ISOLATION AND AMPLIFICATION OF BACTERIA FROM TWO *SOLANACEOUS* CROPS; TOMATO FRUIT AND POTATO TUBER

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ABSTRACT: *The present study had been designed to analyze the molecular characterization of four species of bacteria isolated from Lycopersicon esculentum (tomato) and Solanum tuberosum (potato). The two species of bacteria isolated from the infected tomato were Syntrophospora sp., Arthrobacter sp. and two from infected potato tuber i.e., Oscillospira sp., Acinetobacter sp. They were isolated by streak method. DNA was extracted from them and Polymerase Chain Reaction was applied to them for the analysis of their DNA amplifications. One of these strains of both hosts i.e., tomato and potato did not show any amplifications through simple PCR setup therefore colony PCR was applied to that strain. Colony PCR did not require any DNA extraction. Genus of these isolated bacteria can be identified by analyzing their DNA amplifications. Two strains, 226 and 228 from tomato and 230 and 231 from potato were analyzed in this research work. After their molecular characterization, it was observed that from tomato, strain 226 was Arthrobacter sp. whereas strain 228 was syntrophospora sp. Furthermore, from potato; strain 230 related to genus Oscillospira sp. and strain 231 belonged to Acinetobacter sp. The clones of these strains were then prepared. Clones were further purified by Kit QIAGEN. These clones will be used for further studying the sequences of these bacteria to confirm their species.*

Keywords: *S. lycopersicum*, *S. tuberosum*, *Solanaceae*, Bacterial isolation, PCR.

1. INTRODUCTION

The tomato (*Solanum lycopersicum* L.) and potato (*Solanum tuberosum* L.) both belong to *Solanaceae* family and increased production of these vegetable crops by using different varieties or accessions has played a major role in occurrence of many crop diseases ultimately reducing the final yield. Therefore, such climatic conditions which may be favourable for disease development, bacterial diseases of these crops pose a serious threat to crop production during the whole vegetative season [1-3]. A commonly found genus of bacteria found in soil is *Arthrobacter*; Gram-positive obligate aerobes that are rods during exponential growth and cocci in their stationary phase. Colonies of *Arthrobacter* have a greenish metallic center on mineral salts pyridone broth incubated at 20 °C. This genus is distinctive because of its unusual habit of "snapping division" in which the outer bacterial cell wall ruptures at a joint. Microbiologists refer to the type of cell division in which rods break into cocci as reversion. Under the microscope, these dividing cells appear as chevrons ("V" shapes).

Other notable characteristics are that it can use pyridone as its sole carbon source, and that its cocci are resistant to desiccation and starvation. One species of *Arthrobacter* sp. has been shown to reduce hexavalent chromium levels in contaminated soil, suggesting that it may be useful in bioremediation [4]. Moreover, *Syntrophomonas* was the first described *syntrophic bacsterium* that degraded butyrate in co-culture with methanogens or hydrogen-utilizing sulfate-reducing bacteria. Since then known as *Syntrophospora* sp. [5]. Similarly, the potato crop has several economically important bacterial pathogens including the soft rot *Erwinia* spp., the brown rot pathogen *Ralstonia solanacearum*, the ring rot pathogen *Clavibacter michiganensis* sub sp. *Sepedonicus* and the scab forming *Streptomyces* spp. Pathogen indexing of microplants and high grade seed potatoes e.g. mini-tubers, derived from them is likely to become increasingly important as a method of control of some if not all these pathogens [6]. Brown rot and ring rot are two devastating bacterial diseases and are quarantine organisms. Blackleg and soft rot can cause significant losses especially during storage. Scab could become problematic whenever plants are over-irrigated especially as tubers are close to maturity [7]. Hence, the current study was carried out to isolate the bacteria of the two important crops tomato and potato which may help in disease management study and for sequence analysis of the study.

2. METHODOLOGY

Bacterial strains were isolated both from tomato and potato diseased samples (fruits) collected from local market of Lahore using standard bacteriological procedure on LB (Luria Bertani) media.

2.1 Isolation of Bacteria from specimen

The specimens were brought to the laboratory on the same day they were collected and stored at 4 °C. For isolation of bacteria these specimens were surface sterilized in 70% alcohol for one to two min and then in 0.1% Sodium hypochloride for one minute (min) to eliminate any surface microbial contaminants. After washing by distilled water these infected potato tubers were fragmented into small fragments and bacteria were collected by a sterile inoculating needle and then streaked into different dilution of LB broth poured in a test tube. These samples were placed in a rotatory shaker at 37 °C overnight. Pure cultures were obtained by sub culturing. Slants were prepared after pouring and culturing done in a laminar air flow chamber that was sterilized well. These test tubes were incubated overnight in a shaker at 28°C temperature.

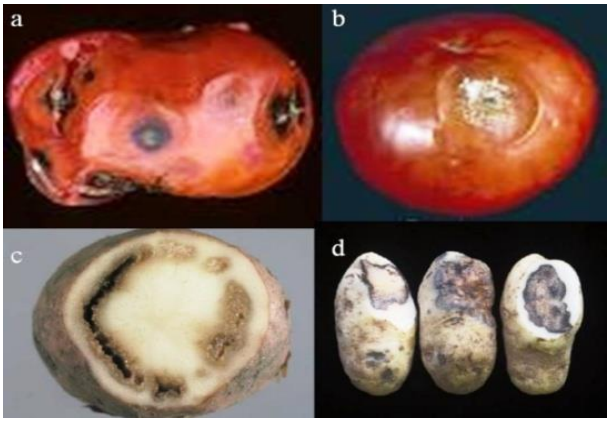

Figure 1: Disease cause by (a) *syntrophospora* sp. (b) *Arthrobacter* sp. in tomato (c) necrosis and rotting of a potato tuber infected by brown rot and (d) potato tubers exhibiting soft rot symptoms.

2.2 DNA Extraction

The bacterial culture grew overnight in rich broth was transfered (2ml of culture) to a micro centrifuge tube and spined for 3 min at 14000 rpm. The supernatant was decanted, drained well and eppendorfs filled with cultures and centrifuged for 2 min at 14000 rpm. Drained the supernatant and inverted the eppendorfs on tissue. Resuspended the pallet in 467 µl TE buffer and dissolved by repeated pipetting. Then added 30 µl of 10% SDS and 3µl of 20 mg/ml proteinase K, mixed it well for incubation at 37 ºC for 1 hr. Added 250 ml of phenol and chloroform each. Mixed it well by moving the tubes up and down gently, to avoid shearing the DNA by inverting the tube until the phases were completely mixed (This step was performed quickly to avoid denaturation of DNA). Carefully, transfered the DNA and phenol mixture into an eppendorf (spined for 10 min at 12000 rpm) and upper aqueous phase was pipetted out into new eppendorfs. Added 1/10 vol. of sodium acetate, mixed it. Added 0.6 volumes of isopropanol i.e. 600 to 700 µl in this case and mixed gently until the DNA precipitated. Centrifuged for 10 min at 14000 rpm. Drained the supernatant and incubated at 37 °C for 40 min and dissolved the pallet in 100 µl double distilled (dd) water.

2.3 Polymerase Chain Reaction

Universal primers were used to amplify bacterial DNA. A final volume of 50 µl amplification reaction mixture comprised of 1X (25ul) PCR buffer (DNA 5 ul, $MgCl_2$ 1.5ul, 10X Taq buffer 2.5ul, Primer reverse 0.5ul, Primer forward 0.5 ul, Taq polymerase 0.25ul, ddH2O 12.25 ul) was used. Master solution for PCR was prepared for two samples. It was then transferred to two labelled eppendorfs in equal amounts and extracted DNA was also added to them and placed in a PCR machine. The PCR protocol adopted consisted of a preheat treatment of 94 ºC for 5 min followed by 25 cycles of 94 ºC for 1min, 48 ºC to 55 ºC for 1 min and 72 ºC for varying times followed by a final incubation of 10 min at 4 ºC. The annealing temperature, extension time and number of cycles were adjusted according to the requirement of the experiment. The whole process for the preparation of reaction mixture was carried out on ice. Eppendorf thermal cycler was used for PCR. The whole process takes about 1hr and 55 min.

2.4 Colony PCR

One of the samples did not show any amplification through Polymerase Chain reaction carried out after DNA extraction therefore a colony PCR was performed to get amplifications for that sample. Took 20 ul of dd. water in an effendorf. Picked 3ul of bacterial colony from streaked plates and dissolved in effendorf containing double distilled water. A final volume of 50µl amplification reaction mixture comprised of 1x (25ul) PCR buffer (DNA 5 ul, $MgCl_2$ 1.5 ul, 10x Taq buffer 2.5 ul,16S A2 Primer reverses 0.5 ul,16S B2 Primer forward 0.5 ul, Taq. polymerase 0.25 ul, dd. H2O 12.25 ul) was prepared. Took 20 ul of master mixture in each PCR effendorfs and added 5 ul template. Started up PCR for 2 hours. Loaded it on gel for 30 min and observed through gel documentation system. Agarose gel was used to observe the amplification obtained through PCR. If amplifications are visible place the gel onto a gel documentation system. Took a snap shot and saved the observed the amplifications on a monitor screen. Excised the portion of the gel containing DNA with a sharp and sterilized scalp. Put it in an eppendorfs tube and label with date and sample number.

2.5 Gene Elution/Gene clean

Weighed eppendorf (W1). Also weighed the removed section of the gel along with the microfuge tube in which it was placed (W2). Subtracted W1 from W2. (mg). Added **Binding buffer** that must be double the amount of gel. Placed it at 55 °C for 10 min and then transfer the liquid to the column. Centrifuged for 1 min and discard the liquid. Added 500 µl **Wash buffer** (Centrifuged for 1 min. Discarded the liquid. Again centrifuged for 1 min. Discarded the liquid. Then again centrifuged for 3 min, this time let the lid remain open. Transfered the column to a new eppendorf. Added 30 µl of **Elution buffer** and kept for 2 min then centrifuged for 1 min.

2.6 Ligation

Ligation was accomplished using the enzyme DNA ligase. It requires ATP and magnesium ions to catalyze the reaction of a 3'-OH and a 5'-P on double-stranded DNA to form a phosphodiester bond. The DNA ends can be cohesive ends, such as those formed between molecules that have been digested with the same restriction endonuclease, or they can be blunt ends. Ligation between cohesive-ended molecules is much more efficient than ligation between blunt-ended molecules. Because of this, when ligating blunt-ended molecules, the DNA and ligase concentrations must be higher than when ligating cohesive-ended molecules. Prepared the master soln. Divided it equally in two eppendorfs, also added 10µl of insert in each eppendorf. Placed the eppendorfs in ligation chamber overnight at 16 °C.

2.7 Transformation

Transformation of the bacteria begins with making the bacterial cells "competent," which simply means that they are made permeable to the foreign DNA. This is normally accomplished by the treatment of cells in the mid-log phase of growth with calcium chloride. Took a competent cell. The competent cells that were used are DH5-α competent cells. Placed the competent cell in ice for 5 minutes. Added 5µl of plasmid in it. Placed on ice for half an hour. Provided a heat shock at 45-50 °C for 2 min. Then again placed on ice for 2 min. Added 1 ml LB broth to eppendorf in Laminar Flow Chamber. Placed on rotatory shaker at 37 °C for one and a

half hour. Centrifuged the eppendorf for 5 min at 14000 rpm. Removed the supernatant, a little amount of it should be kept so as to dissolve transformed cell. Spreaded the suspension on agar media plate and incubate at 37 °C.

2.8 Mini Prep

Culture was filled in labelled eppendorf tubes and entrifuged at 14000rpm for 3 min at room temperature. Discarded supernatant, if pellet is small in size again repeat the step. Inverted tubes on tissue. Added 150 µl Soln. I in each tube and vortex it. Added 150 µl soln. II in tubes, shook it by inverting with hands. Added 200µl soln. III, shook it by inverting with hands. Centrifuged at 14000 rpm for 10 min, at room temperature. Pipetted out the supernatant carefully with the micro pipette. Added this aqueous layer in another labelled eppendorf. Added 1 mL absolute ethanol (chilled) in each eppendorf. Mixed it by inverting hands. Centrifuged at 14000 rpm for 15 min. Discarded the supernatant. Added 150 µL 70% ethanol centrifuge for 3 min. Removed supernatant carefully with the help of micro pipette and pallet was dried at 60 ºC. Then added dd water according to the size of the pallet and mixed it well.

2.9 Restriction

Restriction is done in order to ensure the ligation of plasmid with the vector. Three types of restrictions were done for the samples. After all the master solutions were prepared and were poured into their respective labelled eppendorfs, these eppendorfs were then placed in the PCR machine and reaction protocol. The Restriction PCR products were then analyzed on a gel documentation system after loading it in an agarose gel.

2.10 Gel electrophoresis

After Polymerase chain reaction was completed, samples were loaded in an agarose gel and were observed.

2.11 Clone streaking

Samples showing amplification were then spreaded on an agar media plate containing ampicilin as an anti-biotic. IPTG and X-gal were also spreaded along with the sample. The petri dish was sealed and placed in an incubator for overnight at 37 ºC. Blue and white colonies were observed and white colonies were marked.

2.12 Clones preparation

This procedure was done in a laminar air flow chamber that was pre sterilized. Marked white colonies were picked singly with a sterilized toothpick and were dropped in their respective labelled test tube containing LB media. These test tubes were left overnight in a shaker at 25 ºC.

2.13 Miniprep by kit QIAGEN (QIAPREP SPIN)

Poured culture in eppendorf tube and centrifuge for 3 min at 14000 rpm and supernatant was discarded. Added 250 µl solution I then vortex it. Added 250µl solution II mixed it by inverting the eppendorf. Added 350 µl solution III mixed it by inverting eppendorf. Centrifuged for 10 min, shifted the supernatant to column. Centrifuged for 1 min and then discard the liquid from the collecting tube. Added 700 µl wash buffer into column. Again centrifuged for one min, supernatant was discarded and centrifuged for 3 min at 14000 rpm without covering the lid of centrifuge machine. Added 50 µl elution buffer and centrifuge 3 min. Purified final clone is obtained. This purified clone can further be analysed for sequential studies.

3. RESULTS AND DISCUSSION

3.1: Tomato Strains.

Diseased samples of *S. lycopersicum* (tomato) exhibiting typical bacterial symptoms were collected from local markets of Lahore. Total genome of diseased samples was extracted and DNA present in the genome were amplified by Polymerase Chain Reaction. PCR product was digested with different restriction endonucleases and DNA fragments of 1.5 KB and 600 MB were obtained. The two basic primers were used in the PCR. One was full length which was 16SA1 and 16SB1 and other was partial primer which was 16SA2 and 16SB2. The size of DNA fragment was 1.5 KB by using a full length primer 16SA1 and 16SB1, whereas, DNA fragment of 600 MB length was obtained by using a partial primer 16SA2 and 16SB2. The primers were categorized into forward and reverse. The 16SA1 and 16SB1 were the forward primer and the 16SA2 and 16SB2 were the reverse primer which used in PCR are shown in Figure 2(a) and 2(b). Study of amplification of strain 226 showed that it has typical amplification of genus *Arthrobacter*, whereas, that of strain 228 showed amplification similar to that of genus *Syntrophospora*. The strain of 228 did not show the amplification from the simple PCR amplifications therefore, colony PCR was applied to it. To run this setup, colony of bacteria was directly picked from the petri dish by a micropippette and was placed in a microfuge tube added in it 10µl of dd H_2O. This dilution was then loaded in a gel and was observed on a gel documentation system to look for the presence of DNA in it (Figure 2 c and d). Restriction was done to prevent plasmid's self ligation. It was done by using two enzymes i.e. Eco RI and Pst I and the V3 buffer had used to show restriction of double digestion of plasmid. V3 buffer is the universal buffer which was used in double digestion. The EcoRI and Pst I were used separately with same same buffer in the single digestion. The size of band was 4.3kb because the fragment of V3 buffer was 2.8kb and the size of strain was 1.5kb. The Figure 2(e) shows the single digestion and 2(f) double digestion.

3.2: Potato Strains.

Total genome of diseased samples was extracted and DNA present in the genome was amplified by Polymerase Chain Reaction. PCR product was digested with different restriction endonucleases and DNA fragments of 1.5 KB and 600 MB were obtained. DNA fragments of 1.5 KB were obtained by using full length primers 16SA1 and 16SB1, whereas, DNA fragments of 600 MB length were obtained by using partial primer 16SA2 and 16SB2. The primers 16SA1 and 16SB1 are forward primers whereas; primers 16SA2 and 16SB2 are reverse primers. DNA fragment amplified through full length primers i.e. 16SA1 and 16SB1 are shown in Figure 3(a).

DNA fragment amplified through partial primers i.e. 16SA2 and 16SB2 are shown in Figure 3(b). Amplification of strain 230 showed that it has typical amplification of genus *Acinetobacte*r, whereas, that of strain 231 showed amplification similar to that of genus O*scillospira*. Amplifications of strain 230 were obtained by applying simple PCR setup as shown in Figure 3(d). Strain 231 did not show any amplifications therefore, colony PCR was applied to it. To run this setup, colony of bacteria was directly picked from the Petri dish by a micropipette and was placed in a

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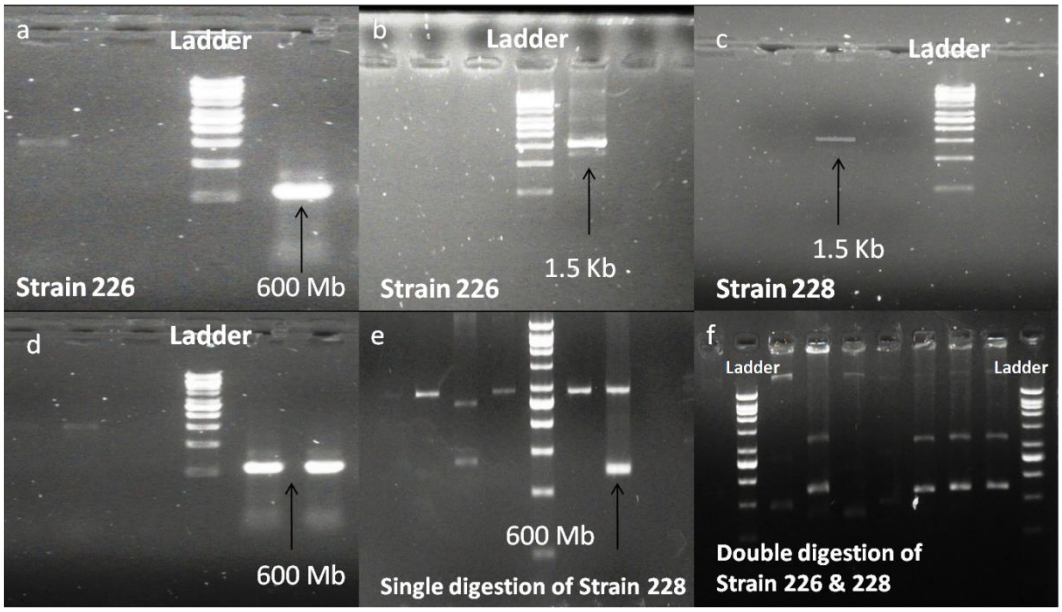

Figure 2: Amplification of (a) strains 226 and 228 by using PCR with partial primer showing the band of 600 MB (b) strain 226 by using PCR with full length primer showing the band of 1.5 Kb (c) strain 228 by using colony PCR showing the band of 1.5 Kb (d) strain 228 by using colony PCR showing the band of 600 Mb (e) single digestion of the strain 226 showing band of 4.3 Kb and (f) double digestion of the strain 226 and 228 in tomato fruit.

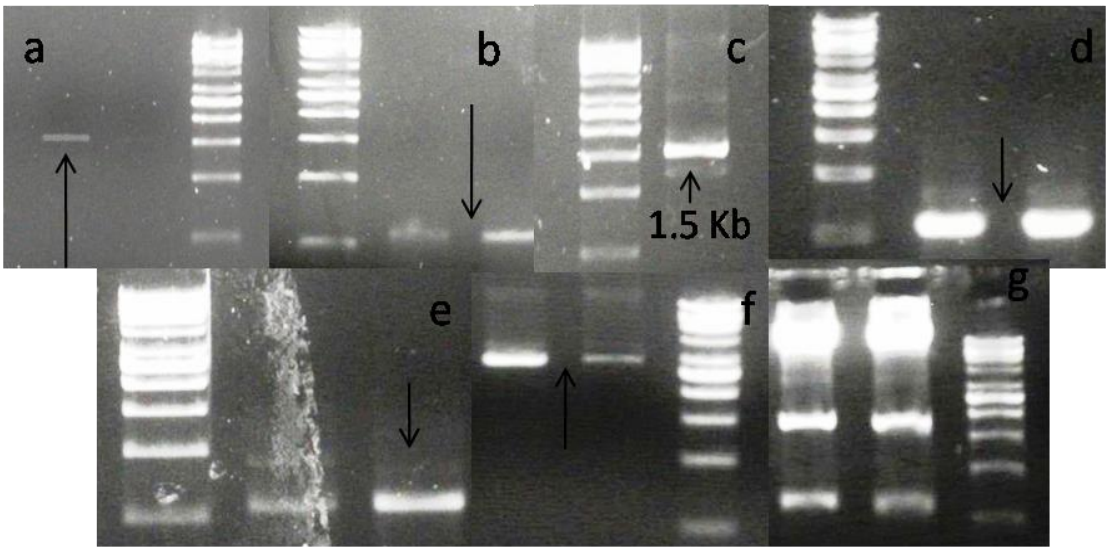

Figure 3: Amplifications by using primers (a) full length (b) partial after colony PCR (c) amplification of strain 230 showing 1.5 Kb band (d) dilution of colony for the detection of presence of DNA (e) amplification of strain 231 amplified by colony PCR (f) bands of restriction by single digestion of plasmid and (g) bands of restriction by double digestion of plasmid in potato.

microfuge tube added in it 10µl of ddH_2O. This dilution was then loaded in a gel and was observed on a gel documentation system to look for the presence of DNA in it. It is shown in fig.4.4. This dilution was then further diluted. 4µl of this dilution was added in 36µl of ddH_2O and was placed in a PCR machine. Amplifications were observed on a gel documentation system as shown in Figure 3(e). Restriction was done to prevent plasmid's self ligation. It was done by using two enzymes i.e. Eco RI and Pst I. Results presented in Figure 3(f and g) depict the restriction by single digestion of plasmid and double digestion of plasmid respectively. Bacterial diseases cause extensive losses to crops including family *Solanaceae* throughout the world thus rapid identification of the pathogen is need of the hour and crucial for diseases management [8]. Previously results were reported by a number of scientists that bacteria *Arthrobacter* and *Syntrophospora* which are the pathogens of *Solanaceae* family mostly found in tomato by using PCR analyses as a tool for analysis and amplification [9,10].

4. CONCLUSION AND SUMMARY

Prevention is better than cure; chemical control is very costly and even is impossible to recover heavily infected, shrivelled and damaged plant. However, cultivation of resistant variety is only the most easy, economical and safe method. Growers are advised to sow improved immune or resistant varieties, because seed or seed treatment with suitable seed dressing these are equal to the susceptible varietirs in yield, adaptation and other cultural characteristics and field practices. Uprooting and burning of diseased plants minimize further spread of the problem. Good drainage improves soil conditions which help to minimize the disease incidence. Use of disease free fungicides before sowing is also recommended. Nevertheless, late and deep sowing reduces disease incidence. Mixed cropping of with non-host crops help to check the disease. Besides, further analysis of sequences of bacterial DNA strains because knowledge of DNA sequences has become indispensable for basic biological research, and in numerous applied fields. It also helps in the phylogenetic studies of species, such as diagnostic, biotechnology, forensic biology, and biological systematic. The advent of DNA sequencing has significantly accelerated biological research and discovery.

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5. REFERENCES

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