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RESEARCH PAPER

Standardization of isolation and genomic DNA isolation protocols of soil actinobacteria

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Abstract: The concepts of ancient microbiology are promising platform for the modern microbiological aspects of identification of isolates. The actinobacterial genera are well known for production of antimicrobial compounds under stress. Identification and selection of actinobacterial isolates on the media will be a challenging task as the growth pattern resembles both bacterial and fungal morphology. The practical knowledge of selection and characterization is limiting due to the inability to select an isolate based on phenotypic observations. The current study is focused on selection, isolation and DNA isolation protocol of actinobacteria isolated from grass rhizosphere. The results were found convincing to select actinobacterial isolate from the pool of other organisms such as bacteria and fungi. The minor changes in the DNA isolation protocol resulted in impressed DNA yield in terms of quality and quantity which can be used for sequencing purpose such that it avoids investing money in DNA isolation kits and improving the basic knowledge of DNA isolation and role reagents used in each step for students.

Key words: Actinobacteria, DNA, Isolation, Rhizosphere, Tryptic soya agar

Introduction

The Biological control has gained its importance due to the neutral residual effect and eco-friendliness. Various microbial isolates of fungal and bacterial origin has been exploited for increasing yield and controlling disease and pest of economic importance. The success of output will be greatly influenced by the efficient selection of starting material. The selection depends on the phenotype of particular organism among diverse population which have similar intra and inter properties that renders the selection difficult at times. The Actinobacteria are known for their plant growth promoting and as well for its antagonistic properties. Identification of major genes responsible for both the characters is the present and interesting scenario. The downstream process of identification of specific characters through molecular approaches needs an effective upstream process of pure actinobacteria isolation and its genomic DNA extraction. The initial step in biological control is isolation selection and characterization of a strain. The isolation of actinobacteria is challenging due its intermediate growing pattern between bacteria and fungus. The characterization is difficult at molecular level due the genetics of actinobacteria. The actinobacterial isolates are gram positive in nature and that implies the dominance of peptidoglycan layer which is the main hinderence in the cell lysis further reducing the efficiencyin genomic DNA extraction. Also they actinobacteria have the tendancy to grow like mycelia mats and form dense clumps making it difficult for lysis leading to low yield (Hopwood *et al*., 1985). The proper selection of pure culture and isolation of good quality genomic DNA maylead to success in the identification and utility of actinobacteria as biological control. Major attempts for DNA isolation from actinobacterial strains have been done (Bull *et al*., 2005, Q in *et al*., 2009, Jami *et al*., 2015, Wei *et al*., 2018) but the challenge depends on type of samples and reagents used in a particular step which may be the reason for failure to isolate good quality prerequisite for DNA sequencing. The conventional methods being less efficient and the commercial kits rendering low yields needs arisies for effective actinobacteria DNA extract. The current study focused on isolation and selection methods of typical actinobacterial strains and to develop a good quality and quantity DNA for molecular characterization.

Material and methods

Collection of soil samples

The soil samples used for isolation of actinobacteria were from Orang National Park, Assam from grass rhizosphere.

The soil samples collected were dried at room temperature for 24 h and 10 g of each sample was diluted in 90 ml of autoclaved water. The samples were kept on shaker at 150 rpm for 4 hours. The samples were kept stationary for 10 min and then serial diluted upto 10^{-5}. Spread plate method was used for isolation using 100 µl of dilutions (10^{-3}, 10^{-4} and 10^{-5}) of respective samples (Suryawanshi *et al*., 2020).

Media components

Since the objective was to isolate maximum number of actinobacterial strains, different media components were used. Five different types of agar media were selected (Starch case in Agar, L-Arginine medium, Streptomyces Agar, Actinomycetes isolation Agar and Trypticsoya Agar). Each dilutions selected was subjected to spread plating, dried and incubated at 30°C for 10 days (Sharma and Thakur, 2020).

Antibiotics to prevent the fungal and bacterial growth

To restrict the growth of bacteria and fungi, Nalidixic acid (25 mg/L) and Nystatin (50 mg/L), respectively were used. The plates without antibiotics were also kept as control to study

the effect antibiotics on number of isolates (Sharma and Thakur, 2020).

Isolation of actinobacterial isolates

Ten days after incubation, each dilution with respective media components were taken separately and the single colonies were picked up based on different phenotypic and morphological characters. Typical phenotypic characters such as non-shiny/ dry, amorphous nature, attractive colours, restrictive growth on media which can even be seen grown underneath the media, powdery appearance, sturdy nature *etc,* were considered to select the individual isolate on different media plates. Growth pattern of actinobacterial isolates on different media components and number of isolates in sampling site and the dilution corresponding to which the maximum number of isolates retrieved were recorded (Borah and Thakur, 2020).

Actinobacterial DNA isolation and quantification

The DNA isolation from actinobacterial isolates was done from one of the isolates as per the protocol (enzyme lysis method) (Pospiech and Neumann, 1995) with minor modifications. The individual bacterial isolates were grown in Tryptic Soya Broth after purification. The actinobacterial isolates usually show slow growth (3-6 days). Freshly grown cells were selected *i.e,* when the broth shows turbidity immediately the cells were harvested by centrifugation at 6,000 rpm for 5 min. Approximately 50 mg of cell mass was used for DNA isolation. The cells were harvested in 2 ml centrifuge tubes and 300 µl of 0.5 MEDTA and 1M Tris, respectively 400 µl of 5 M NaCl were added to cells and incubated for 10 min at room temperature. The mixture containing cells were subjected to enzymatic digestion by adding lysozyme (50 mg/ml) and incubated at 37°C for 30 min in water bath. After incubation, the lysiswas enhanced by adding 20 % SDS and incubating in water bath 55 °C for 1:30 min with intermittent shaking at every 15min. The temperature was increased from 55°C to 95°C and the tube containing cells were continued until the temperature reaches 95°C. The centrifuge tubes were removed from water bath and incubated at room temperature for 10min. The tubes were added with phenol and chloroform (25:24) and centrifuged at 12,000 rpm for 15min and clear supernatant was collected and transferred into new 2 ml centrifuge tubes. The tubes were added with chloroform and isoamyl alcohol in the ratio of (24:1) and centrifuged at 12,000 rpm for 10 min, the supernatant obtained was transferred to 1.5 ml centrifuge tubes. The clear supernatant was subjected to incubation at -20°C for overnight by adding equal volume of ice cold isopropanol. The tubes were subjected to centrifugation at 13,000 rpm for 10 min, the pellets formed were retained and supernatant was discarded. The pellets were washed with 100 µl of 70 % ethanol and dried to eliminate isopropanol for 10-15 min at room temperature by discarding the ethanol and retaining the pellet at the bottom of the tubes. The pellets were then dissolved in nuclease free water and subjected for gel electrophoresis and quantification.

Gel electrophoresis and quantification of DNA

The dissolved DNA was subjected to gel electrophoresis (Wei *et al*., 2018). The agarose gel was prepared at 0.8 per cent concentration by adding 5 µl of Ethidium bromide (10 mg/ml), the mixture of 5 µl of DNA sample and 2 µl of loading dye (bromophenol blue (25 mg/ml+25 mg xylene syanol-FF+3.3 ml glycerol) was loaded in each well and subjected for electrophores is at 70 volts for 30 min. The agarose gel was subjected to DNA visualization under UV light in gel documentation unit (UviTec, Germany). The quantification of DNA obtained was done using Biospectrophotometer, Eppendorf at 260/280 nm and 260/230 nm (Wei *et al*., 2018).

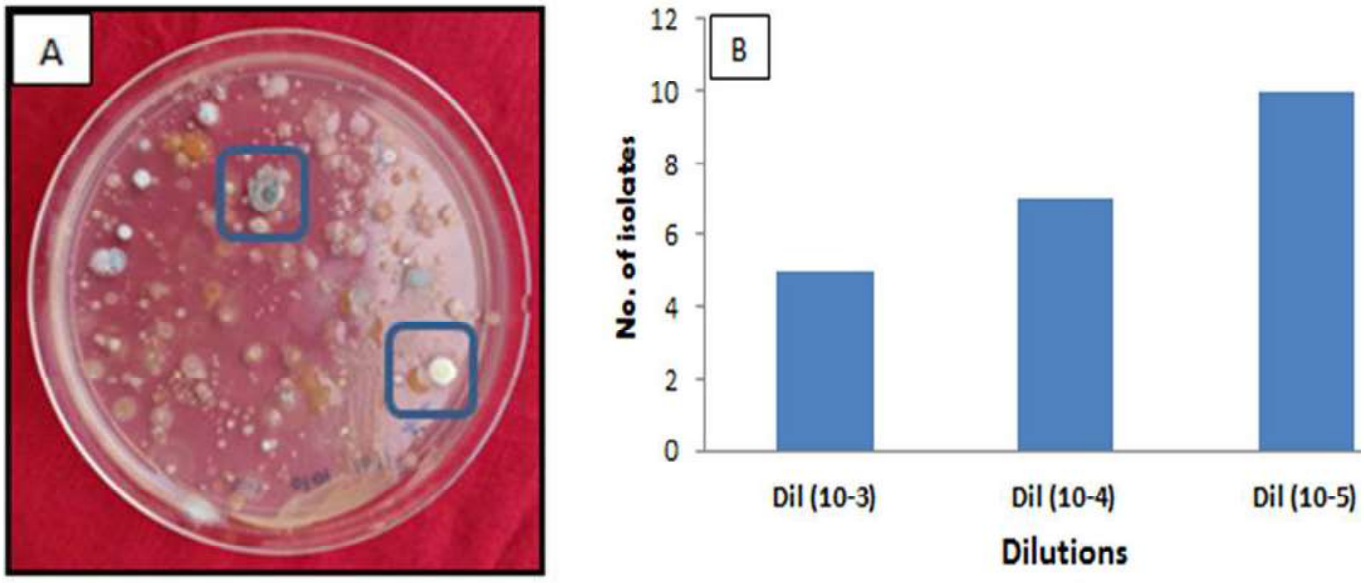

Fig 1. A. The actinobacterial growth in the galaxy of other micro-organisms B. Bar graph representing the actinobacterial isolates retrieved in each dilution

Results and discussion

Selection of actinobacterial isolates

The isolates which were extremely different from morphology of bacteria and fungi were selected from plates after incubation. The isolates with typical phenotypic characters such as non-shiny/dry, amorphous nature, attractive colours, restrictive growth on media which can even be seen as growth underneath the media, powdery appearance, sturdy nature *etc* (Figure 1 A). A total of 22 isolates (Figure 2) were isolated in three dilutions (Figure 1B). The selection of isolates was challenging due to over growth of bacterial and fungal isolates even under the addition of antibiotics due to non-specificity and drug resistance mechanisms. Initially some actinobacterial isolates may appear slimy like bacterial or yeast like colonies which later will turn like powdery growth typical of actinobacteria. In contrast to this character, the dry morphology may also leads to wrong selection of actinobacterial isolate as they may end up in growing as slimy growth corresponding to bacteria. Hence, it is recommended to pick the maximum number of isolates based on colony morphology and while purifying the individual isolates we can select the actual actinobacterial isolates and eliminate the odd ones which are devoid of actinobacterial growth characteristics.

At lower dilutions the numbr of colonies is less due to at lowest dilution of soil, bacterial population will be more on plates since actinobacteria is slow growing compared to

Fig 2.Morphological diversity of actinobacterial isolates from grass rhizosphere soil samples

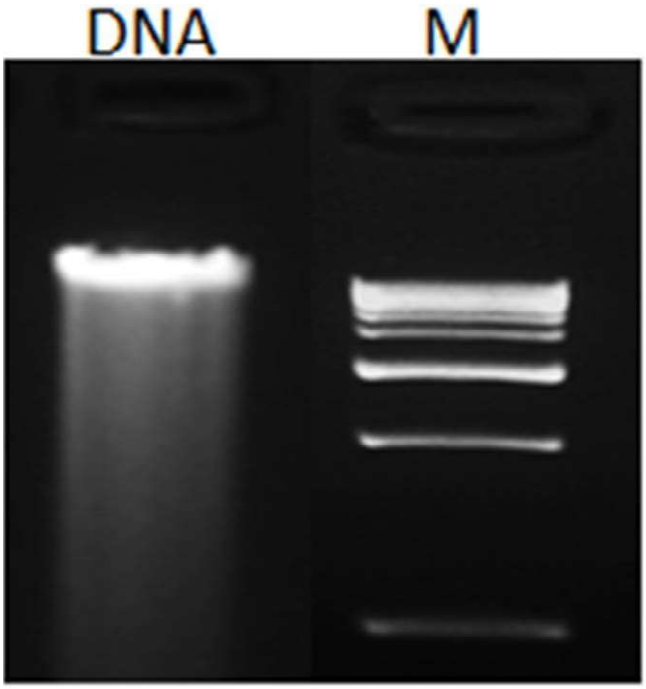

Fig 3. Actinobacterial DNA, M-1kb ladder

bacterial and fungi two observation can be observed. Due to fast growth of bacteria and fungi they will colonize quickly which will cause com petition for space and nutrition leading to less growth of slow growing actinobacteria (Borah and Thakur *et al*., 2020)

DNA quantification

The agarose gel electrophoresis provided DNA band with high intensity (Figure 3). Based on bio spectrophotometer results, the DNA quantity obtained was approximately 1000 ng/ µl with the quality of 1.7-1.8 at 260/280 nm and 1.9-2.0 at 260/230 nm. The DNA visulalized under UV light is depicted in the Figure 2. The DNA isolation from gram positive bacteria is difficult due to the presence of peptidoglycan layer which makes the cell lysis difficult and failure to get good quality and quantity of DNA. The DNA quantity and quality is important based on different down stream objectives like polymerase chain reaction (PCR). For PCR analysis the template required is 50-100 ng with the purity range between 1.6-1.9 at 260-280 nm. But if the purpose is for whole genome sequencing using next generation sequencing platforms, the quality and quantity plays

important role in identifying maximum genes in a genome such that major gene clusters can be identified based on the objective which will the key success in whole genome and metagenome sequencing.

The importance of readings at 260/230 nm at the range of 1.8-2.0 is equally important to the readings at 260/280 nm. The purity of DNA is depicted by the results of 260/280 nm (Glasel, 1995) and the extent of elimination of other impurities can be studied by the results at 260/230 nm. Hence, both the readings help to get good quality DNA and further improve the purification and handling methods during DNA isolation. The lysis of actinobacterial cells in this study is enhanced with increasing the temperature from 55 °C to 95 °C for 10 min actually provided fascinating results based on quantity and quality. Hence, upstream process with effective bench work considering every step of DNA isolation as a critical steps will definitely yields good quality and quantity DNA for further analysis based on defined objective.

Conclusion

The current study was focused on isolation and selection of actinobacterial isolates based on particular characters such that right isolate is selected for further objectives. The study also focused on tackling problems of DNA isolation from actinobacterial isolates from the rhizosphere soil and major steps that has to be followed to get good quality DNA with sufficient quantity for high throughput sequencing which helps scientific community to reduce investing for DNA isolation kits and can improve the knowledge at each step and role of each reagent used such that we can standardize the protocols based on the samples from which the DNA to be isolated.

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