Isolation and Characterization of Amycolatopsis sp. strain CRJ2-11 with Biocontrol and Plant Growth Promoting Potential from Upland Rice Rhizosphere in Manipur, India

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Abstract
Fifty seven actinomycetes isolated from upland rice rhizosphere in Chandel, Manipur were subjected to antifungal assays by Dual Culture technique. Five strains showed biocontrol potential against five major rice fungal pathogens viz. Rhizoctonia oryzae-sativae, Fusarium oxysporum, Bipolaris oryzae, Curvularia oryzae and Pyricularia oryzae. Of the five biocontrol strains listed here, one (CRJ2-11) showed highest antagonistic potential. The bioactive strain was also positive for various plant growth promoting traits such as indole-3-acetic acid (IAA) and siderophore production and phosphate solubilization. The strain produced significant levels of IAA (196 µg/ml) and solubilized substantial amounts of inorganic phosphate (82.49 µg/ml) respectively. Characterization of the strain CRJ2-11 by phenotypic tests and 16s rDNA sequence analysis indicated it to be a species of Amycolatopsis having closest affinity with Amycolatopsis keratiniphila (99%). Hence, the potential biocontrol and PGP (plant growth-promoting) strain CRJ2-11 was designated as Amycolatopsis sp. strain CRJ2-11.

Keywords: Actinomycetes; Biocontrol; IAA; Phosphate Solubilization; Siderophore; Upland Rice Rhizosphere; Manipur

Introduction
Rice constitutes a major cereal crop feeding more than 50% of the world’s population. The global human population is projected to rise to over 9.7 billion by 2050 [1]. The demand for rice is projected to increase by over 70% by 2050 [2]. To meet the burgeoning demands, its yield must be enhanced by over 70% [2]. However, rice yield is compromised by various diseases, pests and other abiotic and biotic stresses. There is public outcry to replace ‘green revolution’ involving massive use of agrochemicals by low-input ‘green’, ‘organic’ and ‘evergreen’ agriculture. In this context, deployment of microbial inoculants in agriculture is an increasingly promising option. Microbial strains with biological control and PGP potential offers an attractive alternative to the use of synthetic fertilizers and fungicides for enhancing crop yield and management of crop pathogens. Deployment of native strains holds greater promise for biological control and plant growth promotion of crops and enhancing their yields [3].

Bacteria especially Bacillus and Pseudomonas species have shown great potential for biocontrol activity against plant diseases and rice fungal pathogens. Microorganisms that can grow in rhizosphere are ideal for use as biocontrol agents as they can provide the frontline defense against the phytopathogens. These rhizobacteria produce antibiotics and other metabolites that protect the roots from pathogen attack [4].

Actinobacteria have shown immense potential for biocontrol against a wide range of phytopathogens [5]. They can also promote plant growth through P (Phosphate) solubilization, and production of phytohormones such as IAA and siderophores [6]. They also elaborate a plethora of secondary metabolites and antibiotics with antifungal and other bioactivities and form an abundant component (10-50%) of the soil microbial community and produce heat resistant spores [7]. Another attractive feature of actinomycetes is that they can survive in extreme environments and withstand various abiotic and biotic stresses.

Despite their abundance in soil, metabolic versatility and survival under extreme conditions actinomycetes are yet largely underexplored for PGP and biocontrol potential as compared to Bacillus and Pseudomonas species [8].

The present study was aimed at isolation of actinobacteria with biocontrol potential from upland rice rhizosphere to screen their PGP traits for potential application in rice cultivation.

Materials and Methods
Sample collection and isolation of actinomycetes
Upland Rice rhizospheric soil samples were collected from different jhum (slash and burn) cultivation sites at Chandel, Manipur. The soils were treated with CaCO3 and kept air dried for one week. The treated soil was serially diluted (10-2 to 10-5) and spread plated on Starch Casein Nitrate Agar (SCNA) medium containing (g/L); Starch soluble: 10, casein: 0.3, KNO3: 2, K2HPO4: 2, NaCl: 2, MgSO4.7H2O: 0.05, CaCO3 : 0.02, FeSO4.7H2O: 0.01 and Agar: 16. Culture plates were kept incubated at 300°C for 4-5 days or longer, if needed. Morphologically distinct isolates were picked up and subcultured on SCNA media till pure cultures were obtained.

Biocontrol assay (Dual culture method)
The selected isolates were screened for biocontrol activity against five fungal pathogens viz. Rhizoctonia oryzae-sativae (MTCC 2162), Fusarium oxysporum (MTCC 287), Bipolaris oryzae (LSMU 1), Curvularia oryzae (MTCC 2605) and Pyricularia oryzae (MTCC 1477). Fungal pathogens were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The strains were grown and maintained on potato dextrose agar (PDA) (HiMedia). Actinomycete agar plugs (8 mm diameter) of five day old cultures grown on SCNA were placed at the corners of the PDA plates leaving 1 cm from the margins. The plates were incubated at 300°C for 24 h. Fungal plugs (8 mm diameter) were then placed at the centers of the plates. Plates containing fungal plugs without the actinomycete isolates were kept as controls.

Colony growth inhibition was calculated using the formula: C-T/C × 100, where C is the colony growth in control (mm), and T is the colony growth of pathogen in dual culture (mm) [9]. Assays were performed in triplicates. The inhibition zones were measured after the fungal mycelia in control plates reached the edges of the plates.
Screening of plant growth promoting (PGP) traits

**Indole-3-acetic acid (IAA) production**: The production of IAA was determined according to the method of Bano and Musarrat [10]. The strain was inoculated in SCNB containing different concentrations (%) of trp (0, 0.1, 0.3, 0.5, 0.8, 1, 1.5, 2) and kept incubated under shaking conditions (150 rpm, 30°C, 6 days). The culture broth was centrifuged at 10,000 rpm for 10 minutes. One ml of the supernatant was mixed with 2 ml of Salkowski reagent. Appearance of pink color indicates IAA production.

Quantitative assay of IAA production at different trp concentrations (%) was also studied by inoculating the strain in SCNB containing different concentrations (%) of trp (0, 0.1, 0.3, 0.5, 0.8, 1, 1.5, 2) and kept incubated under shaking conditions (150 rpm, 30°C, 6 days). The culture broth was centrifuged at 10,000 rpm for 10 minutes. One ml of the supernatant was mixed with 2 ml of Salkowski reagent and incubated for 20 min at room temperature. Optical density (OD) was read at 530 nm and the amount of IAA produced was calculated by comparing with the standard IAA (Rankam) curve.

**Phosphate (P) solubilization**: Phosphate (P) solubilization assay was done using NBRIP-BPB medium [11]. A halo zone surrounding the colony after fourth day of incubation at 30°C indicated P solubilization. Quantitative estimation of P solubilization was done according to Kapri and Tewari [12]. The strain was inoculated in 100 ml of NBRIP medium and kept incubated in a shaker (150 rpm, 30°C, 6 days). The culture broth was centrifuged at 10,000 rpm for 10 min. The amount of P in the culture supernatant was estimated using the method of Fiske and Subbarow [13], and expressed as equivalent P (µg/ml). KH₄PO₄ was used as the standard.

**Siderophore production**: Siderophore production was assayed according to You et al. [14], with few modifications. Agar plug (8 mm) of strain CRJ2-11 was inoculated on SCNB (without iron) amended with CAS-substrate and kept incubated at 30°C for six days. Halo zone with orange color surrounding the colony was considered as positive for siderophore production.

**Ammonia production**: Ammonia production was screened according to Cappuccino and Sherman [15].

**Hydrocyanic acid production**: Hydrocyanic acid (HCN) production was studied as per the procedure of Lorck [16].

**Phylogenetic analysis**: Genomic DNA extraction and PCR amplification of the 16S rRNA gene was performed as described by Li et al. [17]. The almost complete 16S rRNA gene sequence of the strain was identified using the EzTaxon-e server database [18] and aligned with the 16S rRNA gene sequences of related species using CLUSTAL X version 2.1 [19]. Phylogenetic analyses were performed using the software package MEGA version 5 [20]. Phylogenetic distances were calculated with the Kimura two-parameter model [21] and tree topologies were inferred using the neighbor-joining method [22]. To determine the support of each clade, bootstrap analysis was performed with 1000 resamplings [23].

Biochemical tests were performed according to Cappuccino and Sherman [15]. Physiological characterization was performed by studying the growth of CRJ2-11 in different salt concentrations (NaCl, 0-10%) and at different pH values (4 to 10) and utilization of various sugars and amino acids as sole C and N sources respectively [15]. Morphological characteristics of the strain were analyzed by growing it in various International Streptomyces Project (ISP) media (HiMedia) [ISCC-NBS color code, Kelly] [24].

Antibiotic sensitivity tests were performed using a total of six antibiotics viz. neomycin (30 µg), chloramphenicol (30 µg), ampicillin (10 µg), penicillin (10 µg), streptomycin (10 µg) and rifampicin (5 µg) (HiMedia) for the sensitivity / resistance pattern of the isolate against the antibiotics by paper disc method.

**Results**

**Sample collection and isolation of actinomycetes**

A total of 57 actinomycete isolates were recovered from the upland rice rhizosphere.

**Biocontrol assay**

Among the 57 isolates, one strain designated as CRJ2-11 was found effective against all the five fungal pathogens. The mycelial inhibition percentages of the strain CRJ2-11 ranges from 64 to 76 showing highest inhibition against *Fusarium oxysporum* (Figure 1).

**Screening for PGP traits**

The strain CRJ2-11 was found positive for IAA and siderophore production and P solubilization but negative for ammonia and HCN production (Table 1). CRJ2-11 showed highest titer of IAA (196 µg/mL) when amended with 0.8% of trp (Figure 2). Further increase in trp concentration decreased the production of IAA. It also solubilized maximum amount of inorganic phosphate (92.49 µg/mL) (Figure 3).

**Characterization of the strain**

Strain CRJ2-11 showed highest 16S rRNA gene sequence similarity (99%) with *Amycolatopsis keratiniphila*. Based on the phylogenetic and genomic data, the strain was found to represent a strain of the genus *Amycolatopsis* which, therefore, has been

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PS</th>
<th>IAA</th>
<th>SP</th>
<th>AP</th>
<th>HCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRJ2-11</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
designated as *Amycolatopsis* sp. strain CRJ2-11 (Figure 4).

The strain was found positive for catalase and lipase, and it utilized lactose, mannitol and fructose and asparagine, adenine, arginine, leucine, glutamine and tyrosine as sole C and N sources respectively (Tables 2-3). Strain CRJ2-11 grew at wide range of NaCl salt concentrations (0% to 5%) and pH values (5-10) (Table 4). Growth of the strain in various ISP media is shown in Table 5. *Amycolatopsis* sp. strain CRJ2-11 was found to be sensitive to neomycin, streptomycin, ampicillin, penicillin and rifampicin but resistant to chloramphenicol.

**Discussion**

*Amycolatopsis* sp. strain CRJ2-11 was selected out of 57 isolates obtained from *Jhum* cultivated upland rice rhizospheric soil in Manipur, India. The selection of the strain was based on the biocontrol activity against major rice fungal phytopathogens and PGP traits such as IAA production, phosphate solubilization and siderophore production.

Actinomycetes especially those belonging to the genus *Streptomyces* appear to be good candidates for development as biocontrol agents for plant diseases [25]. However, some non *Streptomyces actinomycetes* (NSAs) have also shown great potential for biocontrol of soil-borne fungal plant pathogens and also as plant growth promoters [26].

This possibly is the first report of an *Amycolatopsis* strain with biocontrol and PGP potential from upland rice rhizospheric biotope. There are meager reports of *Amycolatopsis* with biocontrol
and PGP potential in the literature. However, there are some reports of antifungal activities of *Amycolatopsis* species viz. strains associated with attine ant nests [27], strains producing amidin [28] and amychelin [29]. Poomthongdee, et al. [30], had reported an *Amycolatopsis* sp. from rhizospheric habitats in Thailand.

Strain CRJ2-11 produced 196 µg/ml of IAA when supplemented with 0.8 % trp. This is comparable to that of *Pseudomonas* fluorescens CHAO, an IAA hyperproducing strain [195 mg/L] [31]. The strain produced much higher levels of IAA than those reported for bacterial strains reported by various authors such as Hata, et al. [7], Khamna, et al. [32], Shrivastava, et al. [33], Harikrishna, et al. [34] and Jog, et al. [35]. However, the optimal conditions for IAA production differ among various bacterial strains. For example, Jog, et al. [6,7], Hata, et al. [7], and Ghosh, et al. [36] found maximal IAA production in media supplemented with 0.2% trp whereas Jog, et al. [35] reported maximal IAA production at 0.5% trp concentration. In contrast, CRJ2-11 produced maximal IAA at 0.8% trp concentration.

IAA is known to induce rapid cell division and enlargement and

**Figure 4:** Neighbor-joining tree showing phylogenetic relationship of strain CRJ2-11 with its closely related strains.
extension of plant tissues. The abundant production of IAA seems to be a positive feature for CRJ2-11 to be developed as a bioinoculant for rice cultivation.

CRJ2-11 showed maximum P solubilization capacity of 82.49 µg/ml. This is comparable to that of Streptomyces sp C [37] which solubilized P in the range of 92 µg/ml. The strain CRJ2-11 solubilized much higher levels of P than that reported by Passari, et al. [38] but lower than those reported by other research groups e.g. Dougou, et al. [39] and Mehta, et al. [40]. P solubilization is a major PGP trait as phosphate solubilizing bacteria (PSB) can stimulate the growth of plants by releasing soluble P from the insoluble bound form of P in the soil. Hamdali, et al. [41] reported P solubilizing Streptomyces griseus as a plant growth promoting (PGP) bacterium. The significant level of P solubilization besides IAA production by CRJ2-11 makes it a potential bioinoculant for rice and other crops.

The strain CRJ2-11 was also positive for siderophore production. Siderophore production is also a major PGP trait. Siderophore production may promote plant growth directly by supplying Fe⁺ which is deficient in soil to the host plant and indirectly by starving the fungal phytopathogens of iron. Dimpka, et al. [42], Wang, et al. [43], Misk and Franco [44] and Rungin, et al. [45] have reported plant growth promoting potential of siderophore positive bacteria.

The results of the present study indicated that Amycolatopsis sp. strain CRJ2-11 from upland rhizosphere soil holds promise for development as biocontrol and PGP agent for rice cultivation. The utilization of such beneficial rhizobacterial actinomycetes may lead to increased crop yields while reducing the use of agro-chemicals. Such approach is indeed an attractive trend towards introducing sustainable, green, and eco-friendly agriculture [46].

Conclusions

CRJ2-11 has several positive traits for PGP e.g. high IAA production, siderophore production and P solubilization. In addition, it showed good tolerance of wide range of NaCl (up to 5%) and pH (5-9). CRJ2-11 may, therefore, hold promise for development as an inoculant for rice cultivation. Further experiments on rice seedling germination and pot trials of rice with CRJ2-11 are being analyzed and compiled for publication as a separate paper.

Acknowledgments

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References


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Table 5: Growth of CRJ2-11 on different ISP media. ISCC-NBS color code (Kely 1964).

<table>
<thead>
<tr>
<th>ISP Media</th>
<th>Growth</th>
<th>CRJ2-11</th>
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<tbody>
<tr>
<td>ISP2</td>
<td>Aerial Mycelium Soluble pigment</td>
<td>Moderate White</td>
</tr>
<tr>
<td></td>
<td>Substrate Mycelium</td>
<td>Deep Orange Yellow</td>
</tr>
<tr>
<td>ISP3</td>
<td>Aerial Mycelium Soluble pigment</td>
<td>Poor Light Gray</td>
</tr>
<tr>
<td></td>
<td>Substrate Mycelium</td>
<td>Light Gray</td>
</tr>
<tr>
<td>ISP4</td>
<td>Aerial Mycelium Soluble pigment</td>
<td>Poor White</td>
</tr>
<tr>
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<td>Substrate Mycelium</td>
<td>Light Orange Yellow</td>
</tr>
<tr>
<td>ISP5</td>
<td>Aerial Mycelium Soluble pigment</td>
<td>Poor White</td>
</tr>
<tr>
<td></td>
<td>Substrate Mycelium</td>
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</tr>
<tr>
<td>ISP6</td>
<td>Aerial Mycelium Soluble pigment</td>
<td>Poor Pink White</td>
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<tr>
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