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CHARACTERIZATION OF PLANT GROWTH PROMOTING ACTIVITIES OF BACTERIAL ENDOPHYTES AND THEIR ANTIBACTERIAL POTENTIAL ISOLATED FROM CITRUS

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ABSTRACT

In this study screening of novel bacterial endophytes isolated from the leaves of citrus were performed for plant growth promoting activities which include Indole Acetic Acid (IAA), Siderophores and phosphate Solubilization. Isolated bacterial endophytes were also tested for the screening of cell wall hydrolyzing enzymes production such as cellulase, chitinase, protease, pectinase and lipase as well as antibacterial activity. Thirteen genera of different bacteria were isolated and characterized from twelve different varieties of citrus on the basis of morphological, biochemical and molecular 16S rRNA. Neighbor joining phylogenetic dandrogram showed that all the isolates have genetic relatedness among them. According to results 75-80% of the strains were positive for plant growth promoting (PGP) and enzymatic activities of test strains except few of them. However antibacterial activity were found positive in 68.75% of test cultures while five isolates *Enterococcus faecalis*, *Klebsiella pneumonia*, *Staphylococcus haemolyticus*, *Bacillus megaterium* and *Bacillus cereus* did not show any antibacterial activity against any of the targeted bacterial strains. The data presented in this article is unique and this type of work on these bacterial species has not been reported from citrus. The findings will be helpful for the use of these endophytes to enhance the plant growth and yield. On the other hand, the study will be equally beneficial to the scientist and farmers community.

Key words: Antibacterial activity, Siderophores, Indole acetic acid, cell wall degrading enzymes.

INTRODUCTION

Bacterial endophytes isolated from plants have ability to reduce the deleterious effects of certain pathogens. The positive beneficial effects of endophytic bacteria on their host plants happen through similar mechanisms as occurs with rhizobacteria. In depth study of these mechanisms has been performed by (Gray and smith, 2005). Different disease causing agents such as bacteria, fungi, viruses, insects, nematode and other microbes can be managed by the use of bacterial endophytes as inoculants for infected hosts (Ping and Boland, 2004; Berg and Hallmann, 2006). Hence it's generally believed that some endophytic bacteria trigger a phenomenon known as ISR (Induced systemic resistance) that is apparently similar to Systemic acquired resistance (SAR). Frequently when plants primarily infected by a pathogen as response of plant defense activation SAR develops. As a result of hypersensitive response pathogen become limited to a necrotic lesion of brown dead tissue. In comparison ISR is quite different hence the bacterium does not cause any visible sign of infections on host plants (Santoyo *et al*., 2016).There are many reports on the emergence of pathogenic strains of endophytes from the rhizosphere of the plants which includes *Enterobacter*, *Burkholderia*, *Herbasipirillum*, *Pseudomonas*, *Ochrobactrum*, *Ralstonia* and *Staphylococcus* (Berg *et al*., 2005). Hence many facultative endophytes have been recruited from the large population of bacterial endophytes from soil and rhizosphere adapted to live inside plant tissues may include opportunistic human and animal pathogens. There is a report of association of bacterium from the interior of alfalfa plant. So this area should be further investigated to prevent the establishment of risk of pathogen association with plants endophytic bacterial niche that appear by the use of biotechnological applications.

Endophytic bacteria are those bacteria that reside inside plant tissues and showed no visible external signs of invasion or negative effects on plant growth (Schulz and Boyle, 2006). There are nearly 300,000 plants species present on the earth, so each individual plant is host of one or more endophytes out of total, only few of the plant species has been studied completely for their biology. As a result there is increase chances to find new and beneficial endophytes among the diverse hosts in complex ecosystem is considerably possible. These endophytes colonize similarly as phyto-pathogens prevails in plants ecosphere and interact with each other so make it possible their use as biological control agents (Berg *et al*., 2005). There are several reports that represent the ability of these endophytic microbes as a bio control agents to control plant pathogens (Kong *et al*.,

2015), insects (Azevedo *et al*., 2000) and nematodes (Ek-Ramos *et al*., 2019). The endophytic niche provides protection from the external environment for those bacteria that can colonize and establish inside plants. These bacteria generally inhabit in the intercellular spaces and plant parts (Posada and Vega, 2005). These microbes can be isolated from the wide host range from both monocotyledonous and dicotyledonous plants, such as oak and pear, or to filed crop plants such as sugar beet and maize.

Plant growth promoting traits of bacterial endophytes has been studied to check the PGPR abilities of many rhizobacteria. These endophytes differ from biological control agents as they improve the growth of plants not necessarily inhibit pathogens. Although bacterial endophytes found inside the plant tissues also promote plant growth by similar mechanisms. Generally these mechanism includes indole acetic acid production (Lee *et al*., 2004), phosphate solubilization activity (Verma *et al*., 2001; Wakelin *et al*., 2004), production of a siderophores (Pandey *et al*., 2015).These organisms also supply the essential mineral and vitamins to plants (Pirttila *et al*., 2004). However a number of other beneficial aspects on plant growth has been studied which consist of osmotic adjustment, regulation of stomatal openings, root morphology modification, increase of mineral and change of nitrogen deposition inside tissues, or metabolism of plant (Compant *et al*., 2005a, 2005b). In terrestrial plants phosphorous is second most important nutrient that can limit the growth. Although total amount of phosphorous are maximum in agriculture soils but their availability to plants is limited because large amount of phosphorus is present in insoluble form (Azevedo *et al*., 2016). On the other hand, soluble form of inorganic phosphorous applied as a fertilizer is stopped soon its application (Glick, 2012). Plants root zone soil is rich in phosphate solubilizing bacteria which secretes organic acids and phosphatases converts the soluble form of phosphorus to available form to plants (Mesa *et al*., 2017). Now agricultural microbiologists are taking attention in utilization of the P-solubilizing strains for improving P uptake of crops (Stefan *et al*., 2013). Most of the bacterial populations residing in soil are involved in various biotic activities of the ecosystem to make it dynamic for essential nutrients and sustainable crop production. As balanced quantity of nutrients are required for plant growth.

There are various functions and effects of indole acetic acid (IAA) on the physiology of plants that control vegetative growth process; disturbed the plant cell division, extension and differentiation; increase the development of roots and starts the lateral and adventitious root formation; enhance the nutrients uptake through xylem, improve the light responses, gravity and fluorescence; it also alter the biosynthesis of different metabolites, photosynthesis, pigment production and resistance of plant against stress conditions (Spaepen and Vander Leyden, 2011). However reaction of plant for IAA depends on plant tissue type. PGPR bacterial has ability to alter the internal pool of IAA while the endogenous level of IAA in plant roots may be optimum for growth hence more IAA required from soil rhizosphere bacteria may enhance or suppress the plant growth as a result promotion or inhibition happens respectively (Phan *et al*., 2016). Usually a PGPR bacterium secretes IAA which increases the plant root access to nutrients by increasing the surface area of root and length. In returns it increase the root exudates from plant to attracts PGPR and also increases root exudation by loosening plant cell walls which provide nutrients to rhizosphere bacteria (Riera *et al*., 2017).

PGPR produce variety of extracellular and intracellular lytic enzymes such as chitinases, β 1, 3-glucanases, proteases, cellulases, and lipases which have function to lyse the cell wall of many plant pathogens. Several strains of bacteria have found to be produced one or more enzymes and have the ability to control a range of pathogenic fungi (ElTarabily, 2006) ; also affect the spore germination and germ-tube elongation of plant pathogenic fungi (Frankowski *et al*., 2001). On the other hand, the enzyme producing bacteria has been used in synergism with other biocontrol agents to control plant pathogens. (Chen *et al*., 2019).The aim of the current study was to isolate and characterize the endophytic bacterial strains from different varieties of citrus through 6S rRNA and their screening for the plant growth promoting trait IAA, siderophores detection, Phosphate solubilization, cell wall degrading enzymes and antibacterial activities against pathogenic strains of bacteria.

MATERIALS AND METHODS

Survey and sampling: A comprehensive survey of the citrus orchards of Sargodha were conducted and 12 different varieties of citrus showing symptoms of citrus greening were collected and proceeded for isolation of endophytic bacteria from leaves.

Isolation and identification of bacteria: Isolation of endophytic bacteria from 3-4 cm mid rib portions of citrus leaves were performed by surface sterilization of leaf mid ribs with 1 % sodium hypochlorite solution for 3-5 minutes and three consecutive washings with sterilize distilled water. Homogenized mixture of grinded mid rib portion were prepared with distilled water and inoculated on Nutrient agar medium plates, and incubated at 28ºC for 24-48 hours. Further isolated bacterial colonies were purified on nutrient agar plates and incubated at 28ºC for 24-48 hours. Pure cultures of bacterial isolates were characterized on the basis of colony morphology and Gram staining (Garrity, 2005). Glycerol stocks of all

isolated and identified bacterial cultures were prepared for long time preservation and stored at -80ºC.

Molecular characterization of isolates: CTAB (cetyl trimethyl ammonium bromide) method was used for isolation of total genome of DNA (Wilson, 1987). Bacterial culture were grown in 5ml of growth medium (Nutrient agar) for 24 hours and centrifugation at 13000 rpm for 2 minutes to make pellet. The pellet was suspended in 567µL of TAE buffer and 30µL of 10% SDS, 3µL of proteinase k (20 mg/ml) was added and incubated at 37ºC for 1 hour. 100µL of 5M NaCl, 80µL of CTAB were mixed and incubated for 10 minutes at 65ºC followed by addition of 750µL of Chloroform Isoamyl Alcohol (24:1) and centrifuged for 5-10 minutes. 400µL of the upper layer was transferred to a new eppendorf tube. The 700µL of Phenol Chloroform was mixed and centrifuged for 10 minutes and again transferred supernatant to new tube. On the other hand, 20µL of 3M Sodium Acetate and 500µL of Absolute Ethanol (100%) were added and mixed gently to precipitate DNA and placed at -20ºC for overnight. Next day the tubes were centrifuged again at 13000 rpm or 10000 rpm for 5-10 minutes and the supernatant was discarded. Pallet was washed with 70% ethanol and re suspended in 50µL sterile double distilled water. DNA was run on 1% [w/v] agarose gels containing ethidium bromide (0.5 µg/ mL).

Genomic DNA 16 bacterial isolates were subjected to PCR for further DNA sequencing, using the bacterial primers 27-F(5' AGAGTTTGATCMTGGCTCAG 3'), 1492-R(5' ACCTTGTTACGAC TT 3') and previously reported PCR conditions were applied. All PCR products were purified and directly sequenced Macrogen Korea. The gene sequences obtained were compared by aligning the result with the reported sequences in Gene Bank using the Basic Local Alignment Search Tool (BLAST) search program at the National Centre for Biotech Information (NCBI), as well as Ribosomal databased project (RDP Hierarchy Browser) was used to classify the isolated bacterial sequences. Sequences were submitted to NCBI Gene Bank data base and accession numbers were obtained.

Phylogenetic analysis: Multiple sequence alignments were generated and the 16S rDNA gene sequences were phylogenetically analyzed using MEGA 6.0. (Tamura *et al*., 2011). A confidence value for the aligned sequence dataset was obtained by performing a bootstrap analysis of 1000 replications. A phylogenetic tree was constructed using the neighbor joining algorithm to study the evolutionary relationship among organisms.

Characterization of plant growth promotion traits assay

IAA production assay: Indole-3-acetic acid (IAA) production of the selected bacterial strains was measured by following the method of (Patten and Glick, 2002) with slight modification. All the strains were replicated thrice and experiment was performed in sterile conditions. About 20 µl aliquots of an overnight grown bacterial culture were used to inoculate 5 ml TSB without and with tryptophan (500 µg ml-1) and incubated at 37ºC for overnight. After incubation the cultures were centrifuged for 30 minutes and 1 ml supernatant was mixed with 4 ml Salkowski's reagent (Gordon and Weber, 1951). The mixture was incubated for 20 minutes at room temperature and then the absorbance was measured at 535 nm by using spectrophotometer.

Phosphate solubilization: Phosphate solubilizing activity of rhizobacteria was determined qualitatively by using (Nautiyal, 1999) method. Bacterial strains were evaluated for their ability to solubilize inorganic phosphate. Tri-calcium orthophosphate was used in agar medium as insoluble inorganic form of phosphate and was used as a source of indication for phosphate solubilization property of the bacterial strains. The medium used to access the phosphate solubilization property of selected bacterial strains was comprised of agar (15 g), glucose (10 g), NH4Cl (5 g), NaCl (1 g), MgSO4.7H2O (1 g), Ca3 (HPO4) (0.8 g) and yeast extract (0.5 g) per liter while pH of the medium was adjusted to 7.2. On each plate three bacterial strains were checked with triplicate and the plates were incubated at 30±1 °C for 4 days where as non-inoculated medium with tri calcium phosphate source served as control. A clear halo formed around some of the colonies after 4 days indicated that these isolates were positive for phosphate solubilization.

Siderophores detection: To determine siderophores production for selected bacterial strains isolated from citrus leaves method was used. In this assay CAS medium was used which was prepared according to (Schwyn and Neilands, 1987) procedure with some modifications in the absence of nutrients. The CAS medium (1L) contained Chrome azurol S (CAS) 60.5 mg, hexadecyltrimetyl ammonium bromide (HDTMA) 72.9 mg, Piperazine-1,4-bis (2ethanesulfonic acid) (PIPES) 30.24 g, and 1 mM FeCl3·6H2O in 10 mM HCl 10 mL. Agarose (0.9%, w/v) was used as gelling agent. Siderophores detection was achieved after 10 ml (standard, 80 mm diameter Petri dishes) overlays of this medium were applied over those agar plates containing cultivated microorganisms to be tested for siderophores production. After a maximum period of 80 minutes, a change in color was observed in the overlaid medium exclusively surrounding producer microorganisms, from

blue to purple or from blue to orange. All these experiments were performed at least three times each in three replicates.

Enzymatic characterization: Different enzymatic activities of the isolated bacterial strains from citrus leaf samples were conducted and are described below.

Protease activity: In order to determine the protease activity of the selected bacterial isolates from citrus. The protease activities of selected bacterial strains were determined by using skim milk agar medium. All the strains were processed in triplicates and strict measures were taken to avoid any kind of contamination. About 500 ml of modified TSB medium was prepared, while in another flask 1.5% (W/V) of skimmed milk was dissolved in distilled water (100 ml). Both of these flasks were properly plugged, labeled and autoclaved at 121 ºC for 20 minutes. From each of the strains to be tested for protease activity, a colony was picked with a sterile inoculation loop and was spotted on the media plate containing skim milk. On each plate five morphologically different strains were spotted. All the plates were then placed in an incubator at 30ºC and were regularly checked after 24, 48 and 72 hours to find out if there were any protease activity.

Cellulase activity: In order to determine the cellulase activity of the selected bacterial strains, all the strains were processed in triplicates. Cellulase activities of the bacterial strains were analyzed by (Cattelan *et al*., 1999) method. Carboxy Methyl Cellulose medium (0.2%) was prepared (1g CMC and 3 g of TSB dissolved in 500 ml of distilled water). The CMC medium was properly plugged, labeled and autoclaved at 121 ºC for 20 minutes. After autoclaving the media was cooled and poured into plates, five different strains were inoculated onto a single plate. All the plates were then placed in an incubator at 37ºC for 48 hours. After 48 hours incubation all the plates were flooded with 0.1% of Congo red dye solution (0.1 g CRD in 100 ml of distilled water), the plates were shaken carefully in shaker for about 20-30 minutes. After shaking the plates were washed with 1 M NaCl solution. Data was recorded by examining yellow halos against red background.

Lipase activity: In order to determine the Lipase activity of the selected bacterial strains, all the strains were processed in triplicates. For lipase activity 1% of tween 20 was added to TSB medium and was properly plugged, labeled and autoclaved at 121 ºC for 20 minutes. The media was cooled and poured into plates; five different strains were spotted onto a single plate with sterile inoculation loop. All the plates were then placed in an incubator at 25ºC and were regularly checked after 24 and 48 hours to find out if there was any protease activity. Data was recorded by examined white type precipitation surrounding their colony.

Chitinase activity: Chitinase activity was determined by using (Renwick *et al*., 1991) method, in which carbon was the sole source in a defined medium having colloidal chitin. All the strains were processed in triplicates and strict measures were taken to avoid any kind of contamination. TSA medium (0.5g of MgSO4-7H2O, 0.7g of K2HPO4, 0.3 g of KH2PO4, 0.01 g of FeSO4 · H2O, 0.001 g of ZnSO4, 0.001 g of MnCl2) with 0.6% (w/v) colloidal chitin was used. For colloidal chitin two grams of chitin from crab shell (UniChem) was dissolved in concentrated HCl (200 ml), by shaking the mixture overnight at 4ºC in shaker. To decrease the viscosity of mixture it was incubated in water bath at 37ºC. These isolates were screened to determine chitinase production. Each isolate was inoculated on colloidal chitin agar (CCA) and incubated at 28ºC in the dark until (after 7 days incubation) zones of chitin clearing were seen around colonies. Clear zone diameters are measured (mm) and used to indicate the chitinase activity of each isolate.

Pectinase activity: The pectinase activity was determined by (Raju and Divakar, 2013) method. After 48 hours incubation at 28°C, the plates were flooded with iodine solution (50 mM) and incubated for 15 minutes at 37ºC. Strains surrounded by clear halos around colonies were considered positive for pectinase activity. Composition of media used for pectinolytic activity was pectin (0.2%), KH2PO4 (0.3%), MgSO4.7H2O (0.01%), NaCl (0.5%), NH4Cl (0.2%), Na2HPO4 (0.6%). Bacterial isolates were spot inoculated on plates and incubated at 28± 2°C for three days. The medium was properly plugged, labeled and autoclaved at 121ºC for 20 minutes. From each of the strains to be tested for pectinase activity, single colony was picked with a sterile inoculation loop and was spotted on the media plates. After incubation, plates were observed for pectinase activity by flooding plates with iodine solution. Isolates possessing pectinolytic activity formed clear zones around colonies

Antibacterial activity: In order to determine the antibacterial activities by agar well diffusion method (Azoro *et al*., 2002) of the selected bacterial isolates was conducted. All the strains were processed in triplicates. Antibacterial activity of test strains were tested against *Xanthomonas oryzae, Pseudomonas syringae, Bacillus compestris, Acidovorax faecalis, Kluyvera sp., Pseudomonas aeruginosa, Burkholderia pseudomallei, Xanthobacter autotrophicus, Bacillus fortis*. The bacterial strains that were used as test and target strains were grown in LB broth overnight in a shaking incubator at 25ºC. About 50 ul ($1.5x10^5$ CFU) of these selected target strains were spread on solidified LB agar plates with a sterile glass spreader. Then 5 sterile filter disks were placed on top of solidified media plates, the control disk was placed in the center while the other four disks were

placed at equal distance from the center. From each test strains 10 µl was poured onto separate filter disks, while onto the center filter disk 10 µl antibiotic solution (kanamycin) was applied at 20 µg/ml and was considered as positive control. These plates were then incubated at 37°C and were observed clear zones after 24-72 hours. After the incubation period, the diameter of inhibition zones of each well was measured and the values were noted. Replications were maintained and the average values were calculated for the eventual antimicrobial activity.

Statistical analyses: Statistical analysis was performed using one way analysis of variance (ANOVA) followed by least significant difference (LSD) using the Statistics software version 8.1.

RESULTS

Phylogenetic analysis of isolated bacterial strains: In this study molecular characterization of the isolated strains of endophytic bacteria were performed through 16S rDNA universal primer and sequenced. Sequences were assembled and blast in NCBI to identify bacteria. Neighbor joining tree was constructed by using Mega 6.0 with bootstrap value 1000 (figure 1). All the strains have shown maximum similarity of 97-100 percent. According to phylogenetic analysis all strains were laid in different clades along with their type strain and showed geographic relatedness with each other.

Plant growth promotion traits of selected isolates from citrus: Bacterial strains were isolated from leaves of different varieties of citrus. Morphologically different genera of bacterial strains isolated through culturing method were selected for the study of plant growth promoting (PGP) traits. All the tested strains were identified through16S rRNA gene sequencing. Total sixteen bacterial strains were selected for in vitro screening for PGP traits *e.g*., siderophores, phosphate solubilization. The data regarding each activity was given in (Table 1).

Phosphate solubilization: Phosphate solubilizing activity of the selected bacterial strains isolated from leaves of different varieties of citrus were screened on media containing Tricalcium phosphate (TCP) as substrate. Out of sixteen bacterial strains SM-68, SM-57, SM-42, SM-27, SM-82, SM-1, SM-30, SM-58, SM-56, SM-20 and Sm-36 displayed the development of a clear zone around colonies as an indication of phosphate solubilization activity. Five bacterial strains Sm-34, SM-90, SM-15, SM-37, and SM-76 showed phosphate solubilization activity with zone diameter (2-3 mm). Three isolates of GM maize rhizosphere (n=3) were positive for phosphate solubilization, whereas 11 isolates were positive for phosphate solubilization. While five isolate did not show phosphate solubilizing activity.

Siderophores production: Siderophores activities of the selected bacterial strains isolated from different varieties of citrus leaves were assessed on Chrome azurol S agar (CAS) medium. Total sixteen isolates were used to check the siderophores production. SM-34, SM-68, SM-57, SM-90, SM-15, SM-37, SM-1, SM-30, SM-58, SM-56, SM-20 and SM-36 changed the color of CAS medium from blue to orange and were positive for siderophores production. While three isolates showed growth up to some extent but not changed the color of CAS medium, hence these isolates were negative for siderophores production.

Indole acetic acid (IAA): Indole acetic productions of the selected bacterial strains isolated from leaves of different varieties of citrus were screened. Overnight grown bacterial culture was used to inoculate 5 ml TSB without and with tryptophan (500 µg mL−1) and incubated at 30◦C in rotary shaker for 24 hour. The indole acetic acid concentration was detected by spectrophotometer. Majority of the bacterial strains showed IAA production. The culture with no tryptophan, isolate SM-34 (*Bacillus safensis*) produced maximum IAA (0.353 µg mL−1) and isolate SM-58 (*Psychrobacterium pulmonis)* produced maximum IAA (0.226 µg mL−1) as compared to other isolates. Whereas the culture with tryptophan, isolate SM-34 (*Bacillus safensis*) produced maximum IAA (0.355 µg mL−1) and minimum IAA (0.215 µg mL−1) in isolate SM-27 (*Enterobacter hermachei*) as compared to other isolates as shown in (figure 2).

Production of cell wall hydrolyzing enzymes by selected isolates of citrus: All the 16 different bacterial isolates were in vitro characterized for production of fungal cell wall hydrolyzing enzymes such as cellulase, chitinase, protease, pectinase and lipase (Table 1).

Cellulase activities of the isolated bacterial strains: All the 16 bacterial strains were screened for cellulase enzyme production. After two days of incubation and treatments of the individual plates with Congo red dye and NaCl solution, cellulase activity of the strains were estimated by measuring the yellowish brown halo around individual colonies. The bacterial strains isolated from different varieties of citrus were screened, 75% showed cellulase positive activity while 25% did not show any activity. From the diameter of the zone it was concluded that 12 strains SM-34, SM-68, SM-90, SM-42, SM-27, SM-37, SM-82, SM-1, SM-30, SM-58, SM-56 and SM-20 showed positive cellulase activities. Four bacterial strains (25%) not showed cellulase activity.

Chitinase activities of isolated bacterial strains: The selected bacterial isolates were in vitro screen for

chitinase activities. The chitinase activity was screened for all the 16 bacterial strains on TSA medium, in which carbon was the sole source in a defined medium having colloidal chitin. These isolates were screened to determine chitinase production. Each isolate was inoculated on colloidal chitin agar (CCA) and incubated at 28ºC in the dark up to 7 days, zones of chitin clearing were observed around colonies. Results showed that 12 out of 16 selected strains namely; SM-68, SM-57, SM-42, SM-15, SM-37, SM-76, SM-82, SM-1, SM-58, SM-56, SM-20, SM-36 were positive for chitinase production which was 75% of the total isolates. While the remaining 4 (25%) isolates did not show any chitinase activities.

Protease activities of isolated bacterial strains: Bacterial isolates were in vitro screen for protease activity. The protease activities were screened for all the 13 bacterial strains of harvesting stage on their respective medium with added skimmed milk. All the bacterial strains after three days inoculation on medium were screened for protease activities. Strains producing a clear zone around colony were assumed to have positive for protease production. Results showed that 14 out of 16 selected strains SM- 36, SM-20, SM-56, SM-58, SM-1, SM-82, SM-76, SM-37, SM-15, SM-27, SM-42, SM-57, SM-68, SM-34 (87.5%) were proved to possess the protease activity. While 2 (12.5%) isolates SM-30 and SM-90 did not show any protease activity.

Pectinase activities of isolated bacterial strains: Selected isolates were in vitro screen for pectinase activity results showed that 13 out of 16 bacterial strains SM-34, SM-68, SM-57, SM-27, SM-37, SM-76, SM-2, SM-1, SM-30, SM-58, SM-56, SM-20, SM-36 showed pectinase activity. While the remaining 3 (18.25%) SM-90, SM-40, SM-15 isolate did not show any pectinase activities.

Lipase activities isolated bacterial strains: Bacterial isolates of citrus were in vitro screened for lipase activity. Total 16 strains were evaluated for enzymes production. Lipase activity was determined on TSB medium with 1% tween 20. Strains surrounded by white precipitation were considered positive for lipase production. Results showed that maximum isolates showed lipase activity. All the 13(81.25%) isolate SM-34,SM-68,SM-42,SM-27,SM-15,SM-37,SM-76,SM-82,SM-1,SM-30,SM-56,SM-20,SM-36 were able to produced lipase enzyme. Whereas 3(18.75%) SM-57, SM-90, SM-58 isolates do not produced lipase enzyme (Table 1).

Antibacterial activities of isolated bacterial strains from different varieties of citrus: All the selected bacterial strains were assessed for antibacterial activity using disc diffusion method against various pathogenic bacteria such as *Pseudomonas syringae*(FCBP-009) , *Xanthomonas oryzae* (FCBP-133), *Bacillus compestris* (FCBP-324), *Acidovorax faecalis* (FCBP-464), *Kluyvera sp*.(FCBP-642), *Xanthomonas compestris* (FCBP-003), *Burkholderia pseudomallei*(FCBP-460), *Xanthobacter autotrophicus*(FCBP-432), *Bacillus fortis* (FCBP-162).The results were checked for any antibacterial activity at intervals of 24 hours, 48 hours and 72 hours respectively. According to results out of 16 selected strains maximum isolates showed antibacterial potential against selected targeted pathogenic strains. Isolate SM-34 (*Bacillus safensis*) showed positive results for *Xanthomonas oryzae*, *Burkholderia pseudomallei* only, while isolate SM-68(*Pseudomonas aeruginosa*) has potential to control *Xanthomonas compestris*. However isolate SM-57 (*Pseudomonas sp.*) showed good results for *Pseudomonas syringae, Acidovorax faecalis, Kluyvera sp., Xanthomonas compestris* (Table 2).whereas isolate SM-90 (*Staphylococcus scuiri*) has maximum potential to control *Pseudomonas syringae* and *Xanthomonas compestris*. Isolate SM-42(Brevibacterium *borstelensis*) showed antibacterial potential against *Xanthomonas oryzae, Bacillus compestris, Acidovorax faecalis, Xanthomonas compestris, and Burkholderia pseudomallei.* On the other hand Isolate SM-30 (*Bacillus subtilis*) has potential for *Xanthomonas oryzae, pseudomonas syringae, Bacillus fortis,* while SM-20(*Proteus mirabilis*) showed good results for *Pseudomonas syringae, Xanthomonas compestris, Burkholderia pseudomallei*. Isolate SM-58 (*Psychrobacterium pulmonis*) has good potential to control *Bacillus compestris* and *Acidovorax faecalis* respectively. Five isolates SM-76(*Enterococcus faecalis*), SM-82 (*Klebsiella pneumonia*), SM-1 (*Staphylococcus haemolyticus*), SM-56(*Bacillus megaterium*) and SM-36 (*Bacillus cereus*) did not show any antibacterial activity against any of the targeted bacterial strains (figure 3).

Table 1. Screening of entophytic bacterial isolates of citrus for Plant growth promotion traits and cell wall degrading enzymes production.

Strains Code	Bacterial Isolates	Citrus Varieties	Phosphate solubilization	Siderophores detection	Cell wall degrading enzymes				
					Protease	Cellulase	Lipase	Chitinase	Pectinase
SM-34	*Bacillus safensis*	Lemon	-ve	+ve	+ve	+ve	+ve	-ve	+ve
SM-68	*Pseudomonas aeruginosa*	Olinda Valencia	+ve	+ve	+ve	+ve	+ve	+ve	+ve
SM-57	*Pseudomonas sp.*	Dancy	+ve	+ve	+ve	-ve	-ve	+ve	+ve
SM-90	*Staphylococcus scuiri*	Parson brown	-ve	+ve	-ve	+ve	-ve	-ve	-ve
SM-42	*Brevibacterium borstelensis*	Sweet orange	+ve	-ve	+ve	+ve	+ve	+ve	-ve
SM-27	*Enterobacter hermachei*	Grape fruit	+ve	-ve	+ve	+ve	+ve	-ve	+ve
SM-15	*Comamonas terrigena*	Casa grand	-ve	+ve	+ve	-ve	+ve	+ve	-ve
SM-37	*Yersinia mollaretti*	Lemon	-ve	+ve	+ve	+ve	+ve	+ve	+ve
SM-76	*Enterococcus faecalis*	Sour orange	-ve	-ve	+ve	-ve	+ve	+ve	+ve
SM-82	*Klebsiella pneumoniae*	Gada dahi	+ve	+ve	+ve	+ve	+ve	+ve	+ve
SM-1	*Staphylococcus haemolyticus*	Musambi	+ve	+ve	+ve	+ve	+ve	+ve	+ve
SM-30	*Bacillus subtilis*	Grape fruit	+ve	+ve	-ve	+ve	+ve	-ve	+ve
SM-58	*Psychrobacterium pulmonis*	Natal	+ve	+ve	+ve	+ve	-ve	+ve	+ve
SM-56	*Bacillus megaterium*	Dancy 4	+ve	+ve	+ve	+ve	+ve	+ve	+ve
SM-20	*Proteus mirabilis*	Kinnow	+ve	+ve	+ve	+ve	+ve	+ve	+ve
SM-36	*Bacillus cereus*	Lemon	+ve	+ve	+ve	-ve	+ve	+ve	+ve

Table 2. Antibacterial activity of isolated strain of citrus against pathogenic strains of bacteria collected from first fungal culture bank of Pakistan (FCBP).

Isolates	Target Micro organisms	Accession numbers	16srRNA based % identity	Test micro organisms								
				Xanthomonas oryzae	***Pseudomonas syringae***	***Bacillus compestris***	***Acidovorax faecalis***	***Kluyvera sp.***	***xanthomonas compestris***	***Burkholderia pseudomallei***	***Xanthobacter autotrophicus***	***Bacillus fortis***
SM-34	*Bacillus safensis*	MF801628	99%	+ve	- ve	- ve	- ve	- ve	- ve	+ ve	- ve	- ve
SM-68	*Pseudomonas aeruginosa*	MF802727	95%	- ve	- ve	- ve	- ve	- ve	+ ve	- ve	- ve	- ve
SM-57	*Pseudomonas sp.*	MF973203	89%	- ve	+ ve	- ve	+ ve	+ ve	+ ve	- ve	- ve	- ve
SM-90	*Staphylococcus scuiri*	LT745975	99%	- ve	+ ve	- ve	- ve	- ve	+ ve	- ve	- ve	- ve
SM-42	*Brevibacterium borstelensis*	LT745989	93%	+ ve	- ve	+ ve	+ ve	- ve	+ ve	+ ve	- ve	- ve
SM-27	*Enterobacter hermachei*	LT745966	97%	- ve	- ve	- ve	- ve	- ve	+ ve	- ve	- ve	+ ve
SM-15	*Comamonas terrigena*	LT844635	95%	+ ve	+ ve	+ ve	+ ve	- ve	+ ve	+ ve	- ve	+ ve
SM-37	*Yersinia mollaretti*	LT745988	87%	- ve	- ve	+ ve	- ve	- ve	+ ve	- ve	- ve	- ve
SM-76	*Enterococcus faecalis*	LT844634	100%	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve
SM-82	*Klebsiella pneumoniae*	MF966247	98%	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve
SM-1	*Staphylococcus haemolyticus*	MF957708	89%	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve
SM-30	*Bacillus subtilis*	MF977360	99%	+ ve	+ ve	- ve	- ve	- ve	- ve	- ve	- ve	+ ve
SM-58	*Psychrobacterium pulmonis*	LT745968	97%	- ve	- ve	+ ve	+ ve	- ve	- ve	- ve	- ve	- ve
SM-56	*Bacillus megaterium*	MF802485	94%	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve
SM-20	*Proteus mirabilis*	MF958504	99%	- ve	+ ve	- ve	- ve	- ve	+ ve	+ ve	- ve	- ve
SM-36	*Bacillus cereus*	MF801630	97%	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve

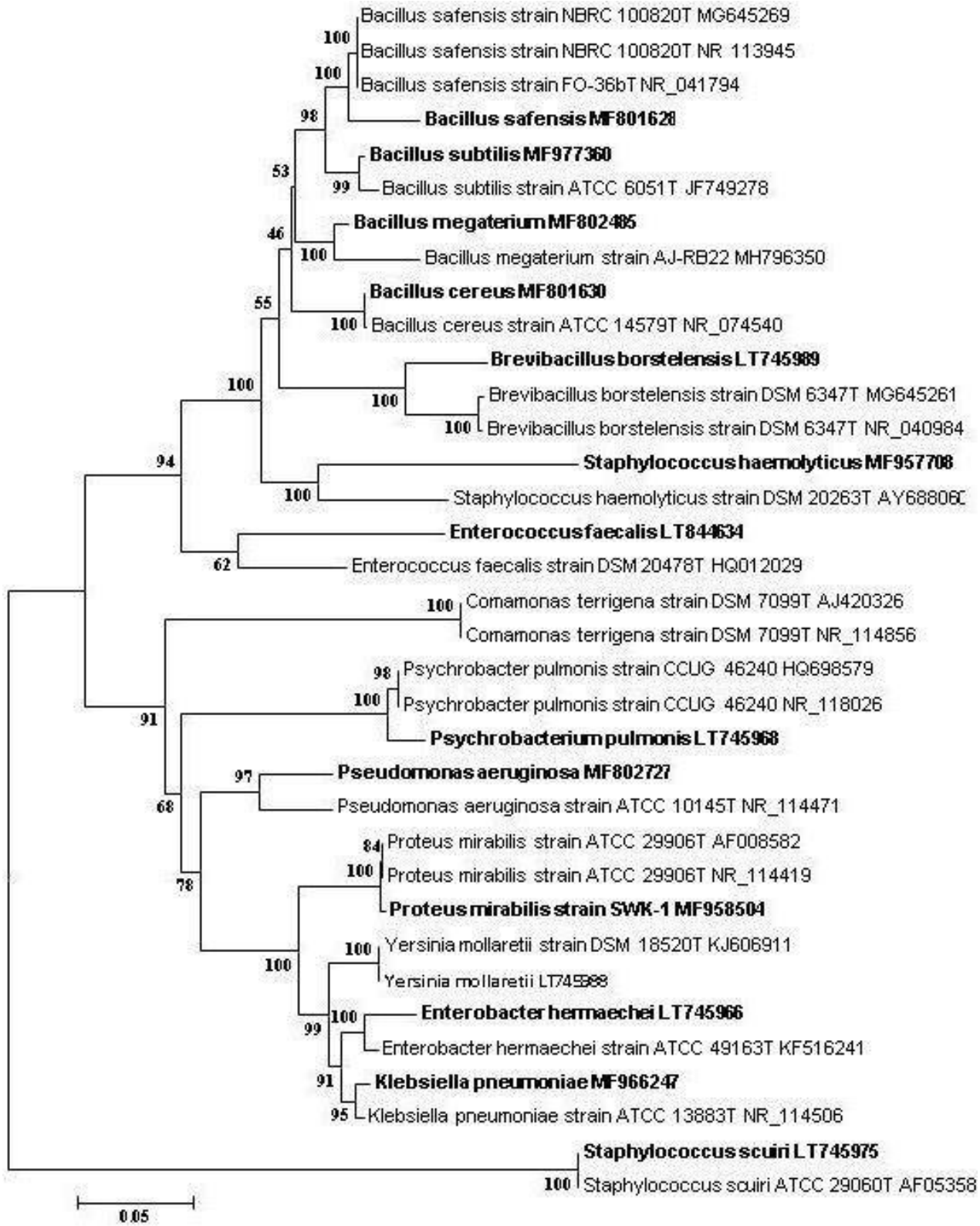

Figure 1. Neighbor joining Phylogenetic tree from analysis of partial 16S rDNA of citrus leaf bacterial endophytes. Level of bootstrap support based on 1000 replication data set are shown greater than 90% the scale bar represents 0.05 substitutions per nucleotide position.

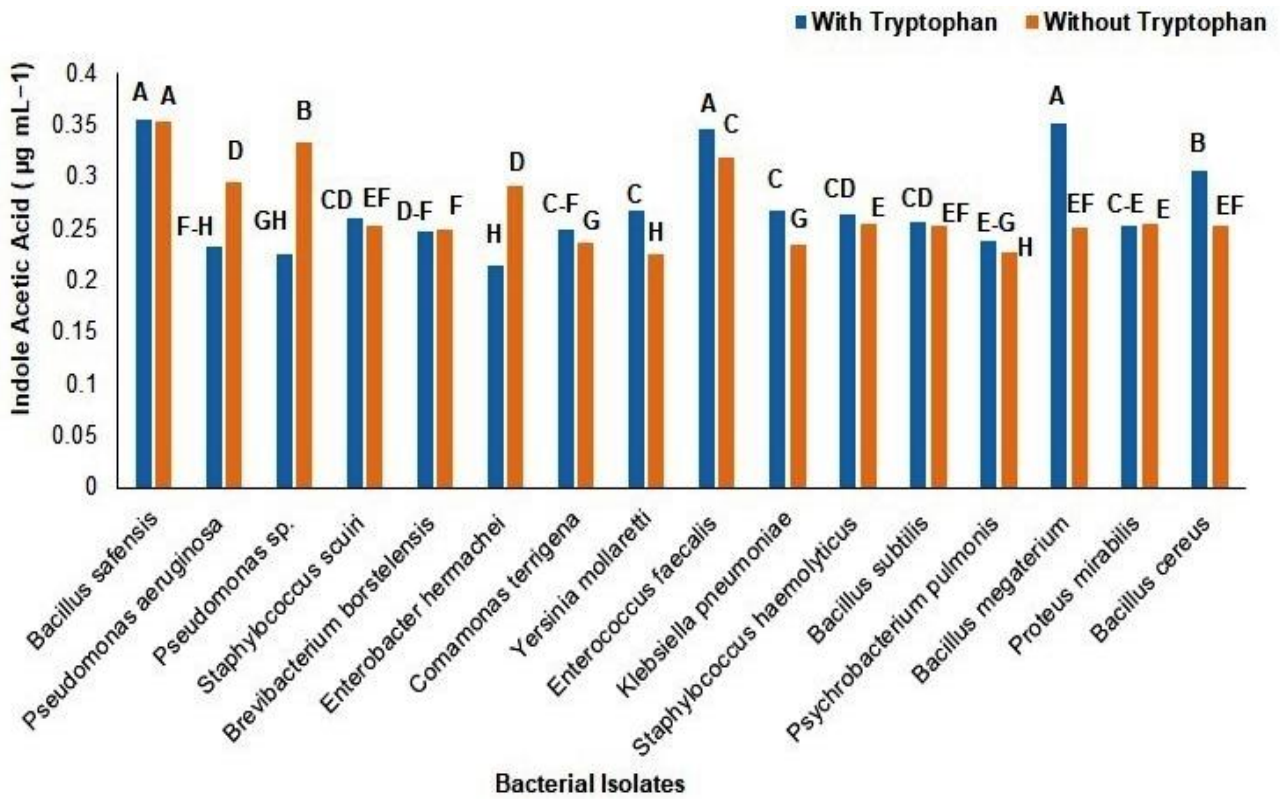

Figure 2. Screening of entophytic bacterial isolates of citrus for Indole acetic acid (µg mL−1) Production

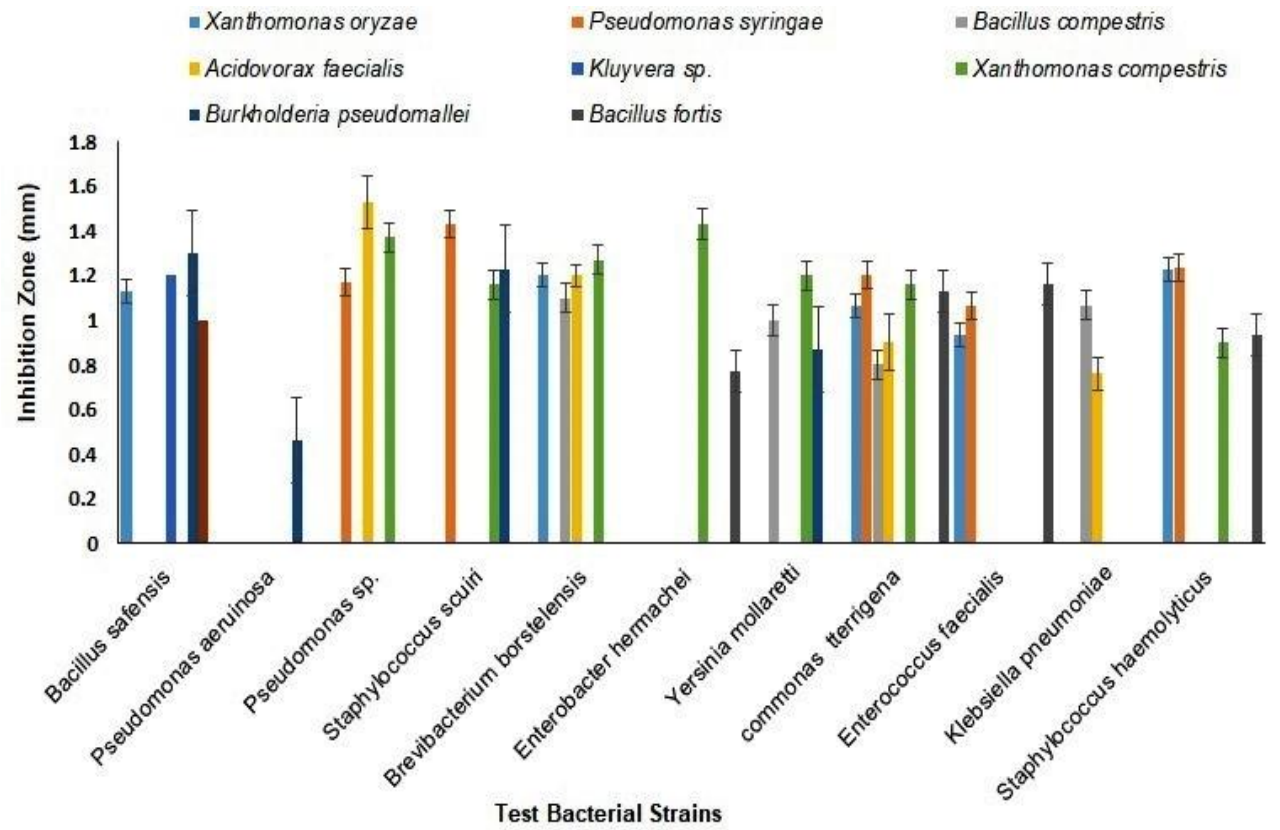

Figure 3. Antibacterial potential of test bacterial strains against isolated strains from this study.

DISCUSSION

The ability of a PGPR to solubilize and make available insoluble forms phosphorus to plants make their application more beneficent and interesting in agriculture systems. The use of phosphate solubilizing bacteria as inoculants increases the P uptake by plants (Chen *et al*., 2006; Alori *et al*., 2017; Nassal *et al*., 2018). Similarly, the plant beneficial effects of PGPR have mainly been attributed to the production of phytohormones like IAA and nitrate reduction (Somers *et al*., 2004; Khan *et al*., 2017). During the current studies, the improvement in plant growth due to PGPR inoculation may be attributed to their secretion of IAA, capacity of phosphate solubilization. Among other isolates, bacillus safensis isolated from lemon exhibited higher IAA production while minimum in *Enterobacter hermachei* while SM-68, SM-57, SM-42, SM-27, SM-82, SM-1, SM-30, SM-58, SM-56, SM-20 and SM-36 were capable of phosphate solubilization activity.

Siderophores are low-molecular-weight molecules secreted by microorganisms under iron limiting conditions (Shah *et al.*, 2018). Previous studies have shown that PGPR capable of phosphate solubilization and IAA production also produced siderophores (Shameer *et al.*, 2018). During present studies, the isolates SM-34, SM-68, SM-57, SM-90, SM-15, SM-37,SM-1,SM-30,SM-58, SM-56,SM-20 and SM-36 were capable of producing siderophores. Previous studies have shown that most of the siderophores producing bacteria are Gram-negative among which *Pseudomonas* and *Enterobacter* genera are common. Whereas, *Bacillus* and *Rhodococcus* genera are the Gram-positive bacteria having potential of siderophores production (Tian *et al*., 2009; Rosales *et al.*, 2017). Siderophores produced by Rhizobacteria scavenge iron in the rhizosphere starving pathogenic organisms of proper nutrition to mount an attack of the crop (Saharan and Nehra, 2011). The siderophores produced by Rhizobacteria not only act as biocontrol but also help in mitigating abiotic stresses in various crop plants.

The beneficial effects of rhizobacteria on plants are also due to their inhibitory effects on soil borne pathogens (Van Loon and Glick, 2004; Parewa *et al.*, 2018). Cellulase production and utilization of substrates available in the rhizosphere are important for controlling the rhizosphere competence (Yadav *et al.*, 2017). During current studies, the cellulase activity was recorded for rhizobacteria strains SM-34, SM-68, SM-90, SM-42, SM-27, SM-37, SM-82, SM-1, SM-30, SM-58, SM-56 and SM-20 isolated from different varieties of citrus were showed positive results for cellulose, lipase, protease, pectinase activities etc. The beneficial rhizobacteria inhibit the growth of phytopathogens by the production of cell wall lytic enzymes like cellulose, chitinase etc. (Kumar *et al*., 2012). The extracellular cell wall degrading enzymes are positively correlated with biocontrol abilities of the producing rhizobacteria (El-Tarabily, 2006; Rizvi *et al.*, 2017).

Identification of chitinase producing bacteria from rhizosphere soil is important for the isolation of bacteria that have antifungal activities. A vast majority of bacteria and fungi produce chitinase enzymes (Bai *et al.*, 2016) which plays important role in the control of fungal diseases (Kamil *et al*., 2007). According to current study, maximum chitinase activity was shown by rhizobacteria belonging to genera *Pseudomonas, Brevibacterium, Comamonas, Yersinia, Enterococcus, Klebsiella, Staphylococcus, Psychrobacterium, Bacillus* , *Proteus* were positive for chitinase inhibit fungal growth by hydrolyzing chitin which is a major component of the fungal cell wall. Moreover, chitinases are of great biotechnological importance for engineering of phyto-pathogenic resistant plants, their use as food and preservative agents for seeds (Kamil *et al*., 2007; Islam and Datta, 2015) isolated 400 isolates from rhizospheric soil in Egypt and tested them for chitinase production. They found that majority of the chitinase producing rhizobacteria belonged to genus Bacillus. Moreover, the members of the genus Bacillus have been previously reported for the production of chitinases (Schallmey *et al*., 2004; Wahyuni *et al.*, 2016).

Lipases are widely distributed among microorganisms and are of great industrial importance. They not only hydrolyse triglycerides to free fatty acids and glycerol but are also used in the production of foods, biodiesel, pharmaceuticals, textiles and detergents etc. (Pascoal *et al*., 2018). Majority of the lipase producing bacteria isolated from citrus strains i.e. SM-34,SM-68,SM-42,SM-27,SM-15,SM-37,SM-76,SM-82,SM-1,SM-30,SM-56,SM-20,SM-36 were positive for lipase production. A large number of bacteria produce lipase which has capacity to hydrolyze triglycerides (Javed *et al.*, 2018). The bacteria belonging to genera *Achromobacter, Alcaligenes, Arthobacter, Bacillus, Burkholderia, Chromo bacterium* and *Pseudomonas* are reported extensively for the production of extracellular lipases (Gupta *et al*., 2004; Ismail *et al*., 2018). Among the different genera, the extracellular lipases produced by *Pseudomonas* and Bacillus are widely used in biotechnological applications (Hasan *et al.*, 2018). It is inferred that bacteria associated with rhizosphere of GM and NON-GM maize can be exploited for commercial scale production of lipases.

On the other hand, microbial proteases may be useful and play an important role in infection of hosts by degrading the host's protective barriers. Microbial proteases have been proposed as virulence factors in their pathogenesis against nematodes (Huang *et al*., 2004; Stach *et al.*, 2018). Majority of the protease producing bacteria isolated from citrus belonged to genera *Klebsiella, Psychrobacter, Proteus, Enterobacter,*

Pseudomonas, Brevibacterium, Enterococcus, Yersinia, Comamonas, and *Enterobacter*. These results are in agreement with previous findings of (Lian *et al*., 2007; Rahman *et al*., 2017) who have reported that bacteria belonging to genus Bacillus are more efficient producer of proteases. Multiple Bacillus sp. can promote crop health by suppressing plant pathogens and pests through the production of antibiotic metabolites or directly through the stimulation of plant host defense before the occurrence of infection (Lian *et al*., 2007; Rahman *et al*., 2017). It could be inferred that various Bacillus species which have capacity of protease production might contribute to their activity as biocontrol agents (Siddiqui *et al*., 2005; Rocha *et al.*, 2017; Santos *et al*., 2018).

Pectinolytic enzymes produced by non-pathogenic bacteria are essential in the decay of dead plant material and thus contribute in the recycling of carbon compounds in biosphere. Among the various bacteria isolated from citrus varieties isolates SM-34, SM-68, SM-57, SM-27, SM-37, SM-76, SM-2, SM-1, SM-30, SM-58, SM-56, SM-20, SM-36 showed highest pectinase activity. The pectinolytic enzymes play crucial role in root invasion by bacteria and thus play important role in plant microbe interaction (Hayat *et al*., 2010; Vardharajula *et al.*, 2017). Moreover, these pectinase producing bacteria isolated from citrus contribute to the nutrient cycling by degrading the pectic compounds present in the cell wall of plants and thus contribute to the fertility of soil.

Antimicrobial activity of isolated endophytic bacteria was studied against Gram positive and Gram negative bacteria. Endophytic bacteria with potent antibacterial activity isolated from roots of *Solanum sp*. and medicinal plants were reported by (Long *et al*., 2003; Khunjamayum *et al.*, 2017). Furthermore, *B. megaterium* and *B. licheniformis* isolated from *P. tenuiflorus* leaves exhibited antibacterial activity against *E. coli, S. aureus* and *S. typhi*. It has been also reported that bacterial endophytes *Bacillus sp*., *B. licheniformis*, *Paenibacillus sp., B. pumilus*, and *B. subtilis* isolated from medicinal plants produce antibiotics (Madigan *et al*., 2005; Egamberdieva *et al.*, 2017). Generally, the extract of endophytic bacteria was significantly effective against both Gram-positive and Gram-negative bacteria. Endophytic bacteria produce antibiotics, which can act against human pathogenic bacteria, have previously been reported (Seo *et al*., 2010; Pina *et al.*, 2018). Thus, endophytes can be a good source for the industrial production of antibiotics.

Conclusion: The present findings conclusively demonstrate that maximum endophytes have good potential for plant growth promoting traits and enzymatic activities except few of them. Altogether the test bacterial endophytes showed a broad spectrum activity against most of the targeted bacterial pathogens. It is concluded that the endophytic bacteria from citrus leaves have potential to reduce the bacterial plant pathogens except five isolates *Enterococcus faecalis*, *Klebsiella pneumonia*, *Staphylococcus haemolyticus*, *Bacillus megaterium* and *Bacillus cereus* did not show any antibacterial activity against any of the targeted bacterial strains. Detailed investigations on citrus endophytic bacteria are required to attest its antimicrobial potential and it will leads to the discovery of numerous valuable antimicrobial compounds that can be helpful in disease management. This study provides baseline for the use of endophytes against the plant pathogens which caused different bacterial diseases and yield loses in crops. Moreover research on other aspects of antibacterial compounds produced by test bacteria and their characterization suggests a better understanding about these biological agents. The bacterial endophytes could be more useful for controlling the different diseases of field crops, less costly, time saving, not harmful for human health and equally beneficial to the scientific and farmer's community for increasing the economy of the country.

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