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Research Article

# Analysis of Cell Surface Properties of *Exiguobacterium aurentiacum* Associated with *Lumbricus terrestris* and its Potency as Biological Inoculants

**Syeda Shazia Bokhari, Aisha Waheed Qurashi*, Roheela Yasmeen*, Fouzia Yasmeen, Nabeela Nayab, Uzma Rafi**

*Department of Biology, Lahore Garrison University Sector C Phase 6 DHA Lahore.*

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**Authors' Contributions**
SSB supervised the research. AWQ co-supervised the research and concieved the idea. RY and AWQ wrote the manuscript. FY and NN perfromed the experiments. RY and UR proofread the manuscript.

**Keywords**
Auto-aggregation, *Exigoubacterium auranticum*, Hydrophobicity, *Lumbricus terrestris*

**Abstract** | Earthworms are called soil engineers as they increase the soil fertility. The study was aimed to see the aggregation potential of microbial communities which are associated with earthworms (*Lumbricus terrestris*) towards adherence and exopolysaccharides productions. Both productions are important parameters of plant growth promoting bacteria. Earthworms, along with soil sample, were collected from different areas of Lahore. Bacteria were isolated, purified and further characterized morphologically, biochemically and at molecular level. Bacteria after isolation were labelled as EPF1 while by molecular confirmation this bacterial strain was named as *Exigoubacterium auranticum*. Optimum bacterial growth response was recorded at pH 7.0, temperature 37°C, LB media and shaking 160 rpm conditions. Cell surface characteristics of *E. auranticum* (EPF1) was determined in terms of bacterial cell surface hydrophobicity and auto-aggregation assay. Highest percentage hydrophobicity values of the strain EPF1 were recorded with toluene (84 %) as compared to other organic solvents such as chloroform (58 %) and xylene (63%). Auto-aggregation response of *E. auranticum* EPF1 was highest at fifth hour of culture incubation. Qualitative analysis of exopolysaccharides (EPS) in congo red supplemented Brain Heart Infusion (BHI) agar medium showed positive results and EPS contents showed higher carbohydrate content as compared to Protein contents. It was found that the microbes associated with earthworm (*L. terrestris)* have significant potential of adherence and can be exploited as a bioinoculant formulation for improving soil fertility.

**Novelty Statement** | The study is novel in its design as it is planned to see the aggregation potential of microbial communities which are associated with earthworms (*Lumbricus terrestris*) for the adherence and exopolysaccharides productions. Both characteristics are important for promoting the growth of plants and soil fertility traits.

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## Introduction

The organisms, which are present in soil, have great influence on plant growth. They enhance organic mineralization in the soil and also have ability to change both physico-chemical properties of soil (Ma *et al*., 2011; Whalen, 2014). Earthworms as soil macrobiotic engineers play an important role in turnover of soil organic material and make the ecosystem functional (Ojha and Devkota, 2014; Lavelle *et al*., 2016). Previously positive impact of earthworms on plant growth and soil quality have been reported (Van-Groenigen *et al*., 2017; Xiao *et al*., 2018). They affect directly or indirectly on the availability of

**Corresponding Author: Aisha Waheed Qurashi and Roheela Yasmeen**
aishawaheedqureshi@lgu.edu.pk, roheelayasmeen@lgu.edu.pk

nutrients in the soil by increasing decomposition of plant residues and by moving soil organic matter. The micro flora in the digestive tract of earthworms makes casts richer with plant nutrients (Xiao *et al*., 2018). The bacteria, which are present in the intestine of earthworms release adhesives substances which are helpful in cementing the casts into water stable aggregates (Munnoli and Bhosle, 2011; Dhakshayani *et al*., 2014). The interactions between earthworms and microbes are of major importance for the decomposition of the organic substances and release of mineral components. Bacterial auto-aggregation phenomenon has been reported as selfish mechanism and offers the cells a competitive advantage resulting in complex microbial communities Nocelli *et al*. (2016). Another parameter that facilitates the cells towards surface colonization is the production of extra polymeric substances that enables the micro-organisms to adhere to different biotic and abiotic surfaces to develop biofilms (Harimawan and Ting, 2016). Along with auto-aggregation and extra polymeric substances release, cell hydrophobicity is another significant parameter that is associated with bacterial adhesiveness and varies within species and surface structures of bacterial cells (Vatsos *et al*., 2001; Krasowska and Sigler, 2014).

Bacterial cell surface hydrophobicity in soil or water involves self-immobilization procedures. The ability of auto-aggregation of bacterial cells increased with the increase of their hydrophobic ability and this aggregation capacity of the cell is proportional to the adhesion abilities (Balakrishna, 2013). Krausova *et al*. (2019) reported no relation in bacterial cell hydrophobicity and its adhesion capacity; however, bacterial auto-aggregation was apparently related with adhesion. Presence of polysaccharides molecules on bacterial cell is related to hydrophilic surfaces (Bauer *et al*., 2013; Habimana *et al*., 2014). Since indigenous earthworms as a natural biological source play important role for soil improvement, this study was aimed to explore the efficacy of earthworm associated bacteria for bacterial cell surface characteristics so that they can be exploited in bio inoculant formulations in the field of agriculture.

## Materials and Methods

*Collection of samples*

Earthworms, along with soil sample, were collected from different areas of Lahore in 2016. Samples were collected in polythene zipper bags which was not tightly packed. Bacteria were isolated by serially diluting the earthworm's skin swabs and finally purified by quadrant streaking on the criteria of gummy and shiny colony characteristics. Colony showing positive results of EPS by qualitative screening and hydrophobicity was finally selected and named as EPF1.

*Bacterial characterization*

Cultural characteristics for isolate was done following Bergey's Manual of Determinative Bacteriology (Murray and Holt, 2005) while biochemical characterization of the bacterial isolate was done by Litmus milk reaction, Citrate utilization, Methyl Red, Vogesproskauer, Catalase test, starch hydrolysis test (Tittsler and Sandholzer, 1936).

Gene sequencing (16S rRNA gene sequencing) of purified bacterial colonies of EPF1 was done using the commercial services of first BASE Laboratories Sdn. Bhd. (Shah Alam, Selangor, Malaysia). The homology of the finally obtained gene sequences were searched in the Gen Bank database (NCBI) using Search Tool (BLAST). Consensus sequences thus obtained were submitted to NCBI GenBank and accession numbers were obtained. For checking the bacterial Phylogeny, phylogenetic tree was constructed using neighbor joining method (Saitou and Nei, 1987) in MEGA 6.0 software with a 1000 bootstrap value.

*Effect of varying physiological conditions (pH and temperatures) on bacterial growth*

Media L-broth (10 ml) Garrett *et al*. (1994) prepared in sterilized test tube was inoculated with a loop full of inoculum from fresh (24 h) culture and incubated at 37°C for 24 h in shaking incubator. After 24 h incubation absorbance was recorded at 600 nm and cell density was adjusted to $10^8$ CFU/ml for using as inoculum. To study the effect of different pHs, test tubes containing sterilized L-Broth adjusted at different pH (pH 6, 7, 8) were inoculated (100 ul) and incubated for 24 h at 37°C. After 24 h of culture incubation, absorbance (OD) of all culture strains were measured at 600 nm and results were recorded. To study the effect of different temperature (4°C, 37°C and 42°C), L broth (10 ml-pH7.0) was inoculated (100ul) and incubated at respective temperatures for 24 h of incubation.

*Hydrophobicity*

This assay was performed and determined (Abdulla *et al*., 2014) using following formula:

$$\% \text{ Hydrophobicity} = (A540 \text{ initial} - A540 \text{ aqueous phase})/A540 \text{ initial}] \times 100$$

*Auto-aggregation*

The Auto-aggregation assaying was performed as described previously (Abdulla *et al*., 2014) at different hourly intervals up to 4 h. Upper suspension (100 μl) was taken and transferred to tube containing phosphate buffer saline (3.9 ml) and absorbance was measured at 600 nm. Auto aggregation was calculated by using following formula:

$$\% \text{ Auto-aggregation} = 1 - (At / A0) \times 100$$


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*EPS production and extraction by* Exiguobacterium aurentiacum *(EPF1)*

Qualitative and quantitative analysis of EPS production was done at varying pHs and media.

*Qualitative test*

Qualitative assessment of Exopolysaccharides (EPS) production was done by using Congo red agar following previous modification (Ferreira *et al*., 2014) except for using 20% glucose instead of sucrose in brain heart infusion medium Mariana *et al*. (2009). Isolates were cultured on plates and incubated for 24 h at 4, 37 and 41°C, respectively. The plates were scored for black colonies for positive result and red colonies for negative results.

*Quantitative analysis under different physiological conditions (media, agitation and pH)*

Quantitative analysis of EPS (exopolysaccharides) production and extraction by bacterial cells was done following De Vuyst *et al*. (1998) and extraction modification by Qurashi and Sabri (2012). EPS was quantified in terms of Carbohydrates (Dubois *et al*., 1956) and Protein contents (Lowry *et al*., 1951). To study the effect of varying media, Exopolysaccharides (EPS) contents were quantified by growing bacterial cultures in different growth media i.e., L-broth and M9 minimal media at shaking (160 rpm) and non-shaking conditions. To study the effect of varying pH (5, 6, 7, 8, 9), bacterial EPS cultures at different pH were incubated and then study the shaking (160 rpm) and non-shaking conditions and extracting EPS from culture supernatant. Dry weight and wet weight of EPS was determined and number of EPS was expressed as mg per ml of bacterial. Protein and glucose contents were determined as mg per gram fresh weight of EPS Qurashi and Sabri (2012).

*Plant-microbe interaction*

Experiment was further proceeded to check the efficacy of EPF1 (*Exiguobacterium aurentiacum*) on plant growth promotion. For this purpose, certified wheat seeds Var. were surface sterilized using disinfectant (0.1 % $HgCl_2$ solution) for 2-3 minutes and followed by repeated rinsing with sterile distilled water. Seeds were inoculated with bacterial cell culture suspension (cfu $10^8$ cells per ml $OD_{600}$=0.5) in sterile water (OD was adjusted to 0.3) for 20 minutes and finally sown on petri plates layered with sterilized moistened cotton and filter paper. For non-salt stressed plates, sterilized distilled water was used. Plates were placed in dark for 3 days for seed germination at room temperature. Watering was done with autoclaved distilled water to avoid moisture loss. After seed germination, seedlings were transferred to light for next seven days. After 10 days of experimental set up, harvesting was done to check plant growth parameters, determined in terms of germination, length cm (shoot length, root length, seedling length) and biomass in grams (fresh weight). To study the effect of salt stress, seeds were also subjected to salt stress, by providing 10 ml of sterile (100 mM) NaCl solution instead of distilled water.

*Statistical analysis*

In all the experiments the data was analyzed statistically following method (Steel and Torrie, 1981). The error bars are represented as standard errors of the mean values in figure.

## Results and Discussion

*Bacterial characterization and growth optimization*

Bacterial colonies were analyzed on agar plates it was found that Strain EPF1 showed orange colored, medium size colonies with mucoidy texture, smooth margins, round shape and flat elevation (Table 1). Strain EPF1 were gram positive, rod shaped bacterial cells showing positive results for capsule staining while negative for spore formation. Biochemical characterization of bacterial isolates were also done and isolated strains showed positive results for catalase, starch hydrolysis, methyl red test while negative results for oxidase, citrate and for VP test. Results of 16 S rRNA nucleotide sequence homology, the isolated EPF1 showed homology with *Exiguobacterium aurentiacum* and gene bank accession number KY435705.1 was acquired (Figure 1). Diversity of microbial communities that exists in soil interacts with other living communities as well Pagano *et al*. (2017). In the present study bacteria associated with earthworms were analysed to study their cell surface characteristics. Bacterial colonies showed bright orange colour and identified on the basis of morphological, biochemical and molecular level as *Exiguobacterium aurentiacum*. Using the gene sequencing services results become reliable for its prompt and trustworthy results of 16S rRNA gene (Figure 1).

**Table 1: Characteristics of bacterial strain EPF1.**

| **Characteristics** | |
|---|---|
| **Morphological characteristics** | |
| Simple staining | Rod |
| Gram | Positive |
| Capsule | Positive |
| Spore | Negative |
| **Cultural characteristics** | |
| Color | Orange |
| Shape | Round |
| Texture | Mucoidy |
| Margin | Smooth |
| Size | Medium |
| Elevation | Flat |
| **Biochemical characteristics** | |
| Catalase | Positive |
| Oxidase | Negative |
| VP | Negative |
| Methyl Red | Positive |
| Citrate | Negative |
| Starch | Positive |



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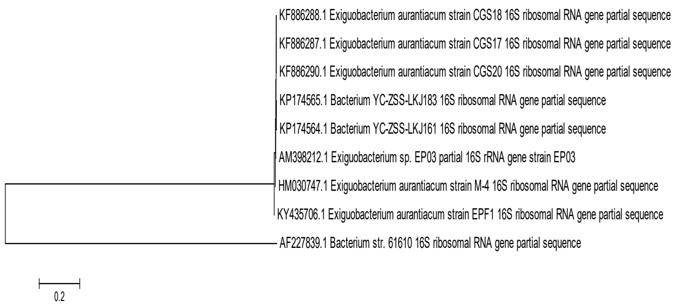


**Figure 1: Phylogenetic tree by neighbor joining method showing evolutionary similarities of this strain EPF1 with related taxa.**

Effect of varying physiological parameters (pHs, temperatures, agitation and media) on bacterial growth was recorded and maximum cell densities were seen at pH 7, temperature 37°C, LB and shaking (160 rpm) conditions (Figure 2). pH along with different environmental factors was significant in formulating the shape of soil microbial communities Cho *et al.* (2016). Different environmental factors like temperature, pH and nutrient availability have been reported to influence the growth of bacteria causing it to spoilage of food (Sumner and Jenson, 2011). Broad range of pH and temperature has been reported for the growth of *Exiguobacterium* species in a recent study Kasana and Pandey (2017).

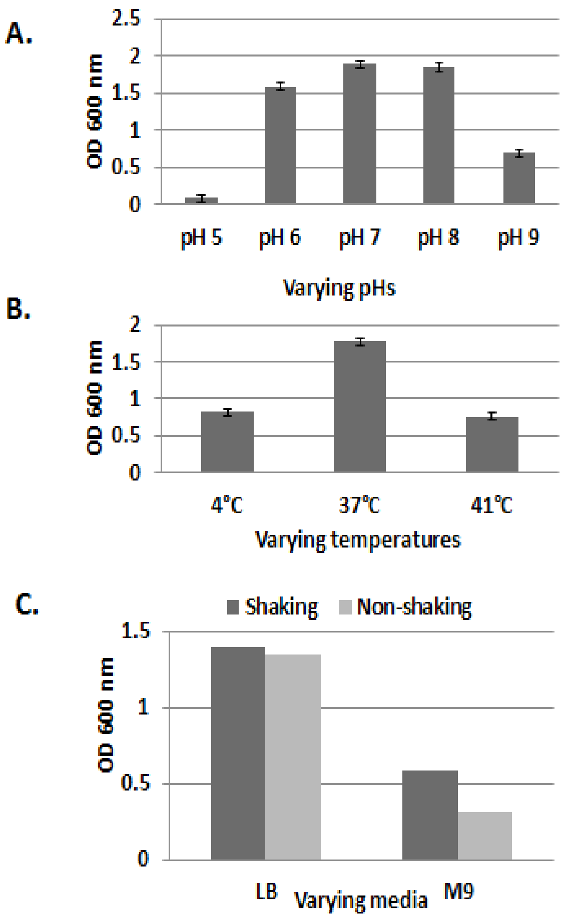


**Figure 2: Effect of varying pH, temperature, media and aeration on the growth of strain EPF1.**

*Hydrophobicity, auto aggregation and EPS production in* Exiguobacterium aurentiacum

Hydrophobicity of isolates were checked and percentages for the tested isolates were found between 84 %, 58 % and 63% with toluene, chloroform, and xylene respectively. EPF1 showed highest affinity toward toluene as compared to rest of organic solvents (Figure 3). A gradual increase in auto-aggregation percentages recorded at hourly interval ranged between 96 % to 99 %. For strain EPF1, after 5 h of culture incubation, highest aggregation for strain EPF1 (99%) was recorded (Figure 4). The higher hydrophobicity of cell is an important characteristic that actually results in attachment of bacterial cells to biotic and abiotic surfaces and ultimately more bacterial cell clumping (Lather *et al.*, 2016). Higher adhesion to chloroform also exhibited higher auto-aggregation of bacterial strains (Lather *et al.*, 2016). Higher cell surface hydrophobicity was recorded with chloroform as compared to other solvents and reported to play an important role in the cell attachment to different surfaces based on different forces like Brownian movement or van der Waals forces etc. (Van Loosdrecht *et al.*, 1990). It has been reported that the distance between cells to surfaces and hydrophobicity of the surfaces, contribute towards colonization of bacteria at surface (Olszewska, 2013). It has been generally stated that lower the degree of microbial cells hydrophobicity then also lowers the adhesive ability (Van Loosdrecht *et al.*, 1990). The concept although still unclear due to many previous reports (Basson *et al.*, 2008; Di *et al.*, 2007), however, many probiotic bacterial species have been reported to be dependent on the hydrophobic characteristics for attachment to epithelial linings (Tareb *et al.*, 2013). Bacterial cell surface hydrophobicity is also determined by the composition of the cell wall (Clarke *et al.*, 2007).

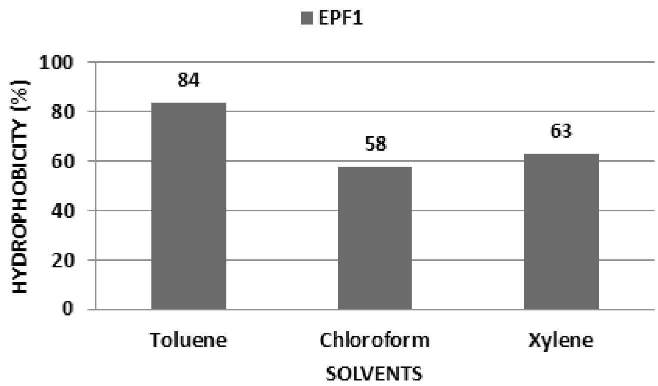


**Figure 3: Hydrophobicity response of EPF1 towards organic solvents (toluene, Chloroform, and xylene).**

Results of the qualitative test showed that EPF1 strain grows at BHI agar plates and light brown colored colonies appeared (Figure 5) that considered as a positive result. EPS was quantified for determination of proteins and carbohydrate content. The analysis showed that protein


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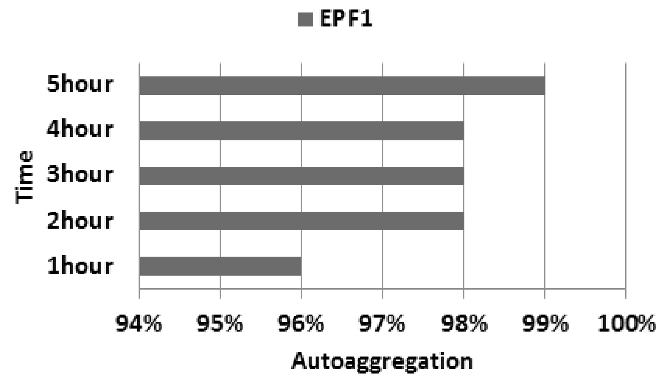


**Figure 4: The observed gradual increase in auto aggregation response in EPF1 till 5th hour.**

**Figure 5: Qualitative test for EPS: Light brown colored colonies as positive result.**

content of EPS was 27 % higher in LB as compared to M9 at shaking condition. However, at non-shaking conditions 26 % higher protein contents were recorded in M9 as compared to LB. The carbohydrate content was 57 % higher in LB as compared to M9 at shaking condition while it was 10 % higher in M9 as compared to LB at non-shaking condition. At varying pHs, protein content was 33 % higher at pH-7.0 as compared to pH-5 and 39 % higher as compared to pH-9 while glucose content was 30 % higher at pH-7 as compared to pH-5 and 50 % higher as compared to pH-9 (Figure 6). Cell adhesion and EPS production are an irreversible process and results in biofilm formation. EPS results in entrapment of bacterial cells and cell multiplication (Myszka and Czaczyk, 2011). Adhesive properties significantly contribute towards the bio- film formation (Garrett *et al*., 2008) and temperature contributes significantly in biofilm formation (Di Bonaventura *et al*., 2008). Production of EPS in bacteria has been reported as a safe process to grow under stress as well as to adhere with different abiotic surfaces. It is also beneficial for the reservation of nutrients and formation of a biofilm under harsh stress conditions (Chimileski *et al*., 2014; Lü *et al*., 2017). The EPS contributes towards biofilm ecology and adhesion (Banat *et al*., 2011) and emulsification of hydrocarbon and finally results in biodegradation (Gutierrez *et al*., 2013). Microbes produce extracellular polysaccharides that facilitate the attachment of bacterial cells towards different surfaces and results in biofilm communities. Microbes produce EPS as an essential criteria when being exposed to different environmental stress condition or this phenomenon may be produced excessive products as a metabolic waste that ensure its survival (Decho *et al*., 2017). Bacterial auto-aggregation in present study increased with the passage of time and reached at its peak at fifth hour. Bacterial cell auto-aggregation has been related to inter and intra-species interactions of bacteria as well as towards different surfaces. Ultimately this increased rate of bacterial cell auto-aggregation results in biofilm development as reported in previous reports for isolate MKK4 (Ray *et al*., 2017). Biofilm formation in bacterial cells enable the cells to cope environmental stress factors. The phenomenon of motility, auto-aggregation, cell hydrophobicity, and EPS have been very important to report for pathogenic strain of *H. pylori* helping the cells to cope with their survival in extreme conditions (Attaran and Falsafi, 2017).

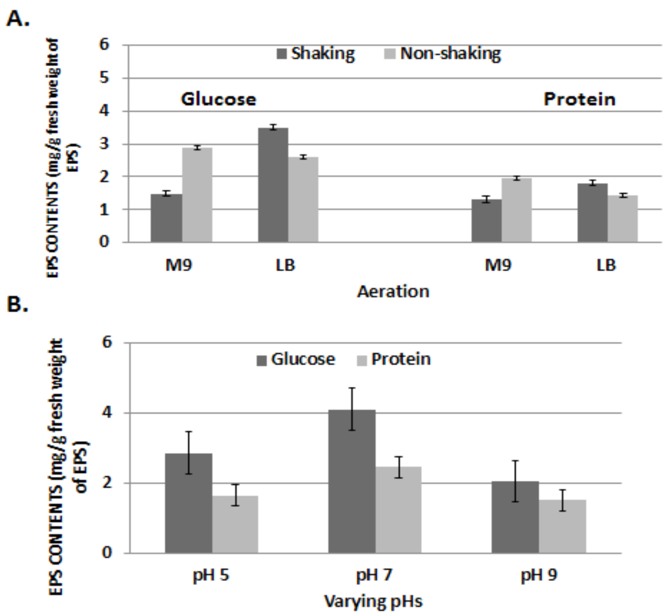


**Figure 6: EPS Quantification under different physiological conditions (media, agitation and pH) of culture media.**

*Plant-microbe interaction*

Salinity resulted in reduced growth of plants in terms of germination, length and biomass. On the basis of germination, non-inoculated control plants in the absence of salinity showed 87.5 % germination however, in the presence of 100 mM salt stress, germination was reduced to 35 % (Figure 7A). The germination was augmented by 5 % in inoculated seedlings, when compared to non-treated control seedlings at 100 mM salt stress. In non-inoculated seedlings under 100 mM NaCl stress showed 35% reduction in root length. It was recorded that roots showed more growth in non-stressed plates. Similarly, 47% reduction in shoots length was recorded under salt stress. It was recorded that; seedlings length showed


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more growth in the absence of salinity than salt treated plants. Inoculation significantly improved length, weight and biochemical parameters i.e., the protein and glucose content as compared to non-inoculated treatments (Figures 7B, 8A, 8B). Significant reduction in plant growth under salt stress has been described (Alom *et al*., 2016; Acemi *et al*., 2017), however, bacterial inoculation significantly improved the plant growth under salt stress. Many previous and recent reports state the potential of different species of Exiguobacterium in plant growth (Montañez *et al*., 2012; Kasana and Pandey, 2017).

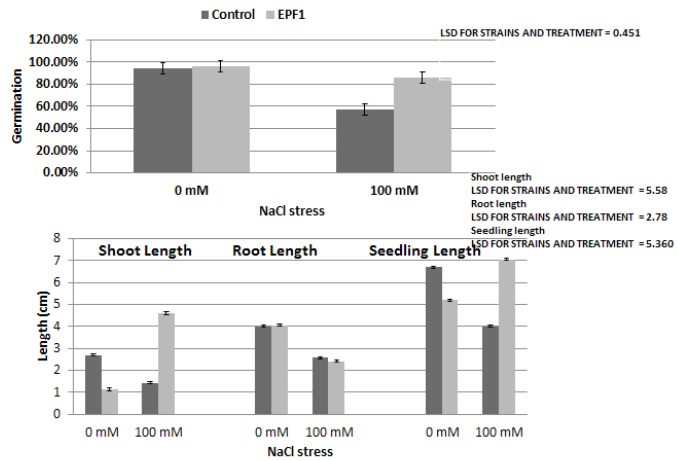


**Figure 7: Growth parameters for plant A: Percentage germination of seedlings. B: Length parameters (cm) of seedlings.**

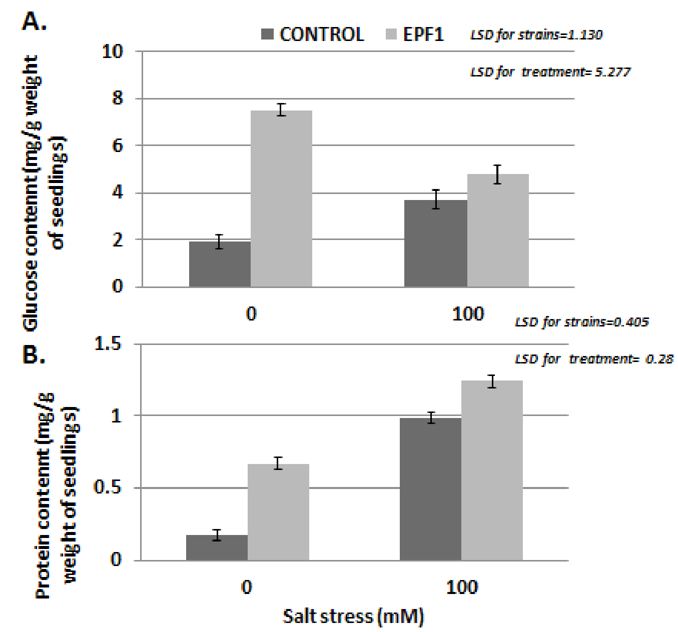


**Figure 8: Biochemical parameters for plant growth A: Glucose content mg $g^{-1}$ weight of seedlings. B: Protein content mg $g^{-1}$ weight of seedlings.**

## Conclusions and Recommendations

It was concluded *Exiguobacterium aurentiacum* was molecular identified strain that isolated from earthworm's associated soil. *Exiguobacterium aurentiacum* was noticed as an efficient strain with significant cell surface characteristics. This strain can be exploited as bioinoculant and may open new avenues in formulation of bio inoculants. In future, further field research work is needed to utilize this bacterial strain as a bio inoculant. This will be helpful in improving plant growth subsequent enhancement of yield for agricultural crops.

*Conflict of interest*

The authors have declared no conflict of interest.

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