BioScientific Review (BSR)
Volume 7 Issue 4, 2025
ISSN$_{(P)}$: 2663-4198, ISSN$_{(E)}$: 2663-4201
Homepage: https://journals.umt.edu.pk/index.php/bsr

Title:	**EPS Analysis and Phytostimulatory Potential of Biofilm-forming Bacteria**
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DOI:	https://doi.org/10.32350/bsr.74.06
History:	Received: May 05, 2025, Revised: June 27, 2025, Accepted: August 18, 2025, Published: September 21, 2025
Citation:	Bibi N, Ahmed A, Tariq A. EPS analysis and phytostimulatory potential of biofilm-forming bacteria. *BioSci Rev.* 2025;7(4):69-84. https://doi.org/10.32350/bsr.74.06
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Conflict of Interest:	Author(s) declared no conflict of interest

A publication of
The Department of Life Sciences, School of Science
University of Management and Technology, Lahore, Pakistan

EPS Analysis and Phytostimulatory Potential of Biofilm-forming Bacteria

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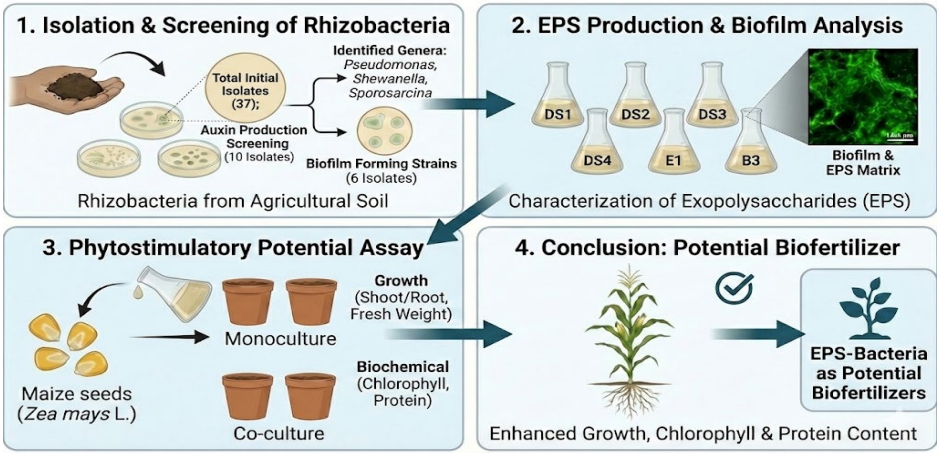
ABSTRACT

Use of plant growth-promoting rhizobacteria (PGPR) reclaims the productivity of the agricultural land through several mechanisms. Biofilm-forming rhizobacteria secrete extracellular polymeric substances (EPS) embedded in their self-produced exopolysaccharides. In the current study, thirty-seven isolates were evaluated or auxin production ability, however, ten isolates displayed significant auxin production ability. Six out of these ten isolates were biofilm-forming rhizobacteria, that is, *Pseudomonas* sp. 1 (DS1), *Shewanella putrefaciens* (DS2), *Pseudomonas* sp. 2 (DS3), *Pseudomonas aeruginosa* (DS4), *Sporosarcina saromensis* (E1), and B3. These were analyzed for their EPS production ability. The EPS production capacity of these rhizobacteria was evaluated under various physiological attributes, such as temperature, pH, and incubation periods. Phytostimulatory potential of the rhizobacterial strains was evaluated using *Zea mays* L. with monoculture and co-culture conditions. Results indicated that bacterial strains significantly enhanced the growth parameters, such as the percentage germination, shoot length, root length, number of leaves as well as fresh weight and biochemical parameters, that is, chlorophyll and soluble protein content of the plants. Distinct from previous PGPR studies, this study is unique in the evaluation of six rhizobacterial isolates with respect to the EPS production ability under varied pH, temperature, and incubation conditions and their combined effects in consortia in correlation with phytostimulatory impacts on *Zea mays* L. Thus, the EPS producing bacteria can be used as a sustainable biofertilizer to promote plant growth.

GRAPHICAL ABSTRACT



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Keywords: Auxin, biofilm, extracellular polymeric substances (EPS), plant growth promoting rhizobacteria (PGPR), PMI, *Pseudomonas* sp.

Highlights

- EPS-producing and biofilm-forming PGPR significantly improved the growth and physiological attributes of *Zea mays* L.
- Six selected bacterial isolates showed strong auxin production, biofilm formation, and EPS synthesis under optimized conditions.
- Bacterial consortia enhanced nutrient uptake, chlorophyll content, and biomass, demonstrating their potential as sustainable biofertilizers.

1. INTRODUCTION

The expanding global population continues to exert pressure on agricultural productivity and soil fertility. The excessive dependence on chemical fertilizers has caused long-term deterioration of soil health, microbial diversity, and ecosystem balance [1]. In this context, the use of plant growth-promoting rhizobacteria (PGPR) represents an eco-friendly and sustainable alternative for improving soil fertility and crop performance [2]. These beneficial bacteria enhance plant growth through mechanisms, such as phytohormone production, nutrient solubilization, and exopolysaccharide (EPS) secretion, which contributes to biofilm formation and soil aggregation [3, 4]. Although numerous studies have demonstrated the role of EPS-producing PGPR in promoting crop growth. There is still limited understanding of how different physiaggregation [rs (pH, temperature, and incubation period) influence EPS production and biofilm stability, and how these attributes relate to the phytostimulatory performance of PGPR under monoculture and consortia conditions [5, 6]. However, most previous reports have focused on individual bacterial strains, while the comparative evaluation of bacterial combinations (consortia) under optimized EPS conditions remains uninvestigated [7]. To address these knowledge gaps, the current study investigated the EPS production, biofilm-forming capacity, and plant growth-promoting potential of six rhizobacterial isolates. These included *Pseudomonas* sp. 1 (DS1), *Shewanella putrefaciens* (DS2), *Pseudomonas* sp. 2 (DS3), *Pseudomonas aeruginosa* (DS4), *Sporosarcina saromensis* (E1), and B3 which were previously screened for auxin production ability from a total of 37 isolates [8].

A combination of bacterial treatments was evaluated further under laboratory conditions and a significant increase in physiological parameters was recorded, such as significant increase in chlorophyll content and higher sugar content [9, 10]. *Zea mays* L. was selected as the test crop due to its global agricultural significance as a staple food and fodder crop. Its high nutrient requirements and remarkable responsiveness towards microbial inoculation makes it suitable for this study [11]. Maize serves as an excellent model plant for evaluating rhizobacterial interactions due to its microbial colonization supported by large rhizosphere area that is already well-studied [12, 13]. This study aimed to evaluate the PGPR or their bioilm and EPS production ability and its effect on growth and biochemical parameters of *Zea mays* L.

2. METHODOLOGY

2.1. Bacterial Isolates and Study Design

The current study was carried out using

thirty-seven bacterial isolates from maize rhizosphere soil. These were screened out for auxin production [8, 11]. Ten out of these bacterial isolates were auxin-producing and were further tested for biofilm-forming and EPS production ability. Six out of ten auxin-producing isolates had biofilm and EPS production potential. These six selected isolates were used to inoculate the *Zea mays* L. in order to determine its impact on the growth and biochemical parameters of the plant. Each treatment, including control, consisted of four replicates, with five surface-sterilized seeds per replicate. Control seeds were treated only with sterile distilled water, while all other seeds were inoculated with individual or consortia bacterial cultures as described above. Plants were grown under identical controlled laboratory conditions.

2.2. Auxin Production Analysis

Auxin production ability of the bacterial isolates was evaluated following Ahmed and Hasnain [8]. Bacterial cultures prepared in L-broth media with addition of 1% tryptophan were incubated for 5 days at 37 °C, and after centrifugation the supernatant was treated with Salkowski reagent (1:2). The detection was based on colorimetric method, where the appearance of pink color indicated auxin production. Absorbance was recorded at 535 nm for quantitative measurement of auxin-producing isolates. The amount of auxin was determined by using the standard curve.

2.3. Biofilm Formation Ability

To analyze the biofilm production potential of isolated bacterial strains, the qualitative and quantitative techniques following Hu et al. [14] were used. For qualitative assessment of biofilm production, the bacterial inoculum was prepared by inoculating each bacterial isolate in L-broth medium and incubated at 37°C for 24 hours. The biofilm was observed on the walls of the test tubes after staining with 1% crystal violet solution by giving a stay of 15-20 minutes. Then, the biofilm was dissolved in 30% acetic acid by pouring it in dried test tube and absorbance was recorded at 600nm.

2.4. EPS Production Ability

The bacterial inoculum was prepared by inoculating each bacterial isolate in L-broth medium and incubated at 37°C for 24 hours for estimation of EPS production potential following Ansari et al. [15]. Then, it was centrifuged at 9000rpm for 10 minutes after supplementation of 1mM EDTA in order to extract the exopolysaccharide (EPS). Acetone was added in the supernatant and kept in refrigerator overnight at 4°C. Then, EPS was extracted after centrifugation at 15000rpm for 20 minutes. Characterization of various physiological parameters for EPS production potential were performed.

Bacterial isolates were grown at 37°C, 25°C, and 45°C in the incubator and accessed for unfavorable temperature variations. The bacterial growth was observed at different pH levels, for instance at 4pH, 7pH, and 9pH. Bacterial strains were grown with varied incubation period and accessed for optimum growth of the bacterial strains for 3, 5, and 7 days.

2.5. Biochemical Analysis of EPS

Estimation of carbohydrate and protein content was done by following Vaishnav et al. [16]. 100mg sample was homogenized with 3ml of 80% methanol and kept at 70°C temperature for 30 minutes. Then, extract was taken and an equal volume of 5% phenol was added in the extract. Then, 1.5ml of 95% H_2SO_4 was added in the solution and incubated in the dark for 15-20 minutes. Then, absorbance was recorded by taking 1ml of sample for each bacterial strain for

variations of temperature, pH, and different incubation periods at 490nm. After that, the carbohydrate content was estimated by applying formula.

0.5g sample was taken in a test tube and 2ml of 1N phosphate buffer was added into it. Then, the solution was centrifuged at 10000rpm for 10 minutes. 0.2ml supernatant was taken out in a test tube and 1ml of Folin mix was added in the supernatant. The sample was kept for 15 minutes at room temperature and then 0.2ml of Ciocalteu's phenol reagent was added in it and kept for 45 minutes at room temperature. Then, absorbance was recorded at 750nm for all the bacterial strains for the variations of temperature, pH, and different incubation periods. Protein content was estimated by applying formula.

2.6. Plant Bacterization Assay

Certified seeds of *Zea mays* L. var. Sdg 2002 were obtained from Punjab Seed Corporation, Lahore, Pakistan. Plant growth experiment was carried out under laboratory conditions. The experiment was conducted in quadruplicates. Seeds were washed with liquid detergent and then washed with tap water for seven to eight times to surface sterilize. Seeds were then treated with 0.1% solution of mercuric chloride ($HgCl_2$) for two minutes following several washings with sterile distilled water to remove all the traces of mercuric chloride. The bacterial inoculum was prepared by inoculating each bacterial isolate in L-broth medium and incubated at 37°C for 24 hours. After incubation, optical density of bacterial cultures was adjusted at ~10^6–10^7 CFU/mL. Seeds were then bio-primed with bacterial inoculum for one hour, while for control treatment, the seeds were treated with sterile distilled water for the same period of time.

Monocultures and bacterial consortia were used for plant inoculation treatment. After 20 days, plants were harvested and various growth parameters, that is, length of shoot and root, fresh weight, and number of leaves per plant were recorded.

2.7. Biochemical Analysis

The biochemical analysis was done by estimation of chlorophyll and protein content. Chlorophyll 'a', chlorophyll 'b', and total chlorophyll content of treated and non-treated plants were estimated by modified method described by Vaishnav et al. [16]. The total soluble protein content of treated and non-treated plants was assessed following Lowry et al. [17].

2.8. Statistical Analysis

The data obtained was statistically analyzed using Post Hoc Duncan Multiple Range Test (DMRT) to determine significant differences (p<0.05) using SPSS ver. 16.3. The data was presented as mean ± standard error (SE). Error bars in the graphs represent standard error.

3. RESULTS

3.1. Auxin Production Analysis

Ten out of thirty-seven isolates were auxin-producing. For instance, *Pseudomonas sp.* 1 (DS1), *Shewanella putrefaciens* (DS2), *Pseudomonas sp.* 2 (DS3), *Pseudomonas aeruginosa* (DS4), *Sporosarcina saromensis* (E1), and B3 produced 132,108, 314, 279, and 224 µg/ml of auxin, respectively. While DR1, DR2, DR3, and DR4 produced 87, 64, 57, and 73 µg/ml, respectively (Table 1).

3.2. Biofilm Formation Ability

Auxin-producing bacterial isolates were analyzed for their biofilm-forming ability. Six out of ten bacterial isolates, that is, *Pseudomonas sp.* 1 (DS1), *Shewanella putrefaciens* (DS2), *Pseudomonas sp.* 2

(DS3), *Pseudomonas aeruginosa* (DS4), *Sporosarcina saromensis* (E1), and B3 were screened out as biofilm formers. In the current research, the biofilm formers were selected for further analysis. Bacterial isolates were categorized as strong, moderate, and weak biofilm formers bacteria. *Pseudomonas aeruginosa* (DS4), B3, and *Sporosarcina saromensis* (E1) proved as strong biofilm formers based on their biofilm-forming ability.

Table 2. Auxin Content of Bacterial Isolates

Bacterial Strains	Auxin
DS1	132 ± 16.27
DS2	108 ± 5.42
DS3	314 ± 9.32
DS4	279 ± 11.59
DR1	87 ± 8.43
DR2	64 ± 8.89
DR3	57 ± 4.29
DR4	73 ± 5.61
B3	124 ± 9.75
E1	224 ± 8.22

3.3. EPS Production Ability

All the bacterial strains, that is, *Pseudomonas sp.* 1 (DS1), *Shewanella putrefaciens* (DS2), *Pseudomonas sp.* 2 (DS3), *Pseudomonas aeruginosa* (DS4), *Sporosarcina saromensis* (E1), and B3 showed EPS production potential. The extracted EPS was weighed for different physiological conditions, for instance temperature (25, 37, and 45°C), pH (4, 7, and 9), and incubation period (3, 5, 7 days) and was recorded. At 25°C, bacterial strains were found to be least efficient in their ability to produce EPS. The maximum quantity of EPS extracted at this temperature by the bacterial strains DS3 and E1 was recorded as 0.018 g for both of bacterial strains. At 37°C, the maximum weight of EPS extracted by bacterial strains B3, and E1 was 0.109, and 0.11 g, respectively. At 45°C, the quantity of EPS extracted by bacterial strains DS4 and E1 was 0.019g for both of the bacterial strains.

At acidic pH (4), bacterial strains showed weak potential to produce EPS. The quantity of extracted EPS produced by both the bacterial strains DS1 and DS4 was recorded as 0.0091g for both of bacterial strains. At neutral pH (7), all the bacterial strains showed efficient EPS production. The quantity of extracted EPS by the bacterial strains DS2, DS4, and E1 was recorded as 0.092, 0.097, and 0.086 g, respectively. At basic pH (9), the maximum quantity of EPS extracted by bacterial strains DS1 and DS4 was recorded as 0.019g for both. At incubation period of three days, the maximum quantity of EPS extracted from bacterial strains DS1 and DS4 was 0.099g for both. At incubation period of five days, the maximum quantity of EPS extracted from bacterial strains DS2, B3, and E1 was 0.042, 0.050, and 0.044g, respectively. At incubation period of seven days, the maximum quantity of EPS extracted from bacterial strains DS1 and E1 was 0.0099g for both of these strains.

3.4. Biochemical Analysis of EPS

The highest carbohydrate content was recorded as 1.12 and 2.22 µg/g by DS2 and E1, respectively at 25°C. However, at 37°C, the maximum carbohydrate content was recorded as 12.53 and 12.69 µg/g by DS4 and E1, respectively. The maximum carbohydrate content was recorded as 4.38 and 2.41 µg/g by DS4 and E1, respectively at 45°C. The maximum carbohydrate content was recorded as 1.79, 1.62, and 1.42 µg/g by DS1, DS2, and E1, respectively at acidic pH (4). At neutral pH (7), the maximum carbohydrate content was recorded as 3.76 and 4.20, µg/g by DS1 and DS4, respectively. The maximum carbohydrate

content was recorded as 1.74, 1.71, and 1.75µg/g by DS1, DS4, and E1, respectively at basic pH (9). The maximum carbohydrate content was recorded as 12.5367 and 12.69µg/g by DS4 and E1, respectively at incubation period of three days. The highest carbohydrate content was recorded as 2.92 and 2.37 µg/g by DS1 and E1, respectively at incubation period of five days. At incubation period of seven days, the maximum carbohydrate content was recorded as 5.02and 7.56 µg/g by B3 and E1, respectively.

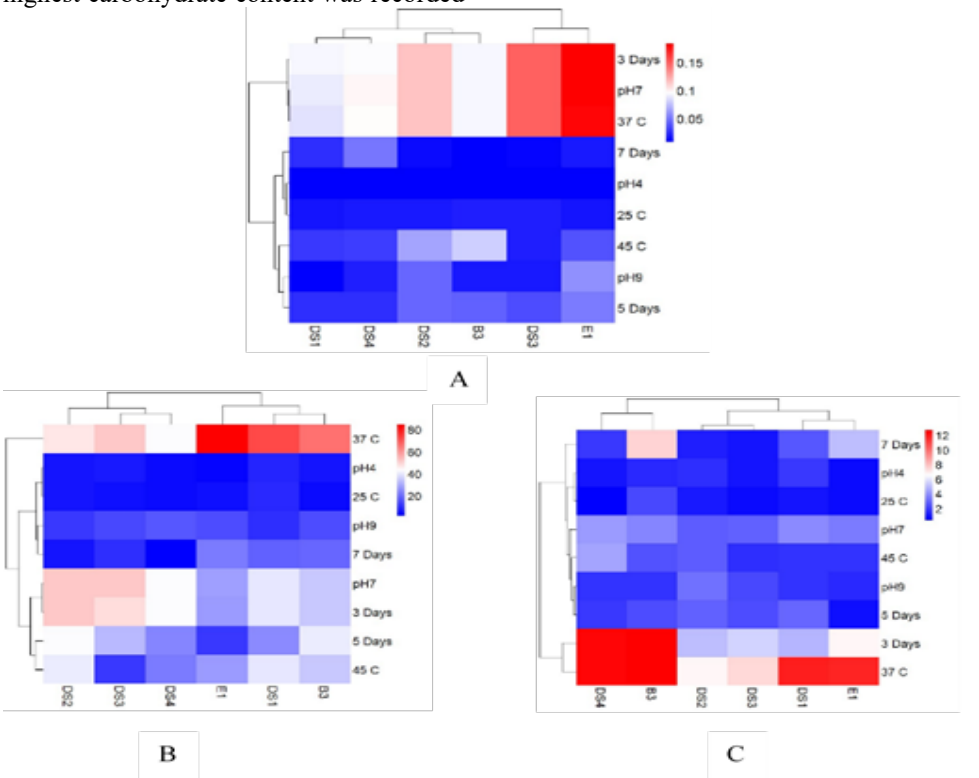

Figure 1. Heatmap and Hierarchical Clustering Displaying Carbohydrate/ Protein /Weight of EPS Produced by the Bacterial Strains under Various Physiological Parameters. A) Weight of EPS Extracted by Bacterial Strains DS1, DS2, DS3, DS4, E1 and B3 at various Physiological Parameters; Variations in Temperatures, i.e., 25, 37 and 45°C; pH, i.e., 4,7 and 9 and Incubation Period, i.e., 3, 5 and 7 days. B) Carbohydrate Content in EPS Extracted by Various Bacterial Strains DS1, DS2, DS3, DS4, B3 and E1 at Different Temperature Variations, i.e., 25, 37 and 45°C; pH, i.e., 4,7 and 9 and Incubation Period, i.e., 3, 5 and 7 days. C) Protein Content in EPS Extracted by Various Bacterial Strains DS1, DS2, DS3, DS4, B3 and E1 at Different Temperature Variations, i.e., 25, 37 and 45°C; pH, i.e., 4,7 and 9 and Incubation Period, i.e., 3, 5 and 7 days.

The maximum protein content at 25°C was recorded as 9.05, 5.67, and 5.07 µg/g by DS1, DS2, and B3, respectively. The maximum protein content was recorded as 72.42 and 84.73 µg/g by DS1 and B3, respectively at 37°C. The maximum protein

content was recorded as 40.03, 40.63, and 34.99µg/g by DS1, DS2, and E1, respectively at 45°C. The highest protein content was recorded as 8.41, 6.10, 6.95µg/g by DS1, DS2, and DS3, respectively at acidic pH (4). The highest protein content was recorded as 52.42 and 52.25 µg/g by DS2 and DS3, respectively at neutral pH (7). The highest protein content was recorded as 16.53, 14.99, and 14.58 µg/g by DS4, B3, and E1, respectively at basic pH (9). The highest protein content was recorded as 52.42 and 48.49µg/g by DS2 and DS3, respectively at incubation period of three days. The maximum protein content was recorded 43.11and 40.71 µg/g by DS2 and E1, respectively at incubation period of five days. The highest protein content was recorded as 22.51 and 19.09 µg/g by B3 and E1, respectively at incubation period of seven days (Figure1).

3.5. Plant Bacterization Assay

Plants were treated with the monocultures using (*Pseudomonas sp*. 1 (DS1), *Shewanella putrefaciens* (DS2), *Pseudomonas sp.* 2 (DS3), *Pseudomonas aeruginosa* (DS4), *Sporosarcina saromensis* (E1), and B3) and consortia treatment using (B3+DS1, B3+DS2, B3+DS3, B3+DS4, B3+E1, DS1+DS2, DS1+DS3, DS1+DS4, DS1+E1, DS2+DS3, DS2+DS4, DS2+E1, DS3+DS4, DS3+E1 and DS4+E1) of bacterial strains. In case of monocultures, maximum increase of 23.16% in germination percentage was shown by DS3 bacterial-treated plants as compared to control in *Zea mays* L. B3+DS4 bacterial consortia showed the maximum increase in percentage germination, that is, 41.17% as compared to control plants. On the other hand, 41.18 and 26.32% increment in percentage germination was observed when compared with the plants treated with B3 and DS4, respectively.

Figure 2. Effect of Bacterial Treatments on *Zea mays* L. Under Laboratory Conditions. **[A**-Control (Sterile Distilled Water), **B-**DS4 (*Pseudomonas aeruginosa*), **C**-B3+DS4 (B3 + *Pseudomonas aeruginosa*), and **D**-DS4+E1 (*Pseudomonas aeruginosa* + *Sporosarcina saromensis*)**]**.

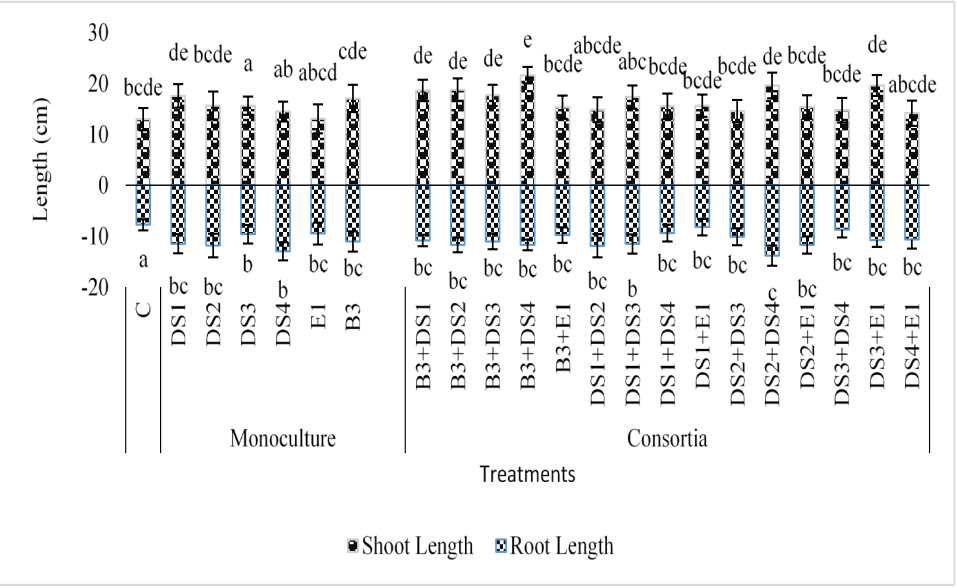

Figure 3. Effect of Bacterial Inoculation of Monocultures and Consortia on Shoot and Root Length of *Zea mays* L. [C-Control; Pure Cultures = DS1, DS2, DS3, DS4, B3, E1, Consortia treatments- B3+DS1, B3+DS2, B3+DS3, B3+DS4, B3+E1, DS1+DS2, DS1+DS3, DS1+DS4, DS1+E1, DS2+DS3, DS2+DS4, DS2+E1, DS3+DS4, DS3+E1, DS4+E1]. Different letters Indicate Significant Differences Between Treatments Using Duncan's Multiple Range Test ($p< 0.05$)

Shoot length of bacterially-inoculated plants increased as compared to non-inoculated plants. DS1 and B3 showed the maximum increase of 35.46 and 31.61%, respectively in shoot length as compared to the control. The consortia treatment B3+DS4 showed a maximum increase of 67.16% in shoot length as compared to the control. The increase in shoot length in consortia was 26.67 and 49.24% as compared to the monocultures B3 and DS4 treated plants, respectively. Among monocultures, the DS4 increased the root length of the plants of 67.1% as compared to control. Among the consortia cultures, the maximum escalation in root length was shown by the bacterial strain DS2+DS4 and DS1+DS as 77.99% and 52.83, respectively as compared to control. While, increase in root length was recorded as 16.96 and 6.61% as compared to the monocultures DS2 and DS4 treated Zea mays L, respectively (Figure 2 & 3). Different letters on the bars indicate statistically significant differences among treatments for shoot length and root length, as determined by Duncan's Multiple Range Test (DMRT), while bars sharing the same letter represent non-significant differencesThe bacterial monocultures DS4 and DS1 showed an increase of 83.12% and inoculation B3+DS4 showed 106.2% enhanced number of leaves in comparison with control. While, the increase in number of leaves shown by the consortia treatment was 32 and 66.01% as compared to monocultures B3 and DS4, respectively. The maximum increase in fresh weight of the plants was measured in B3, that is, 46.2% increase in weight as compared to control. The rise in fresh weight of Zea mays L. was

44.05% in DS4+E1 bacterial consortia treatment and the increase in fresh weight was 26.43 and 30.95% in comparison to DS4 and E1 monoculture-treated plants, respectively (Figure 4). Different letters on the bars indicate statistically significant differences among treatments for number of leaves and fresh weight, as determined by DMRT; bars sharing the same letter represent non-significant differences.

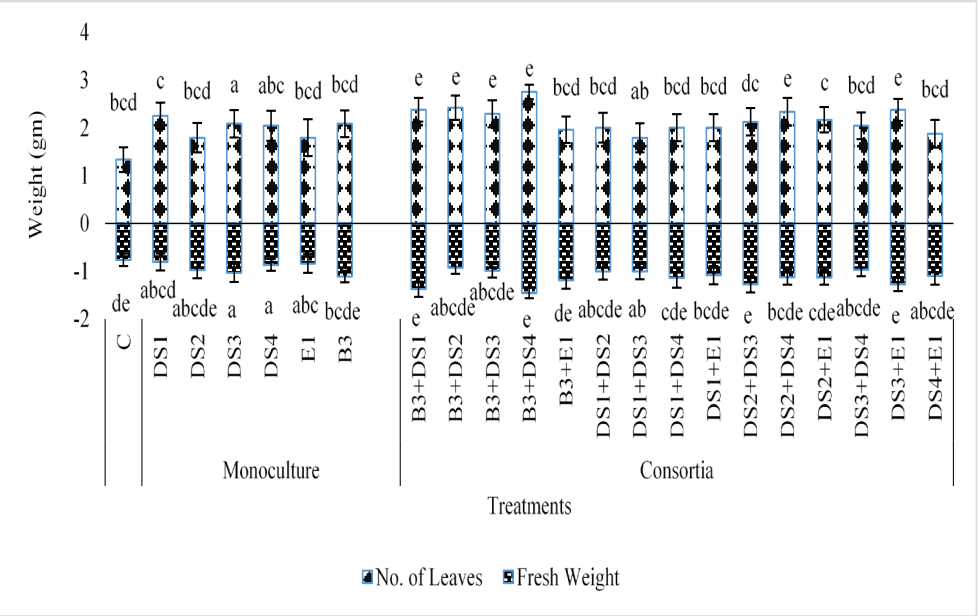

Figure 4. Effect of Bacterial Inoculation of Monocultures and Consortia on Number of Leaves and Fresh Weight of *Zea mays* L. [C-Control; Pure Cultures = DS1, DS2, DS3, DS4, B3, E1, Consortia treatments- B3+DS1, B3+DS2, B3+DS3, B3+DS4, B3+E1, DS1+DS2, DS1+DS3, DS1+DS4, DS1+E1, DS2+DS3, DS2+DS4, DS2+E1, DS3+DS4, DS3+E1, DS4+E1]. Different Letters Indicate Significant Differences Between Treatments Using Duncan's Multiple Range Test (p<0.05).

3.6. Biochemical Analysis

In the case of monocultures, the bacterial strain DS2 and DS3 showed the maximum increase of 128.2 and 117.75% in chlorophyll 'a' content, respectively as compared to the control. Among the consortia treatment, the maximum increase in chlorophyll 'a' content was 96.43 and 93.03% observed in DS4+E1and DS3+E1, respectively in comparison to control. The results indicated that the maximum increase in chlorophyll 'a' content was estimated in DS4+E1 in case of consortia treatment, while 1.3 and 4.6% increase occurred as compared to DS4 and E1, respectively. In monoculture treatment, the maximum increase in chlorophyll 'b' content was 77.82 and 77.18% by the treatment of B3 and DS1 bacterial isolate, respectively. Among consortia treatments, the maximum enhanced chlorophyll 'b' content was 90 and 83.49% in DS4+E1 and DS1+DS3 bacterial strain, respectively. While, 29.96 and 58.59% increase was observed when compared with treatment of monocultures DS4 and E1, respectively and 1.59 and 17.34% increase

was observed when compared with treatments of monocultures DS1 and DS3, respectively.

In the case of bacterial monoculture treatment, 73.64 and 72.89% increase in total chlorophyll content was observed in B3 and DS1 bacterial treatments as compared to control. Among bacterial consortia treatment, the highest increase in total chlorophyll content was 90.43% in DS4+E1 bacterial treatment as compared to control. While, the increase was observed as 24.05 and 47.78% as compared to the monocultures DS4 and E1, respectively (Figure 5). Different letters on the bars indicate statistically significant differences among treatments for chlorophyll a, chlorophyll b, and total chlorophyll content, as determined by DMRT; bars with the same letter do not differ significantly.

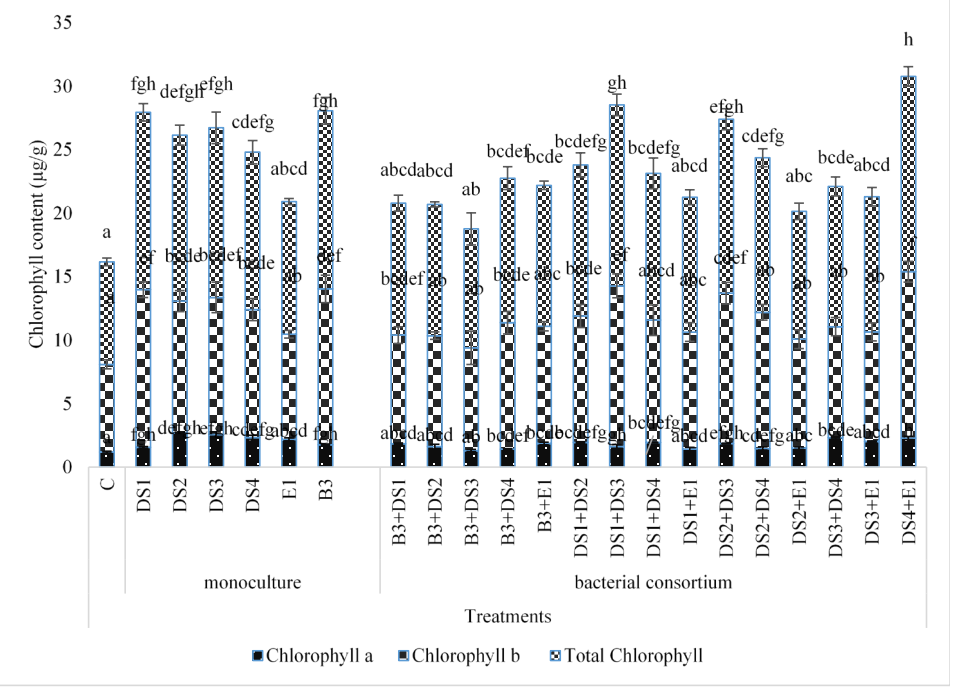

Figure 5. Effect of Bacterial Inoculation of Monocultures and Consortia on Total Chlorophyll Content of *Zea mays* L. [C-Control; Pure Cultures = DS1, DS2, DS3, DS4, B3, E1, Consortia treatments – B3+DS1, B3+DS2, B3+DS3, B3+DS4, B3+E1, DS1+DS2, DS1+DS3, DS1+DS4, DS1+E1, DS2+DS3, DS2+DS4, DS2+E1, DS3+DS4, DS3+E1, DS4+E1]. Different Letters Indicate Significant Differences Between Treatments Using Duncan's Multiple Range Test (p<0.05).

In the monoculture, the protein content of strain DS3 increased by 23.43% as compared to control. The value of soluble protein content after monoculture bacterial treatment of DS4, DS1, DS2, E1, and B3 showed an increase of 23, 21.79, 20.49, 20.3, and 15.66%, respectively in contrast to the control. The highest increase in soluble protein content was 39.51 and 39.3% in B3+DS3 and DS3+DS4, respectively in

comparison to control among consortia treatment. While, 10.92 and 3.93% increased as compared to B3 and DS3, however, 12.85 and 13.25% as compared to DS3 and DS4 bacterial monoculture treatment, respectively (Fig.6). Different letters on the bars indicate statistically significant differences among treatments for protein content, as determined by DMRT however, bars sharing the same letter indicate non-significant differences.

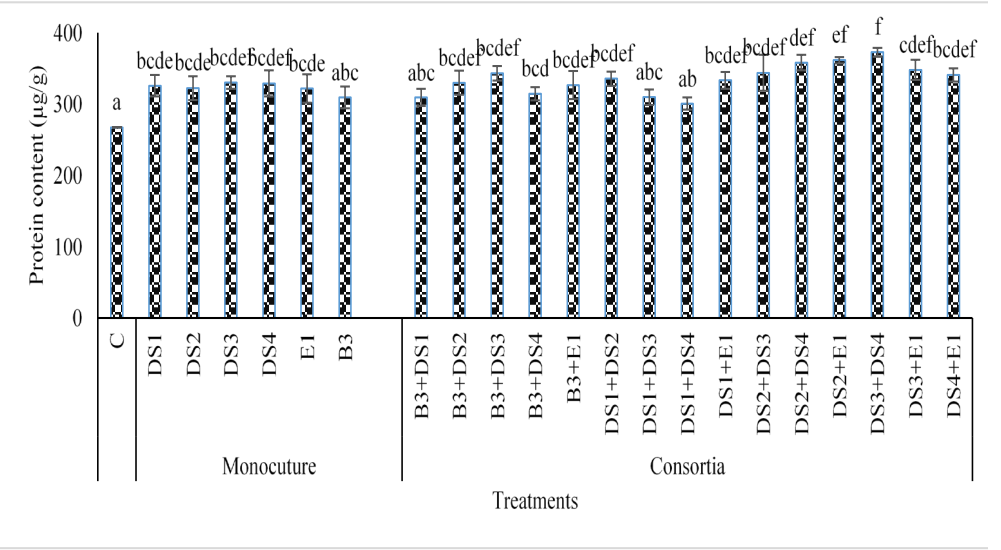

Figure 6. Effect of Bacterial Inoculation of Monocultures and Consortia on Soluble Protein Content of *Zea mays* L. [C-Control; Pure cultures -DS1, DS2, DS3, DS4, B3, E1, Consortia treatments- B3+DS1, B3+DS2, B3+DS3, B3+DS4, B3+E1, DS1+DS2, DS1+DS3, DS1+DS4, DS1+E1, DS2+DS3, DS2+DS4, DS2+E1, DS3+DS4, DS3+E1, DS4+E1]. Different Letters Indicate Significant Differences Between Treatments Using Duncan's Multiple Range Test (p<0.05)

4. DISCUSSION

The current study demonstrated that EPS-producing and biofilm-forming PGPR significantly enhanced the morphological and biochemical attributes of *Zea mays* L. under laboratory conditions. Among the six isolates evaluated, DS4, B3, and E1, along with consortia, such as B3+DS4 and DS4+E1, exhibited the strongest phytostimulatory effects. Improvements in germination percentage, shoot and root development, chlorophyll content, and soluble protein accumulation indicated the multifaceted role of PGPR in promoting plant growth, consistent with earlier reports on the plant-beneficial attributes of rhizobacteria [2]. Biofilm-forming PGPR with auxin production ability plays a crucial role in improving soil health by secreting EPS [18]. EPS is composed of proteins, lipids, and carbohydrates which create a sticky environment that causes adhesion of bacterial cells to the root vicinity, enhancing soil aggregation [19]. The polysaccharides present in EPS form a biofilm which binds soil particles and bacterial cells. The biofilms help in maintaining soil moisture and ensuring water availability for plants to facilitate plant growth [20]. Moreover, the components of EPS served as nutrients, absorbed

by plant roots. These extracellular polymeric substances also support plant growth besides providing them protection against harsh environmental conditions. Bacterial isolates capable of producing EPS are more competitive as they can survive unfavorable conditions by embedding themselves in extracellular polymeric substances. Therefore, EPS producing bacteria can enhance plant growth more effeciently [21]. The maximum EPS production conditions were 20°C temperature, pH 7 for *Pseudomonas aeruginosa* and *Micrococcus* sp., and pH 8 for *Ochrobactrum* sp. The incubation periods were 96 hours for *P. aeruginosa*, 72 hours for *Micrococcus sp*., and 48 hours for *Ochrobactrum* sp [5]. EPS secretion plays a central role in improving soil aggregation, water retention, and root-microbe adhesion, thereby facilitating greater nutrient mobilization towards the plant. These functions of EPS-rich biofilms have been widely reported, emphasizing their ability to enhance soil structure and microbial stability [3, 6]. The isolates used in this study produced auxin, which likely contributed to enhanced root length and improved nutrient uptake. Auxin-mediated stimulation of root architecture is a well-known mechanism through which PGPR promotes plant vigor, as also observed by Khan and Bano [4]. The strains used in this study exhibited biofilm-forming ability which is challenging to remove once formed [22]. Nawaz et al., 2020 reported that the bacterial inoculation increases Zea *may*s biomass along with shoot and root length, fresh weight, and leaf count of *Zea mays* due to increased peroxidase, acid phosphatase, and auxin content [23]. The increased biomass and physiological parameters in *Zea mays* were likely due to improved nutrient uptake by biofilm-forming PGPR, which enhanced chlorophyll content and photosynthesis [24]. Siderophores released by these microbes increase the solubility and availability of iron, supporting chlorophyll synthesis and overall photosynthetic efficiency [25]. Additionally, zinc-solubilizing PGPR improves Zn availability for plants, contributing to proper enzyme activation, chlorophyll formation, hormone regulation, and growth enhancement [26]. The findings of this study align with the previous research. Studies by Nayak et al. demonstrated that EPS-producing PGPR improves soil's structural stability and increases nutrient availability, supporting the improved chlorophyll and protein content observed here [3]. Khan and Bano reported that auxin-producing PGPR significantly enhances shoot biomass in cereals, which agrees with the improved shoot growth observed in maize treatments [4]. Biofilms facilitated the uptake of key nutrients, including N, P, K, Fe, and Zn thereby supporting the overall plant vigor [27]. Bacterial consortia further strengthened these effects through positive microbial interactions that enhanced root colonization [28]. The superior performance of bacterial consortia in this study is also consistent with the observations of Behera et al. who showed that microbial combinations promote synergistic nutrient mobilization and enhance plant growth more effectively than individual strains [7]. EPS production recorded at 37°C and neutral pH matches earlier findings by Kılıç and Dönmez (2008), who reported optimal EPS synthesis under similar conditions [5]. The comparative evaluation of PGPR under varying physiological conditions and the correlation of EPS production with plant responses is a strong aspect of this study. Unlike studies focusing solely on single strains, this work demonstrated that bacterial consortia not only improved growth parameters but also enhanced biochemical attributes, such as chlorophyll and protein accumulation. This highlights the potential of mixed inoculants as robust biofertilizers for

sustainable agriculture, particularly in nutrient-poor soils [7, 11]. These results show the importance of EPS-mediated interactions in plant resilience and overall physiological functioning. Increased chlorophyll content suggests improved photosynthetic efficiency, whereas higher soluble protein levels reflect enhanced metabolic activity. Such improvements are vital for sustaining crop productivity in soils degraded due to long-term chemical fertilizer use [1]. While the findings are promising, this study was conducted under controlled laboratory conditions. Future studies can be conducted to evaluate these isolates under field environments and examine their efficiency under abiotic stress conditions. Additionally, molecular analysis of EPS biosynthesis genes would provide deeper insights into the mechanisms governing EPS production and biofilm formation [29]. Overall, the study concluded that EPS-producing PGPR, particularly DS4, B3, and E1, along with their consortia, hold strong potential as sustainable biofertilizers capable of improving plant growth, biochemical performance, and soil health.

4.1. Conclusion

This study confirmed the efficacy of six EPS-producing PGPR strains *Pseudomonas sp*. 1 (DS1), *Shewanella putrefaciens* (DS2), *Pseudomonas sp.* 2 (DS3), *Pseudomonas aeruginosa (*DS4), *Sporosarcina saromensis* (E1), and B3, in enhancing *Zea mays* (L.) growth, with consortia (e.g., B3+DS4) outperforming monocultures through synergistic effects on key growth and biochemical traits. Strains, such as DS4, B3, and E1, along with consortia including DS4+E1 and DS1+DS3, notably improved plant vigor and nutrient dynamics. These findings advocate for the use of EPS-mediated PGPR as viable biofertilizers, offering a sustainable approach to agricultural productivity enhancement.

Author Contribution

Naseem Bibi: data curation formal analysis writing – original draft. **Ambreen Ahmed:** conceptualization project administration resources software validation visualization. **Aqsa Tariq:** formal analysis investigation methodology writing review & editing.

Conflict of Interest

The authors of the manuscript have no financial or non-financial conflict of interest in the subject matter or materials discussed in this manuscript.

Data Availability Statement

Data supporting the findings of this study will be made available by the corresponding author upon request.

Funding Details

No funding has been received for this research.

Generative AI Disclosure Statement

The authors did not used any type of generative artificial intelligence software for this research.

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