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Isolation, Identification and Screening of Endophytic Bacteria Antagonistic to Biofilm Formers

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Abstract.- An array of 76 endophytic bacterial isolates obtained from different plant tissues including root, stem and fresh and wilted leaves of various plants in Punjab, Pakistan were investigated for their antagonistic ability to biofilm formers. The morphological, biochemical and physiological characterization and 16S rRNA gene sequence analysis of the selected endophytic isolates leads to the identification of different bacterial species belonging to the genera *Bacillus, Pseudomonas, Serratia, Stenotrophomonas* and *Micromonospora*. The isolates exhibited potent antimicrobial activity against biofilm forming bacterial strains in biological screening by agar diffusion assay. In chemical screening, the crude extracts obtained from each of the isolate and analyzed by thin layer chromatography (TLC) using various spraying reagents and by HPLC-UV, exhibited an impressive chemical diversity of the bioactive secondary metabolites. The antimicrobial activity and the chemical diversity exhibited by these endophytic bacterial strains demonstrate that they are a promising source of useful/novel antimicrobial agents, to control the infections caused by biofilm forming bacterial species.

Key words: Endophytic bacteria, bioactive secondary metabolites, biofilm formers, biological and chemical screening.

INTRODUCTION

The need for new and useful compounds to provide assistance and relief in all aspects of the human health is ever growing with the passage of time. Drug resistance in bacteria, the appearance of life-threatening viruses, the recurring problems with disease in persons with organ transplants, and the tremendous increase in the incidence of fungal infections in the world's population each day only underscore our inadequacy to cope with these medical problems (Berdy, 2005). As a result of these problems, there is a general call for new antibiotics, chemotherapeutic agents, and agrochemicals that are highly effective, possess low toxicity, and have a minor environmental impact. Natural products from endophytic microbes have been observed to inhibit or kill a wide variety of harmful disease causing agents including phytopathogens and other bacteria, fungi, viruses, and protozoa that affect humans and animals (Strobel and Daisy, 2003). Endophytes are 'fungi or bacteria, which for all or part of their life cycle invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues, but cause no symptoms of disease'(Wilson, 1995). It is noteworthy that, of the nearly 300,000 plant species that exist on the earth, each individual plant is host to one or more endophytes. Only a few of these plants have ever been completely studied relative to their endophytic biology. Consequently, the opportunity to find new and interesting endophytic microorganisms among plants in different settings and ecosystems is significant.

Biofilms consist of microcolonies embedded in a polysaccharide matrix produced by the bacteria. The polysaccharide slime protects the bacteria against hostile environmental factors. Chronic bacterial infections are frequently caused by biofilm forming bacteria and the biofilms cannot be eradicated by currently available antibiotics (Hoiby *et al*., 1994). The need for new antibiotics and novel bioactive compounds is also driven by the development of resistance in these biofilm forming pathogenic bacteria and by the presence of naturally resistant organisms. The novel antibiotics or bioactive compounds may be obtained from a

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number of different and unique sources. Such sources usually provide organisms and their biotopes that are subjected to constant metabolic and environmental interactions and finally produce even more diverse biomolecules and secondary metabolites. Endophytes that inhabit such biotopes, namely higher plants, are currently considered as a wellspring of novel secondary metabolites offering the potential for medical, agricultural and/or industrial exploitation.

Screening for bioactive compounds is based on some major factors like selection of a proper microorganism, isolation and culture methods and the detection and identification of their metabolites. Usually the secondary metabolites are considered more important in chemical screening for the formation of the patterns of the metabolites and the detection of the metabolic pathways of the microorganisms. Currently various chemical procedures have been developed for the screening of active metabolites from the microbial extracts (Grabley *et al.,* 1992; Magome *et al.,* 1996). Separation of natural products by TLC and HPLC followed by the detection of secondary metabolites by a detector provides an easy way to screen the bioactive compounds (Kishimoto *et al.,* 1996). The TLC and HPLC-UV are lab scale chromatographic techniques and are frequently used for the detection, isolation and purification of all types of biomolecules and secondary metabolites (Burkhardt *et al.,* 1996; Taddei *et al.,* 2006). In this study, we selected various plants for the isolation of endophytic bacteria and screened the endophytic isolates for the production of bioactive compounds. A total of 76 endophytic bacterial strains were recovered from different plant tissues and the selected isolates were identified by morphological, biochemical and physiological characterization and by 16S rRNA gene sequencing. The selected endophytic isolates were screened for antimicrobial activity by agar diffusion assay against a set of biofilm forming bacterial strains. In chemical screening the crude extracts obtained from the culture broth of these endophytic isolates were analyzed by TLC and HPLC-UV. Based on the results of biological and chemical screening 10 competent strains were detected which showed promising capability to produce useful compounds.

MATERIALS AND METHODS

Sample collection and selective isolation of endophytic bacteria

Plant tissues including roots, stem and leaves of different plants were collected from agricultural farmlands at the university campus. The selected plants include sun flower (*Helianthus annus* L.), banana (*Musa accuminata* Colla), lemon (*Citrus limon* L. Burm. f) and bathu (*Chenopodium album* L.). The samples were taken and shifted to lab in sterile sampling bags and processed within 4 hours after collection. The plant tissues were washed in running water to remove soil particles and were surface sterilized by sequential immersion in 70% ethanol for 5 minutes and a solution of sodium hypochlorite (0.9% available chlorine) for 20 minutes. Then the samples were washed three times in sterile distilled water to remove surface sterilizing agents before being soaked in 10% $NaHCO_3$ solution to disrupt the plant tissues and to inhibit the growth of fungi (Cao *et al*., 2003). Surface sterility test was performed for each of the sample to ensure the elimination of surface microorganisms. Each of the sample was divided into small fragments (1-3 cm) under aseptic conditions and was plated on LB-agar (tryptone 10g/L, yeast extract 5g/L, sodium chloride 5g/L, agar 15 g/L) and rice agar (rice water 18 ml/L, agar 18g/L). The LB-agar plates were incubated at 37°C, while the rice agar plates were incubated at 28°C for 2-3 weeks. Endophytic bacterial strains growing on selective media plates were isolated, purified and were preserved as agar slants for further studies.

Biological screening

Determination of Antimicrobial activity

The test organisms *i.e.* biofilm forming bacterial strains were obtained from the culture collection of the department of Microbiology and Molecular Genetics (MMG), University of the Punjab, Lahore, Pakistan. The endophytic bacterial isolates were cultivated as shaking cultures in LB-broth for 48 hour at 37°C. The culture broth of each of the isolate was centrifuged at 3000 rpm to get cell free supernatants. The test plates (Petridishes**:** 94mm diameter, 16mm height, Fa. Greiner Labortechnik, Nürtingen), of biofilm forming

bacteria were prepared by pouring 14 mL of LB-agar as base layer; after it has solidified, it was overlayed with 4 ml of the inoculated seed layer. The agar wells (5 mm diameter) were made by using sterile cork borer and 60 µl of the supernatant was loaded in each well. The test plates were left at room temperature for 2 hours to diffuse the supernatant and were incubated at 37°C for 18-24 hours. After 18-24 hours the diameter of the zones of inhibition was measured in mm.

Identification of endophytic bacterial strains

The selected endophytic bacterial strains were identified by morphological, biochemical and physiologically characterization and by 16S rRNA gene sequencing. In morphological characterization, macroscopic and microscopic features of the selected isolates were studied, additionally an array of biochemical and physiological tests including catalase test, oxidase test, starch utilization test, nitrate reduction test, motility test, H_2S production test and sugar utilization test were performed following the methods described by Gerhardt *et al*. (1994).

16S rRNA gene sequencing

High-molecular weight DNA of the 10 selected strains was isolated by using a genomic DNA isolation kit (Promega Wizard Genomic DNA Purification Kit, Cat # A1125). The PCR amplification of 16S rRNA gene of the selected strains was done by using the forward and reverse primers including 27f 5′-AGAGTTTGATCCT GGCTCAG-3′ and 1522r 5′-AAGGAGGTGATCC ARCCGCA-3′. Amplification was performed in an automated thermocycler with the amplification profile as: 94°C for 5 min followed by 36 cycles of 94°C for 20 sec, 50°C for 20 sec, 72°C for 2 min each and followed by a final extension step of 5 min at 72°C. The amplified PCR product was extracted by using gel extraction kit (Fermentas Gene JET™ purification column; Cat # K0691). The pure extracted DNA was sequenced, the sequence data obtained was refined and was BLAST analyzed at NCBI website: http://www.ncbi.nlm.nih.gov to determine the similarity of the sequence with already reported gene sequences in the GenBank. The nucleotide sequence data obtained was deposited to the GenBank and the accession numbers were obtained.

Chemical screening

The selected endophytic isolates were grown as subcultures on LB agar plates for 24 hours. The resulting subculture of each of the isolates was used to inoculate 2 x 250 mL LB broth 1-L Erlenmyer flasks (the PH was adjusted to 6.8 before sterilization) and the flasks were incubated as shaking cultures (100 rpm) at 37°C for 48 hours. The culture broth was sonicated on a sonicating water bath (Ultrasons Medi-II, J.P Selecta, s.a) for 30 min to break the cells and was extracted with ethyl acetate in a separating funnel by shaking vigorously for 10 minutes. The mixture was allowed to settle until the appearance of two distinct layers, the upper solvent layer was separated from the lower aqueous layer and the extraction process was repeated three times. The solvent (ethyl acetate) was evaporated on a rotary evaporator (Heidolph Laborata 4000) and the powder crude extract obtained was dissolved in 2-5 mL methanol. The methanolic crude extracts were subjected to a combinatory of thin layer chromatography (TLC) and high performance liquid chromatograph (HPLC) analysis to get the metabolic fingerprints of each of the isolate.

TLC analysis

The thin layer chromatography (TLC) analysis was performed using TLC aluminium sheets (20 x 20cm silica gel F_{254}: Merck KGaA Darmstadt, Germany). A small drop of sample was spotted on the TLC plate with a capillary and air dried, the spotting process was repeated by superimposing more drops on the original spot for obtaining appropriate quantity (2-5µg) of the sample on the plate. The TLC plates were developed with CH_2Cl_2/10%MeOH solvent system and the air dried plates were visualized under UV light (at 254 and 366 nm). The components showing UV absorbance and fluorescence were marked and scanned, later the plates were stained with Anisaldehyde/H_2SO_4 and Ehrlich's reagents separately. After air drying, the plates were dried under hot air and were observed for the appearance of different color bands.

HPLC analysis

The HPLC analysis was performed on clarity chromatography data system, the HPLC system consisted of two pressure pumps (Syknm S1122 delivery system), the injection port with a 20 µl loop (Syknm S 5111 injector valve bracket), a UV detector (Syknm S 3210 UV/Vis detector). For chromatographic separation, C18 column (Thermo Hypersil Keystone, 250 x 4.6 mm 5µm Hypersil) was used. Methanol and water (90:10) was used as mobile phase and flow rate was adjusted to 1mL/min. The crude extract was dissolved in methanol and 20µl of sample was injected with the help of a micro syringe, the run time was adjusted to 15 min and UV absorbance was determined at 254 nm.

RESULTS AND DISCUSSION

Endophytic bacteria residing in different tissues of living plants are relatively unstudied and are being considered as potential source of novel natural products to be used in medicine, agriculture, and industry. Although known since long time, the importance of endophytic bacteria become evident only more recently when it was shown that they play specific role as, protecting the host plants against insects and diseases (Strobel and Long, 1998; Strobel *et al.,* 1996). In another study by Zinniel *et al.* (2002)**,** some endophytic strains with this specific role have been isolated from aerial tissues of four agronomic crops and from prairie plant species. In this study a total of 76 endophytic bacterial strains were isolated from internal tissues of different plants. The surface sterilization of the excised tissues after rinsing with sterilized distilled water and by sequential immersion in 70% ethanol, 0.9% sodium hypochlorite and 10% sodium bicarbonate ensured the removal of surface microbial flora. A variety of chemical disinfectants have been employed for surface sterilization of the excised plant tissues to remove epiphytes however immersion of the tissues in ethanol, sodium hypochlorite and sodium bicarbonate have shown significant success in different studies (Bacon and Hinton, 1996; Coombs and Franco, 2003; Larran *et al.,* 2001). The processed tissues after dividing into small pieces (1-3 cm) with sterile surgical blades under aseptic conditions were shifted to the isolation media. The endophytic bacterial colonies were selected and the cultures were purified by repeated sub culturing on LB agar. Subjection of the selected endophytic bacterial isolates to a combinatory of morphological, biochemical and physiological characterization and 16S rRNA gene sequencing provided the taxonomic status of the isolates.

In morphological characterization the endophytic bacterial isolates exhibited the diverse colony shapes, colors, margins and texture including round to irregular colonies, off white, pink and yellow colonies with regular or wavy margins. The endophytic bacterial isolates recovered from LB agar usually showed soft and sometime mucoid colonies while most of the isolates obtained on rice agar produced rough, dry and embedded colonies which are the characteristics colony pattern of actinobacteria. In biochemical and physiological characterization all the isolates gave positive results for catalase and oxidase tests while negative results for H_2S production test. These results indicated that they can produce catalase and oxidase enzymes but can't produce H_2S by using media components. The isolates UL3, BS2A, SL3G and LS7 were negative for citrate test exhibiting their inability to utilize citrate while the remaining exhibited utilization of citrate as sole carbon source. Similarly the isolates UL3, MR3 and LS7 showed positive results in starch utilization test indicating their ability to utilize starch from the media, while only the isolates BR3 and LML6 showed positive results for nitrate reduction tests exhibiting their ability to reduce nitrate to nitrite or ammonia. Likewise the isolates UL3, BW8, LML6, SL3G, LS7 and SL6 showed positive results for motility test (Table I). In the test for utilization of different sugars as sole carbon source, it was seen that all the isolates exhibited growth on glucose and fructose. However all the strains were negative for lactose, arabinose and rhamnose indicating their inability to utilize these sugars as sole carbon source. A variable behavior was observed for the ability of the isolates to utilize different sugars as carbon sources e.g. the isolate SL3A was negative for sucrose but positive for galactose, while all the remaining isolates were positive for sucrose and negative for galactose (Table II). These wide ranging biochemical and

Table I.- Results of the biochemical and physiological characteristics of the selected endophytic bacteria.

Endophytic bacterial isolate	Catalase test	Oxidase test	Citrate utilization	Starch utilization	Nitrate reduction	Motility test	H_2S production
LML5	+	+	+	-	-	-	-
BR3	+	+	+	-	+	-	-
UL3	+	++	-	+	-	+	-
BW8	+	++	+	-	-	+	-
BS2A	+	++	-	-	-	-	-
LML6	+	++	+	-	+	+	-
MR3	+	+	+	+	-	-	-
SL3G	+	++	-	-	-	+	-
LS7	+	++	-	+	-	+	-
SL6	+	++	+	-	-	+	-

(++), strong positive; (+), positive; (-), negative.

Table II.- Results of sugar utilization test of the selected endophytic actinomycetes.

Isolate	Glucose	Sucrose	Lactose	Galactose	Fructose	Arabinose	Xylose	Mannitol	Rhamnose
SL3A	+	-	-	+	+	-	-	-	-
BW6	+	+	-	-	+	-	-	-	-
SL4	+	+	-	-	+	-	-	-	-
SL1	+	+	-	-	+	-	-	-	-
SL7	+	+	-	-	+	-	-	-	-
SC1	+	+	+	-	+	-	-	-	-
LS6	+	+	-	-	+	-	-	+	-
SC2	+	+	-	-	+	-	-	-	-
BB1	+	+	-	-	+	-	-	-	-

(+), exhibited growth; (-), no growth.

physiological characteristics of the endophytic bacterial isolates indicate that they are different bacterial species. The final taxonomic status of ten selected endophytic bacterial isolates was determined by 16S rRNA gene sequencing. The BLAST analysis of 16S rRNA gene sequence data of the selected endophytic bacterial strains showed alignments of these sequences with reported 16S rRNA gene sequences in GenBank. The highest similarities found with different bacterial genera and GenBank accession numbers for 10 selected strains are summarized in Table III.

In biological screening for antimicrobial activity against biofilm formers, the promising antimicrobial activity against the test organism E4 (*Enterobacter*) was exhibited by the endophytic isolates MR3, SL3G, SL3A, SL1 and BW5, the zone of inhibition of 19, 15, 18, 14 and 16mm respectively were recorded. Similarly the isolates

Table III.- Gene Bank accession numbers along with the alignments of sequences obtained with reported 16S rRNA gene sequences in Gene Bank and highest similarity with different bacterial genera.

Isolate	%age similarity with		Gene bank accession number
SL3G	*Bacillus cereus*	97%	HQ283476
UL3	*Bacillus megatarium*	95%	HQ283477
LS7	*Bacillus subtilus*	98%	HQ283478
BW8	*Serratia marcescens*	97%	HQ283479
SL6	*Pseudomonas* spp.	98%	HQ283480
MR3	*Bacillus* spp.	91%	HQ283481
BS2A	*Pseudomonas* spp.	97%	HQ283482
SL3A	*Stenotrophomonas* spp.	92%	HQ283483
BW5	*Stenotrophomonas* spp.	98%	HQ283484
BW6	*Micromonospora* spp.	98%	HQ283485

BR3, LML6, SL3G and BW5 showed zones of inhibition of 35, 17, 12 and 18 mm respectively against the test organism X4 (*Pseudomonas*). The isolates LML5 showed 20 mm, LML6 showed 18 mm, MR3 showed 15 mm, SL3G showed 19 mm, SL1 showed 15 mm and BW5 showed 18 mm zone of inhibition against the test organism M9 (*Enterobacter*). The most promising antimicrobial activity against the test organism S2 (*Bacillus*) was exhibited by the endophytic isolates LML5, BR3, LS7, SL3A, SL1 and BW5 which showed 12, 12, 12, 15, 17 and 20 mm zones of inhibition respectively. In the same way highest activity against the biofilm forming test organism S3 (*Bacillus*) was exhibited by the endophytic isolates UL3, BW8, MR3, SL3G, LS7, SL6, SL3A, BW6, SL1 and BW5 which showed zones of inhibition of 13, 15, 18, 18, 15, 15, 26, 15, 19 and 17 mm respectively (Table IV). The isolates MR3 and SL3G, UL3, LS7 were identified as members of the genus *Bacillus* by 16S rRNA gene sequencing (Table III), the antimicrobial activity of the endophytic *Bacillus* species against various bacterial and fungal pathogens have been reported by many workers (Laorpaksa *et al.,* 2008; Melnick *et al.,* 2008; Wang *et al.,* 2009). Bacon and Hinton (2002) demonstrated that endophytic *Bacillus* species may have the utility as biological controls for plant diseases and confer other beneficial traits to plants, they further highlighted that how molecular modifications of endophytic *Bacillus* species might be used to improve the nutritional value of their hosts. The isolates SL6 and BS2A exhibited 97% and 92% similarity with *Pseudomonas* sp. in 16S rRNA gene sequence analysis, these two isolates exhibited promising antimicrobial activity against the biofilm forming *Bacillus* species (Table IV). The antimicrobial potential of *Pseudomonas* is well established (Cartwright *et al.,* 1995; Kaleli *et al.,* 2007; Rosales *et al.,* 1995), however, the activity of these endophytic *Pseudomonas* isolates against biofilm forming bacterial strains is fascinating and could be exploited for the isolation of lead antimicrobial agents. The isolates SL3A and BW5 were identified as the members of genus *Stenotrophomonas* (Table III), they exhibited potent antimicrobial activity against almost all the tested biofilm forming bacterial strains. The gram negative rod *Stenotrophomonas* is opportunistic human pathogen (Drancourt *et al.,* 1997), however it has been frequently isolated from different plant tissues and exhibit antibacterial and antifungal activity and have plant growth promoting properties (Jeonga *et al.,* 2010; Wolf *et al.,* 2002). Among the endophytic actinomycete isolates recovered on rice agar, the isolate BW6 was identified as *Micromonospora* sp., it exhibited growth inhibition of almost all the biofilm forming test strains, which shows its promising potential for the production of useful compounds.

In chemical screening performed by TLC and HPLC-UV, an impressive diversity of the chemical constituents of the crude extracts obtained from culture broth of endophytic bacterial isolates was observed. In TLC analysis, different UV absorbing bands were observed under short and long wavelengths of UV i.e. 254 nm and 366 nm (Fig. 1). Prominent colored bands of the components of different crude extracts were observed by staining with anisaldehyde/H_2SO_4 and Ehrlich's reagents. The colored bands like brownish to yellow, yellow, orange, red to brown bands were seen by staining with Ehrlich's reagent, indicating the presence of different functional groups like amines and indole derivatives etc in the extracts. Similarly yellow, purple, blue and brown colored bands were observed by staining the TLC plates with anisaldehyde/H_2SO_4 reagent indicating the presence of the functional groups like phenols, steroids, and terpenes in the crude extracts. The most promising diversity of the colored bands were seen in the crude extracts of the endophytic isolates LML6, LS7, BS2A, LML5, SL3, MR3, SL6, BW8, BR3 and UL3 (Fig 1). In HPLC-UV analysis the crude extracts showed a number of peaks in their chromatograms at different retention time (t_R) e.g. the crude extract of the isolate BW8 gave five distinct peaks at retention time of 2.80, 3.06, 3.57, 4.65 and 5.40 min, similarly the chromatograms of the isolates SL3G, BW5 and BW6 showed a number of major peaks at different retention time. The HPLC-UV chromatogram of the isolate SL6 exhibited most exciting diversity of the chemical constituents in its crude extract which showed eight different peaks at retention time (t_R) 1.75, 2.72, 3.09, 3.52, 4.10, 4.59, 5.31 and 7.69 min (Fig. 2). The

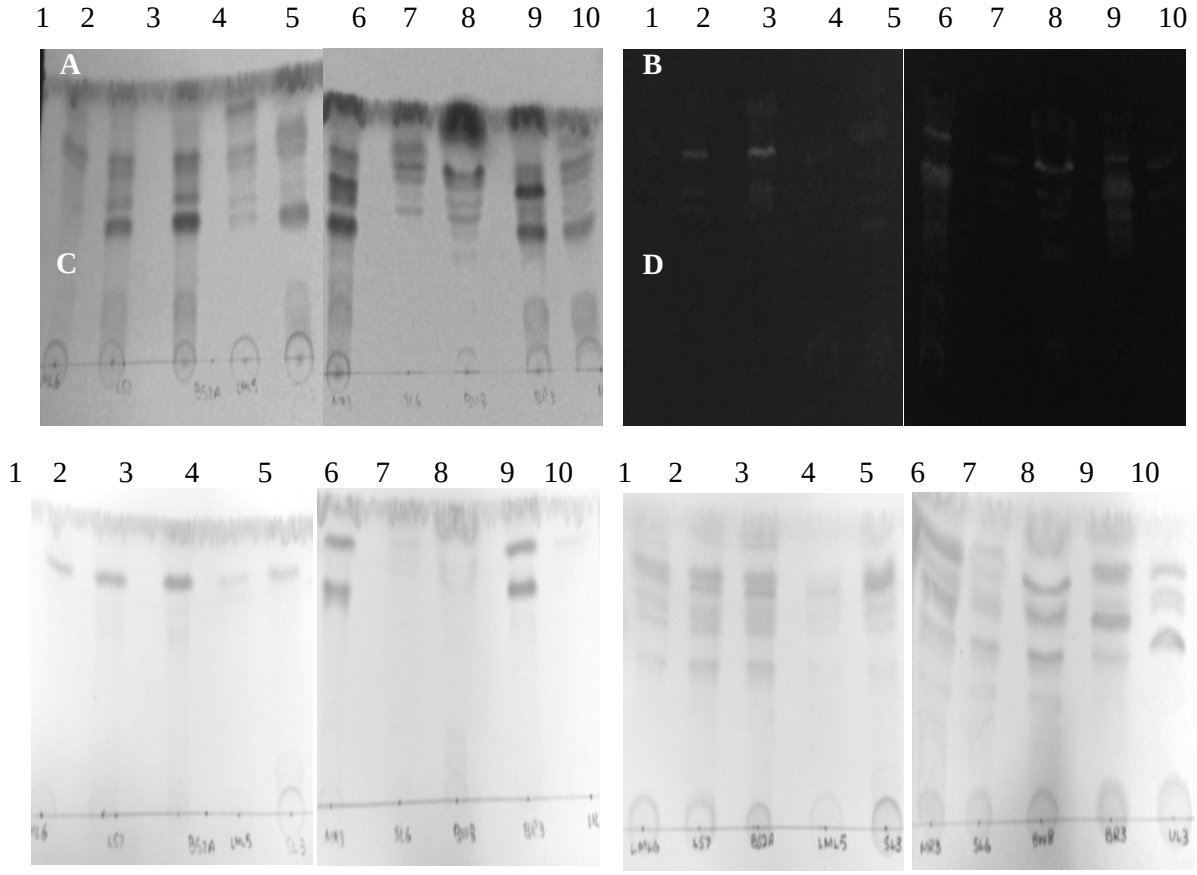

Fig. 1. Chemical screening using TLC detection. A) under UV at 254 nm, B) under 366 nm, C) after staining with Ehrlich reagent, D) after staining with Anisaldehyde/H_2SO_4 reagent. Numbers 1-10: Crude extract of isolates 1, LML6; 2, LS7; 3, BS2A; 4, LML5; 5, SL3; 6, MR3; 7, SL6; 8, BW8; 9, BR3; 10, UL3.

Table IV.- Antimicrobial activity of the selected endophytic bacterial isolates against biofilm formers.

Endophytic bacterial isolate	Antimicrobial activity (Zones of inhibition in mm)				
	Enterobacter	***Pseudomonas* spp.**	***Enterobacter***	***Bacillus* sp.**	***Bacillus* sp.**
LML5	10	12	20	12	10
BR3	10	35	0	12	10
UL3	10	10	10	0	13
BW8	10	0	7	0	15
BS2A	0	10	0	12	15
LML6	10	17	18	10	0
MR3	19	10	15	0	18
SL3G	15	12	19	10	18
LS7	13	9	0	12	15
SL6	0	12	13	15	15
SL3A	18	0	10	15	26
BW6	12	0	12	10	15
SL1	14	13	15	17	19
BW5	16	18	20	20	17

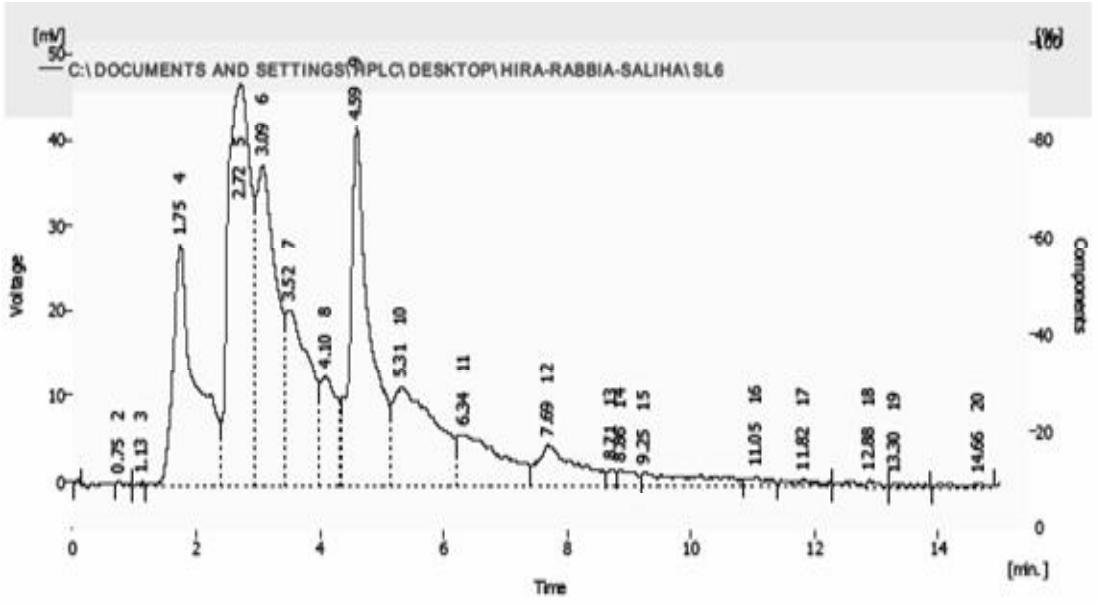

Fig. 2. HPLC-UV chromatogram of crude extract of the isolate SL6, detected at 254 nm using methanol/ water (90:10) as mobile phase

chromatograms with significant number of well separated peaks indicate that these endophytic bacterial isolates have the ability to produce diverse bioactive compounds simultaneously at relatively good concentrations.

CONCLUSIONS

A total of 76 endophytic bacteria were isolated from different plants at the province Punjab, Pakistan, out of which 10 potential candidates were detected which showed promising capability to produce useful compounds. On the whole based on biological and chemical screening results it can be conferred that different plants of this area Punjab, Pakistan can be a promising source of inimitable bioactive endophytic bacteria, which should be continuously isolated, characterized and investigated for the discovery of lead antimicrobial agents and to control the infections caused by biofilm forming bacterial species.

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Table IV.- Antimicrobial activity of the selected endophytic bacterial isolates against biofilm formers.

Endophytic bacterial isolate	Antimicrobial activity (Zones of inhibition in mm)				
	E4	**X4**	**M9**	**S2**	**S3**
LML5	10	12	20	12	10
BR3	10	35	0	12	10
UL3	10	10	10	0	13
BW8	10	0	7	0	15
BS2A	0	10	0	12	15
LML6	10	17	18	10	0
MR3	19	10	15	0	18
SL3G	15	12	19	10	18
LS7	13	9	0	12	15
SL6	0	12	13	15	15
SL3A	18	0	10	15	26
BW6	12	0	12	10	15
SL1	14	13	15	17	19
BW5	16	18	20	20	17

Key: E4 = *Enterobacter*, **X4** = *Pseudomonas spp.* **M9**= *Enterobacter*, **S2** = *Bacillus* sp., **S3** = *Bacillus* sp.

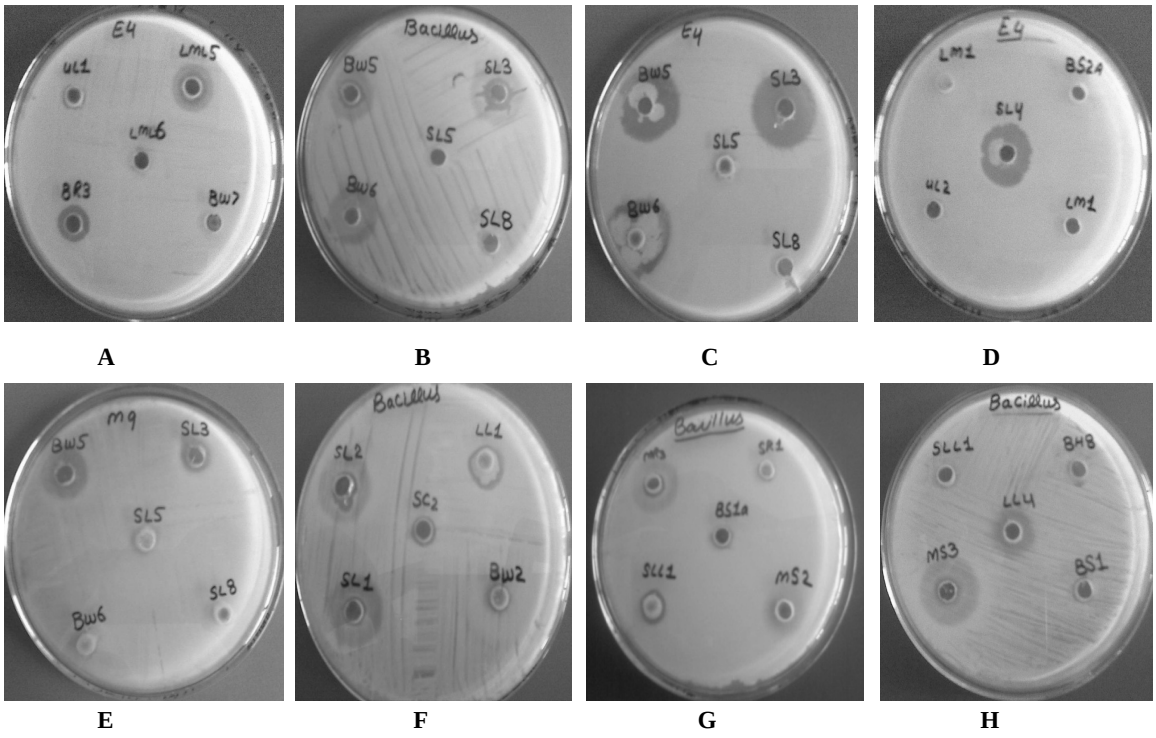

Fig. 1. Antimicrobial activity of the selected endophytic strains against biofilm formers determined by agar diffusion method (60 µl culture supernatant was loaded in each 5mm well).