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Isolation and screening of soil bacteria for EPS production

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Abstract
Exopolysaccharides are the high molecular weight polymeric substances, which have been secreted by the microorganisms as tightly bound capsule or loosely attached slime layer. EPS have the potential to preserve the structural integrity of the soil, stimulate soil fertility, and optimize soil health. In this study, exopolysaccharides producing microbes were isolated from the drought soil. The biochemical characterization and screening of the isolates for the production of exopolysaccharides by phenol sulfuric acid method were carried out. The obtained results revealed that 3 out of 19 cultures (*Rhizobium* spp., *Bacillus megaterium* and *Lysinibacillus sphaericus*), were known to produce consummate amount of EPS at the rate of 50.00 mg/ml, 49.89 mg/ml and 49.67 mg/ml respectively. Also, these isolates ensured to produce higher quantity of IAA, GA3, PO_4 solubilization and K release. Hence, these cultures were considered for EPS production, thereby improving soil aggregation and stimulating crop growth.

Keywords: Exopolysaccharides, soil aggregation, drought, phenol sulfuric acid assay, PGP traits

1. Introduction
With the insistence of persistently expanding agricultural production has come an incessant turndown in soil fertility. This could be a major challenge that Indian agriculture is currently coping with. The productive capacity of the soil is being harmed by soil degradation and loss of fertility, which have been brought forth by chemical fertilisers and insecticides used excessively on the soil, moving away from traditional organic soil revitalization methods that are ineffective. The degradation of soil chemical, physical, and biological health causes low nutrient utilisation efficiency (Aleminew *et al*., 2020) [1]. Research recommendations have been provided to establish an alternative technology to promote a more sustainable and resource-efficient method of sustaining fertility in agricultural soils. Microbial polysaccharides are soluble or insoluble high molecular weight extracellular polymeric compounds produced by bacteria, yeast, algae, fungus and other microorganisms (Staudt *et al*., 2004) [15]. Exopolysaccharides have a prominent influence on desiccation resistance, maintenance of soil moisture content, microbial aggregation, plant-microbe interaction, surface adhesion, bioremediation, soil aggregate stabilization, thereby restoring soil structure and soil health. Under water deficiency stress, EPS-producing bacteria accumulated higher amounts of proline, carbohydrates and free amino acids (Naseem *et al*., 2018) [13]. The practice of applying exopolysaccharides to the soil can be used as an alternative methodology for improvement of soil fertility and health.

2. Materials and Methods
2.1 Sampling and sampling location
Drought soils were collected for the isolation of EPS producing organisms from distinct areas in and around the districts of Ramnad and Tuticorin. Pallamerkkulam (9.18^{O}N, 78.49^{O}E), Kannirajpuram (9.05^{O}N, 78.22^{O}E), Sivaperumkuntram (9.05^{O}N, 78.20^{O}E), Velayuthapuram (9.07^{O}N, 78.25^{O}E) and Nariyur (9.14^{O}N, 78.54^{O}E) are among the six places in the Ramnad and Tuticorin districts of Tamil Nadu, India.

2.2 Isolation of bacteria from drought soil
Bacterial species were isolated from drought soil by serially diluting and culturing them on Trypticase Soya agar media with dilution factors of 10^5 and 10^6 and incubating them at 28 °C for 48 hours. A total of 19 isolates were selected based on the mucoid morphology and then subjected to morphological and biochemical analysis and for exopolysaccharide screening (Sandhya *et al*., 2009).

2.3 Quantitative assay for indole-3-acetic acid (IAA) production

Indole production by the bacterial isolates was assessed using nutrient broth supplemented with 100 µg/mL tryptophan (Glickamann and Dessaux., 1995). The cultures were kept under incubation at 28±1 °C at an agitation rate of 125 rpm for 48-72 hours. After the completion of the incubation period, the broth cultures were centrifuged at 10000 rpm for 20 min, and the cell free extract was used for the further estimation. In this experiment, 4.0 ml of Salkowski reagent (1 ml of 0.5 M $FeCl_3$ dissolved in 50 ml of 35% perchloric acid) was added in to the cell free extract and incubated in the dark for 30 min. The pink colour development was read at 530 nm using a UV-Vis spectrophotometer and expressed as µg ml^{-1}. The quantity of IAA produced by the cultures were calculated using IAA standard.

2.4 Quantitative assay for GA3 production

For the quantification of GA3 in the selected isolates, 7-day old cultures grown in the nutrient broth were centrifuged at a rpm of 10,000 for 15 min. GA3 production was estimated in the cell free extract as per Horace *et al*., 1961 [6]. Collected supernatant was mixed with equal amount of ethyl acetate and the aqueous layer was separated and evaporated at room temperature. The residue was dissolved in 1.0 mL of alcohol. To this mixture, DNPH reagent was added and incubated at room temperature for 5 min. There after 10% KOH was added and again incubated for 5 min. Development of red wine colour was read at 430 nm using UV-Vis spectrometer with gibberellic acid as standard, which was expressed as µg ml^{-1}.

2.5 Quantitative assay for phosphate solubilization

The quantitative estimation of PO_4 solubilization was carried out by inoculating the selected cultures into Pikovskaya's broth supplemented with 0.5% tricalcium phosphate. Inoculated broth was kept at incubation for 120 hours at 30±2 °C with KH_2PO_4 as the standard solution. The cultures were centrifuged at the agitation of 10,000 rpm for 5 min. From the mixture, 1ml of the supernatant was taken in the tube to which an equal quantity of distilled water was added. Then 2 ml of the colour reagent was added to the suspension and the volume was made up to 6 ml, which was then kept for incubation at 15 min. The development of blue colour was observed and the absorbance was read at 882 nm in a spectrophotometer and the total P was calculated and expressed as mg/ml (Murphy and Riley, 1962) [11].

2.6 Quantitative assay for Potassium release

The purified cultures were estimated for K release by inoculating the 2 day old cultures in Aleksandrov's broth and incubating them for about 10 days at 28±1 °C. After the incubation period, the culture solutions were centrifuged at 7000 rpm for about 10 min for the separation of the cell pellet. To the culture filtrate obtained, 25 ml of 1N ammonium acetate was added and kept in the shaker for 10 min. The solutions were filtered through Whatman No. 1 filter paper and then fed into the flame photometer. Using KCl as a standard solution, the K content of cultures was quantified and expressed as mg/ml. (Stanford and English, 1949) [17]

2.7 Screening for exopolysaccharides production

The log phase cultures of bacterial isolates were inoculated in the nutrient broth and kept in the incubator shaker at 200 rpm for 48 hours for allowing the cultures to attain stationary phase (Deka *et al*., 2018). These cells were harvested by centrifugation at 10000 rpm for 20 min. To the supernatant collected, thrice its volume of cold acetone was added and kept overnight for precipitation at 4 °C. After the incubation, precipitated EPS were recovered at the agitation rate of 6000 rpm for 10 min. The recovered EPS were processed by the phenol sulfuric acid assay for the estimation of EPS. 50 µL of the EPS precipitate was dissolved in sterile water to which 150 µL of conc. sulphuric acid and 30 µL of 5% aqueous phenol solution were added in a microtitre plate. The plate was subjected to 90 °C in a water bath and cooled to 35 °C. The plate was read in ELISA plate reader at 490 nm. The EPS produced was quantified using the formula y=0.0608x (where y represents OD values and x represents EPS produced by the organism), which was obtained from the graph of glucose standard solution. The obtained OD values of the cultures (y) were substituted in the given formula and the corresponding quantity of EPS produced by the organisms (x values) were calculated and expressed as mg/ml.

2.8 Molecular identification and characterization of bacterial cultures

The selected EPS producing isolates were purified and subjected to molecular characterization. The three isolates were grown in LB broth overnight and they were used for the DNA isolation. Using hexadecyl trimethyl ammonium bromide (CTAB) method, the isolation of genomic DNA from the purified bacterial isolates were carried out. By the method of Polymerase chain reaction, the 16S rRNA gene present in the isolated genomic DNA was amplified. The products of PCR were resolved by agarose gel electrophoresis (Sambrook, 1989). Finally, the gel was examined and photographed with Alpha imager TM 1200 gel documentation system. The amplified 16S rRNA gene was sequenced in both the directions which includes 5' and 3' with Applied biosystems automated sequencer. The nucleotide sequence so obtained was subjected to similarity check against the GenBank database for BLAST analysis. CLUSTAL W version 1.8 was used for the analysis of multiple sequence alignments.

3. Results and Discussion

3.1 Isolation of bacteria from drought soil

A total of 19 isolated cultures from the drought soil were selected for further purification based on their mucoid morphology (Table 1). Most isolates were found to be elevated colonies and dull white in colour. D9 produces yellow-coloured colonies, in contrast to other isolates. Gram's reaction classified two isolates out of the 19 pure cultures, as Gram-positive cultures. Rest of them are Gram negative.

3.2 Biochemical characterization of the bacterial isolates

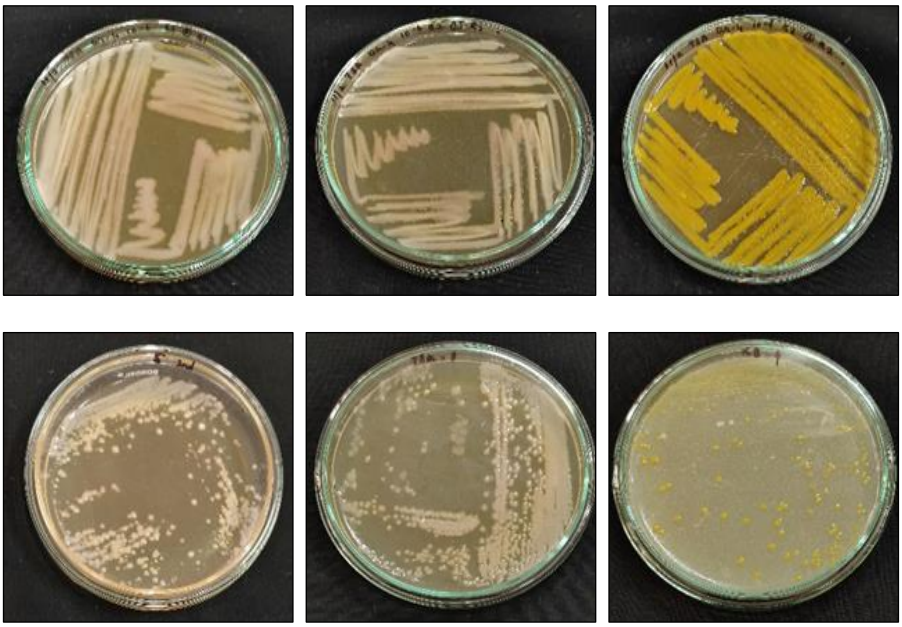
All the isolates were subjected to biochemical characterization reactions.

D12, D17, D6, D22 and D2 isolates showed positive for starch hydrolysis. All the isolates tested positive for catalase activity and urease test. Most of the isolates were tested positive for the citrate utilization and MR test. Few isolates were found positive for VP test. The development of a red or violet ring at the top of the culture solution was a consistent sign of IAA production in all of the isolates.

Isolates D2, D5, D8, D9, D17, D18, D19 and D22 were able to utilise glucose and sucrose sugars and the isolates D6, D7, D12, D14 and D16 did not utilise either glucose or sucrose. Rest of the isolates able to use either glucose or sucrose. D8, D9 and D11 showed positive result for Gram's reaction and rest were Gram negative.

Table 1: Biochemical characterization of the bacterial isolates

Isolates	Gram's Reaction	Colony colour and colony morphology	Fermentation of carbohydrates				IAA test	MR test	VP test	Urease test	Citrate Utilization test	Starch hydrolysis test	Catalase test
			Glucose		Sucrose								
			A	G	A	G							
D2	-	Transparent white colony	+	+	-	+	+	+	-	+	+	+	+
D3	-	Small transparent white colony	+	+	-	-	+	-	+	+	+	-	+
D5	-	Pure white raised colony	+	-	+	-	+	+	-	+	+	-	+
D6	-	Pure white raised colony	-	-	-	-	+	+	-	+	+	+	+
D7	-	Dotted yellow colour slimy colony	-	-	-	-	+	-	+	+	+	-	+
D8	+	Pure white raised colony with even margin	+	-	-	-	+	+	-	+	+	-	+
D9	+	Yellow colour colony with even margin	+	-	-	-	+	+	-	+	+	-	+
D11	+	Dull yellow colour raised colony with even margin	+	-	-	-	+	+	-	+	+	-	+
D12	-	Small white colour raised colony	-	-	-	-	+	+	-	+	+	+	+
D14	-	Yellow colour raised colony with even margin	-	-	-	-	+	+	-	+	+	-	+
D15	-	Small transparent white colony	-	-	-	+	+	+	-	+	+	-	+
D16	-	Pure white raised colony	-	-	-	-	+	-	+	+	+	-	+
D17	-	Yellow colony with even margin	+	+	+	-	+	+	-	+	+	+	+
D18	-	Yellow slimy colony	+	-	+	+	+	+	-	+	+	-	+
D19	-	White slimy colonies	+	-	+	+	+	+	-	+	+	-	+
D20	-	Dotted white colony	+	+	-	-	+	+	-	+	+	-	+
D22	-	Yellow slimy colony	+	+	+	+	+	+	-	+	+	+	+
D23	-	Dull yellow transparent colony	+	-	-	-	+	+	-	+	-	-	+
D24	-	Dull yellow transparent colony	+	-	-	-	+	+	-	+	-	-	+



Rhizobium pusense *Bacillus megaterium* *Lysinibacillus sphaericus*

Fig 1: Purified cultures of the bacterial isolates

3.3 Quantitative assay for Indole-3-Acetic Acid (IAA) production

Plants respond to drought stress by producing auxin indole-3-acetic acid (IAA), which is critical for plant adaptation to drought stress. It has been established that a bacterial strain's local distribution of IAA to plant root nodules altered root IAA polar transport and encouraged the growth of more branching root systems with more secondary roots and nodules (Defez *et al*., 2017) [4]. With the addition of 100 µg/mL tryptophan to the cultures, the quantitative estimation of IAA was carried out. All the cultures showed a considerable production of IAA in the range of 12 to 15 µg/ml with observable significant variation among them. The maximum of IAA production was given by D5 (15.7 µg/ml) followed by D8 & D9. The minimum production was by D2 (12.3 µg/ml). The cultures D14, D18, D19 were at par with each other. D5, D8 & D9 were found to produce satisfactory results for IAA production in comparison with the other isolates. (Table 2)

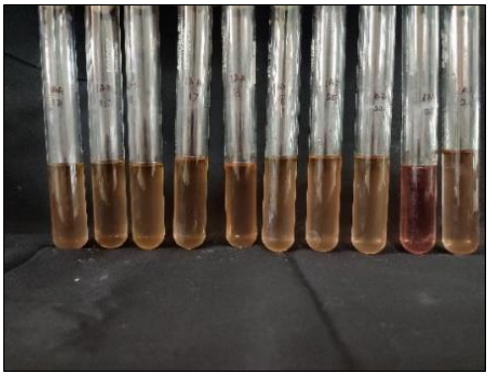


Fig 2: IAA produced by the bacterial isolates

3.4 Quantitative assay for GA3 production

The culture solutions were grown in nutrient broth and the GA3 production was quantified by the addition of DNPH reagent. In comparison with the other cultures, D8 showed significantly higher production of gibberellic acid, with a value of 19.70 g/ml, followed by D4 & D9. It was reported that the exopolysaccharide production has been enhanced by the presence of gibberellic acid in addition to the other parameters, which include the growth, biomass, and pigment production (Prathipa *et al*., 2021) [14]. Additionally, it has been revealed that the isolates which produces significant amount of exopolysaccharides under minimum water potential were found to exhibit PGPR characteristics which includes IAA, gibberellic acid and P solubilization (Sandhya *et al*., 2009). The isolates D16, D18, D19, and D23 yielded the lowest production. The isolates which showed on-par results include D12, D13, D14, D15, D16 and D17.

3.5 Quantitative assay for phosphate solubilization

According to Mahmood *et al.* (2004) [2], EPS synthesis in the rhizosphere region could improve water availability and nutrient content, which includes phosphorus and potassium, and also help plants tolerate salinity. The cultures were cultivated in Pikovskaya's broth supplemented with 0.5% tricalcium phosphate. The results obtained were in accordance with this study, which revealed that D5 and D9 could solubilize phosphorus at a maximum rate of 98.4 and 97.34 mg/ml, respectively than the others. The minimum solubilization was observed in D2. The results of D7, D8, D9, D11 and D12 isolates were at par (Fig 3). It has been revealed in a study that 48 out of 86 isolated strains from the drought soil showed solubilization of phosphorus in the range of 41.7 to 226.35 mg/ml (Kour *et al*., 2019) [9]. Additionally, it has been shown that P-solubilizing microorganisms provide many benefits, which include nitrogen fixation, availability of trace elements, increased production of iron chelating compounds, thereby giving overall protection against soil-borne pathogens and encouraging plant growth.

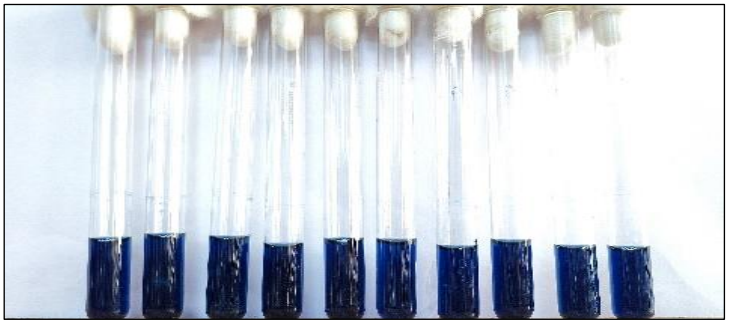


Fig 3: Phosphate solubilization by bacterial isolates

3.6 Quantitative assay of Potassium release

K is the essential determinant of cell turgor (White, 2013) [20], needed for cell expansion and for promoting growth in plants. For the maintenance of turgidity and water uptake of crops being cultivated in drought soil, there is a criteria of reduction in the osmotic potential by the increase in the cellular osmolyte concentration. To enable osmotic adjustment and maintain cell proliferation at low soil water potentials in the field, a substantial K supply is necessary (Grzebisz *et al*., 2013) [5]. For the estimation of potassium release, the cultures were inoculated into Aleksandrov's broth. In accordance with this study, significantly higher amount of K content has been released by the isolate D5 (3.44 mg/ml) and the lowest level of release by D6 (2.23 mg/ml). D8 and D9 were also comparatively good in K release (3.15 and 3.27 mg/ml K) with that of D5. D11, D19, and D24 exhibited on-par results of 2.54, 2.52 and 2.56 mg/ml, respectively. Plant growth-promoting bioinoculants are expected to have greater significance in increasing crop production by providing the enhancement of shelf life and tolerance to adverse conditions (Suman *et al*., 2016). Additionally, it plays a major role in the improvement of plant growth, yield, and soil fertility. The bacterial cultures isolated from drought soil produced potassium in the range of 1875.0 µg/ml to 1812.5 µg/ml. The process of solubilising fixed potassium into the soil by the microbes was considered to be the vital mechanism of potassium release by chelation, acidolysis, exchange reactions and production of organic acids (Kumar *et al*., 2021).

Table 2: PGP traits of the bacterial isolates

Isolates	IAA (µg/ml)	GA 3 (µg/ml)	PO_4 Solubilization (mg/ml)	K Solubilization (mg/ml)
D2	12.3±0.05^{l}	17.90±0.36^{h}	93.78±0.09^{de}	3.15±0.00^{c}
D3	13.1±0.25^{i}	18.34±0.06^{cd}	94.35±1.30^{de}	2.28±0.03^{l}
D5	15.7±0.30^{a}	18.90±0.19^{c}	98.40±1.98^{a}	3.44±0.07^{a}
D6	14.1±0.12^{e}	17.90±0.06^{f}	93.45±0.06^{de}	2.23±0.01^{m}
D7	13.5±0.06^{h}	18.23±0.16^{e}	94.16±0.34^{bc}	2.72±0.01^{f}
D8	14.9±0.11^{b}	19.70±0.02^{a}	96.70±0.44^{cd}	3.15±0.03^{c}
D9	14.6±0.28^{c}	19.10±0.30^{b}	97.34±1.55^{b}	3.27±0.01^{b}
D11	12.8±0.00^{k}	18.56±0.43^{d}	95.70±0.05^{ab}	2.54±0.01^{g}
D12	13.6±0.04^{g}	18.10±0.01^{ef}	94.56±0.71^{de}	2.92±0.04^{d}
D14	12.5±0.03^{kl}	17.43±0.00^{hi}	95.19±1.22^{bc}	2.98±0.01^{d}
D15	12.7±0.05^{j}	18.60±0.13^{cd}	95.61±0.04^{bc}	2.82±0.02^{e}
D16	12.4±0.04^{l}	17.30±0.19^{ij}	94.40±0.05^{de}	2.94±0.01^{d}
D17	14.0±0.31^{f}	17.78±0.07^{gh}	94.60±0.17^{d}	2.39±0.00^{jk}
D18	13.9±0.01^{de}	17.00±0.02^{ij}	95.78±0.08^{bc}	2.41±0.02^{ij}
D19	12.5±0.02^{kl}	17.17±0.08^{ij}	92.56±0.88^{e}	2.52±0.00^{gh}
D20	14.3±0.11^{d}	18.00±0.10^{ef}	94.36±1.03^{de}	2.34±0.01^{kl}
D22	14.0±0.15^{de}	18.21±0.23^{de}	93.63±0.43^{cd}	2.49±0.01^{hi}
D23	13.7±0.26^{fg}	17.10±0.06^{j}	93.88±0.28^{de}	2.45±0.03^{ij}
D24	12.4±0.04^{jk}	17.89±0.02^{g}	95.21±0.91^{bd}	2.56±0.04^{g}
S.Ed.	0.22	0.25	1.18	0.03
CD(0.05)	0.44	0.51	2.39	0.07

3.7 Screening the isolates for exopolysaccharides production

Polysaccharides are involved in the maintenance of soil structure even though they are not the primary aggregating agents. Other molecules, such as humic acids, are also responsible for soil structure. The importance of biopolymers in soil structure may explain their resistance to degradation. The higher the resistance, the longer the EPS remains in soils. The association of polymers with metal ions and colloids, such as clay, may also influence the degradation rates of polymers because of their influence on enzymatic activity. Since the addition of polymers to soil started to be investigated, it has been demonstrated that the binding power of plant and microbial polysaccharides is variable. However, characteristics of the soil such as pH also influence the action of polysaccharides, because the charges of molecules are essential for binding particles (Martin, 1971) [12]. Ilyas *et al*., (2020) [7] reported that *Bacillus subtilis* and *Azospirillum brasilense* were known to withstand drought stress and produce considerable quantity of EPS individually. Even actually, the combination of these strains produced higher amount of exopolysaccharides (sugar 6976 µg/g, 731.5 µg/g protein and 1.1 mg/g uronic acid). Extracellular polymeric substances (EPS) production can confer advantages to microorganisms in environments under drought stress. A high water-holding capacity was observed for an EPS produced by a *Pseudomonas* strain isolated from soil. This EPS can hold several times its weight in water. When added to a sandy soil, the EPS altered its moisture by allowing the amended soil to hold more water than unamended soil. The production of exopolysaccharides by lactic acid bacteria ranges from 10 mg/L to 400 mg/L (Jurášková *et al*., 2022) [8]. When using the phenol-sulphuric acid assay for the quantification of EPS produced by the isolated bacterial cultures, the cultures at stationary phases were taken into the ELISA plate after centrifugation and precipitation procedures along with the conc. sulfuric acid and phenol solution (Deka *et al*., 2018). The isolates D5, D8 and D9 were known to secrete a maximum amount of exopolysaccharides at a rate of 50.00, 49.89 and 49.67 mg/ml, respectively. Other cultures produced EPS in the range of 44 to 48 mg/mL. The best cultures were taken for further processing (Table 3).

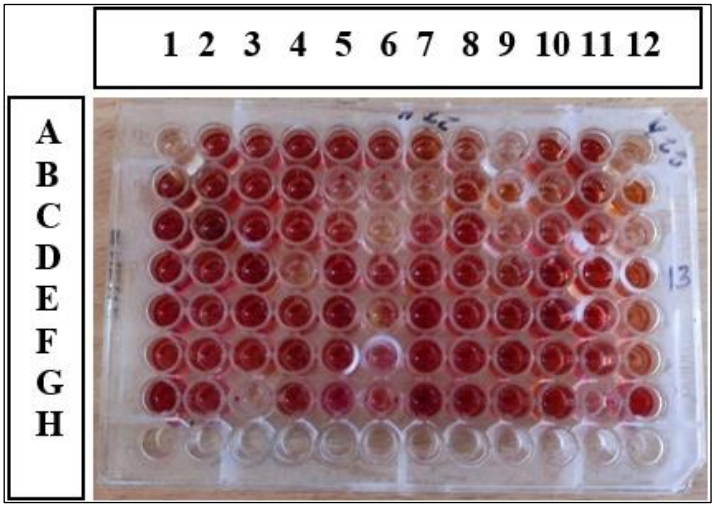


Fig 4: Quantification of EPS in ELISA plate reader

Table 3: Quantification of EPS produced by the bacterial isolates

Isolates	EPS produced (mg/ml)
D2	45.36±0.95^{g}
D3	45.58±0.08^{e}
D5	50.00±0.96^{a}
D6	46.12±0.46^{de}
D7	44.29±0.91^{ij}
D8	49.89±0.08^{a}
D9	49.67±0.16^{a}
D11	47.19±0.47^{d}
D12	46.09±0.66^{ef}
D14	47.17±0.16^{de}
D15	44.19±0.70^{j}
D16	47.74±0.96^{c}
D17	45.15±0.05^{h}
D18	46.24±0.94^{e}
D19	48.54±0.61^{b}
D20	44.71±0.73^{i}
D22	45.49±0.02^{f}
D23	45.42±0.21^{f}
D24	48.39±0.11^{b}
SEd	0.61
CD (0.05)	1.24

3.8 Molecular identification of bacterial isolates

The molecular characterization through 16S rRNA sequencing was carried out for the three promising, maximum exopolysaccharides producing isolates. Further the BLAST analysis of 16S rRNA sequence revealed that D5 isolate exhibited 99.24% identity to *Rhizobium pusense*, D8 isolate exhibited 98.20% identity to *Bacillus megaterium* and D9 isolate exhibited 96.71% identity to *Lysinibacillus sphaericus*.

4. Conclusion

Based on the above results, among the studied isolates, *Rhizobium pusense*, *Bacillus megaterium* and *Lysinibacillus sphaericus* were considered to be the best performing and most promising cultures with the significant secretion of exopolysaccharides, which is the sole compound of this study. In addition, these cultures exhibited better performances in IAA, GA3 production, phosphate solubilization and K release, giving supportive results. Hence, these cultures have been proposed for further enhanced production of EPS and extraction of EPS from the cultures, in order to use it for sustainable plant growth and crop production with consistent productivity.

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