedures can aid in the isolation of members of the novel taxa[2]. Most of them predominantly produced by the Genus of *Streptomyces* sp[3]. Rare *Actinomycetes* are usually regarded as strains whose isolation frequency much lower than those strains isolated by conventional methods. Efforts on isolation of rare Actinomycetes have discovered some genera, such as *Actinomadura*, *Actinoplanes*, *Micromonospora*, and *Microtetraspora* that had been recovered from many soil samples[4]. Selective isolation of industrially important actinomycetes is not effective as simple isolation technique[5]. When conventional isolation techniques were applied, most of the isolates recovered on agar plates have been identified as genus *Streptomyces*, which are the dominant actinomycetes in soil[6]. For the purpose of screening novel bioactive molecules, several factors must be considered: choice of screening source, pretreatment, selective medium, culture condition, and recognition of candidate colonies on a primary isolation plate[7]. The role of rare actinomycetes as bioactive molecule sources became apparent as these organisms provided about 25% of the antibiotics of actinomycete origin reported during 1975 to 1980. Rare actinomycetes have usually been regarded as strains of actinomycetes whose isolation frequency by conventional methods is much lower than that of *Streptomycete* strains. Consequently basic knowledge of the habitat, physiology and productivity of molecules of rare actinomycetes gradually increased. An alternative approach was to make the isolation procedure more selective by adding chemicals such as phenol to the soil suspension. Specialized growth media were developed to isolate specific actinomycete genera. 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[8] . 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 and relatively unknown or unstudied areas[9] .

Materials and method

Sampling and study area

Marine sediments were collected from Auroville, Pondicherry east coast region by Zig Zag manner between 25 m distance at 10 different sites. About ten gram air dried soil samples were mixed well and divided in to four parts and used for isolation.

Heat treatment:

About 10 gm of air dried soil sample was heated at 60° C for 30 min and then serially diluted up to 10^{-7} by normal saline. One ml of 10^{-6} was poured on sterile plates and overlaid with Actinomycetes isolation agar. All the plates were incubated at 28° C for 15 days.

Phenol treatment[10]

1gm of soil was mixed with 10 ml of 0.1% phenol and stirred well for 30 min. Then the aliquot was serially diluted up to 10^{-7} and one ml of 10^{-6} was poured on sterile plates and overlaid with Actinomycetes isolation agar. All the plates were incubated at 28° C for 15 days.

SDS treatment

1gm of soil was mixed with 10 ml of 1% SDS and stirred well for 30 min. Then the aliquots was serially diluted up to 10^{-8} using 1% SDS and one ml of 10^{-6} was poured on sterile plates and overlaid with Actinomycetes isolation agar. All the plates were incubated at 28° C for 15 days

Sucrose gradient centrifugation

Soil sample was serially diluted with 0.2, 0.4, 0.6, 0.8 and 1M sucrose of sucrose solution and centrifuged at 10,000 RPM for 30 minutes. One ml of 10^{-6} aqueous suspension of different concentration was poured

on sterile plates and overlaid with Actinomycetes isolation agar. All the plates were incubated at 28° C for 15 days.

Spore and mycelia Morphology

Spore ornamentation was observed by a Nikon photo microscope. Mycelia production was identified by Slide culture method and the nature of mycelium was determined by staining with Sudan black.

Results and discussion

Fine powdery, granular and leathery colonies were isolated from marine sediment and identified based on their spore and mycelia study. Genera identification studied by presence of aerial mycelium, fragmentation or non fragmentation of substrate and aerial mycelium, presence of sclerotia, spore chain morphology and color of spore mass according to Hayakawa[11] Diffusible pigmentation was observed in *Micromonospora* sp and *Planomonospra* sp only (image 1). The number of genera isolated from the sample by different method was given in figure 1. It denotes that maximum of 4 different rare genera was isolated by simple sucrose gradient technique. *Streptomyces* sp with spiral chain of retractile spores was most frequently isolated from the untreated soil sample and *Micromonospora* sp with monospore on substrate mycelium was less frequently isolated. Besides the treated sediments, phenol treatment significantly found to be selective for isolation of *Streptomyces* sp. It was observed that 87.5 % of the isolated colonies were belongs to *Streptomyces* sp. Treatment of sample with SDS showed maximum colony forming unit of actinomycetes[12] than untreated soil and also enhanced the recovery of *Micromonospora* sp. Similarly least number of actinomycetes was observed in chlorinated sediments. Among the nine different selective isolation methods it was observed that the heat treated and sucrose gradient centrifugation are found to be most effective methods for selective isolation of rare actinomycetes. Centrifugation permits the isolation of motile actinomycetes. The frequency of isolated actinomycetes was given in table1. It was noted that the heat treatment reduced the frequency of Streptomyces than control of from 72 to 0 and enhanced by phenol treatment to 87%. Similarly maximum

of 12 rare actinobacteria were isolated by sucrose gradient method. Characteristics of the spore bearing hyphae and spore chains was determined by phase contrast microscopy using slide culture techniques[13]. Based on spore morphology the isolates were identified as *Streptomyces* sp, *Micromonospora* sp, *Micropolyspora* sp, *Nocardia* sp, *Streptosporangium* sp and *Planomonospora* sp. The diversity and frequency of non Streptomyces was given in table 2. Soil sample treated at 60°C showed reduction on recovery of *Streptomyces* sp. Soil suspension treated at 70°C for 15 min inhibited the fungal and bacterial colonies, thus the recovery of actinomycetes, specifically, rare actinomycetes, increased up to 50% of the total microorganisms. Phenol treatment of soil suspension lowered the number of fungi and other bacteria, but the actinomycetes were less affected, thus 65% of the colonies belonged to rare actinomycetes[14] . The maximum frequency of *Micromonospora* sp (70%) and *Nocardia* sp was 20 % were isolated from heat treated soil. Other non streptomyces like *Micropolyspora* sp and *Planomonospora* sp were predominant in 1M sucrose solution treated sediments. Sample treated with 5% sodium chloride showed the isolation of *Streptosporangium* sp. The significant interest of search for rare and new actinomycetes is a key for drug discovery due to a growing need for the development of new and potent therapeutic agents. Challenges on isolation of rare actinomycetes due to the requirement of appropriate isolation procedures was explored in this study and found a simple heat treatment is effective[15] and optimized selection methods. Novel genera isolation like *Herbidospora*, *Microbispora, Microtetraspora* and *Streptosporangium previously* done by chloramines treatment as chlorination is known to suppress growth of contaminant bacteria and promotes the growth of these rare actinomycetes produced rare metabolites [16-17]. Antimicrobial, larvicidal, and antioxidant producing morphologically different actinobacterial cultures isolated from mangrove rhizosphere sediment[18] and 42 strains of actinobacteria were isolated from south-east coast of India[19]. The chances of isolating a novel *actinomycetes* strain have substantially diminished, and so the probability of discovering a novel compound also less. Therefore the study is focused to isolate some rare actinomycetes from marine sample. Isolation of rare actinomycetes and its bioinformatic analysis of genomes further reveals silent gene clusters, which are not expressed under standard laboratory condition needed for novel compound discovery [20]

Conclusion

We have evaluated the different isolation methods for selective isolation of rare actinomycetes. Isolation of actinomycetes followed by the treatment of sucrose gradient centrifugation was a fast and suitable method for the recovery of nonstreptomyces. Similarly, elimination of unwanted bacteria by heat, chlorine and SDS treatment also enhanced the recovery of nonstreptomyces colonies. Diverse genera of rare actinomycetes are successfully recovered incidentally by conventional dilution-plating techniques.

Table 1: Frequency of isolated actinomycetes from soil sample

S.No	Sample	No. of. Streptomyces	Streptomyces (%)	No. of. Non Streptomyces	Total
1.	Untreated soil	8	72.7	3	11
2.	Heat treated	0	0	6	6
3.	Phenol treated (0.1%)	14	87.5	2	16
4.	Chlorinated sample (10 ppm)	1	33.33	2	3
5.	SDS (5%)	7	63.63	4	11
6.	Sucrose Gradient centrifugation (0.25M)	6	54.54	5	11
7.	Sucrose Gradient centrifugation (0.5M)	2	13.33	12	15
8.	Sucrose Gradient centrifugation (0.75M)	1	7.69	12	13
9.	Sucrose Gradient centrifugation (1M)	1	11.11	8	9

Table 2: Frequency of non Streptomyces isolates from soil

S.No	Sample	Genus name	Frequency percentage
1.	Untreated soil	*Micromonospora sp*	27.2
2.	Heat treated	*Micromonospora sp*	70
		Nocardia sp	20
		Micropolyspora sp	10
3.	Phenol treated (0.1%)	*Micromonospora sp*	12.5
4.	Na Cl treatment (5%)	*Streptosporangium sp*	66.66
5.	SDS (5%)	*Micromonospora sp*	36.36
6.	Sucrose Gradient centrifugation (0.25M)	*Micromonospora sp*	27.27
		Planomonospora sp	18.18
7.	Sucrose Gradient centrifugation (0.5M)	*Micromonospora sp*	20
		Micropolyspora sp	20
		Nocardia sp	20
		Planomonospora sp	26.6
8.	Sucrose Gradient centrifugation (0.75M%)	*Micromonospora sp*	38.46
		Micropolyspora sp	23.07
		Planomonospora sp	30.76
9.	Sucrose Gradient centrifugation (1M)	*Micropolyspora sp*	33.33
		Planomonospora sp	55.55

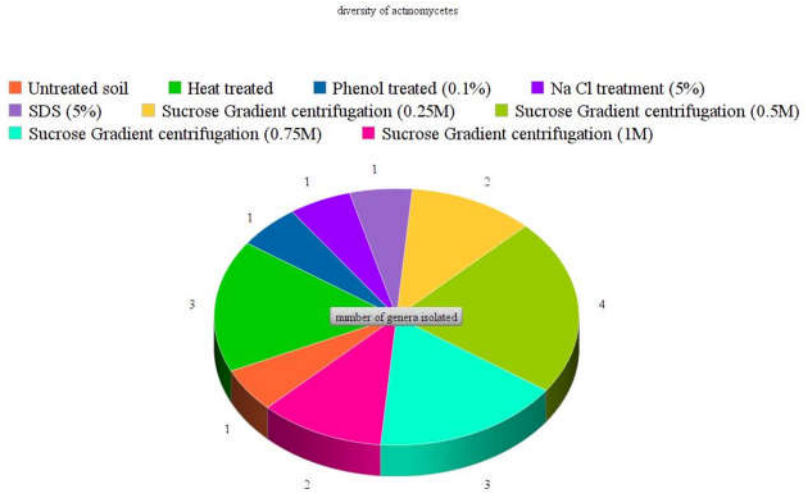

Figure 1. Number of genera recovered by modified conventional method

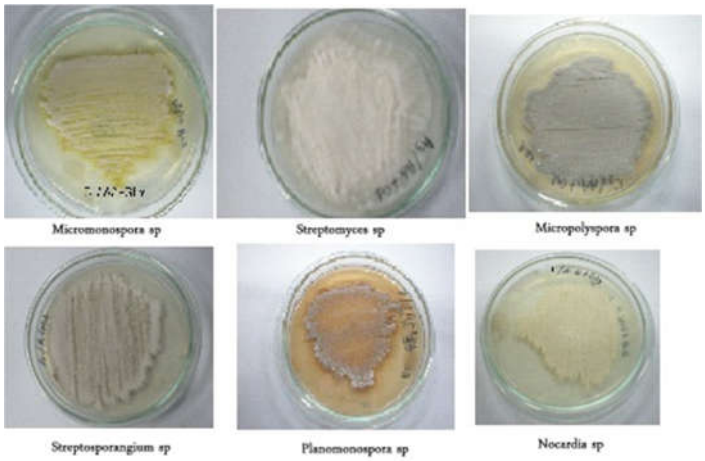

Image 1. Isolated rare actinomycetes colony morphology

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