Characterization of Salt Tolerant *Enterobacter hormaechei* Strain associated with Tomato Root Grown in Arid Saline Soil

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(Received: 15 August 2014; accepted: 02 October 2014)

A salt tolerant bacterium was isolated from the rhizosphere of tomato grown in saline arid soil of Uzbekistan. 16S rRNA gene sequence analysis indicated that the strain was most closely related to *Enterobacter hormaechei* with sequence similarities of 99%. The strain utilizes various carbon sources and showed multidrug resistance to various antibiotics. *E. hormaechei* NUU10 was able to stimulate root and shoot growth of tomato in both non saline and saline soil conditions. The strain was shown to have some PGP activities, such as production of IAA and phosphate solubilization ability that, together or alone, might explain the capacity of this strain to alleviate stress of salt-affected tomato plant. The bacterial isolate was able to survive in soil, rhizosphere and phyllosphere of tomato plant grown under arid, saline soil condition. The results of this study indicated that *Enterobacter hormaechei* NUU10 has the potential to produce different biological active compounds such as cell wall degrading enzymes, IAA and utilize a wide range of carbohydrates as carbon as energy sources. They may positively effect on plant growth and has the ability to colonize and survive in tomato root grown in saline soils with hot summer temperature.

Key words: Saline soil, Enterobacter hormaechei, tomato, colonization, auxin.

The rhizosphere is the habitat for a large diversity of microorganisms, and some of bacteria possess beneficial interactions with plant, while others may be opportunistic pathogens (Berg *et al.* 2005; 2013; Egamberdieva 2005, 2011, 2012). The role of root associated bacteria in plant growth development and protection from various diseases

has been intensively discussed (Lugtenberg and Kamilova 2009; Pliego *et al.* 2011; Egamberdieva *et al.* 2011, 2013). The best-known bacterial species such as *Pseudomonas, Bacillus, Azospirillum* spp. and *Pantoea* spp. have been indicated as effective PGPR organisms (Kampfer *et al.* 2005; Shoebitz *et al.* 2008; Berg *et al.* 2010). Some other bacterial genera, including *Burkholderia, Enterobacter, Ochrobactrum, Ralstonia, Staphylococcus* and *Stenotrophomonas* can encounter bivalent interactions with both plant and human hosts (Berg *et al.* 2005; Egamberdieva 2010, 2012, Egamberdieva *et al.* 2008).

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Bacteria belonging to the genera Enterobacter have been found in the rhizoshere of wheat (Kampfer et al., 2005; Egamberdieva et al. 2008), rice (Hassen et al., 2007) tomato (Glick et al. 1995), sugarcane (Zakria et al., 2008) plants. Quadt Hallmann and Kloepper (1996) observed colonization of E. asburiae JM22 in the rhizosphere of cotton plants. Peng et al. (2009) isolated Enterobacter oryzae from surface-sterilized roots of the wild rice species Oryza latifolia. Most of Enterobacter species showed plant beneficial properties such as plant growth stimulation and biological control of fungal disease (Ruppel 2000; Kämpfer et al. 2005). For example E. cloacae is described as an antagonist of Pythium sp., which causes cucumber root rot (Howell 1989), Enterobacter sp. stimulated plant growth of broccoli (Zakria et al. 2008), whereas E. *radicincitans* showed plant growth promotion for wheat (Kampfer et al. 2005). Park et al. (2005) isolated E. hormaechei from a sand dune plant. Enterobacter species have been also isolated from blood, wounds and sputum of adult hospital patients (O'Hara et al. 1989). For example E. hormaechei has been recognized increasingly frequent as the cause of nosocomial infections. Wenger et al. (1997) found that E. hormaechei is a nosocomial pathogen that can infect vulnerable hospitalized patients and can spread between patients.

Knowledge about the characterization and possible function of *Enterobacter hormaechei* in the environment such as soil, water or associated with plants is limited. In the present study, *E. hormaechei* strain isolated from the rhizosphere of tomato grown in saline arid soil was examined and their potential role within that ecosystem discussed. Such studies may provide valuable information on the physiology and activity of these microbes within plant environment and improve our knowledge on their ecology under hostile environment.

MATERIALS AND METHODS

Isolation of bacterial strain

Tomato plant was grown in saline soil of Syrdarya province, open field condition, (41°00'N, 64°00'E), north-east of Uzbekistan. The main soil chemical properties are: organic matter 0.79 %; Ct

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2.39 %; Nt 0.07 %; $CO_3^{2-}C$ 1.59%, Ca^{2+} 54.3 g/kg; Mg²⁺ 26.1 g/kg; K⁺ 6.7 g/kg; P 1.2 g/kg; Cl⁻ 0.1 g/kg; Na⁺ 0.8 g/kg; pH 8.0. The high concentration of Ca²⁺, K⁺, and Na⁺ are associated with CO_3^{2-} and Cl⁻ reflecting the dominance of carbonate and chloride in saline soil. An electrical conductivity (EC) value of saline soil was 6.2 dS/m.

The roots of 2 months old tomato were separated from soil (10 g) and were shaken for 1.5 h in 100 ml of phosphate buffered saline (PBS; 20mM sodium phosphate, 150 mM NaCl, pH 7.4). The soil suspension was diluted and aliquot was plated on TSA/20 (One- twentieth of Tryptic Soya Broth (Difco Laboratories, Deitroit, USA) supplemented with 4 % NaCl. Cycloheximide (Sigma, St. Louis, USA) at final concentration of 100 mg/ml was used to prevent fungal growth. The plates incubated at 28°C and after 48 hours, bacteria was randomly picked up from plate and purified. Morphologically distinct colonies were subcultured to obtain pure cultures. Purified strains were checked for different salt tolerance in Luria-Bertani broth supplemented with 4, 5 and 6 % NaCl. The most salt tolerant strain was selected for the further study.

Identification of strain

Total DNA from strain was isolated using technique of de Souza *et al.* (2003). A fragment encoding part of 16SrDNA sequence of approximately 1,440 bp DNA was amplified. Total DNA of strains were used as source of template DNA. The nucleotide sequence of the PCR fragments was determined by ServiceXS (Leiden, The Netherlands). Sequences were assembled with DNAMAN Software. Homology searches with 16S rDNA sequences in GeneBank were performed with the BLASTN program (version 2.2.1) (Altschul *et al.* 1997).

Phenotypic characterization of strain

The salt tolerant bacterial strain was tested for a number of key characteristics by using standard procedures (Smibert and Krieg, 1994). Production of oxidase was determined as described by Cappuccino and Sherman (2001). The catalase production were determined by adding the H_2O_2 (3% vol/vol) to a bacterial culture and the present of catalase indicated by bubbles of free oxygen gas (O_2) (2001). The Voges-Proskauer test was performed according to the guidelines given by Chapin and Lauderdale (2003). Hugh–Leifson

medium (Hugh and Leifson, 1953) was used to test for glucose oxidation/fermentation.

The production of acid from carbohydrates was tested in nutrient broth containing 1% of D-glucose, D-mannose, Dtrehalose, D-mannitol, D-arabitol, D-sorbitol, DLglycerate, galactose, glycerol, L-arabinose, maltose, malat, xylose, xylitol, and Andrade's indicator (Cowan 1974). Arginine dihydrolase activities were tested in Moeller's broth (pH 6.5), consisting of 0.5% peptone, 0.5% meat extract, 0.05% glucose, 0.5% pyridoxal, bromcresol purple, cresol red, and 1% respective amino acids (Burkhardt 1992). Urease activity was tested in Christensen's agar (Burkhardt 1992). Citrate utilization was tested on Simmon's agar (Oxoid, United Kingdom). Hydrolysis of Tween 20 and 60 was determined on modified sierra agar, containing 10 g peptone, 3 g of meat extract, 5 g of NaCl, 0.2 g of Fe-citrate, 0.1 g of CaCl₂.H₂O and 15 g of agar in 1 L of distilled water. Ten ml of sterile Tween 20, 60 and 50 ml of 0.067 % (w/v) Victoria Blue B solution were added to the medium after autoclaving. Hydrolysis of Tween was recorded as white precipitation around the colonies. Indole production from tryptophan was tested using the method of Clarke & Cowan (1952). The HCN production by bacterial strain was determined according to the method of Castric (1975), lipase activity as described by Howe and Ward (1976), protease activity by Brown and Foster (1970), glucanase activity by Walsh et al. (1995) and cellulase activity was detected using the substrate carboxymethylcellulose in top-agar plates (Hankin and Anagnostakis 1977).

Antagonistic activity

The bacterial strain was tested in vitro against *Fusarium oxysporum* f.sp. *radicislycopersici* (Forl), *F. solani, Gaeumannomyces graminis pv. tritici* (Ggt), *Pythium ultimum, Alternaria alternate* and *Botrytis cinerea* using a plate bioassay with PDA agar. Fungal strains grown in agar plate at 28°C for 5 days and disks of fresh culture of the fungus (5 mm diameter) were cut out and placed in the centre of a 9 cm petri plate. A bacterium (grown in peptone agar plates) was streaked on the test plates perpendicular to the fungi. Plates were incubated at 30°C for 7 days, until the fungi had grown over control plates without bacteria. Antifungal activity was recorded as the width of the zone of growth inhibition between the fungus and the test bacterium. **Production of IAA**

The production of IAA (indole 3-acetic acid) was determined according to the method of Bano and Musarrat (2003). Tested bacterial strains were grown in LC medium, a modification of Luria broth base Miller (Difco), amended with 1 - 4% NaCl without and with tryptophan (100 mg/ml). After three days of cultivation, aliquots of bacterial cultures were centrifuged at 13 000 xg for 10 min. One ml of supernatant was transferred to a fresh tube to which 100 ml of 10 mM orthophosphoric acid and 2 ml of reagent (1 ml of 0.5 M FeCl, in 50 ml of 35% HClO₄) were added. After 25 min, the absorbance of the developed pink color was read at 530 nm. The IAA concentration in culture was calculated by using a calibration curve of pure IAA as a standard.

Phosphate solubilizing activity

The phosphate solubilizing activity of *E. hormaechei* was determined using Sperber's agar (Sperber 1957) containing 0.5% tricalcium phosphate $[Ca_3(PO_4)_2]$ and supplemented with 2 and 3% of NaCl. Strain was plated onto agar and incubated at 28°C for five days. Solubilization of mineral phosphate was characterized by a clear halo around the bacterial colonies.

1-aminociclopropane-1-carboxylacid (ACC) deaminase activity

In order to measure growth on ACC as the sole N-source, bacterial isolates were incubated in BM medium (Lugtenberg *et al.* 1999) minimal medium supplemented with 3.0 mM ACC (Sigma Chemical Co., St. Louis, Missouri, USA) (to test ACC utilization) 3.0 mM (NH_4)2SO₄ (positive control) as the sole N source or without added Nsource (negative control).

Antibiotic resistance patterns

Antibiotic resistance of *E. hormaechei* strain against the antibiotics of human and veterinary significance was assayed using a modified Kirby-Bauer disk-diffusion method (Bauer et al. 1966). The Neo-Sensitab (Rosco Diagnostica A/S) antibiotic discs were used to determine the susceptibility of the strains to ampicillin (AMP, 10 μ g), amoxicillin/clavulanic acid (AMC/20 + 10 μ g), penicillin (PEN, 10 μ g), Chloramphenicol (CLR/30 μ g), cefotaxime (CTX/30), ciprofloxacin (CIRP/5), piperacillin (PIPRA/30), ceftazidime (CAZ/30), Sulphonamides (SULFA/240

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μg), Kanamycin (KAN/30 μg), Tetracycline (TET/ 30 µg), Streptomycin (STR/100 µg), Erythromycin (ERY/15 µg), Tobramycin (TOB/10 µg), Trimethoprim-Sulphamethoxazole (SXT/25 µg), Neomycin (NEOM/100 µg), Gentamicin (GEN/10 µg). Bacterial strain was grown at 30°C in Mueller-Hinton broth (Difco Laboratories, Detroit, MI), and after 24 hours 1.0 ml of culture was sedimented by centrifugation and the supernatant discarded. The cells were washed with 1 ml phosphate buffered saline and re-suspended in PBS. Then 100 µl of cell suspension was spread on agar plate and left to dry before placing the antibiotic disc on the surface of the plate.

After incubation at 30°C for 24 h the diameter of inhibition haloes around the colonies was measured. Characterization of strains as sensitive, intermediate or resistant was based on the size of the inhibition zones around each disk according to the National Committee for Clinical Laboratory Standards guidelines (NCCLS, 2001).

Plant beneficial properties

Soils were taken for pot experiments from irrigated agricultural site of Syrdarya province, north-east of Uzbekistan. Soil properties are described above. Bacteria were grown in LB medium for 24 hours and seeds were inoculated with different bacterial densities: 106, 107 and 108 cells/ml. Sterilized seeds were germinated in sterile petri plates and were placed in the bacterial suspension with sterile forceps and shaken gently and after approximately 10 min the inoculated seedlings were sown in the plastic pots (9 cm diameter; 12 cm deep) containing 350 g of soil. The inoculation treatments were set-up in a randomised design with ten replications. Plants were grown at 24 - 26°C during the day and 16 - 18°C at night and after 1 month the shoot, root length and dry matter of plants were measured.

Survival of bacterial strain in soil, rhizosphere and phyllosphere

Spontaneous rifampicin (200 µg/ml) resistant mutant of the strain was used for the colonization studies. Rifampicin resistant mutant of E. hormaechei were obtained by plating the parental strain onto LC agar amended with 200 µg/ ml rifampicin. After incubation, isolates were selected based on similarities in colony morphology and growth rate with the parent strain, and were recultured on medium containing rifampicin to

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ensure stability of the antibiotic resistance marker. Plants were grown in plastic pots (9 cm diameter; 12 cm deep) containing 350 g of soil. Tomato seeds sterilized, allowed germinate, then seedlings were coated with bacteria by dipping the seedlings in bacterial suspensions that resulted in 108 CFU ml-¹ seeds. Plants were grown in saline soil, open field condition, temperature ranged between 22-26°C day and 16-20°C night. After two months, plants with its surrounding soil was collected from field and taken for laboratory. After harvesting, adhering soil was removed from roots and shoots were separated. For determination of the soil of the root zone colonization with the inoculated bacteria, 1 g soil was shaken with 9 ml sterile PBS with Cycloheximide (100 g/ml, Sigma, St. Louis, USA) for 30 min. For determination of rhizosphere colonization 1 g washed roots was macerated and shacked with 9 ml sterile PBS. To determine the phyllosphere colonization 1 g leaves were macerated and shacked with 9 ml sterile water. The resulting suspensions were evaluated for colony forming units (cfu) according to the dilutionplate method in LB agar with addition of 200 µg/ ml rifampicin. After an incubation time of 3 days at 28°C the reisolated, rifampicin resistant strains were identified for their colony characteristics (Egamberdieva and Hoflich 2002).

Statistical analysis

Data were tested for statistical significance using the analysis of variance package included in Microsoft Excel 2007 and mean comparisons were conducted using a least significant difference (LSD) test (P=0.05). Standard error and a LSD result were calculated.

RESULTS

Identification of strain

The bacterial strain was isolated from the rhizosphere of tomato grown in saline arid soil of Uzbekistan. Molecular characterization based on 16SrDNA homology of a partial sequence (1,440 bp) with the sequences in GeneBank Nucleotide sequencing of amplified 16S rDNA fragments, obtained after colony polymerase chain reaction (PCR), and comparative analysis with the DNA databases, indicated that this strain was related to members of the genus Enterobacter. The 16S rRNA gene sequence of strain NUU10 showed highest

similarities to *Enterobacter hormaechei ssp. Steigerwaltii* EN-562 (99%).

Physiological characterization

E. hormaechei strain is gram-negative rods which are motile, catalase positive, and oxidase negative and ferment D-glucose. The strain show positive Voges-Proskauer reactions. A detailed biochemical profiling of the isolate is given in Table 1. Acid is produced from the following compounds: D-glucose, D-mannose, D-trehalose, D-mannitol, D-arabitol, D-sorbitol, galactose, glycerol, Larabinose, maltose, and xylose. The following compounds are not utilized as sole sources of carbon: malat and xylitol. E. hormaechei has also been shown to produce arginine dihydrolase, urease, but was negative for Tween 20 and 60 hydrolase. Tests for indole production were negative. The strain was resistance to ampicillin, amoxicillin plus clavulanic acid, cefotaxime, ceftazidime, erythromycin, kanamycin, neomycin, penicillin, sulphonamides, trimethoprim plus sulfamethoxazole, but not to piperacillin, chloramphenicol, gentamicin, tetracycline, streptomycin and tobramycin.

Plant beneficial traits

The strain E. hormaechei NUU 10 can grow at NaCl concentration up to 6% NaCl (Figure 1). The strain produced glucanase, cellulase and was negative for lipase, protease activity and did not produce HCN. The strain did not show antagonistic activity towards the phytopathogenic fungi F. oxysporum f.sp. radicis-lycopersici, F. solani, G. graminis pv. tritici, P. ultimum, A. alternate and B. cinerea. The strains grow well in the presence of high salt up to 6%. Auxin production was tested in the absence and presence of 100 ug/ml of the auxin precursor tryptophan and results obtained from 4 days old cultures showed that E. hormaechei NUU10 produce IAA (9.2 μ g/ml) in media contained 2% NaCl. The presence of tryptophan strongly stimulated auxin production (19.4 μ g/ml) by the strain (Table 1). The

 Table 1. Characteristics of Enterobacter hormaechei strain NUU10 isolated from tomato root grown in saline soil

Properties E	E. hormaechei strain NUU10
Acid production	
D-glucose	+
D-mannose	+
D-trehalose	+
D-mannitol	+
D-arabitol	+
D-sorbitol	+
Galactose	+
Glycerol	+
L-arabinose	+
Maltose	+
Xylose	+
PGP traits	
IAA	+
Phosphate solubilization	+
ACC deaminase	-
Enzymes	
lipase	-
glucanase	+
protease	-
cellulase	+
Antibiotic resistance	
Ampicillin	+
Amoxicillin/clavulanic ac	id +
Penicillin	+
Cefotaxime	+
Ciprofloxacin	+
Ceftazidime	+
Sulphonamides	+
Kanamycin	+
Erythromycin	+
Neomycin	+

Table 2. The effect of *E. hormaechei* NUU10 on the root and shoot

 length and dry matter of tomato in non saline and saline soil conditions

Parameters		Non saline NUU10, CFU/ml				Saline NUU10, CFU/ml		
	Control	106	107	108	Control	106	107	108
Shoot length. cm Root length, cm Dry matterg/plant	6.3±1.2 5.2±1.1 0.12±0.02	6.4±1.0 4.8±0.9 0.11±0.01	6.7±0.9 5.4±0.7 0.12±0.02	6.9±1.1 5.5±1.2 0.13±0.02	5.1±0.7 3.7±0.5 0.09±0.01	5.6±0.9 3.9±0.5 0.09±0.01	6.0±1.1* 4.7±0.9* 0.11±0.01	4.5±1.0

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strain was able to solubilize mineral phosphate in the presence with 2% NaCl, but not with 3% NaCl. The bacterial strain was negative for ACC deaminase activity (Table 1).

Plant growth promotion and survival

Plant growth promoting properties of E. hormaechei NUU10 showed that the strain affected positively on shoot, root growth of tomato in both non saline and saline soil conditions (Table 2). There was no significant difference in plant growth stimulation by bacterial isolate in non saline soil. The data showed that root, shoot length of tomato treated with NUU10 strain significantly higher in comparison to the non-inoculated control under saline soil condition (Table 2). We found that bacterial effects on plants (e.g increased shoot, root length and dry matter) dependent on initial inoculum levels (106, 107 and 108 cells/ml). Bacterial stimulation of plant growth was more pronounced when plant inoculated with bacterial density 107 and 108 cells/ml.

Rifampicin resistant mutant, obtained from bacterial strain was tested for its survival in soil, rhizosphere and phyllosphere of tomato grown in saline soil. The results showed that bacterial strain was able to colonize in the soil, root and leaves of two months old tomato. The results showed that this isolate exhibited colonization level of 6.2 + 1.6, 90 ± 0.7 , 1.5 ± 1.2 (10^{3} CFU/g soil, fresh root, leaves) for soil, rhizosphere and phyllosphere respectively. The strain showed lower colonization efficiency in the phyllosphere.

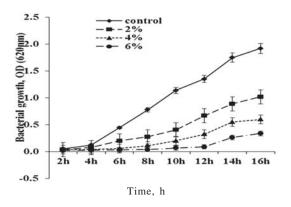


Fig. 1. Growth curve of *Enterobacter hormaechei* NUU10 incubated under various NaCl concentrations (2, 4, 6 %, w/v)

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DISCUSSION

We showed here that the rhizosphere of tomato grown in saline soil was colonized by salt tolerant *E. hormaechei*. To our knowledge this is the first report on the isolation and identification of *E. hormaechei* as a free-living tomato associated rhizobacterium. Previous reports have shown the colonization of *E. hormaechei* in the root of sand dune (Park *et al.* 2005). Prajapati and Modi (2012) confirmed existence of *E. hormaechei* in ceramic industry soil. Gupta *et al.* (2012) isolated and identified phosphate solubilizng *E. hormaechei* strain from *Aloe barbadensis* Miller.

We found that isolated E. hormaechei strain utilize various carbon sources and showed multidrug resistance to ampicillin, amoxicillin plus clavulanic acid, cefotaxime, ceftazidime, erythromycin, kanamycin, neomycin, penicillin, sulphonamides, and susceptibility to other tested antibiotics by disc diffusion test. The presence and persistence of antibiotic resistance bacteria in ground water, soil, root or leaves of plants is a growing public health concern. According McKeon et al (1995) antibiotic resistant bacteria are a cause for concern because of possible conjugal transfer of antibiotic resistance to the normal flora, and subsequent shedding of even more multi resistant organisms, serving to further amplify the number introduced into surrounding environments.

The strain did not show antagonistic activity towards the phytopathogenic fungi. Shoebitz *et al.* (2009) in their work found that *Enterobacter ludwigii* BNM 0357 had the capability to antagonize *F. solani* mycelial growth and spore germination.

E. hormaechey was shown to have some PGP activities, such as production of IAA and phosphate solubilization ability that, together or alone, might explain the capacity of this strain to alleviate stress of salt-affected tomato plant. The positive effect of IAA producing PGPR strains on root, shoot growth and dry weight of plants has been reported by several authors (Spaepen *et al.* 2008; Egamberdieva 2005, 2012, 2009; Berg *et al.* 2013). Perhaps the production of IAA by *E. hormaechei* represents a beneficial mechanism that promoted enlargement of root system, enhancing nutrient uptake, and growth of tomato in salinated soil. The phosphate solubilizing bacteria could increase the effectiveness of mineral phosphorus fertilization through solubilizing the phosphorus sources (Mikanova and Novakova 2002). Similar results observed by Gupta *et al.* (2012) that after inoculation of aloe plant with PSB *E. hormaechei*, the soil available P, P uptake and plant growth was increased. In other study potassium solubilizing *E. hormaechei* increased root, shoot growth of okra and also increased K content in plant components (Prajapati *et al.* 2013).

E. hormaechei NUU10 was able to stimulate root and shoot growth of tomato in both non saline and saline soil conditions. The plant growth promotion abilities of other species such as *Enterobacter* sp. on broccoli (Zakria *et al.* 2008), *E. radicincitans* on wheat (Kämpfer *et al.* 2005) and *E. cloacae* on rice (Hassen *et al.* 2007) were also reported. Pereira *et al.* (2010) reported that *E. hormaechei* may improve quality of maize grains obtained at harvest by reducing toxin content.

The bacterial isolate was salt tolerant, and was able to survive in soil, rhizosphere and phyllosphere of tomato plant grown under arid, saline soil condition. According McArthur and Tuckfield (2000), Kummerer (2004) bacteria can quickly adapt to the presence of ecological stressed factors in the ecosystem, acquiring the features that allow them to survive, such as resistance to antibiotics, xenobiotics, etc. Similar observations made by some other authors that the antibiotic resistant bacteria is more fit than its nonresistant counterpart and is therefore able to survive under harsh conditions (Bouma and Lenski, 1988). Zakria et al. (2008) also observed more intense colonization of the broccoli roots by Enterobacter sp. 35 (8.1×106 CFU/ g of fresh weight). According Morales et al. (1996) root colonizing bacteria may reach and utilize a large number of carbon sources and nitrogen compounds as the roots grow.

In conclusion, the results of this study indicated that *Enterobacter hormaechei* NUU10 has the potential to produce different biological active compounds such as cell wall degrading enzymes, IAA and utilize a wide range of carbohydrates as carbon as energy sources. They may positively effect on plant growth and has the ability to colonize and survive in tomato root grown in saline soils with hot summer temperature.

ACKNOWLEDGEMENTS

Research works were supported by the grant received from the Department of Scientific Research at King Saud University, Research Group Project no. RG-1435-014.

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