Characterization of ACTINOMYCETES Isolated From Rhizosphere of PISUM SATIVUM L.

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Abstract: In this study Actinomycetes spp were isolated from soil samples collected from Soil Science Research Farm, SHUATS-U.P. Allahabad their provisional identification was done following the criteria of Bergey’s Manual of Systematic Bacteriology. By the molecular approach the identification process was easy and can obtain results in less time. The phylogenetic analysis based on the 120 bp sequence bearing the variable and region is useful for streptomyces sp. Identification. The isolated sps subjected to 16S rRNA sequence analysis and identified as Streptomyces liciensis (AS1), Streptomyces griseus (AS2) and Streptomyces olivaceus (AS3) and biochemical and physical tests were done for all strains to identify and tolerance to conditions growth Salt, pH, and Temperature Tolerance. The strains AS, AS2 and AS3 were growth well at 28°C, 1.5% NaCl and pH 6 for all strains isolated, where the strains may effect on the plant growth.

Keywords: Actinomycetes, Rhizosphere, Salt, pH, Temperature Tolerance and 16S rRNA

1. Introduction

The Actinomycetes are gram positive, free living saprophytic bacteria and ubiquitous in nature. Majority of them are found in soil, fresh waters, and surface of water bodies and also in sea water, odor of freshly turned soil comes from volatile compounds produced by these bacteria. Colonies have pastel colors, soil-like odor, are hard and stick into agar (Goodfellow and Williams, 1983).

Plant roots produce organic compounds into the rhizosphere soils, contains high microbial biomass and activity when compare to non rhizospheric soils. These organic compounds may inhibit the growth of some microbial population (Li et al., 2010). Plant rhizospheric soils represent aunique biological niche with a diverse micro flora comprised of bacteria, fungi, actinomycetes, protozoa and algae. This community was supported nutritionally by a high input of organic material derived from the plant roots and root exudates that are necessary for microbial growth (Lynch, 1990). However, the composition of quantity of root exudates varies depending on the plant species (Smith, 1976) and physical environment such as humidity and temperature (Martin and Kemp., 1980).

The Rhizosphere contains a large and majority of the soil biota. The plant-microbe interaction in the rhizosphere is one of the major factors regulating the health and growth of plants. Soil bacteria living in the rhizosphere can enhance plant growth by several mechanisms like antagonism against plant pathogens, solubilization of phosphates (De Freitas et al., 1997) production of phytohormones (Arshad et al., 1998), siderophores production (Kloepper et al., 1980), antibiotic production (Schneider et al., 1996), inhibition of plant ethylene synthesis (Glick et al., 1998) and induction of plant systemic resistance to pathogens (Kloepper et al., 1999) The study of rhizosphere is important as far as control of soil pathogens which pass through the rhizosphere and infect root system.

The actinomycetes are well known important saprophytic bacteria in the rhizosphere, where they may influence the plant growth and protect plant roots against the invasion of root pathogenic fungi (Yilmaz et al., 2008). Filamentous soil bacteria belonging to the genus streptomyces are rich source of antibiotics, which are used in pharmaceuticals and agrochemicals (Mantea et al., 2008).

2. Materials and Methods

Study Area: This study was conducted in the district during 2015/16 and 2016/17 the sample was collected from the Farm Department of soil science, Allahabad School of Agriculture (SHUATS) Allahabad.

Purification and storage

Morphological Characteristics: Circular, Raised with smooth edges and musky odor of the colony were observed under low power microscope, similarly using gram staining technique as purple colored gram positive filamentous were observed.

Biochemical Tests: The bio-chemical activities of the selected species were determined by a series of biochemical tests such as IMVIC tests, Nitrate Reduction test, Test for gelatin hydrolysis, Test for starch hydrolysis, Lipid hydrolysis test, Test for Esquiline hydrolysis, Catalase test, Fermentation tests as general fermentation test etc. The utilization of different carbon sources as well as production of melamin was also studied Hydrogen sulfide reduction, Indole production test, Physiological test Range of pH for

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growth optimum temperature for growth. Molecular methods PCR-Amplification, 16S rRNA sequencing.

Salt, pH and Temperature Tolerance: The ability of the isolated *Rhizobial* strain to grow in different concentration of salt was tested by streaking them on YEM medium containing 1.0%, 1.0%, 2.0%, 3.0%, 4.0%, 4.5% and 5.0% (wt/v) NaCl. Differences in pH tolerance were tested in YEM agar by adjusting the pH to 4.0, 5.0, 6.0, 7.0 and 8.0. All the plates were incubated at 28°C for 72 hours and YEM medium plates were used as controls. Differences in the range of growth temperature were investigated by incubation of bacterial cultures in YEM agar at 5°C, 10°C, 15°C, 20°C, 28°C, 38°C, 40°C, 45°C and 50°C. Control plates were incubated at 28°C. Strains were considered salt tolerant, resistant to acidity and temperature resistant when growth was similar to the growth in the control.

DNA extraction from pure cultures: Total genomic DNA was extracted from bacteria samples using a modified method described by (Petersen and Scheie, 2000).

Identification of the isolated bacteria by sequencing of the amplified 16S rRNA gene: The most powerful tool to identify the unknown bacteria is to sequence the gene (DNA) coding for 16S rRNA, which is present in the chromosome of the bacteria. The prokaryotic specific primers used for 16S rRNA gene.

3. Result and Discussion

The microscopic studies and staining properties of selected *Streptomyces* spp. (AS1, AS2 and AS3) showed that both of them were gram positive and non-acid fast and they had filamentous, branched and coenocytic mycelia. The growth patterns, amount of growth, aerial mass color, reverse color and soluble color of the selected species on different media were observed and recorded. The biochemical test result showed that they were catalase positive and responded positively to nitrate reduction and both were starch hydrolyzer. There was negative response in indole production and Voges-Proskauer tests in case of both species. In general fermentation tests, several carbohydrates such as lactose, mannitol, maltose, sucrose and glucose were added to the nutrient broth in presence of phenyl red indicator to observe the fermenting capability of the *Streptomyces* spp. and the observations were summarized. The tests for carbon utilization by the organisms were performed. In (Table 1) three isolates bacteria were isolated from *Rhizospher of Pisum sativum L.* collected from different locations in Allahabad. All strains tested were found to have growth on at 28°C. On the basis of morphological isolates (Table 2).

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name of the Isolate</th>
<th>Location of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AS1- <em>Streptomyces</em></td>
<td>Soil Science Research Farm, SHUATS, ALLD, U.P.</td>
</tr>
<tr>
<td></td>
<td>lucensis gene</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>AS2- <em>Streptomyces</em></td>
<td>Soil Science Research Farm, SHUATS, ALLD, U.P.</td>
</tr>
<tr>
<td></td>
<td>griseus gene</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>AS3- <em>Streptomyces</em></td>
<td>Soil Science Research Farm, SHUATS, ALLD, U.P.</td>
</tr>
<tr>
<td></td>
<td>olivaceae gene</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Isolates of Bacteria from Rhizospherica of *Pisum sativum L.*

The morphology of their substrate mycelium and aerial mycelium was studied. The vegetative mycelium was coenocytic. These species were found to produce filamentous, profusely branched mycelium with net like structure. Species of *Streptomyces* were characterized by the production of typical aerial mycelium super imposed upon the substrate growth. Aerial hyphae were found to vary considerably in length. Biochemical characteristics of the selected (AS1, AS2 and AS3) were analyzed. Species were tested for their capability to ferment different types of carbohydrate such as lactose, mannitol, maltose, sucrose, glucose and salicin. Different *Streptomyces* spp. gave different substrate colors.
Table 3: Effected temperature on Actinomycetes isolated Rhizospherica of Pisum sativum L.

<table>
<thead>
<tr>
<th>Strains</th>
<th>5°C</th>
<th>10°C</th>
<th>15°C</th>
<th>20°C</th>
<th>25°C</th>
<th>30°C</th>
<th>35°C</th>
<th>40°C</th>
<th>45°C</th>
<th>50°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS1</td>
<td>0.00</td>
<td>0.07</td>
<td>0.16</td>
<td>0.26</td>
<td>1.25</td>
<td>1.20</td>
<td>0.69</td>
<td>0.29</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>AS2</td>
<td>0.06</td>
<td>0.19</td>
<td>0.34</td>
<td>0.44</td>
<td>0.67</td>
<td>0.53</td>
<td>0.35</td>
<td>0.30</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>AS3</td>
<td>0.09</td>
<td>0.21</td>
<td>0.38</td>
<td>0.46</td>
<td>0.75</td>
<td>0.70</td>
<td>0.63</td>
<td>0.61</td>
<td>0.57</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1: Effected of temperature on actinomycetes isolated from pea

The Streptomyces spp were grown at different temperature all strains were able to grow at 10°C and 45°C. They were mesospheric, growing best at 30° to 37°C. The selected species behaved as neutrophilic in culture, growing between pH 4.0 and 9.0 with an optimum closeness to neutrality. Most species of them were mesophiles, growing at temperature between 10°C and 37°C. There were also thermo tolerant and thermopile species.

Table 4: Tolerance of Actinomycetes strains to pH and NaCl concentrations

<table>
<thead>
<tr>
<th>Strains</th>
<th>pH 4</th>
<th>pH 5</th>
<th>pH 6</th>
<th>pH 7</th>
<th>pH 8</th>
<th>NaCl (w/v) 1.0</th>
<th>NaCl (w/v) 2.0</th>
<th>NaCl (w/v) 3.0</th>
<th>NaCl (w/v) 4.0</th>
<th>NaCl (w/v) 5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS1</td>
<td>0.37</td>
<td>0.40</td>
<td>0.42</td>
<td>0.45</td>
<td>0.49</td>
<td>0.65</td>
<td>0.43</td>
<td>0.42</td>
<td>0.40</td>
<td>0.37</td>
</tr>
<tr>
<td>AS2</td>
<td>0.73</td>
<td>0.79</td>
<td>0.83</td>
<td>0.90</td>
<td>0.97</td>
<td>0.44</td>
<td>0.40</td>
<td>0.37</td>
<td>0.35</td>
<td>0.30</td>
</tr>
<tr>
<td>AS3</td>
<td>0.57</td>
<td>0.60</td>
<td>0.64</td>
<td>0.70</td>
<td>0.75</td>
<td>0.56</td>
<td>0.47</td>
<td>0.43</td>
<td>0.40</td>
<td>0.38</td>
</tr>
</tbody>
</table>

A few species grew slowly at 4°C. In our study, the selected Streptomyces spp. was grown at different temperature. Both were able to grow at 10°C and 45°C. Data revealed that they were mesophilic, growing best at 30° to 37°C (Table 3). Most Streptomyces spp. behaved as neutrophilic in culture, growing between pH 5.0 and 9.0 with an

Optimum closeness to neutrality, Acidophilic and acidonuric strains had been isolated from acidoic soils and other materials. In our study, the species were grown on Bennett agar adjusted at different pH ranging from 4.0 to 9.0 (Table 4). Further research is necessary for species identification as well as enzyme activity determination.

Microorganisms isolated from the soils of Allahabad region was identified as Streptomyces lucensis (AS1), Streptomyces griseus (AS2) and Streptomyces olivaceus (AS3) have been reported as notably producer of antibiotics. In our case these strain showed the strong antifungal activity against the various fungal pathogens with broad spectrum antibacterial activity which shows the novelty of active metabolite produced by our isolate. Therefore these can be employed as a target to search for a new active metabolite or drug to satisfy public demands.
In this study, we focused on the optimization of culture conditions for production of antibiotics by a new isolate. The optimization of fermentation mediums as important as selection of an organism to obtain antibiotic production, the source of carbon and nitrogen in the Fermentation media plays an important role, since microbial and fermented products are largely composed of these elements. It is usual that the production of antibiotic is promoted after readily utilisable sugars as a carbon source. It has been reported in literature that the high strain bacterial activity of Streptomyces sp., was obtained when glucose at 1 % (w/v) was used as a carbon source followed by xylose and arabinose. It is well known that changes in the kind and concentration of nitrogen source influence greatly antibiotic production. The presence of tryptophan in soybean meal concentration of the solution was also tested in order to increases antibiotic production up to a certain level, other based on the 120 active strain and characterization of bioactive metabolite produced by the ARD 1 (5). According to the results, the strain Streptomycyces lucensis, Streptomycyces griseus, and Streptomycyces olivaceus were subjected to 16SrRNA sequence analysis and identified as Streptomyces lucensis (AS1), Streptomyces griseus (AS2), and Streptomyces olivaceus (AS3) (Table 5). By the secondary structure prediction and restriction site analysis, one can calculate the free energy and percentage of GC and AT contents by using restriction site enzymes.

4. Acknowledgement

I am highly indebted to my advisor for his guidance and constant supervision as well as for providing necessary information regarding the study. I express a heartfelt thanks to The authors are thankful to the Hon’ble Vice Chancellor, HOD and Advisor, Department of Soil Science, Sam Higginbottom University of Agriculture, Technology and Sciences, Allahabad, U. P., and Cytogene Research and Development Indira Nagar, Lucknow for providing all necessary facilities.

References


Table 5: Analysis of the 16S rRNA gene sequences for comparison of isolates with other Actinomycetes spp using NCBI BLAST

<table>
<thead>
<tr>
<th>S/No</th>
<th>Isolates</th>
<th>Type of Actinomycetes</th>
<th>Name of Actinomycetes</th>
<th>Gen Bank accession No</th>
<th>Sequence ID</th>
<th>Query ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AS1</td>
<td>Streptomyces lucensis gene</td>
<td>ABDR4</td>
<td>LC176426.1</td>
<td>2555</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>AS2</td>
<td>Streptomyces griseus gene</td>
<td>ABDR5</td>
<td>LC176427.1</td>
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<tr>
<td>3</td>
<td>AS3</td>
<td>Streptomyces olivaceusgene</td>
<td>ABDR6</td>
<td>LC176428.1</td>
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