Isolation and Identification of Actinomycetes from the Rhizosphere of Peach Plant in District Swat, Khyber Pakhtoon Khwa, Pakistan

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***Abstract*- This study focuses on the isolation and characterization of actinomycetes from the rhizosphere of peach plants in Swat District, Khyber Pakhtun Khwa, Pakistan. The identification process includes assessment of cell morphology, Gram staining, and Biochemical tests. Notable genera isolated include Arthrobacter, Bacillus, Citrobacter, Corynebacterium, Erwinia, Enterobacter, Micrococcus, Pseudomonas, Staphylococci and E. coli. Through detailed analysis, Arthrobacter and Bacillus show rod and coccus shapes, while Citrobacter and Enterobacter appear as rods. Corynebacterium has a rod-shaped morphology, and Micrococcus Present as a cocci. Additionally, Erwinia and Pseudomonas are rod-shaped, while Staphylococci have cocci morphology. E. coli is rod-shaped. Catalase tests reveal a variety of enzyme activities: positive results for Arthrobacter, Bacillus, Corynebacterium, Micrococcus and Staphylococci and negative results for Citrobacter, Erwinia, Enterobacter, Pseudomonas and E. coli. This study expands our understanding of the diverse actinomycete community in the peach rhizosphere, offering insight into potential applications in agricultural and soil management practices.**

Key Words: **Gram staining, Cocci, Catalase, Micrococcus, Actinobacteria.**

I. INTRODUCTION:

Actinomycetes are a group of branching single cell organisms that closely related to bacteria and considered as higher, filamentous bacteria. They are gram positive with guanine and cytosine (G+C) content and hyphae is usually 0.5-10μm in diameter Ventura et al., 2007. Taxonomic classification, they are placed in actinomycetes, usually propagate through fission or through specialized spore or conidia. Ecologically actinomycetes are widely distributed in different habitats i.e., fresh water, marine, composites, soil etc George et al., 2012. However, soil is their favorable habitat. In soil they are found mostly in surface layer of soil. Soil actinomycetes are usually neutrophillic but in nature acidophilic. Actinomycetes are also frequently distributed. The optimum temperature range for their normal growth and metabolism is 20-30°C and also dependent upon seasonal variation. The class actinobacteria, taxonomically contain16 orders and 53 families where as actinomycetes are well studied. The morphological and chemotaxonomy as important feature for actinomycetes. Mycelia and spore morphology are extremely important taxonomic features. Spore may form directly on substrate or on aerial mycelium as single or chain. actinomycetes have produce various types of metabolites that have vital application Demain and Fang, 1995. These metabolites have many enzymes used in the fields of biotechnology and biomedicine. Actinomycetes produce amylase and cellulose, which are mainly used in detergents, textiles and food additives Solans and Vobis, 2003. Streptomyces produces protease enzymes. The chitinase enzyme produced by various Streptomyces species has the potential to be used as a biological control agent for plant pathogens Reguera and Leschine 2001; El-Tarabily et al., 2000. The situation is similar in the biomedical field of antibiotic production. It is well known that about 80% of available antibiotics are obtained from actinomycetes Jensen et al., 2007; Hassan et al., 2011. Enzymes such as cellulose and hemicellulose can decompose soil residues and help form and compose soil. The constant use of pesticides and fertilizers leads to the accumulation of heavy metals in the soil. especially rhizosphere of peach orchard. These heavy metals create various environmental and health hazards. It may cause toxicities to actinomycetes and produce the secondary metabolites that use vital role in biodegradation and bioremediation of these heavy metals from the soil, there are various mechanisms like bio sorption (metal sorption to surface by physiochemical mechanism), bioleaching (heavy metals mobilization through the formation of insoluble sulphides or polymeric complexes) and intracellular accumulation and enzyme catalyzed transformation. Streptomyces a well studies actinomycetes that help in recycling of polymeric carbon molecules Radwan et al., 1998; Barabas et al., 2001. The species of Streptomyces detoxify HG+2 to volatile HgO by means of a mercuric reductase enzyme. Similarly, Streptomyces species CG252 efficiently remove (Cr vi) by promoting reduction CR(iii). Soil microbes are important biological control agents for plant diseases. These organisms are either parasites or predator pathogens that reduce the post population. Biological control is a slow process and environmentally friendly strategies. Among actinomycetes, there is one common microorganism that lives in the soil, which produces biocompounds that have antagonistic fungal

agents, that is, Alternaria, Phythum specie, Rhizoctonia, Phytopthora, Fusarium, etc Cao et al., 2004; Chattopadhyay and Nandi 1982; Hussain et al., 1990; Merriman et al., 1974; Valois et al., 1996. Sreevidya et al., 2016; Lu et al., 2008 reported that Streptomyces specie are important bio control agent. Actinomycetes are high producers of medicinal antibiotics Behal 2000. It has high potential of natural alternative of chemical antibiotics. About 2/3 of the medicinal antibiotics was produce from Streptomyces. Secondary metabolites produce from Streptomyces have characteristics like antibiotics, anticancer etc Berdy 2005. Louis Pasteur in early 19th century extract certain metabolites that has antibiotic for actinomycetes; which was later Purified by Alexander Fleming in 1929 and characterized modern antibiotics. These Streptomyces are soil inhabitant and can be grown on different nutritional medium like Nutrient Agar, Muller-Hinton Agar, Triptocave Soy Agar. Antibiotics produced by Streptomyces are Streptomycin, Cyclohexamide Tetracycline, Aminoglycosides, and Rifamycin.

II. Objectives:

Some of the Objectives of this study are 1) Isolation of actinomycetes from the peach rhizosphere. 2) Culturing and characterization using different isolation techniques. 3) Morphological characterization of Isolates. 4)Biochemical Testing for Actinomycetes identification.

III. Materials and Methods: The present work was steered in the laboratory of Centre for Biotechnology and Microbiology, University of Swat, from mid of February to the mid of August, 2019. First of all, different areas of district Swat was selected as shown in Table 1 for the collection of soil sample from peach orchards. Soil sample were collected from the rhizosphere of peach plants.

How to collect a soil sample: Before collecting soil sample mark, a target place from which sample was collected, remove the wastes from that area like rotten leaves, grass and other herbs. Remove surface soil and sample were collected from soil below 5mm. Sample was collected is seal packed plastic bags, and carried directly to the laboratory. Samples was stored till the next step starting.

Sterilization of Apparatus: Devices such as scissors, inoculation loop, Flasks, Petri dishes were sterilized in the autoclave at above 121°C for 1 hour before sampling. Before storing in the autoclave, all equipment wrapped in aluminum foil. Media were prepared for these soil samples culturing.

Nutrient Agar: Nutrient agar was used in this process for sample culturing. A 28g of nutrient agar was dissolved in 1 liter of distilled water. The media was then sterilized at 121°C for 15 minutes by using an Autoclave. When the media is cool down nearly to room temperature before pouring to the petri plates, Broad spectrum antifungal i.e. Nystatin is added to it. And then poured to petri dish in the laminar flow hood. After pouring to petri plates media is incubated for 24 hours to check contamination. Contaminated plates are discarded.

Potato Dextrose Agar: First, extract will be prepared from boiled potato solution. For 1 liter of potato dextrose media, 400 ml of potato extract is required, while about 600 ml of distilled water is added to the 1000 ml glass flask. Technical agar was used in this process to grow samples. 20 g of technical agar was dissolved in this solution with 400 ml of extract and 600 ml of distilled water. Shake the bottle well to properly dissolve the technical agar. Keep the bottle in the oven for autoclave. The temperature of the autoclave must be at 121°C. This sterilization is completed in about 45 to 55 minutes. After the sterilization is complete, take the media bottle to the Laminar Flow Hood. Keep this for cooling to room temperature. Almost 45 plates will be cast. After pouring onto plates, the media is incubated for 24 hours to detect the contamination. Contaminated plates will be separated and disposed of with care.

Inoculation of sample: Solid soil sample were inoculated on nutrient agar and potato dextrose agar plates at the same time, and were kept for 24 hours for growing. After 24 hours' different microbial growth as shown in Table 2 will be appeared on potato dextrose plates while there was only bacterial growth on nutrient agar plates.

Sub culturing: After 24 hours of incubation the growth appeared on inoculated plates, colonies were selected but only from nutrient agar plates and again sub cultured on nutrient agar plates, and plates were kept for growth for 24 hours.

Pure Culturing: After 24 hours from the sub cultured plates, pure culture was obtained which consist of only single type of growth, i.e. actinomycetes or fungal growth.

Morphological Identification: To study the morphology of the actinomycetes Gram staining and Lacto phenol blue staining were used in the laboratory and then examined with the help of light microscope.

Gram staining: In 1967 Merchant and packer introduced a process by which microorganisms were identified by simple gram staining as shown in Table 3. Sterilized inoculation loop was used. Small drop of sterile distilled water was added to a slide. After cooling of a loop single colony were taken from a sample plates and were smeared on a glass slide. Heat were used for its fixation. A crystal violet solution was added for minute and then washed by tap water. Iodine solution were added for a minute and washed one more time. Discoloring agent were used for 30 seconds and washed again the same slide. Sefranin were put on this slide for a minute and slide were washed. After all these steps slide was dried by air and observed by a microscope via oil immersion lens for identification purpose.

Lacto phenol Cotton Blue staining: A small drop of Lacto phenol Cotton Blue stain were put on a clean micro-concave glass plate. The fungal culture was then teased through a sterilized needle and stored in the droplet of stain. Coverslip was placed on the slide with forming no air bubble and the slide was checked.
Characterization of Actinomycetes: After secondary screening of actinomycetes, these species were characterized by morphological and biochemical test. Simple microscopic examination was used for morphological characterization. It was carried out through cover slip culture method Kawato and Sinobu, 1979. Oil immersion (100X) were used for the observing the sequence of conidiophores and arthrospore present on the mycelium. These structure were then examined by comparing with Berge's Manual of Determinative Bacteriology, ninth edition (2000) and identification of organism were done.
Physiological and cultural characterization: High power magnifying glass were used for checking a colony morphology of actinomycetes isolated by the observation of the color, mycelium's nature, surface of the spore and consistency with loop already sterilized.
Biochemical characterization: Different biochemical tests were conducted in a lab like Indole test, Triple sugar test, Catalase test, Citrate test, Nitrate reductase test for the characterization of actinomycetes.
Triple Sugar Test: Gram-negative enteric bacilli are identified on the basis of sucrose fermentation, lactose, dextrose and hydrogen sulfide production using triple sugar, iron-agar medium. Media preparation is done and then autoclaved at 121°C for 15 minutes. Add approximately 5 ml of media to each tube. Media solidification was achieved in an oblique position and the slopes were found obliquely. In the test tube the isolates were attached one after the other with the help of a straight wire loop together with a line on slope. The tubes were incubated for 24 hours at 37°C after inoculation.
Indole Test: To lower the amino acid tryptophan and produce indole depends on the ability of the organism tested for the indole test. Kovacs reagent contains dimethyl amino benzaldehyde and hydrochloric acid in amyl alcohol is mixed with indole causing the cherry red solution to change its yellow form.
Citrate: This test was used to use the bacterial ability to use sodium, since carbon and nitrogen citrate are used as the carbon source and sodium ammonium ions are present as the nitrogen source in simmon citrate agar oblique alkaline compounds produced as a by-product of bacteria that are energized by citrate.
Catalase: Organism that produces catalase was confirmed by catalase test hydrogen is converted per oxide by catalase enzyme by breaking water and oxygen. Positive results are given by producing bubble-positive catalase are staphylococcus specie and micrococcus specie and enterococcus specie as shown in Table 3.
Nitrate reduction test: Nitrate reduction test is used for differentiation of members of Enterobacteriaceae based on their ability to produce nitrate reductase enzyme that hydrolyses nitrate (NO3–) to nitrite (NO2–) which can then be broken down into various nitrogen products such as nitric oxide, nitric oxide and ammonia (NH3).

IV. Result and Discussion:

Actinomycetes are important soil edaphon that can perform various activities in soil Bérdy J, et al., 2005. They have potential to degrade and remediate heavy metals in soil Khosro Issazadeh et al., 2013. They act as biological control agent against various soil borne plant pathogen and pests. The first ever antibiotics are extracted from Actinomycetes and even now a day 2/3 of antibiotics are extracted from them, the genus Actinomycetes are soil inhabitant and usually associated with root rhizosphere. Our study revealed that number of Actinomycetes are associated with peach plant rhizosphere. Our present study showed that 11 different genera of bacteria were present in peach root rhizosphere. Bacillus specie was isolated from maximum locations. Our results are in line with Gayathramma K et al., 2013, reported that Bacillus were present in six locations. Similar result was obtained by M Ahemad et al., 2014. Jaharamma M et al., 2009 reported that pseudomonas was present in soil rhizosphere. Our results also showed that pseudomonas was associated with 5 locations. Other important soil borne bacteria like Agrobacterium, Erwinia, Corynebacterium also well reported. Kers, J. A et al., 2005. Our results also supported the literature. The most effective soil borne microbes associated with root rhizosphere is fungi that has both useful and harmful impacts on plant roots. A total of 9 number of genera were isolated from different locations in our present study. Penicillium were present in maximum locations i.e, 9 out of 19 as shown in Table 4. Similar result was obtained by Pitt, 1991; Houbraken & Samson, 2011; Taniwaki et al., 2015. Kucuk C et al., 2003 reported that Trichoderma specie has high antagonistic potential against soil borne pathogens. It was also reported in 4 locations in our present research as shown in Table 4. Soil borne pathogens like Fusarium, Rhizoctonia, phythium, were also isolated in our study as shown in Table 4. Same result was reported by Khendker and Deshpande 2014. Similarly, fungi also have a potential of heavy metals degradation Brookes et al., 1986; Doelman and Haanstra, 1985; Kandeler et al., 1996. Actinomycetes are well reported for their antibiotics production potential. Other bioactivities are bio antagonism and biodegradation of heavy metals, bio decomposition are well reported Bérdy J, 2005; Tanaka and Omura 1993. Usually indigenous actinomycetes has high effective in their activities. In present studies actinomycetes were present in peach root rhizosphere of district Swat. It is reported from 12 out of 19 locations as shown in Table 4. Similarly, reported by Bibb, M.J 2005 they reported twenty for thirty-five locations. Waksman conducted research in 1961 to study the production of antibiotics in actinomycetes. Similar report was obtained by Remya M, 2008; Miyadoh S. 1993; Okami & Hotta, 1988. Bio antagonistic potential of Actinomycetes was

well documented against soil borne fungus like Phythium, Phytopthora and Rhizoctonia etc. In our study, it was observed that both plant pathogenic fungus and Actinomycetes were present in same rhizosphere of healthy plants as shown in Table 4. It showed that Actinomycetes has considerably reduced the population of pathogens. Similarly, in our study most of the actinomycetes are reported from rhizosphere where soil has high decomposed farm yard man use. It showed that actinomycetes had decomposed that and provide nutrient to plants. Our study was in support of Nanjwade et al., 2010; Khan and Scullion 2000.

V. Summary:

Actinomycetes are important soil edaphon that can perform various activities in soil i.e., have potential to degrade and remediate heavy metals and act as biological control agent against various soil borne plant pathogen and pests. About 2/3 of antibiotics are extracted from them. Our study revealed that number of actinomycetes are associated with peach plant rhizosphere. Our present study were sabout 11 different genera i.e. Bacillus, Corynebacterium, Pseudomonas, Citrobacter, Arthrobacter, Agrobacterium, Micrococcus, E. coli, Staphylococcus, Enterobacter and Erwinia were present in peach root rhizosphere with high prevelecne of Bacillus spp. that is followed by Pseudomonas spp. The most effective soil borne microbes associated with root rhizosphere is fungi that has both useful and harmful impacts on plant roots. A total of 6 genera of fungi were isolated from different locations in our present study. These fungi are Geotrichum spp, Fusarium spp, Aspergillus niger, Trichoderma spp, Penicillium spp, Rhizopus. Penicillium were present in maximum locations i.e. nine out of nineteen locations. Trichoderma spp. has high antagonistic potential against soil borne pathogens and reported in 4 locations in our present research. Soil-borne pathogens like Fusarium, Rhizoctonia, Phythium, were also isolated in our study. Actinomycetes are well reported are well reported in plant rhizosphere. In present studies actinomycetes were present in peach root rhizosphere of district Swat. It is reported from Twelve out of Nineteen locations. Bio-antagonistic potential of actinomycetes was well documented against soil borne fungus like Pythium, Phytopthora and Rhizoctonia etc. In our study, it was observed that both plant pathogenic fungus and actinomycetes were present in same rhizosphere of healthy plants. It showed that actinomycetes has considerably reduced the population of pathogens. Similarly, in our study most of the actinomycetes are reported from rhizosphere where soil has high decomposed farm yard manure. It showed that actinomycetes had decomposed that and provide nutrient to plants.

VI. Conclusion:

From our all present work and study, different bacteria and fungi were isolated from peach orchards. Among all the bacterial isolates. Bacillus was the most occurring pathogen. Aside Bacillus, Pseudomonas were also present in abundant quantity, and is the most effective genera in soil of peach root rhizosphere. Optimum temperature is 30-40^{o}C. The occurrence rate of the remaining bacterial population is almost lower than these two genera. Among all fungal groups obtained Trichoderma, Fusarium and Aspergillus were in high ratio, which shows resistance or tolerance, against other soil borne pathogen as compared to other pathogens.

VII. Recommendations:

Now a day more resistant species of bacteria are present that shows more resistant to antibiotics obtained earlier. So for obtaining more potent antibiotics study is required to be conducted to overcome these problems. Remediation capability also be enhanced by genes transferring or may be some biotechnological means.

VIII. CONFLICT OF INTEREST: Authors have no conflict of interest.

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Table 1: Samples were only collected from peach plant farms.

S/No	Areas	S/No	Areas	S/No	Areas
1	Bara Bandai	8	Bahrain	15	Thana
2	Charbagh	9	Chakdara	16	Madian
3	Dureshkhela	10	Gulkada	17	Matta
4	Islampur	11	Kota Aboha	18	Shingardar Stupa
5	Khwazakhela	12	Landakay	19	Shamozo
6	Malamjaba	13	Miadam		
7	Marghazar	14	Maniar		

Table 2: Different types of bacterial species were present in a peach rhizosphere.

S.No	Name of Samples sites	Name of Isolates
1	Bara Bandai	Bacillus, Corynebacterium, Pseudomonas
2	Bahrain	Citrobacter, Arthrobacter
3	Charbagh	Agrobacterium, Pseudomonas
4	Chakdara	Citrobacter, Bacillus
5	Dureshkhela	Arthrobacter, E. coli
6	Gulkada	Bacillus, Agrobacterium
7	Islampur	Corynebacterium, Citrobacter
8	Kota Aboha	Erwinia, Staphylococcus, Bacillus
9	Khwazakhela	Micrococcus, Pseudomonas
10	Landakay	Agrobacterium, Enterobacter,
11	Malamjaba	Bacillus, Citrobacter

12	Miadam	Arthrobacter, Pseudomonas,
13	Marghazar	Bacillus, Arthrobacter,
14	Maniar	Pseudomonas, Citrobacter, Arthrobacter
15	Madian	Agrobacterium, Bacillus
16	Matta	Arthrobacter ,Citrobacter,
17	Shingardar Stupa	Arthrobacter, Corynebacterium,
18	Shamozo	Bacillus, Agrobacterium, Citrobacter
19	Thana	Corynebacterium, Bacillus

Table 3: Identification on the basis of cell morphology, Gram staining and Catalase test.

S.No	Name of Isolates	Size (length * Diameter	Shape	Gram staining	Catalase Test
1	Arthrobacter	2.5* 2µm	Rod and Cocci	+	+
2	Bacillus	6 * 1µm	Rod	+	+
3	Citrobacter	5* 2µm	Rod	-	+
4	Corynebacterium	4* 0.5µm	Rod	+	+
5	Erwinia	2* 0.8µm	Rod	-	+
6	Enterobacter	2.5* 0.8µm	Rod	-	+
7	Micrococcus	2* 0.5µm	Cocci	+	+
8	Pseudomonas	3* 7µm	Rod	-	+
9	Staphylococci	1.7* 0.6µm	Cocci	+	+
10	E. coli	1.9* 0.7µm	Rod	-	+

Table 4: Isolates of fungi obtained from the rhizosphere of peach orchards.

S.No	Name of sample location	Name of Isolates
1	Bara Bandai	Fusarium spp, Geotrichum spp
2	Bahrain	Alternaria solani, Trichoderma spp
3	Charbagh	Penicillium spp, Rhizopus spp
4	Chakdara	Aspergillus flavus, Penicillin spp
5	Dureshkhela	Geotrichum spp, Rhizopus spp
6	Gulkada	Aspergilus spp, Trichoderma spp
7	Islampur	Penicillin spp, Aspergillus flavus
8	Kota Aboha	Fusarium solani, Aspergillus flavus
9	Khwazakhela	Penicillin spp, Alternaria solani
10	Landakay	Trichoderma spp, Fusarium spp
11	Malamjaba	Penicillin spp, Aspergillus flavus
12	Miadam	Aspergilus niger, Penicillin spp
13	Marghazar	Rhizopus spp, Trichoderma spp
14	Maniar	Fusarium spp, Penicillin spp
15	Madian	Penicillin spp, Alternaria solani
16	Matta	Aspergillus flavus, Geotrichum spp
17	Shingardar Stupa	Alternaria solani, Fusarium spp
18	Shamozo	Fusarium solani, Rhizopus spp
19	Thana	Penicillin spp, Aspergilus niger