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Sugar Beet Leaf Culturable Endophytic Bacterial Composition from the Major Sugar Beet Growing Areas in the West of Iran

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ABSTRACT

Endophytic bacteria refer to the bacteria that live within plants. Fresh leaves of 23 sugar beet (*Beta vulgaris* L.) plants were collected from the west of Iran. After superficial disinfection, endophytic bacteria were isolated from the host tissue. Isolates were grouped based on their whole-cell protein electrophoresis patterns. One representative from each electrotype was selected and its morphological feature characterized according to the standard bacteriological criteria. The *16S rRNA* encoding gene from these representatives was amplified using fD1 and rD1 universal primers, subjected to sequencing and aligned in the NCBI. The major occurring fingerprint types (electrotypes) were identified as *Acinetobacter calcoaceticus, Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia* (25, 23, and 22 strains respectively). The other minor occurring electrotypes were identified as *Streptomyces* spp, *Acetobacter* spp, and *Agrobacterium* spp. This is the first report of *A. calcoaceticus*, *P. aeruginosa*, and *S. maltophilia* as an endophyte in the leaves of sugar beet.

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Introduction

Plants generally associate with diverse microorganisms. In the same way, phyllosphere is described as the territory for a large diversity of bacteria. Thus, different bacterial species can interact and equilibrium inside the plant micro-ecosystem (Fisher *et al*., 1992). In broad biological terms, endophytes described as endosymbiont micro-organisms existing inner parts of the plant tissues without causing any apparent disease symptoms in the host (Wilson, 1995). As can be expected, bacterial endophytes can be isolated from internal plant tissue. Some of these bacteria can be considered to be dominant species and may be represented by those that are most frequently, and in large numbers, isolated from the host plant (Van Peer *et al*., 1990). Approximately, each plant in the globe is the host of one or more bacterial species as endophytes (Strobel *et al*., 2004). Rosenblueth and Martinez-Romero (2006), and Berg and Hallmann (2006) provided an inclusive list of bacterial endophytes isolated from plants. A large and growing body of literature has investigated the isolation of gram-positive and gram-negative bacterial endophytes from different tissues in several plant species; conversely, a single plant harbored different endophytic bacterial species (Kobayashi and Palumbo, 2000). Recently, researchers have shown an increased interest in endophytes, due to their potential for biotechnological applications. In the other words, microbial products originated from the endophytes could be applied as antifungal, antiviral, insecticidal agents (Ryan *et al*., 2008). Plant tissues are naturally colonized by bacterial endophytes similar to phytopathogens, which makes them an appropriate candidate for biocontrol applications (Berg *et al*., 2005). There is a large volume of published studies describing the potential of endophytic bacteria to suppress plant pathogens (Sturz and Matheson, 1996; Duijff *et al*., 1997; Krishnamurthy *et al*.,

1997). Also, several attempts have been made to show the ability to accelerate seedling emergence, promote and enhance plant growth (Ryan *et al*., 2008). In this regard, Shi et al (Shi *et al*., 2009; 2010; 2011) have revealed that endophytic bacteria could increase sugar content due to increased chlorophyll in sugar beet leaves. According to literature, various methods have been developed and endophytic bacteria have been studied mainly after culturing in laboratory media (Rosenblueth and Martinez-Romero, 2006); Therefore, the objectives of this study were to 1) isolate the culturable, endophytic bacteria from the leaves of sugar beet (Beta vulgaris L.) in the major growing regions in the west of Iran, 2) characterize and group the community of culturable endophytic bacteria using SDS-polyacrylamide gel electrophoresis, 3) identify major occurring bacteria on the basis of biochemical tests and *16S rRNA* gene sequence analysis.

Materials and Methods

Sampling

The samples were collected from a total of 12 sites from 7 major sugar beet growing regions (Malayer, Hamedan, Islamabad-e Gharb, Kermanshah, Mahidasht, Miandoab and Shahin Dezh) in the west of Iran. Plants were evaluated for physical appearance and only those with a healthy appearance were selected. The leaves of the superior portion of the plant were collected, and transported to the laboratory into plastic bags, then stored at 4°C. The isolation of endophytic bacteria performed one day after.

Isolation of endophytic bacteria

Plant leaves were washed in running tap water, and those with symptoms of the disease or superficial damage were discarded. Microbes present on the plant surface have to be fully eliminated. The leaves were surface sterilized with 70% alcohol for 1 min, sodium hypochlorite (2.5% Cl^-) for 4 min, and ethanol for 30 s. Finally, disinfectant was removed by rinsing three times rinses in sterile, distilled water and dried in the sterile paper towel. To confirm the disinfection process was successful, fifty microliters of the sterile water used in the final rinse were plated in 10% TSA w/v (1.5 g/l of triptone, 0.5 g/l of soy peptone, 1.5 g/l of NaCl, 15 g/l of agar, pH 7.3) at 28 ºC for 14 days and the plates were observed daily for growth of micro-organismal colony. Initially, the leaves were cut into pieces 1-3 mm-long and macerated in 6 mL of aqueous solution (0.9 % NaCl) using a sterile mortar and pestle. The suspension was subsequently incubated at 28 ºC for 5 hours to allow the complete release of endophytic microorganisms from the host tissue. Fifty microliters of the suspension were spread on five 10% TSA plates for each dilution (10^{-1} and 10^{-2}). The plates were incubated and observed daily for up to 14 days at 28ºC. Endophytic bacterial strains were defined as strains with differentiable colony morphologies. For each petri dish evaluated, morphologically distinct colonies (color, size, and shape) were selected on days 2, 5, 10, and 14 of incubation and purified in 10% TSA. Bacterial isolates were stored on nutrient agar slants for further studies (Zinniel *et al.,* 2003).

Isolation of total cell protein

Bacterial strains were cultured on nutrient agar medium. After 48 h grown fresh culture was inoculated into 50 ml King B broth and incubated with shaking at 28±2 °C (180 rpm) for 48 h. The bacterial cells were harvested in their logarithmic growth phase by centrifugation at 6000 rpm for 10 min. The harvested cells were washed twice in cold Tris-buffer (3.3 mM, pH 7.4), lysed by suspending in lysis buffer (10mM Tris-HCl, pH 7.4, 0.75 M sucrose, and 100 µg/ml lysozyme) and incubated on ice for 10 min and vortexed. The resulting cell suspension was incubated for 30 min. at 4°C before centrifugation at 10000 rpm for 20 min. (Dristig and Dianese, 1990). The supernatant was used as the total cell protein source for electrophoresis

Protein analysis through SDS-PAGE

SDS-PAGE analysis of the proteins was carried out in polyacrylamide slab gels consisting of 4% stacking gel and 12% separating gel using the Hoefer mini-electrophoresis system (Amersham Biosciences, Sweden). Samples with an equal amount of protein (50 µg) were dissolved in sample buffer and denatured by boiling for

4 min before loading onto the gel. From each sample, 30µl was loaded on a discontinuous polyacrylamide gel (12% acrylamide, 0.09% SDS) similar to the one described by Laemmli (1970). Electrophoresis was carried out at a constant voltage of 65 V for approximately 2h. Gels were stained overnight in a solution containing 40% methanol (v/v) and 10% acetic acid (v/v) and 1% Coomassie brilliant blue and destained in a solution containing 40% methanol (v/v) and 10% acetic acid (v/v). Standardization of gel length was obtained by photography. The reproducibility of the SDS-PAGE technique was estimated by including duplicate runs of a single protein extract on separate gels. The photographs of the protein patterns were compared visually.

Biochemical tests and *16S rRNA* gene amplification

One isolate of each fingerprint type was further characterized by series of biochemical tests using the criteria of Bergey's Manual of Systematic Bacteriology (Brenner *et al.,* 2005). The *16S rRNA* gene was amplified using fD1 (5′-CCGAATTCGTCGACAACAGAGTTTGAT CCTGGCTCAG-3′) and rD1 (5′ CCCGGGATCCAAGCTTAAGGAGGTGAT CCAGCC-3′) primers (Weisburg *et al.,* 1991). These primers are designed to yield nearly full-length *16S rRNA* gene from most bacteria. Each vial contained 50 µl of reaction mixture containing 34.75 µl nano-pure water, 5 µl of 1X PCR buffer, 6 µl of 25 mM $MgCl_2$, and 0.25 µl of 1U *Taq* polymerase, 1 µl of 2 mM dNTPs, 1 µl of each primer (fD1, rD1, 100 pmol/µl) and 1 µl template DNA, respectively. PCR conditions were as follows: Initial denaturation temperature at 94°C for 2 min and 30 cycles, including a denaturation step at 95°C for 1 min, an annealing step at 61°C for 1 min, an extension step at 72°C for 2 min and a final extension step at 72°C for 6 min. Finally, the PCR product was analyzed on 1% agarose gel, stained with ethidium bromide, and visualized under UV illumination and sequencing of the *16S rRNA* gene was performed by Bioneer Company, South Korea.

Phylogenetic tree

Nucleotide sequences were aligned through the CLUSTAL W algorithm using the BioEdit program. Automatically aligned sequences were checked manually. Phylogenetic trees were constructed using the Neighbor-Joining (NJ) algorithm in *MEGA* version 7 with 1000 bootstrap replications. The *16S* rRNA gene sequences determined in this study have been deposited with GenBank under the accession numbers.

Results

Based on colony morphology and color, a total of 85 bacteria were isolated from 23 sugar beet plants in seven different regions in the west of Iran (Fig. 1, Table 1). Polyacrylamide gel electrophoresis of whole-cell proteins (PAGE) were used to group the bacterial strains**.** In this paper, the term that will be used to describe a set of similar protein profiles obtained under standardized conditions is a protein fingerprint type or protein electrotype (Lambert *et al.,* 1987; Aeini and Khodakaramian, 2017). Thus, all electrotypes were designated SBT showing different and reproducible patterns (Fig. 2). In order to further identify the strains, one strain from each electrotype was selected as representative. Total of 13 different electrotypes were shown from sugar beet leaf endophytic area. Therefore, leaf endophytic bacteria were represented by three phyla that comprised 77% of the community (major occurring electrotypes), while the remaining (23%) involved three low-abundant phyla (minor occurring electrotypes) (Fig. 3, Table 2). In this regard, for accurate identification, major electrotypes were identified to species level based on the biochemical tests and sequencing *16s rRNA* gene (Table 4, Fig. 4). Remaining minor electrotypes were identified to genus level according to Bergey's Manual of Determinative Bacteriology (Brenner *et al.,* 2005) (*Acetobacter, Agrobacterium,* and *Streptomyces*). Relative frequency of the major electrotypes (fingerprint types) according to the sampling regions are illustrated in table 3. Minor occurring gram-negative electrotypes identified as *Agrobacterium* spp and *Acetobacter* spp comprised 13% of entire isolates.

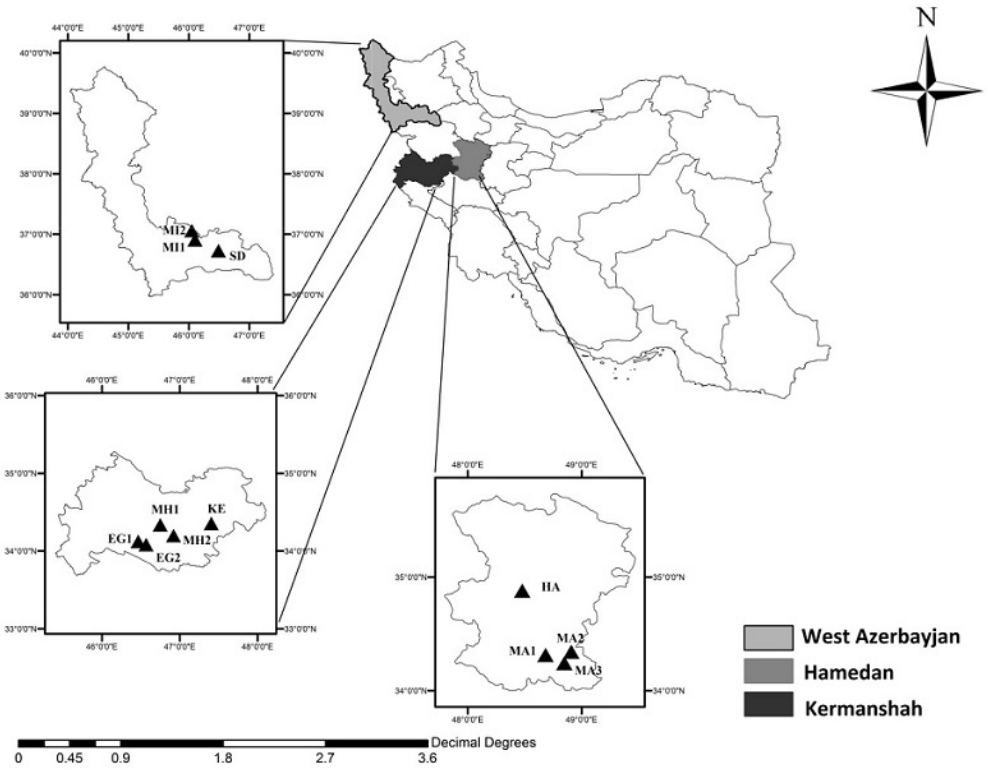

Fig.1. Map of Iran showing sampling sites.

Table 1. Overview of major sugar beet growing areas in the west of Iran.

Location	Sampling code	Varieties	Number of analyzed plants
Kermanshah	KE	Muraille	2
Mahidasht	MH1	Rusta	4
	MH2		
Islamabad-e Gharb	EG1	Rusta	4
	EG2		
Hamedan	HA	Ekbatan	2
Malayer	MA1	Poma	5
	MA2		
	MA3		
Shahin Dezh	SD	Dorothea	2
Miandoab	MI1	Dorothea	4
	MI2		

Table 2. SDS-PAGE fingerprint types and number of endophyte isolates in the leaves of sugar beet.

Major fingerprint types	Fingerprint types[a]	Number of isolates
Acinetobacter calcoaceticus	SBT05	36
Pseudomoas aeruginosa	SBT21	17
Stenotrophomonas maltophilia	SBT13	13
Minor fingerprint types (Gram-negative)		
Acetobacter spp	4	7
Agrobacterium spp	2	4
Gram-positive		
Streptomyces spp	4	8
Total	13	85

[a]For the minor fingerprint types, the total number of different fingerprint types is given.

Table 3. Relative frequency of the major fingerprint types according to the sampling regions.

Major fingerprints	Kermanshah	Mahidasht	Islamabad -e Gharb	Hamedan	Malayer	Shahin Dezh	Miandoab
SBT05	8	4	5	6	4	3	6
SBT13	2	0	3	1	2	2	3
SBT21	2	3	0	4	2	3	3
Total	12	7	8	11	8	8	12

Table 4. Biochemical and molecular analysis of the major fingerprint types (electrotypes).

Biochemical tests	SBT13	SBT05	SBT21
Gram reaction	-	-	-
Motility	+	-	+
Growth in 4°C	-	-	-
Growth in 37°C	+	+	+
Growth on 7% NaCl	-	-	-
Production of fluorescent pigment	-	-	-
Catalase	+	+	+
Oxidase	-	-	+
Urease	-	Nd	+
Lecithinase	+	Nd	nd
Nitrate reduction	+	-	-
Methionine Requirement	+	-	-
Indole production	-	-	+
Argenine dehydrolase	-	nd	+
Citrate utilization	+	+	+
Starch hydrolysis	-	nd	+
Gelatin hydrolysis	+	-	+
Casein hydrolysis	+	+	+
Lipid hydrolysis	Nd	+	-
Acid from			
Glucose	+	+	+
Fructose	-	-	+
Sucrose	Nd	+	-
Xylose	Nd	-	+
Manose	+	+	-
Sorbitol	-	-	+
Growth on			
Cellobiose	+	-	-
Lactose	+	+	-
Trehalose	+	-	-
Arginine	-	+	+
Molecular analysis			
Identification based on *16s rRNA* gene sequencing	*Stenotrophomonas maltophilia*	*Acinetobacter calcoaceticus*	*Pseudomonas aeruginosa*

+ representing the positive reaction, and – shows the negative reaction. nd: not done. The experiment was repeated twice for each representative isolate.

Only one minor occurring gram-positive genus identified as *Streptomyces* spp which comprised 10% of isolates showing four different electrotypes. Major occurring electrotype designated as SBT05 was identified as *A. calcoaceticus* and recovered from all sampling areas in the west of Iran. This electrotype considered as highly-abundant phyla and accounting for 42% of leaf endophytic community. From the data in fig. 3, one-fifth of the bacterial community belonged to SBT 21. This major electrotype was identified as *Pseudomonas aeruginosa*. The last major occurring electrotype (SBT13) identified as *Stenotrophomonas maltophilia*, represented 17% of leaf bacterial endophytic

community. Other minor electrotypes comprised three phyla including *Sterptomyces* spp, *Acetobacter* spp and *Agrobacterium* spp which represented 10%, 8% and 5% percent of leaf endophytic community, respectively.

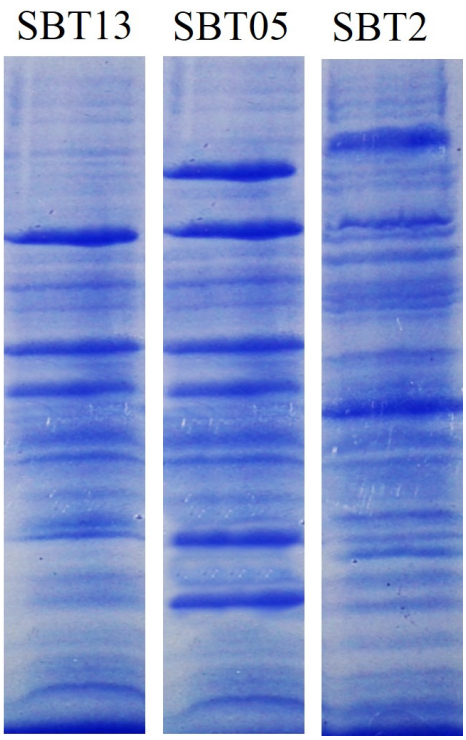

Fig. 2. Whole-cell protein fingerprints of the major occurring endophytic electrotypes isolated from the sugar beet leaves.

The *16s rRNA* gene sequence analysis was performed by using the algorithms BLAST (National Center for Biotechnology Information). The *16S rRNA* gene sequences of endophytic bacteria reported in this article have been deposited in the GenBank database under accession numbers: KX018311 (SBT21), KX232141 (SBT05) and KX018308 (SBT13). Neighbors joining phylogenetic tree of the representative sequences and the reference showed the high percentage of similarities with our strains (Fig. 5).

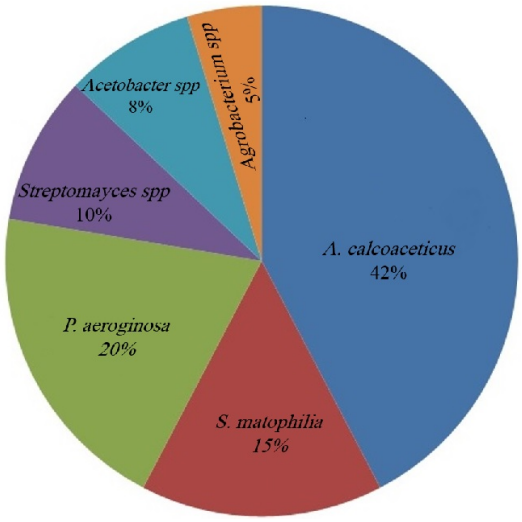

Fig. 3. The pie chart showing the frequency of endophytic isolates in sugar beet leaves.

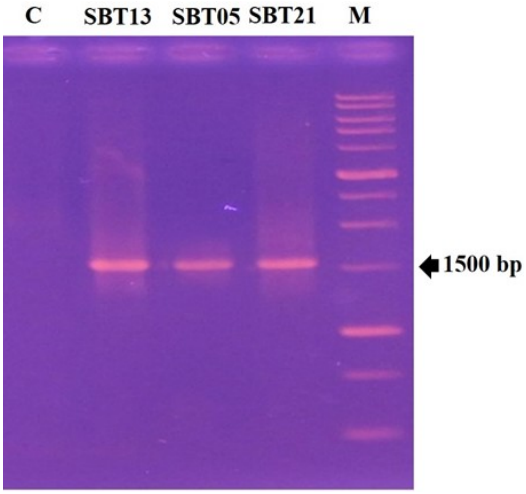

Fig. 4. Agarose gel electrophoresis of PCR-products of representative strains with primers rD1 and fD1: M= 1 KB ladder; SBT05; SBT13; SBT21 and C as negative control.

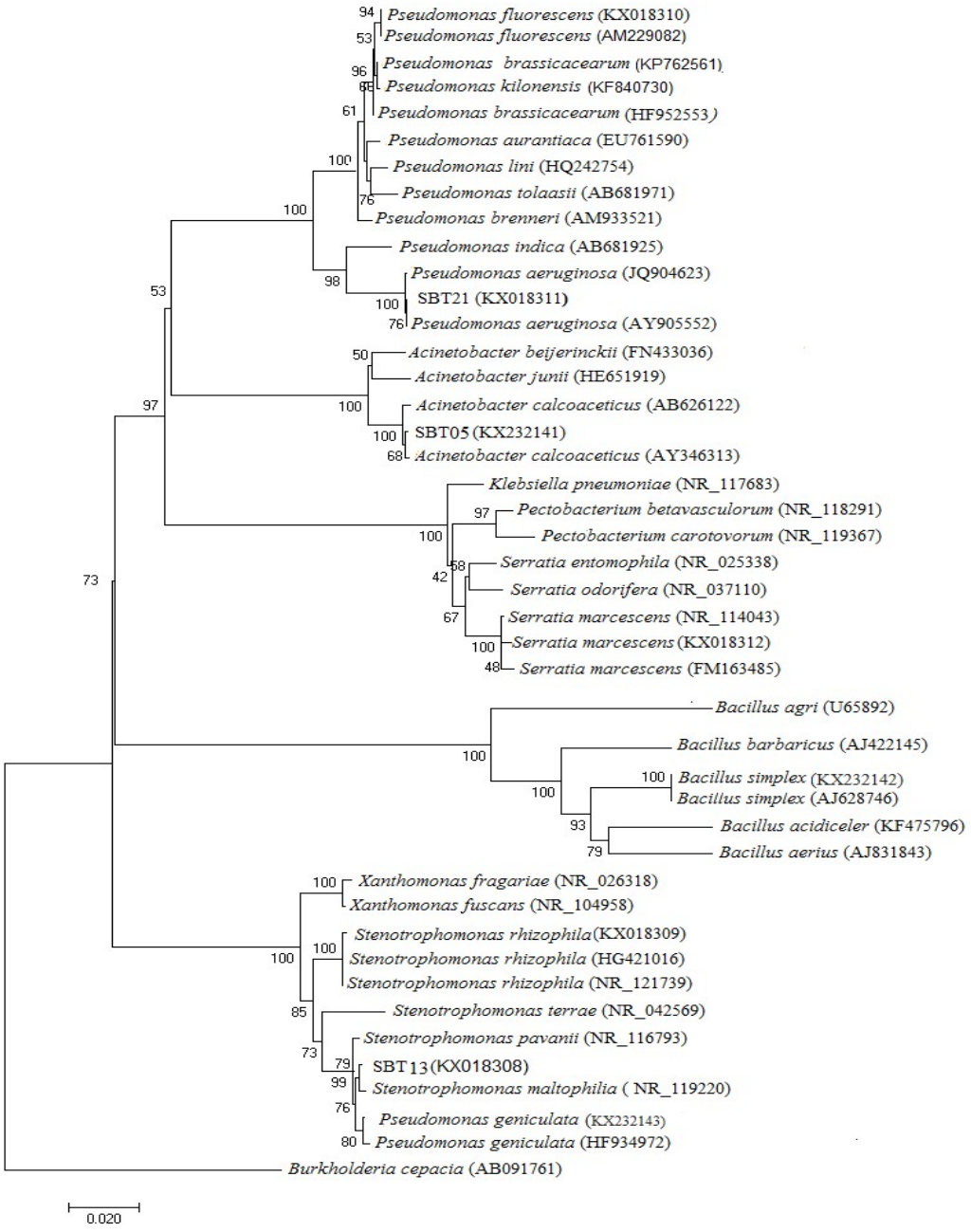

Fig. 5. Phylogenetic analysis of SBT05, SBT13 and SBT21 and related species based on neighbor-joining tree and sequence data from *16s rRNA* gene: The value on each branch is the percentage of bootstrap replications supporting the branch. The GenBank accession number for each microorganism used in the analysis is shown in parentheses after the species name.

Discussion

This study set out with the aim of reporting the leaf associated bacteria in the major sugar beet growing regions in the west of Iran. To the best of the author's knowledge, no research has been performed on the leaf associated bacteria composition in Iran. In this study, we found sugar beet harbored an abundance of culturable bacteria. Previous research findings showed that strains with highly similar protein patterns share a high DNA homology and belong to the same species (Kersters and De Ley, 1980). Thus, we used the bacterial whole-cell protein electrophoresis method for grouping the bacteria. The results of this study

indicate that three bacterial species are particularly well adapted to colonize inner plant tissues .These species are most frequently, and in large numbers and could be regarded as leaf endophytic dominant. Therefore, it could conceivably be hypothesized that mentioned bacteria are resident in the leaves of sugar beet. One of the major occurring bacteria identified as *A. calcoaceticus*. Bacterium *A.calcoaceticus* seems to be highly abundant in the leaf endophytic communities analyzed so far. This species has the positive role in plant growth enhancement and biologically active metabolites production (Indiragandhi *et al.,* 2008, Kang *et al.,* 2009). In addition, this species was reported as major components of the endophytic communities of *Plectranthus tenuiflorus* medicinal plant in Saudi Arabia (El-Deeb *et al.,* 2013). The second dominant bacteria were identified as *P. aeruginosa*. *P. aeruginosa* is the human opportunistic pathogen and has been described as endophytic colonizers of black pepper Due to the biocontrol potential of *P. aeruginosa*, some strains have been advised in integrated pest management programmes (Kumar *et al.,* 2013). *S. maltophilia* as the last major occurring endophytic bacteria, has been previously reported as the resident of rhizosphere in the west of Iran (Aeini and Khodakaramian, 2017). The present findings seem to be consistent with other research found that rhizosphere bacteria colonize the roots firstly and then spread through xylem vessels to the upper part of the plant (Compant *et al.,* 2011). Beside the dominants, some of the isolated bacteria were regarded as minor occurring which cannot be isolated easily because of their low numerical consistency (Lodewyckx *et al.,* 2002). These endophytic bacteria are regarded as rare species and can be isolated occasionally. This study has shown that PAGE can be an ideal method to screen endophytic bacterial population in the leaves of sugar beet. Moreover, this research extends our knowledge of dominant phyla residing as the endophytes in the leaves of sugar beet. One of the more significant findings to emerge from this study is the first report of *A. calcoaceticus*, *P. aeruginosa*, and *S. maltophilia* as endophyte in the leaves of sugar beet. This research has thrown up many questions in need of further investigation. Further investigation and experimentation into the effects of endophytic bacteria on biological controls, growth promotion in plants, and discovering new pharmaceutical drugs is strongly recommended.

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