INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
21–0143/2021/26–2–309–316
DOI: 10.17957/IJAB/15.1839
http://www.fspublishers.org

Full Length Article

Screening of Bacteria for Biosurfactants, Exopolysaccharides and Biofilms and their Impact on Growth Stimulation of *Zea mays* Grown under Petrol Stress

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Received 04 February 2021; Accepted 27 April 2021; Published 10 July 2021

Abstract

Environmental stress imposed by petroleum hydrocarbons can compensate by use of auxin-producing bacteria having potential for biosurfactants production, to assist improved plants' growth in petrol contaminated areas. In the present work, four auxin-producing bacteria were screened for biosurfactants, exopolysaccharides (EPS) and biofilms production capability. We hypothesized that *Enterobacter* sp. (A5C) was the most efficient strain with respect to biosurfactant production and can accumulate EPS as well as biofilms. This strain was attributed to exhibit emulsification index, percentage of hydrophobicity and percentage of hydrocarbon degradation more than 50%. Also, it produced 9.27 mg of EPS per 100 mL of culture while Fourier transform infrared spectroscopy (FTIR) confirmed the presence of alcoholic and carboxylic groups, ketone and sugars in it. Results of *in vitro* plant microbe interaction assay revealed its potential to stimulate the growth of *Zea mays* L. plants under 1 and 2% of petrol stress by improving physio-chemical attributes of treated plants, over control. Thus, it is concluded that the test organism i.e., *Enterobacter* sp. (A5C) might be involved in developing bacterial community (EPS and biofilms) that helped to colonize the bacteria to the plant roots and soil particles that ultimately encouraged the more access to nutrients and protection of plant roots from toxins in soil ecosystem. © 2021 Friends Science Publishers

Keywords: *Enterobacter* sp.; Exopolysaccharides; FTIR; Biofilm; *Zea mays*

Introduction

Worldwide increasing hydrocarbons and petrol pollution in soil ecosystem reduces the potentiality of plants to grow in petrol contaminated areas. Inadvertent release of petroleum products and leakage of petroleum hydrocarbons from oil spills causing damage to soil and ultimate retardation in cultivation of crop plants in such areas. To meet this challenge, synthetic approaches are being used but they are causing severe threat to the environment. Thus, the usage of biosurfactants is considered as effective way to reduce hydrocarbons contamination from soil ecosystem (Joy *et al.* 2017). It has been reported that petroleum hydrocarbons are deemed as serious environmental threat not only for soil community but plant community as well. Owing to the sessile nature of plants, to escape out from such stresses is not possible for plants. Traditional and synthetic means for remediation of petroleum pollutants from soil ecosystem are ecologically damaging, thus, use of biological approaches i.e., microbes can compensate this issue (Gull *et al.* 2019; Xia *et al.* 2020).

According to Pendse and Aruna (2018), bacteria are reported as biosurfactant and bioemulsifier producers and considered as natural tool to remediate petrol contamination. Biosurfactants are bacterial metabolites that have both hydrophobic and hydrophilic domains and tend to accumulate at the interface of hydrocarbons (HC). They can tremendously decrease the surface tension and interfacial tension of growing media. These biosurfactants may contain fatty acids, lipopeptides, lipids, polysaccharides, proteins and some other compounds having amino, phosphate and carboxyl groups (Nishanthi *et al.* 2010; Joy *et al.* 2017).

On the other side, bacteria produce extracellular components along with biosurfactants i.e., plant growth promoting agents, exopolysaccharides (EPS) and biofilms. Bacterial EPS are considered as biomolecules secreted by bacteria having diverse functions like environmental safety, adherence to biotic or abiotic surfaces and cellular interactions (Escárcega-González *et al.* 2018). Bacterial colonization either with biotic surfaces or abiotic materials provide them several survival strategies like improved access to nutrients, preservation of extracellular enzymatic activities and protection from toxins etc. EPS play an important role in the attachment of bacteria to different substrates that ultimately make biofilms. These extracellular EPS can be used as gelling and thickening agents, flocculants, stabilizers,

adhesives and emulsifying agents (Kumar *et al.* 2011). According to Kırmusaoğlu (2019), EPS help attach the bacteria to biotic or abiotic surfaces and after attachment, bacteria are getting aggregated via cell to cell adhesion and this aggregation continues till the biofilms become mature.

The present study was aimed at screening of auxin producing rhizospheric bacteria for the production of biosurfactants, EPS and biofilms. The best strain was further used to assess its potential for growth stimulation in maize (*Zea mays* L.) plants after bacterial inoculation under petrol stress.

Materials and Methods

Bacterial cultural conditions

The present work dealt with the evaluation of bacterial attributes i.e., biosurfactant production potential, EPS and biofilm production. For this purpose, four already isolated and identified rhizospheric bacterial strains were used, which were previously reported for auxin production i.e., [A5C = *Enterobacter* sp. (HQ179967)], [A9G = *Enterobacter cloacae* (HQ202888)], A11E [*Enterobacter* sp. (HQ533177)] and A13G (*Exiguobacterium* sp. (HQ202890)] (Ahmed and Hasnain 2020). Isolates were maintained on LB-agar media by incubating at 37^{o}C for 24 h and further screened for biosurfactants production, EPS and biofilm synthesis.

Bacterial profiling for biosurfactant production potential

Isolates were observed for biosurfactant production potentiality via emulsification index test, bacterial adhesion to hydrocarbons (BATH) assay, penetration assay, hydrocarbon overlay agar method and hydrocarbon (HC) degradation assay. The *n*-hexane was used as HC for all assays.

Emulsification index test was done following Shoeb *et al.* (2015). For this, cell-free broth (1.5 mL) was mixed with equal amount of HC, vortexed for two min and then allowed to stand for 24 h for developing emulsion layer. Emulsification index (E_{24}) was determined following the equation: E_{24} = Emulsion layer height/total height of solution × 100.

BATH assay was performed following the method of Thavasi *et al.* (2011). Bacterial cell pellets were washed with sterilized water and resuspended in 25 mM phosphate buffer and diluted to OD ~0.5 at 600 nm with the same buffer. After that, 2 mL of cells were mixed with 100 µL of HC, vortexed for 3 min and allowed to stand for one h. Decrease in absorbance was recorded to calculate the percentage of cells adherence to HC using the following formula:

$$\text{Percentage adherence to HC} = 1-(OD_{\text{aqueous phase}}/OD_{\text{initial cell suspension}}) \times 100.$$

Penetration assay was done following Walter *et al.* (2010). For this assay, hydrophilic phase was prepared by mixing same amount of cell-free bacterial supernatant and red staining solution while hydrophobic paste was prepared by mixing silica gel in HC (1 mL). Reaction mixture was primed by adding hydrophilic and hydrophobic mixtures in test tubes separated by 100 µL of HC. Results were recorded after fifteen min by observing the color change.

Hydrocarbon overlay agar method was done following Shoeb *et al.* (2015). For which, bacterial spot inoculation was done on HC coated L-agar plates and appearance of halo areas were observed qualitatively after 48 h of incubation. HC degradation assay was performed following 2, 6-dichlorophenol indophenol (DCPIP) method using 1 and 2% of HC as sole carbon source following Habib *et al.* (2017).

Bacterial profiling for EPS detection

Bacterial EPS biosynthesis was examined following Welman and Maddox (2003) and Mu'minah *et al.* (2015) via mucoidy, ropiness and acetoin production assays qualitatively. Bacteria were grown using the growth media ATCC no. 14 and colonies forming thick slime (mucoid) were noted. Bacterial ropiness was observed using inoculation loop while acetoin production was checked via Voges-proskauer test. Quantitatively, bacteria were grown in 100 mL nutrient agar media amended with sucrose as carbon source and incubated for three days. Cells were harvested via centrifugation for 20 min at 10,000 rpm and then twofold isopropanol was added into it following incubating at 4°C overnight. Precipitates were then collected by centrifugation and pellets were dried at 100°C. Weight of dried EPS was recorded. Fourier transform infrared spectroscopy (FTIR) was done for extracted EPS and IR spectra were obtained in the range of 650 to 4000 cm^{-1} of absorption using 32 scans.

Bacterial profiling for biofilm production

For bacterial biofilm assessment, isolates were inoculated in liquid Bushnell Haas (BH) minimal media supplemented with 0.2% dextrose and 0.5% tyrptone and after 72 h of incubation, cultures were discarded and 1% crystal violet solution was poured in the test tubes for staining purpose. After 15 to 20 min, excess stain was washed out and biomass of attached bacterial cells (biofilm) was quantified by solubilization of dye in 2 mL of 95% ethanol. Absorbance was recorded at 600 nm.

In vitro plant microbe interaction assay

On the basis of bacterial screening assays, the most efficient bacteria were further used for plant growth observation under 1 and 2% of petrol stress on the basis of soil dry weight. Petrol (Gasoline) used for the current study was in the liquid state, colorless to pale brown, molecular weight 108 g/mol, density 0.7–0.8 g/cm^3 and insoluble in water.

Maize was used as test plant and the experiment was conducted in March, 2020. Certified seeds (hybrid DK-6714, Monsanto, USA) were procured from Punjab Seed Corporation, Lahore, Pakistan and were surface sterilized and inoculated with bacterial cultures after adjusting optical densities at 600 nm to ~10^6–10^7 CFU/mL for one h. Seven seeds were sown per pot containing 165 g of sterilized sieved soil and experiment was done in triplicates for each treatment i.e., control (without petrol stress and bacterial treatment), negative control (with 1 and 2% petrol stress without bacterial treatment), bacterial treatment (without petrol stress) and bacterial treatment with 1 and 2% petrol. Petrol treatment was given to soil at seedling stage of maize plants, after three days of germination. Pots were placed in light (10 Klux, 16 h duration) at 25±2^{o}C and after 24 days of growth period, seedlings were harvested and analyzed for growth attributes i.e., height of plant, fresh weight, root length and leaves number while for biochemical analysis, plants were observed for protein content, auxin content, chlorophyll content, total soluble sugars (TSS), total free amino acids, free proline and glycine betaine content. Protein content was determined following Lowry *et al.* (1951), 1 g of crushed plant material was mixed with 4 mL of phosphate buffer following centrifugation at 10,000 rpm. Reaction mixture was prepared by adding 0.4 mL extract, 2 mL Folin's mixture 0.2 mL of Folin's ciocalteu's reagent, then kept at room temperature for 45 min to build out the color and absorbance was taken at 750 nm. Standard curve of bovine serum albumin (BSA) was used to interpret the results.

Auxin content was observed following Mahadevan (1984), for which 0.5 g plant material was mixed with 1 mL diethyl ether and kept at 4^{o}C for 2–3 h. Extract was again mixed with 0.5 mL diethyl ether and then 500 µL of 5% sodium hydrogen carbonate was added and shaken well. Bicarbonate layer was acidified with 6N HCl to pH 3. Then, 2 mL of salkowski reagent was added following incubation in dark for 30 min. Absorbance was read at 535 nm. Standard curve of auxin was used to interpret the results.

Chlorophyll content was determined following Lichtenthaler and Wellburn (1983) for which 1 g of squeezed plant material was soaked in 10 mL of 80% acetone solution following overnight incubation in dark. Absorbance was recorded at 663, 646 and 470 nm to determine the concentrations of chlorophyll 'a', chlorophyll 'b' and carotenoid contents.

Total soluble sugars (TSS) were estimated by phenol-sulphuric acid method following Tiwari *et al.* (2017). Similarly, total free amino acid content was determined following Khanna *et al.* (2019) for which 0.1 g plant material was homogenized in 80% alcohol followed by incubation for fifteen min using water bath. Then, 0.2 mL supernatant was mixed with 3.8 mL of ninhydrin reagent and again boiled using water bath. Mixture was allowed to cool till the development of purplish color and absorbance was recorded at 570 nm. Results were inferred by using standard curve of leucine.

Proline content was detected following Karthik *et al.* (2016). Briefly, 0.5 g plant material was homogenized in 3% sulphosalicylic acid. Filtrate was mixed in acid ninhydrin reagent and glacial acetic acid in 1:1:1 which was then heated using water bath at 100^{o}C for 1 h followed by placing on ice bath for twenty min. Further, 1.5 mL of toluene was added and optical density (OD) was measured at 520 nm. Standard curve of proline was used to interpret the results.

Glycine betaine accumulation was recorded following Sadak *et al.* (2019). For this, 0.5 g dried plant sample was homogenized in 5 mL distilled water containing 0.05% toluene for 24 h using shaker. Reaction mixture was prepared by mixing 0.5 mL filtrate, 1 mL 2N hydrochloric acid and 0.1 mL potassium iodide solution. Tubes were chilled, shaken and gently mixed with 2 mL ice-cold distilled water and then with 1, 2-dichloroethane. Two layers were formed and absorbance of bottom pink layer was recorded at 365 nm. Standard curve of glycine betaine was used for inferring the results.

Statistical analysis

Three replicates were taken for determining the biosurfactant production potential, EPS production and biofilm formation by the bacterial isolates. Also, for *in vitro* plant microbe interaction assay twenty four replicates were taken for observing various parameters. The above replicates were used to statistically analyze the data using statistical package, SPSS (version; 16.0). Duncan's multiple range test (post hoc test) was applied to determine the significant differences among means of the treatments at 5% level of significance (P = 0.05).

Results

Bacterial profiling for biosurfactant production potential

Isolates were examined for biosurfactant production potential. Emulsification index test indicated 54.9±2.6, 53.8±2.1, 53.0±2.7 and 50.9±3.1% emulsification by isolates *Enterobacter* sp. (A5C), *Exiguobacterium* sp. (A13G), *Enterobacter* sp. (A11E) and *E. cloacae* (A9G) using *n*-hexane as hydrophobic substrate respectively, while data for BATH assay showed that percentage of hydrophobicity of isolates *Enterobacter* sp. (A5C), *E. cloacae* (A9G), *Exiguobacterium* sp. (A13G) and *Enterobacter* sp. (A11E) with HC was 64.7±5.1, 56.2±3.5, 54.5±5.1 and 46.2±6.6% respectively (Fig. 1A). Penetration assay revealed that all isolates were positive for this assay showing color change from clear red to cloudy white (Fig. 1B). Positive test considered when silica entered from hydrophobic paste to hydrophilic phase by breaking the barrier of hydrocarbon in between both phases. This is because of production of biosurfactants. Likewise, HC over

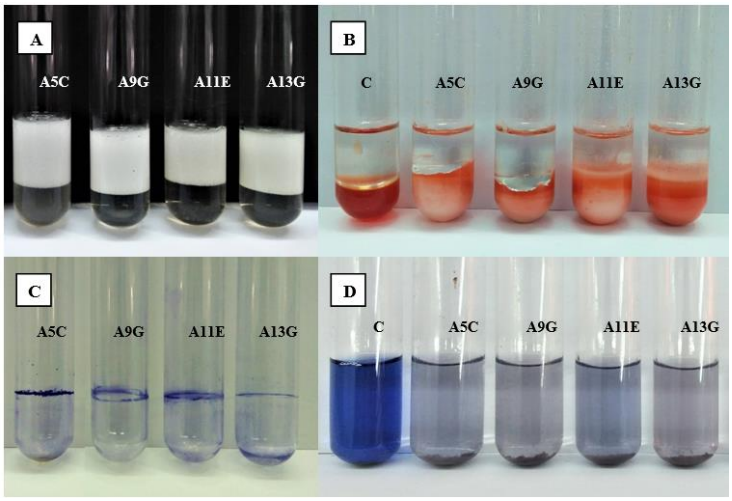

Fig. 1: Emulsification index test (A), penetration assay (B), biofilm production test (C) and hydrocarbon (HC) degradation test using 2% *n*-hexane [C = Control, A5C = *Enterobacter* sp., A11E = *Enterobacter* sp., A9G = *E. cloacae* and A13G = *Exiguobacterium* sp.]

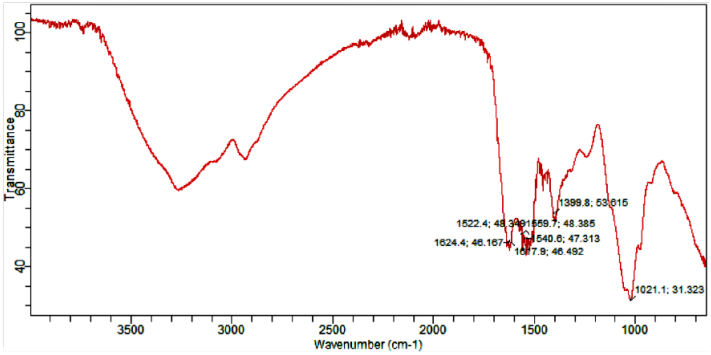

Fig. 2: IR spectra of EPS extracted from *Enterobacter* sp. (A5C)

lay agar method showed potentiality of isolates to make halo areas around bacterial colonies. *Enterobacter* sp. (A5C) exhibited clear halo around bacterial colony, while rest of the isolates were poor in making halo zones. Further, HC degradation assay indicated that with 1% HC, isolates *E. cloacae* (A9G), *Exiguobacterium* sp. (A13G), *Enterobacter* sp. (A11E) and *Enterobacter* sp. (A5C) showed 78.4±1.8, 75.7±2.3, 75.5±1.2 and 73.0±1.4% degradation potential, respectively whereas, 73.8±2.6, 69.6±0.7, 66.8±1.7 and 56.5±3.1% degradation potential was shown by isolates *E. cloacae* (A9G), *Exiguobacterium* sp. (A13G), *Enterobacter* sp. (A5C) and *Enterobacter* sp. (A11E) respectively with 2% HC (Fig. 1D).

Bacterial profiling for EPS detection

Isolates *Enterobacter* sp. (A5C) and *Enterobacter* sp. (A11E) were positive for EPS production. These isolates showed thick slimy colonies (mucoid) and ability of ropiness indicating cohesive and sticky appearance due to presence of EPS while Voges-proskauer test for acetoin production was positive for isolates *Enterobacter* sp. (A5C), *E. cloacae* (A9G) and *Enterobacter* sp. (A11E). Isolates *Enterobacter* sp. (A5C) and *Enterobacter* sp. (A11E) produced 9.27 and 4.13 mg of EPS per 100 mL of culture. On the basis of these assays, IR spectra was obtained through FTIR for the most efficient EPS producing bacteria i.e., *Enterobacter* sp. (A5C). IR spectra showed that peak at 1522.4 cm^{-1} corresponded to strong N–O stretching showing nitro compound while peaks at 1624.4 and 1617.9 cm^{-1} attributed to C=C stretching with medium absorption intensity indicating conjugated alkene and strong C=C stretching indicating α, β-unsaturated ketone respectively. Also, absorption at 1540.6 cm^{-1} was due to stretching vibration of N–O group with strong absorption intensity, whereas, peaks at 1399.8 and 1021.1 cm^{-1} was assigned to O–H bending carboxylic acids with medium absorption intensity which is a characteristic of carbohydrate ring and C–N stretching cyanide group respectively (Fig. 2).

Bacterial profiling for biofilm production

All isolates were positive for biofilm production. A visible film layer the wall of test tubes was observed visually while

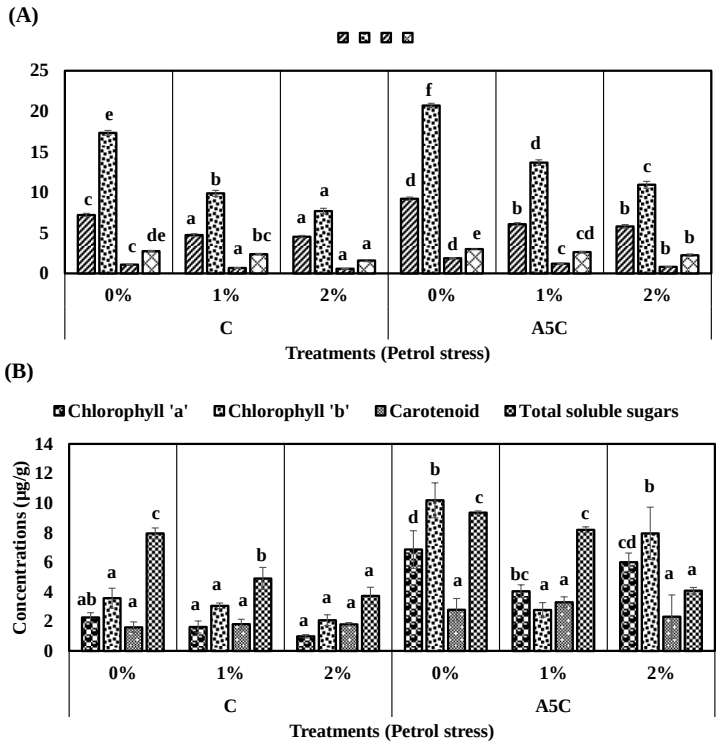

Fig. 3: Effect of bacterial treatment with and without petrol stress (0, 1 and 2%) on height of plant, root length, fresh weight and leaves number (A) and chlorophyll 'a', chlorophyll 'b', carotenoid content and total soluble sugars (B) of *Zea mays*. Data represent mean of twenty four replicates. Different letters indicate significant differences between treatments using Duncan's multiple range test (P = 0.05) [Control – C, Bacterial strain: *Enterobacter* sp. (A5C)]

biofilms was quantified spectrophotometrically (Fig. 1C). It was found that isolates *Enterobacter* sp. (A5C), *Enterobacter* sp. (A11E), *E. cloacae* (A9G) and *Exiguobacterium* sp. (A13G) exhibited 0.51±0.05, 0.40±0.03, 0.35±0.04 and 0.31±0.04 absorbance at 600 nm, respectively.

In vitro plant microbe interaction assay

On the basis of screening profile for biosurfactants, EPS and biofilm production potential, the best isolate i.e., *Enterobacter* sp. (A5C) was selected for *in vitro* plant microbe interaction assay. It was found that 28.1, 19.4, 73.0 and 9.0% increase in height of plant, root length, fresh weight and leaf number was counted respectively, over control when grown without petrol stress. In the presence of 1% petrol stress, 28.7, 38.3, 80.3 and 10.5% increment while in the presence of 2% petrol stress, 28.1, 42.1, 43.0 and 42.1% increase in height of plant, root length, fresh weight and leaves number was noted respectively, when compared with respective control plants (Fig. 3A).

Among the biochemical attributes, significant increase in protein content was recorded up to 40.7% in bacterially treated plants without petrol stress, 60.2 and 48.8% increase in bacterial inoculated plants in the presence of 1 and 2% petrol stress respectively, over respective control treatments. Similarly, prominent rise in chlorophyll 'a', chlorophyll 'b' and carotenoid content of inoculated plants was noted up to 203.0, 185.5 and 74.2% respectively, over control. Under 1% petrol stress, 148.4 and 82.3% increase in chlorophyll 'a' and carotenoid content while 9.1% decrease in chlorophyll 'b' content was detected, over respective control. On the contrary, 508.0, 282.1 and 28.3% increment was observed in chlorophyll 'a', chlorophyll 'b' and carotenoid content of treated plants, respectively over respective control treatment. Similarly, TSS, free aminoacids, proline and glycine betaine tended to increase upto 17.6, 96.1, 51.5 and 9.0% respectively over control, when plants were grown without petrol stress. On the contrary, under 1% petrol stress, TSS, total free aminoacids, proline and glycine betaine contents were increased up to 67.2, 113.0, 97.4 and 16.1% while under 2% petrol stress, 9.1, 113.2, 87.2 and 3.54% increment was recorded in bacterially treated plants respectively, over respective control plants (Fig. 3B and 4).

Discussion

The present study signifies plant-bacterial partnership by secreting biosurfactants, EPS and biofilms to remediate petrol contamination from the rhizosphere of maize plants.

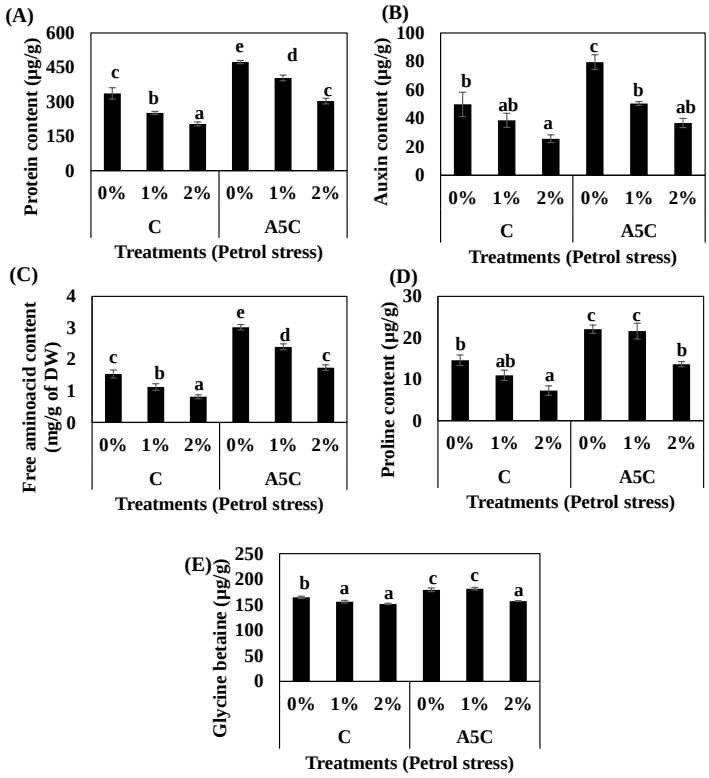

Fig. 4: Effect of bacterial treatment with and without petrol stress (0, 1 and 2%) on protein content (A), auxin content (B), free aminoacid content (C), proline (D) and glycine betaine content (E) of *Zea mays*. Data represent mean of twenty four replicates. Different letters indicate significant differences between treatments using Duncan's multiple range test (P = 0.05) [Control – C, Bacterial strain: *Enterobacter* sp. (A5C)]

Screening profile of bacterial strains provided collective data of the most efficient biosurfactant and EPS producing as well as biofilm making bacterial strain i.e., *Enterobacter* sp. (A5C), for its surviving potential in petrol contaminated soil and bacterial-assisted plant microbe interaction. Screening results in this study suggested the ability of selected bacteria to enhance bioavailability of hydrocarbons to bacterial cells as they ultimately enhanced the rate of biodegradation and consumption of HC. These characteristics of bacteria were verified in the studies of Joy *et al.* (2017) and Ashitha *et al.* (2020).

On the other side, EPS and biofilm synthesizing ability was also checked to analyze bacterial behavior towards surface adhesions or colonizing potential with plant roots. FTIR analysis was made to determine the functional groups that exclusively confirmed the presence of alcoholic and carboxylic groups in extracted EPS. Kumar *et al.* (2011) have reported the presence of polysaccharide group in the EPS extracted from biofilm forming bacteria via FTIR analysis. Also, Qurashi and Sabri (2012) studied biofilm formation and EPS accumulation by salt-tolerant bacteria i.e., *Halomonas variabilis (HT1)* and *Planococcus rifietoensis* (RT4) and observed plant (*Cicer arietinum*) growth enhancement and improved soil aggregation through these bacterial application. In addition, Mostefaoui *et al.* (2014) screened thirty EPS producing bacterial strains on the basis of mucilaginous colony. Our data is in excellent agreement of all the above mentioned studies.

Enterobacter sp. (A5C) was further used in *in vitro* plant-microbe interaction assay due to its potential for biosurfactants, EPS and biofilms production. Significant increase in growth and biochemical parameters was observed in bacterially inoculated plants when grown without and with 1 and 2% of petrol stress (Fig. 5). This increase in physiological and biochemical attributes of maize plants might be due to the bacterial ability to produce biosurfactants that accumulate at soil-petrol interface due to its amphiphilic nature.

Khanna *et al.* (2019) reported increased level of secondary metabolites, proline, total soluble sugars, free amino acid and glycine betaine content in *Solanum lycopersicum* grown under cadmium stress using *Pseudomonas aeruginosa* and *Burkholderia gladioli* strains. Also, Sadak *et al.* (2019) conducted a study that highlighted the increased level of biochemical parameters in *Chenopodium quinoa* plants when foliar application of trehalose was used. Similarly, Habib *et al.* (2019) have also reported a significant increase in protein and chlorophyll

Fig. 5: Effect of *Enterobacter* sp. (A5C) inoculation on the growth of maize with and without petrol stress (0, 1 and 2%) [A =non-inoculated control without bacterial treatment, B = non-inoculated control with 1% petrol stress, C = non-inoculated control with 2% petrol stress, D = bacterial treatment *Enterobacter* sp. (A5C), E = bacterial treatment *Enterobacter* sp. (A5C) + 1% petrol stress and F = bacterial treatment *Enterobacter* sp. (A5C) + 2% petrol stress]

content under chromium stress using bacterial application. Similarly, Naseem and Bano (2014) conducted a study in which they selected *Proteus penneri* (Pp1), *P. aeruginosa* (Pa2), and *Alcaligenes faecalis* (AF3) strains as EPS-producing bacteria on the basis of mucoid appearance of colonies. Authors observed tremendous increase in plant biomass production, root and shoot lengths, TSS, protein and proline content of bacterially treated maize plants under drought stress conditions. Increase in all studied parameters even in the presence of petrol might be due to the fact that isolate *Enterobacter* sp. (A5C) which was auxin-producing, biosurfactant and EPS producing and also possessed biofilm making ability, might have initiated biofilms on plant roots by secreting EPS. It might be possible that these biofilms colonized the roots and by synthesizing auxin and biosurfactants, it would have stimulated plant growth in the presence of petrol stress. Thus, nutrient uptake, solubilization of minerals and protection from toxins or petrol pollutants made the bacterially treated plants able to grow well in that petrol stressed conditions.

Conclusion

Present study revealed the multitrait potential of auxin producing *Enterobacter* sp. (A5C) as biosurfactant production vis-à-vis EPS and biofilm synthesizing ability. The bacterial treatments have shown significant improvement in growth and biochemical attributes of treated maize plants under petrol stress owing to the presence of above mentioned multitrait potential. It may be concluded that bacteria might have produced extracellular polymeric substances (EPS) that constructed biofilm matrix, which ultimately promoted root colonization that has been effective in growth enhancement due to improved nutrient uptake and water absorption. The same explanation was also reported by Qurashi and Sabri (2012) who worked on biofilm producing bacteria under salt stress.

Acknowledgements

This work was supported by University of the Punjab, Lahore 54590, Pakistan.

Author Contributions

Both authors contributed to the study conception and design. AA conceived and designed the research study. Material preparation, data collection and analysis were performed by SH. The first draft of the manuscript was written by SH. AA commented on previous versions of the manuscript, read and approved the final manuscript.

Conflict of Interest

Authors declare no conflict of interests to disclose.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable in this paper.

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