SCREENING OF MULTI-TRAIT PLANT GROWTH AND HEALTH PROMOTING ACTINOMYCETES FROM RHIZOSPHERE SOIL

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Received July 19, 2015

Accepted October 10, 2015

ABSTRACT

Extensive screening was carried out for the novel actinomycetes with diverse metabolic potential with respect to multiple plant growth promotion activities. The isolation was carried out by direct as well as by enrichment methods. Total 52 isolates were obtained from 10 samples using four different selective medium. Primary screening of these isolates were carried out for multiple PGPR and antimicrobial activities. Results showed that nine potential actinomycetes isolates which were further screened for their quantitative abilities. Actinomycetes isolate C13 was also found to produce hydrolytic enzymes such as amylase (0.08 EU), lipase (0.026 EU), protease (12.12 EU), cellulase (1.08 EU), chitinase (0.002 EU). It was found to show antibacterial activity against S. aureus and antifungal activity against T. viridae and F. oxysporium. Beside this, it was also found to show potential PGPR activities such as siderophore production of 36.83 % IAA production 9 mg/l and phosphate solubilization attributes.

Key Words: Actinomycetes, PGPR, Antimicrobial activities, Hydrolytic enzyme, Biotechnological aspects

INTRODUCTION

Actinomycetes are gram positive, filamentous bacteria which comprise a group of branching unicellular microorganisms and are best known for their ability to produce antibiotics. The non streptomycetes are called rare actinomycetes, comprising approximately 100 genera. Actinomycetes are excellent elaborators of biotechnological products such as antibiotics, industrial enzymes and other bioactive compounds1-3. They, especially Streptomyces species, account for more than 70 % of the total antibiotic production.4-5 Hydrolytic enzymes that are essential for the turnover and recycling of the carbon locked in plant polymers. For this reason, actinomycetes and particularly Streptomyces species are considered as important decomposers of plant and plant components.6-8 Cellulases, xylanases and other hemicellulolytic enzymes have received attention worldwide due to their potential applications especially in the biodegradation of agricultural wastes.

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Beside, this siderophore producing actinomycetes have been reported includes (Actinomadur amadurade), Nocardia asteroids and Streptomyces griseus. Actinomycetes produce both hydroxymate and salicylate types of siderophores. Streptomyces albovinaceus, Streptomyces caviscabies, Streptomyces griseus, Streptomyces setonii and Streptomyces virginiiae selected as antagonists of (Moniliphthora) (ex Crinipellis) perniciosa, the causal agent of ‘cacao Witches’ broom, were examined in vitro to detect production of chitinases, β-1, 3-glucanases and cellulases. Streptomyces occur in the rhizosphere of plants and can enhance plant growth by producing plant growth promoter substances e.g. auxin or gibberellins. Several streptomycetes species, such as S. olivaceoviridis, S. rimosus, S. rochei and Streptomyces spp. from the tomato rhizosphere have the ability to produce IAA and improve plant growth by increased seed germination, root elongation and root dry weight.9-11
The use of actinomycetes for agricultural have also been studied by researchers. According to Lo et al., there are about 100 genera of actinomycetes inhabiting the soil. Intense screening of actinobacteria especially rare actinomycetes is taking place all over the world.

**AIMS AND OBJECTIVES**

To search novel actinomycetes isolates with diverse functions which could be utilized for the crop improvement and crop protection as a PGPR.

**MATERIAL AND METHODS**

**Isolation of actinomycetes**

Soil samples were collected from the rhizosphere region of sugar cane, rice and maize field. Actinomycetes were isolated by plating serially diluted samples on a Aspargine Glycerol Salt agar (AGS) medium (pH 7.4), Bennet’s agar medium (pH 7.3), Czapek’s agar medium (pH 7.3) and Starch-Casein agar medium plates (pH 7.3). All medium were supplemented with cyclohexamide 20 μg/ml. After incubation at 30°C for 4 weeks the isolated colonies were sub cultured until pure cultures were obtained.

**Screening of actinomycetes for various biomolecules**

All isolates obtained from rhizosphere soil were studied for screening of diverse potential such as production of hydrolytic enzymes, antimicrobial activities and PGPR activities.

**Antimicrobial activities**

**Antibacterial activities**

Actinomycetes were tested for antibacterial activity against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* by agar well method. Actinomycetes isolates were grown in Basal Salt Medium (BSM) for 74 h at 30°C with 100 rpm on shaker. Antibacterial compound was recovered from the filtrate by solvent extraction method following the process described by Westley et al. and Liu et al. Thus, obtained compound was used to determine antimicrobial activity. The antibacterial activity was determined by agar well method.

**Antifungal activities**

For the screening of antifungal actinomycetes, different test organisms were evaluated against *Aspergillus niger*, *Trichoderma viride* and *Fusarium oxysporium*. These test organisms were grown in sterile potato dextrose broth for 74 h. Then, 1 ml culture of each test organism was seeded in molten potato dextrose agar which was poured in sterile petridish. After solidification of media spot inoculation of each actinomycetes isolate was carried out and incubated at 28°C for 72 h. The antifungal actinomycetes were found to show the zone of inhibition of the test organisms.

**Hydrolitic enzymes**

**Amylase**

The primary screening for protease producing actinomycetes was carried out using inorganic salts-starch agar medium containing 1 % soluble starch. After solidification of medium spot inoculation of each actinomycetes isolate was done. The plates were incubated for 48 h at 30°C. The halo around colonies confirmed the amylase production by actinomycetes. Furthermore, it was re-confirmed by flooding the plates with iodine. The halo of starch hydrolysis against the blue colored background confirms the amylolytic actinomycetes. The potential amylolytic actinomycetes were also evaluated quantitatively. A loopful culture was inoculated into the inorganic salt starch broth. Then it was incubated for 48 h at 30°C in shaking condition at 120 rpm. Filtrate was tested for enzyme activity as described by Bernfield using DNS method. Enzyme activity may be defined as the 1 μmol of glucose produced per ml in the reaction mixture per unit time.

**Protease**

The primary screening for protease producing actinomycetes isolates were carried out using skim milk agar medium. Spot inoculation of each actinomycetes isolate was carried out. The plates were incubated for 48 h at 30°C. The halo around colonies confirmed the protease production ability of actinomycetes. Further these protease producers were evaluated for its quantitative production abilities. A loopful culture was inoculated into protease production medium followed by incubation at 48 h at 30°C in shaking condition. Filtrate was used for enzyme activity as described by McDonald and Chen method. A unit of protease activity may be defined as the amount of enzyme in 1 ml of filtrate which under the conditions described hydrolyzes...
casein at such a rate that amount of hydrolysis products formed per min have the same optical
density on reaction with phenol reagent as 1 μg/l tyrosine.

**Lipase**

Lipase production was determined by using tributyrin agar plate. The cultures were inoculated on to the tributyrin agar plate and incubated at 30°C for 48 h. The plates were examined for clear zone around the colonies. The potential lipolytic actinomycetes were also evaluated quantitatively. A loopful culture was inoculated in lipase production medium (mineral agar medium+1 % tributyrin) and incubated for 72 h at 30°C in shaking condition. Filtrate was used for enzyme activity by determination of the rate of free fatty acid release from tributyrin. One unit of lipase activity was defined as the amount of enzyme required to release 1 μmol of fatty acid per min under these conditions.

**Cellulase**

Each of the isolates was spot-seeded on mineral agar medium containing 1% carboxymethyl cellulose to detect cellulases. Those isolates which produced zone of clearance undergo were reconfirmed by quantitative assay. The quantitative estimation was carried out by inoculating a loopful culture into the cellulase production medium (mineral agar medium+1% CMC). Incubation was carried out at 30°C for 72 h in shaking condition at 120 rpm. Then the filtrate was for determination of enzyme activity. Enzyme activity was measured using DNS method. One unit of cellulase activity was defined as the amount of enzyme required to release 1 μmol of glucose per min under these conditions.

**Chitinase**

Each of the isolates was spot-seeded on a mineral agar medium containing 0.08% colloidal chitin to detect chitinases. Those isolates which produced zone of clearance undergo for quantitative assay. A loopful culture was inoculated into the chitinase production medium consisting of (g/l) colloidal chitin, 10; peptone, 3; KNO₃, 3; K₂HPO₄, 0.7; MgSO₄, 0.5; KCL, 1.0. Then it was incubated at 30°C for 72 h in shaking condition at 120 rpm. Filtrate was used for enzyme activity. Chitinase activity was determined by a DNSA method. This method works on the concentration of N-acetyl glucosamine (NAG), which is released as a result of enzymatic action.

**Potential for PGPR activities**

**Siderophore production**

The screening for siderophore producing actinomycetes isolate were carried out by inoculating onto chrome azurol S (CAS) blue plates with the modifications described previously. The siderophore test was analyzed for the presence or absence of the orange-yellow halo surrounding the colonies, which indicated the presence or absence of a siderophore, respectively. Further, quantitative estimation of siderophore was done by CAS-shuttle assay. In which cultures were inoculated (1% v/v) in sterile succinate medium separately and incubated on rotary shaker at 30°C, 120 rpm. After 36 h of incubation, 0.5 ml of culture supernatant was mixed with 0.5 ml of CAS reagent and absorbance was measured at 630 nm.

\[
\% \text{ Siderophore Unit} = \frac{Ar - As}{Ar} \times 100
\]

Where, \(Ar\) = absorbance of reference at 630 nm and \(As\) = absorbance of sample at 630 nm

**IAA production**

IAA production by actinomycetes isolates were studied by colorimetric technique using Salkowski reagent and orthophosphoric acid. Sterile nutrient broth containing tryptophan (2 mg/ml) was inoculated with loopful culture of each actinomycetes isolate and incubated at 30°C in shaking condition at 120 rpm for 48 h. After that it was centrifuged at1000 rpm for 10 min. 2 drop of orthophosphoric acid and 4 ml Salkowski reagent (50 ml 35 % perchloric acid mixed with 1 ml of 0.5 % FeCl₃) was added in 2 ml of supernatant. It was incubated for 20 min at room temperature. Then the development of pink color was measured at 530 nm spectroscopy to confirm the IAA production.

**Phosphate solubilization**

Primary screening for phosphate solubilization was carried out on Pikovskaya’s agar plate as described by Gaur. Quantitative analysis of solubilization of tricalcium phosphate in liquid medium was carried out as described by King.
The potential isolates were inoculated in 25 ml Pikovskaya’s broth and incubated for 5 days at 30°C. The actinomycetes cultures were centrifuged at 15,000 rpm for 30 min. 1 ml supernatant was mixed with 10 ml of chloromolibdic acid and volume was made up to 45 ml with distilled water. The absorbance of developing blue color was read at 600 nm. The amount of P solubilized was calculated using the calibration curve of KH$_2$PO$_4$. The change in pH following tri-calciumphosphate (TCP) solubilization was also recorded.

**HCN production**

All actinomycetes isolates were screened for the production of hydrogen cyanide by method described by Lorck. All the nutrient broth was amended with 4.4 g glycine/l and the isolates were streaked on modified agar plates. A Whatman filter paper no. 1 soaked in 2 % sodium carbonate in 0.5 % picric acid was placed on the top of the plate. The plates were sealed with parafilm and incubated 30°C for 4 days. Development of orange to red colour indicated HCN production.

**RESULTS AND DISCUSSION**

**Isolation of actinomycetes**

Total 52 isolates were obtained from 10 samples using four different selective medium (Table 1). Sugarcane rhizosphere has better actinomycetes diversity as compared to rice and maize field.

<table>
<thead>
<tr>
<th>Medium use for isolation</th>
<th>Actinomycetes isolates from different rhizosperic soil</th>
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<tr>
<td>AGS</td>
<td>Sugar cane field 9</td>
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<td></td>
<td>Rice field 3</td>
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<td>Maize field 1</td>
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<td>Bennet’s agar</td>
<td>Sugar cane field 11</td>
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<td>Rice field 4</td>
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<td></td>
<td>Rice field 3</td>
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<td></td>
<td>Maize field 1</td>
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<td>Starch-casein agar</td>
<td>Sugar cane field 6</td>
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<tr>
<td></td>
<td>Rice field 1</td>
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<td></td>
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<td>12</td>
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In maize field, it was found to have low number and diversity of actinomycetes even after extensive screening. Also Bennet’s agar medium found to be better selective medium for screening of actinomycetes diversity.

**Screening of actinomycetes for various biomolecules**

The selection of efficient actinomycetes out of 52 isolates obtained during isolation was carried out based on the multiple potential such as antimicrobial activities, production of hydrolytic enzymes and selective PGPR activities.

**Antimicrobial activities**

The antibacterial and antifungal activities of all actinomycetes were studied. Twenty three isolates were found to have potential antibacterial and antifungal activity.

**Antibacterial activities**

52 isolates were studied for antibacterial factor. Nineteen isolates were found to potential against the Gram positive organism S. aureus (Table 2). None of the actinomycetes isolate was found to have inhibitory activity against the E. coli.

**Antifungal activities**

All isolates were also evaluated against three fungi for its antifungal activity. The fungi studied were A. niger, T. viridae and F. oxysporium. Results of primary screening are summarized in Table 2. Isolate A$_8$, C$_5$, C$_{13}$, C$_{16}$, B$_1$, B$_2$, B$_3$ and B$_5$ were found to have inhibitory activity against F. oxysporium. Isolate B$_1$ was found to have inhibitory activity against A. niger. Isolate C$_3$, C$_8$, C$_{13}$, C$_{16}$ and B$_9$ were found to have inhibitory activity against T. viridae.

Out of fifty-two isolates evaluated, 23 isolates were found to be antibacterial or antifungal. Based on antibacterial and antifungal potential, C$_5$, C$_{13}$ and C$_{16}$ actinomycetes isolates were found to show antibacterial activity against S. aureus as well as antifungal activity against T. viridae and F. oxysporium, while, A$_8$ and B$_3$ isolates were found to be effective against S. aureus and F. oxysporium. (Fig. 1)
Table 2: Results of screening of actinomycetes for antimicrobial activity

<table>
<thead>
<tr>
<th>Isolates</th>
<th>S. aureus</th>
<th>E. coli</th>
<th>B. subtilis</th>
<th>P. aeruginosa</th>
<th>T. viridae</th>
<th>A. niger</th>
<th>F. oxysporium</th>
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Fig. 1: Primary screening for antimicrobial activity: 1(a) showing antibacterial activity against S. aureus by C13, C15, C16 and C14 strains, 1(b) showing antibacterial activity against S. aureus by S2 strain, 1(c) showing antifungal activity against F. oxysporium by B1, B2 and B3 strains and 1(d) showing antifungal activity against T. viridae by C16 strain
**Primary screening of actinomycetes**

*Streptomyces* species is a source of thousands of bioactive compounds that attracted the researchers many years ago. Enzymes are one of the important products of this unique group of bacteria and among these are the fiber hydrolytic enzymes that are essential for the turnover and recycling of the carbon locked in plant polymers. Many actinomycetes are reported to produce hydrolytic enzymes such as amylase, lipase, protease, chitinase, cellulase, xylanase and other hemicellulolytic enzymes. In present study, evaluation of the production of hydrolytic enzymes including amylase, lipase, protease, cellulase, chitinase and plant growth promoting activities was carried out to screen the potential actinomycetes. The result of primary screening for selected PGPR activities are summarized in Table 3. *S. griseus* isolates were found to exhibit multi trait potentials. These were selected for further studies.

**Table 3 : Screening for Hydrolytic enzyme and selected PGPR activities**

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Amylase</th>
<th>Lipase</th>
<th>Protease</th>
<th>Cellulase</th>
<th>Chitinase</th>
<th>Siderophore</th>
<th>IAA Production</th>
<th>P-Solubilization</th>
<th>HCN</th>
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**Selection of most potential of actinomycetes**

Based on the multi-trait potential, nine isolates were selected. These were further evaluated quantitatively for screening out the most potential multi trait isolates of actinomycetes. (Fig. 2)

**Amylase**

These secondary screening for amylase production reveals that *C₁₃* strain of actinomycetes produce maximum of 0.2 EU, while *S₄* isolate is corporately producing significant amylase. Whereas *S₈*, *B₁₅*, *B₈* was comparatively inferior (Fig. 2(a)).

**Protease**

Most of the proteases have been produced as by-product during fermentation e.g. production of streptomycin and pronase by *Streptomyces griseus*. It was reported by Dion that certain species of *Streptomyces* might prove valuable for the production of protease. The secondary screening for protease production reveals that *B₈*, *C₁₃* strains were superior and producing the protease in the range of 12-15 EU. (Fig. 2(b))

**Lipase**

Secondary screening for lipase production shows that *C₁₃* strain was the best so far as lipase production. It was found to produce 0.026 EU of lipase. Whereas *S₈*, *B₁₅*, *S₄* were the good lipase producer in the range of 0.001-0.002 EU of lipase. (Fig. 2(c))

**Cellulase**

Cellulases enzymes has received attention worldwide due to their potential applications especially in the biodegradation of agricultural wastes which undergo recycling and facilitate the carbon requirement for the agriculture crop. Also, the digestibility of plant materials has been increased by applying this enzyme, thus the animal feed industry has developed. Selected potential actinomycetes isolates were further evaluated on the basis of their quantitative ability for cellulase production.
production. C_{13} and C_{16} strain were found to produce cellulase above 2.0 EU. Whereas other strains B_{16}, B_{15}, B_{8} and C_{17} were also good cellulase producer. (Fig. 2(d))

**Chitinase**

Due to multiple applications of chitinases in biocontrol and waste management, they have become interesting enzymes for study\textsuperscript{41}. *Streptomyces viridificans* was found to be a good chitinase producer and its crude and purified enzyme has potential cell wall lysis of many fungal pathogens.\textsuperscript{42} Only four isolates of actino imycetes were shown to produce chitinases. These were further evaluated for chitinase production. C_{13} strain was found to produce maximum of 0.008 EU. Whereas S_4, B_8 and S_8 were comparably producing less chitinase than C_{13}. (Fig. 2(e))

![Fig 2](image)

**Siderophore production**

The role of siderophore is to scavenge iron from the environment and to make the mineral, which is almost always essential, available to the microbial cell. In addition, they have applications in clinical, agricultural and environmental fields. Siderophore producing actinomycetes includes *Actinomaduram-
adurae), Nocardia asteroids and Streptomyces griseus. **Fig. 3** Actinomycetes produce both hydroxymate and salicylate types of siderophores. Nine isolates were found to produce siderophore during primary screening. These were further evaluated for siderophore production. C_{13}, B_{15}, C_{17} and C_{15} were the superior in siderophore production. These were producing siderophore in the range of 50-70%. Whereas S_{2}, S_{8}, B_{8} and B_{11} isolates were the good siderophore producer. C_{16}, C_{5} and B_{12} were inferior in the siderophore production (**Fig. 3(a)**).

### IAA production

Indole acetic acid (IAA) is a common natural auxin and is a product of L-tryptophan metabolism in microorganisms. Streptomyces spp., inhabiting the rhizospheres of various plants, also serves as good source of IAA. The rich supply of substrates available in root exudates creates the potential for the streptomycetes to synthesize and release IAA.\textsuperscript{43-46} Several Streptomyces species, such as S. olivaceoviridis, S. rimosus, S. rochei and Streptomyces spp.

from the tomato rhizosphere, have the ability to produce IAA and improve plant growth by increased seed germination, root elongation and root dry weight.\textsuperscript{47-52} Nine potential IAA producing actinomycetes were further screened out for IAA production. C_{13}, C_{16} and S_{8} were found to produce it in the range of 60-80 mg/l. whereas S_{2} and B_{15} was the good IAA producer. Whereas, C_{5}, S_{4} and B_{12} strains were found to be inferior in the IAA production (**Fig. 3(b)**).

### Phosphate solubilization

From selected actinomycetes, eight strains showed abilities to solubilize phosphate were further evaluated quantitatively by their ability to release soluble phosphate (**Fig 3(c)**).

![Fig. 3](image-url) **Fig. 3**: Secondary screening of selected actinomycetes by their quantitative ability for production of (a) siderophore, (b) IAA and (c) phosphate release.
Phosphate release was ranging from 60 to 190 µg/ml. C13 was the most efficient strains releasing 190 µg/ml soluble P in the growth medium after 5 days of incubation. Other two isolates B15 and C16 also showed to release comparable significant P i.e. 180 and 170 µg/ml respectively.

**HCN production**

All actinomycetes isolates were screened for the production of hydrogen cyanide and none of the isolates produced HCN61,62.

**CONCLUSION**

Extensive screening of actinomycetes from 10 samples procured from sugar cane, rice field and maize field yielded 52 isolates. These were investigated further for the novel actinomycetes with diverse metabolic potential with respect to multiple plant growth promotion activities. Nine most potential actinomycetes isolates were studied for their quantitative abilities for production of multiple plant growth promoting bioactive molecules of importance. The multiple plant growth and health promoting features is revealed after exhaustive screening in C13 isolate. This isolate C13 was also found to produce hydrolytic enzymes such as amylase (0.08 EU), lipase (0.026 EU), protease (12.12 EU), cellulase (1.08 EU), chitinase (0.002 EU). It was found to show antibacterial activity against S. aureus and antifungal activity against T. viridae and F. oxysporium. The result indicated C13 is the efficient producer of multiple plant growth promoting factors and could be exploited as PGPR being the versatile inhabitant of rhizosphere soil.

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