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Characterization of *ACTINOMYCETES* Isolated From Rhizosphere of *PISUM SATIVUM* L.

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Abstract: ***In this study Actinomycetesspp 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 16SrRNA sequence analysis and identified as Streptomyces lucensis (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 % NaCl and pH 6 for all strains isolated, where the strains may effect on the plant growth.***

Keywords: *Actinomycets*, 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 a*re rich source of antibiotics, which are used in pharmaceuticals and agrochemicals **(Manteca *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 melanin was also studied Hydrogen sulfide reduction, Indole production test, Physiological test Range of pH for

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°Cfor 72 hours and YEM medium plates were used as controls. Difference 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 coenocyte 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 1: Isolates of Bacteria from Rhizospherica of *Pisum sativum* L.

S.No	Name of the Isolate	Location of isolates
1	AS1- *Streptomyces lucensis* gene	Soil Science Research Farm, SHUATS, ALLD, U.P.
2	AS2- *Streptomyces griseus* gene	Soil Science Research Farm, SHUATS, ALLD, U.P.
3	AS3- *Streptomyces olivaceus*gene	Soil Science Research Farm, SHUATS, ALLD, U.P.

Streptomyces spp. from the soil samples were selected and characterized based on their morphological, physical, cultural and biochemical properties with the help of Bergey's Manual of Systematic Bacteriology (Table 2). These selected species produce aerial mycelium of various colors such as gray, white, ash, brown which can be easily detected with naked eyes. They were found to be gram positive and non-acid fast, which is one of the important criteria of the *Streptomyces* spp. all of the species were studied morphologically and microscopically following cover slip culture on solid medium. Microscopic observation revealed that the selected species showed better performance in the production of aerial or reproductive mycelia as well as sporulation on solid media.

The morphology of their substrate mycelium and aerial mycelium was studied. The vegetative mycelium was coenocyte. 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 2: Morphological and Biochemical characterization of *Actinomycetes* isolated from Pea.

S.No	Particalars	Strain of *Actinomycetes*		
		AS1	AS2	AS3
1	Gram stain –reaction	+ve	+ve	+ve
2	Colony morphology	Filamentous	Spores arranged in straight chain	Filamentous
4	Casein hydrolysis test	-	+	-
5	Citrate utilization test	-	-	-
6	Indole production test	+	+	+
7	Amylase Test	+	+	+
8	Urease test	-	-	-
9	Starch hydrolysis test		+	+
10	Methyl-red (MR)	+	+	+
11	Voges-Proskauer tests (VP)	+	-	-
12	Glucose fermentation	+	+	+
13	Mannitol fermentation	-	+	+
14	Catalase Test	-	+	+

Table 3: Effected temperature on *Actinomycetes* isolated Rhizosphericaof *Pisum sativum* L.

Strains	Absorbance of Strain Actinomycetes								
	5°C	10°C	15°C	20°C	28°C	38°C	40°C	45°C	50°C
AS1	0.00	0.07	0.16	0.26	1.25	1.20	0.69	0.29	0.20
AS2	0.06	0.19	0.34	0.44	0.67	0.53	0.35	0.30	0.26
AS3	0.09	0.21	0.38	0.46	0.75	0.70	0.63	0.61	0.57

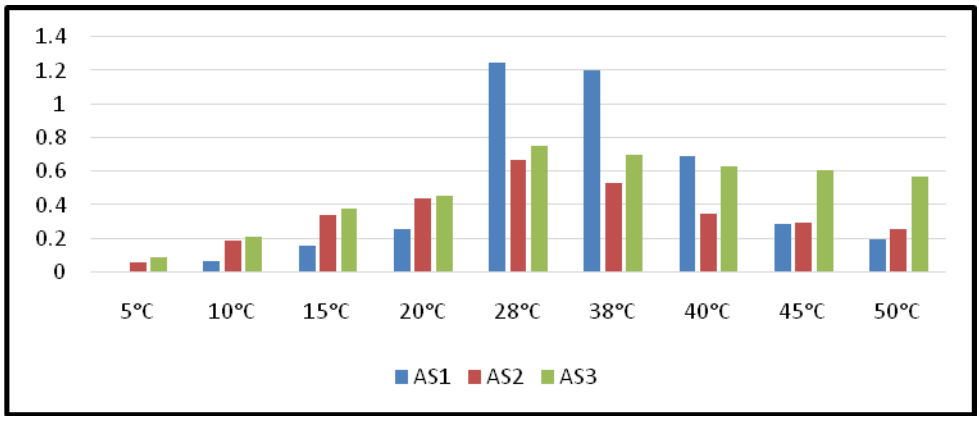

Figure 1: Effected of temperature on *actinomycetes* isolated from pea

The *Streptomyces* sps 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 specie

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

Strains	pH					NaCl (w/v)					
	4	5	6	7	8	1.0	2.0	3.0	4.0	4.5	5.0
AS1	0.37	0.40	0.42	0.45	0.49	0.65	0.43	0.42	0.40	0.37	0.35
AS2	0.73	0.79	0.83	0.90	0.97	0.44	0.40	0.37	0.35	0.30	0.25
AS3	0.57	0.60	0.64	0.70	0.75	0.56	0.47	0.43	0.40	0.38	0.33

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.

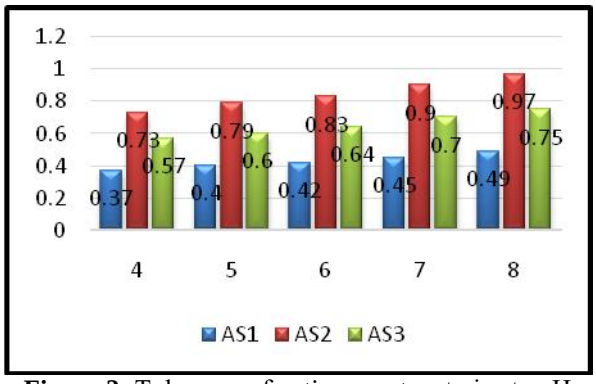

Figure 2: Tolerance of *actinomycetes* strains to pH

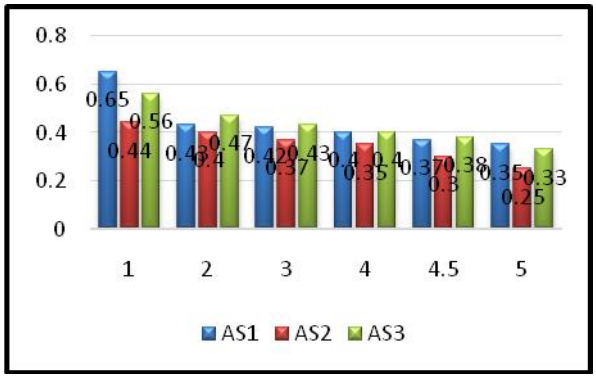

Figure 3: Tolerance of *actinomycetes* strains to NaCl concentrations

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.

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

S/No	Isolates	Type of *Actinomycetes*	Name of *Actinomycetes*	Gen Bank accession No Sequence ID	Query ID
1	AS1	*Streptomyces lucensis* gene	ABDR4	LC176426.1	2555
2	AS2	*Streptomyces griseus* gene	ABDR5	LC176427.1	142655
3	AS3	*Streptomyces olivaceus*gene	ABDR6	LC176428.1	167997

In this study, we focused on the optimization of culture conditions for production of antibiotics by a new isolate. The optimization of fermentation mediumis 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 utilizable 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 increases antibiotic production up to a certain level, other environmental factor such as temperature, pH and NaCl concentration of the solution was also tested in order to establish the suitable cultural conditions for the optimal production of antibiotics. However, more studies should be conducted with regardto statistical optimization, purification and characterization of bioactive metabolite produced by the active strain *Streptomyces* sps. The phylogenetic analysis based on the 120 bp sequence bearing the variable and region is useful for *streptomyces sp*. Identification. They conclude that this type of the phylogenetic tree will serve as a useful tool for rapid identification of the phylogenetic localization of newly isolated Streptomyces strains and it is more effective than the conventional methods, the isolated sps. 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.

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