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RESEARCH ARTICLE

RAPID ISOLATION OF GENOMIC DNA FROM MANGROVE SOIL ACTINOBACTERIA OF COASTAL ANDHRA PRADESH

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ABSTRACT

Actinobacteria isolated from mangrove soil performed by plating technique using Starch Casein agar medium. The inoculated plates were incubated at $30 \pm 2^\circ\text{C}$ for 7 days. The isolates purified by streak plate method. The antimicrobial potent purified isolates were subjected to molecular screening by isolation of genomic DNA. The extraction was designed to be carried out using liquid nitrogen for rapid isolation of high purity and quantity of DNA. Extraction of purified genomic DNA is very crucial step for amplification of 16S rDNA for identification. The present method is also useful and suitable for genetic diversity studies in actinobacteria by randomly amplified polymorphic DNA (RAPD).

Key words:

Liquid Nitrogen, 16SrDNA, PCR (Polymerase Chain Reaction), Agarose gel Electrophoresis, Randomly Amplified Polymorphic DNA (RAPD).

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INTRODUCTION

Actinomycetes are gram positive filamentous bacteria. Characteristics of actinomycetes are similar to both bacteria and fungi. Actinomycetes are similar to fungi in their shape and branching properties and spore formation (Rao et al., 1999). These are rod shaped bacteria and colonies appear to be fungus like branched networks of hyphae (Holt et al., 1994). Generally actinomycetes are present in soil (Martin et al., 1989). Case and Carter (2013) suggested the estimates of the given values range from 10^{-4} to 10^{-8} of actinomycetes per grams of soil, compost etc. These are sensitive to acidic and low pH (optimum pH range is 6.5 to 8.0).

After screening and isolation of pure culture of actinomycetes from soil, identification of strains is carried out by morphological, physiological, biochemical methods. But some of the isolated stains share similar characteristics in their morphological and physiological and biochemically. Hence identification of such actinomycetes is more critical. Therefore the identification based on the molecular characteristics is at

demand which requires good quality and quantity of the genomic DNA.

The identification requires isolation of genomic DNA and amplification of the 16S rDNA gene because the 16S rDNA gene is highly conserved within the strain. Pure DNA which is having high content of GC sequence (69-78%) is essential (Korn-Wendisch et al., 1992; Ventura et al., 2007; Stackebrandt et al., 1991 a, b and 1992).

Most of the methods employed for isolating the genomic DNA from actinomycetes encounter many hurdles, such as poor yield of DNA and high polysaccharide contaminates (Hopwood et al., 1985). The present study was designed to use liquid nitrogen that gives good quality of the DNA. Liquid nitrogen is essential to break the bacteria cell wall and mycelial pellets should be properly grinded. The present method is a modification of Hintermann et al., 1981; Vijay Kumar et al., 2010 for the isolation of total chromosomal DNA from actinomycetes.

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MATERIAL AND METHODS

Selection of soil sample and isolation of microorganisms

Soil sediments were collected from the mangrove ecosystem of Konaseema spread across coastal Andhra Pradesh. Soil sediments were collected from different areas of the ecosystem from a depth one feet using sterile spatula. The samples were sealed in sterile container with sterile zip lock bags (Davies and Williams, 1970) and were transported immediately to the laboratory, numbered and stored at 4°C.

Isolation of pure actinobacteria was carried out according to the Arifuzzaman et al., (2010). The collected samples were dried at room temperature and made into fine powder with the motor and pestle under sterile conditions. The graded soil samples were pre-treated with calcium carbonate followed by heat treatment for 2 hours at 60°C. Each soil sample (25 grams) was added to 100 ml distilled water in a conical flask. For uniform mixing of the soil sample the flasks were kept on orbital shaker for 30 min. Then samples were subjected to serial dilution. The diluted soil (10^{-4} and 10^{-5}) samples were spread over starch-casein agar plates and incubated at 30°C for 10 days for growth of actinobacteria. The medium was supplemented with streptomycin and fluconazole as antibacterial and antifungal agents to prevent the growth of the bacteria and fungi.

The isolated colonies were identified based on colony morphology, colour of aerial spores and pigmentation of substrate mycelium. The isolates were sub-cultured on starch-casein agar plates and incubated at 30°C for 10 days. The morphological characteristics of the isolates were observed under light microscope. These pure cultures were stored at -20°C in 20% glycerol for further studies (Kamil et al., 2014).

Genomic DNA isolation from the actinobacterial isolates

Pure actinomycetes strains were inoculated aseptically from mature slant culture into 100 ml conical flask containing nutrient broth (Beef extract 2g/l, yeast extract 2 g/l, peptone 5 g/l and sodium chloride 8 g/l, pH 7.0) and incubated in rotary shaker at 37°C for three days at 180 rpm. After growth, culture was transferred in Oakridge tube aseptically and centrifuged at 9000 rpm for 10 minutes. The supernatant was discarded and the pellet was retained in 1.5 ml eppendorf tube aseptically and the eppendorf tube is kept in the liquid nitrogen for 4-5 min to freeze the total culture. The frozen sample is then ground with micro pestles to which 500 µl of TE buffer (10mM Tris and 1 mM EDTA) is added and the grinding is repeated till the pellet is dissolved completely. The sample tubes were incubated at 37°C for 45 minutes after which 20 µl of 10% SDS (w/v) and 20µl of proteinase-K is added and incubated at 60°C for 20 minutes. The lysate obtained was cooled and extracted once with equal volume of tris saturated phenol and chloroform solution (1:1) at 10000 rpm for 10 min at 16°C. The aqueous phase was transferred carefully to a fresh tube and 5-8 µl of RNase (10mg/ml) is added and incubate at 37°C for 40 minutes. The extraction with equal volume of phenol and chloroform solution (1:1) at 10000 rpm for 10 min at 16°C is again repeated. The aqueous phase carefully transferred in to

a fresh tube and DNA precipitated by adding 0.6 volumes of isopropanol followed by centrifugation at 10000 rpm for 8 min at 4°C and the pellet is washed with 70% ethanol. The pellet is dissolved in 80 µl TE buffer. The purity and concentration of DNA analysed by UV-spectrophotometer (Shimadzu, Japan) (wave length, A260/A280) and agarose gel electrophoresis (0.8%).

Amplification of the 16S rDNA gene sequence

Amplification of 16S rDNA from actinomycetes was carried out using specific set of primers which generates 1.4 Kb amplification (F'-5'-GGATGAGCCCGCGCCTA-3' and R'-5'-CGGTGTGTACAAGGCCCGGGAACG-3'). 25µl of polymerase chain reaction mixture components were shown in **Table 1**. The reaction was carried out PCR (Eppendorf vapo. Protect, Germany) with the following programme of initial denaturation at 94°C for 5 min, denaturation for 1 min and annealing at 62°C for 1 min and extension at 72°C for 1 min with total 30 cycles, final extension at 72°C for 5 min.

Table 1 PCR reaction components.

S.no	Components	Reaction volume
1	DNA	1 µl (50ng/ µl)
2	Taq buffer (10X)	2.5 µl
3	dNTPs	1 µl (5mM)
4	Forward primer	0.5µl (15pico moles)
5	Reverse primer	0.5µl (15pico moles)
6	Taq enzyme	0.5 µl
7	Nuclease free water	19µl
	Total volume	25 µl

RESULTS AND DISCUSSION

To evaluate the diversity of actinomycetes several selective isolation techniques have been performed. The present study include various pre-treatment techniques have been used to isolate actinomycetes from mangrove soil samples. Starch-casein agar medium was supplemented with antibiotics streptomycin and fluconazole to avoid the growth of bacteria and fungi. After incubation period the isolates were selected based on the colony morphology and pigmentation. Rare actinomycetes were selected for molecular studies. The pure cultures were maintained as starch-casein agar slants with 3% NaCl (**Fig 1 & 2**) and as glycerol stock (20%) at -20°C.

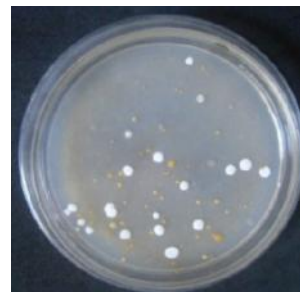


Fig 1 Actinobacteria was grown on petri plate.

The pure isolates were inoculated into nutrient broth and the genomic DNA was isolated from the centrifuged pellet obtained and the purity and quantification of genomic DNA was assayed by UV Vis spectrophotometer at A260/A280 nm and by nano-spectrophotometer. The absorption values shown

in **Table 2**. The genomic DNA was also subjected to electrophoresis in 0.8% agarose gel (**Fig 3**). By using PCR method the 16S ribosomal DNA gene was amplified with Taq DNA polymerase and forward and reverse primers. The gene level molecular based studies using polymerase chain reaction is sufficient for taxonomic studies.

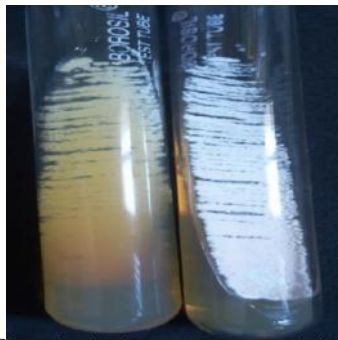


Fig 2 Pure actinobacteria was grown in slant culture.

Table 2 Quantification of actinomycetes genomic DNA.

S.NO	Actinomycetes Samples (A.S)	Absortion at 260nm	Absortion at 280nm	Quantity of DNA (µg/µl)	Purity of DNA (A260/A280)
1	A.S-1	0.007	0.004	0.350	1.75
2	A.S-2	0.006	0.004	0.3	1.5
3	A.S-3	0.009	0.005	0.45	1.8
4	A.S-4	0.009	0.005	0.45	1.8
5	A.S-5	0.006	0.004	0.3	1.5
6	A.S-6	0.005	0.003	0.25	1.6
7	A.S-7	0.005	0.003	0.250	1.6
8	A.S-8	0.006	0.004	0.3	1.5

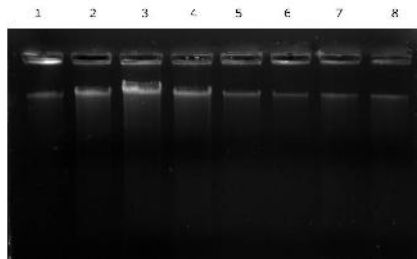


Fig 3 Genomic DNA from Actinomycetes (4µl loaded in gel)

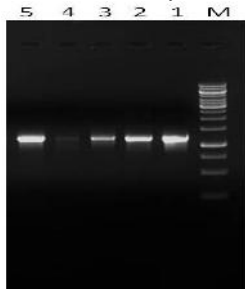


Fig 4 PCR amplification at 1.4 kb from Actinomycetes DNA sample (M-DNA Marker, Lane 1-5)

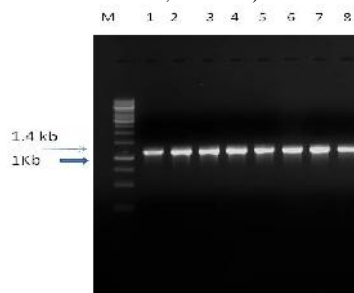


Fig 5 Purification of product after PCR amplification (M- 1 Kb Marker, 1,2,3,4,5,6,7,8 purified DNA samples)

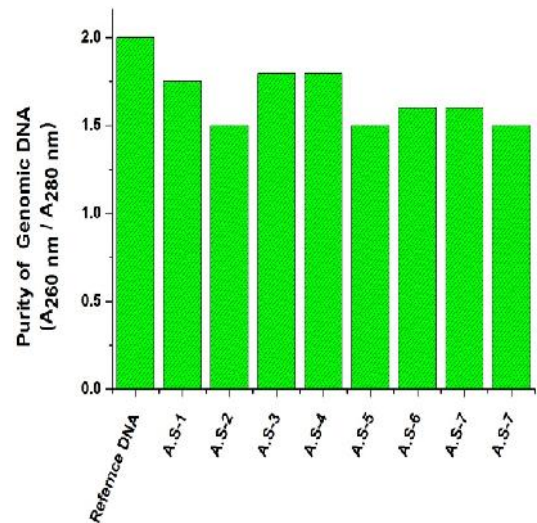


Figure 6 Graphical representation of genomic DNA purity

PCR Product was purified by using Helini DNA purification kit as per the manufacturer's instructions protocol. Total 40 µl of PCR product was eluted. This isolation protocol was repeated 3-5 times to check the quantity and quality of the genomic DNA. The quantity of DNA obtained is 250-450 ng/µl and the purity was found to be 1.5-1.8 at A260/A280. After amplification, 1.4 kb PCR product was obtained (**Fig 4**). Subsequent to purification of PCR product, 4 µl of eluted product was subjected to 1% agarose gel electrophoresis (**Fig 5**). The advantage of this method is lysozyme enzyme is not necessary for cell wall lysis.

This method is simple and less time consuming to obtain pure DNA from actinobacteria. DNA thus obtained is suitable and sufficient for 16s rDNA sequence analysis. Present DNA isolation method is effectively useful for the study of molecular and genetic diversity in actinobacteria by randomly amplified polymorphic DNA (RAPD) method. The quality genomic DNA is pure when the ratio of absorption at A260/A280 is 1.8-2 (*Katara et al., 2013*) but with this cost effective method ratio of absorption was observed between 1.5-1.8 at same conditions. Purity of the DNA of the method adopted and with that of the slandered methods is shown in figure (**Fig 6**).

Conventional methods for the identification of the actinomycetes are not satisfactory, hence 16S rDNA sequence analysis has become evident and popular. The gene encoding the 16S rDNA is highly conserved and to study the phylogenetic association of 16S rDNA sequence is has become a universal marker for their identity (*Kamil et al., 2014*). 16S rDNA gene studies have led to a wealth of information on prokaryotic diversity and have played a vital role in microbiology, taxonomy and ecology.

Since the molecular level identification is necessary the quality of the DNA along with economics is important, the present method employed for the genomic DNA isolation produced pure DNA in required quantities and also the cost for the isolation is very less and time employed for the isolation has greatly reduced.

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