Exploration of exopolysaccharide producing bacterial cultures from various drought affected region

ABSTRACT

Exopolysaccharides (EPS) are extracellular macromolecules secreted by microorganisms play a vital role in improving soil aggregation as well as enhancing the water holding capacity of the soils. This study focuses onto the isolation and characterization of bacterial strains capable of producing Exopolysaccharides (EPS) from drought-affected soil regions across different districts of Tamil Nadu. The core objective revolves around the molecular identification of elite cultures and pivotal genes, responsible for EPS production namely *dex* (dextransucrase) by using the primer pair *WConDex fw* and *WConDex* rev at an amplification size of 1037 *bp* size. A total of 72 bacterial cultures were isolated from 67 soil samples that are capable of secreting EPS using specific medium.

Keywords: *Exopolysaccharides, Phenol sulfuric acid assay, dextran gene*.

1. Introduction

Exopolysaccharides (EPS) are complex carbohydrates with a high molecular weight. They play pivotal roles in enabling bacteria to create biofilms, which are structured microbial communities and attach to different surfaces, including plant roots and soil particles. This attachment capability is significant in facilitating interactions between bacteria and their environment. These functions collectively contribute to the intricate dynamics of bacteria in various contexts. Extensive evidence supports the notion that plants treated with microbes that produce exopolysaccharides (EPS) exhibit enhanced resilience to water stress, primarily attributed to improvements in soil structure [2] and aggregation [3].This study aims to isolate the bacterial cultures having the potential of EPS production, as the EPS producing cultures are considered alternative technology for enhanced root development and easy access of soil moisture and nutrients by its role of aggregation of soil particles.

2. Materials and methods

2.1 Sampling and sampling location

The collection of soil samples from diverse drought-affected areas from the districts of Madurai, Sivagangai, Virudhunagar, Ramanathapuram, Pudukkottai, Dharmapuri, Krishnagiri, Salem, and Tiruchirappalli, marks a crucial step in the isolation of bacterial strains with the ability to produce EPS (extracellular polymeric substances). The study involves a comprehensive collection of 67 distinct soil samples, each obtained from these targeted locations which are impacted by drought conditions, the

study was carried out under laboratory in Department of Agricultural Microbiology of Agricultural College and Research Institute, Madurai.

2.2 Isolation of bacteria from drought soil

The isolation of cultivable bacterial communities from the soil was done by following the spread plate technique. This method includes a series of soil dilutions, extending up to 10^{-6}. 0.1ml of sample from 10^{-5} and 10^{-6} dilutions were spread over the TSA plate (Trypticase soy agar) [15] containing tryptone 17.00g, soya peptone 3.00g, sodium chloride 5.00g, dextrose(Glucose) 2.5g,dipotassium hydrogen phosphate 2.g, agar 15.0g at a pH 7.3 ± 0.2 and incubated at 30^{o}C for 3-5 days. After incubation the mucoid colonies were picked up.

2.3 Screening isolates for exopolysaccharides (EPS) production

To screen the EPS producing bacterial colonies a specific medium called salt agar base (SAB) was used [4]. The isolated cultures were tested for growth in SAB medium. The strains that were capable of producing EPS exhibit good growth, wherein the other colonies failed to grow. This process facilitated the identification of bacterial strains that exhibited EPS production ability. The bacterial isolates grown on the media were further quantified for EPS production.

2.4 Extraction of the exopolysaccharides (EPS) of bacterial isolates

1ml of the bacterial isolates in the log phase were introduced into 10 ml nutrient broth and placed in an incubator shaker at a speed of 200 rpm. This setup was maintained for 48 hours, allowing the cultures to progress to the stationary phase. EPS extraction and seperation of cells was done by centrifugation at 10000 rpm for 20 minutes . Cold acetone was added thrice the volume of the recovered supernatant and left overnight at 4°C for precipitation. Following an overnight incubation, the precipitated EPS was recovered by centrifugation at 6000 rpm for 10 minutes [14]. The recovery of the EPS was about 1.5 µl ml^{-1}.

2.5 Quantification of the exopolysaccharides (EPS) of bacterial isolates

The recovered EPS was estimated using the phenol sulfuric acid assay. In a microtitre plate, 50 microlitre of the EPS precipitate that had been dissolved in sterile water, 150 microlitre of concentrated sulfuric acid, and 30 microlitre of 5% aqueous phenol solution were added. The plate was heated to 90^{o} Celsius in a water bath and then cooled to 35^{o} Celsius. The plate was examined at 490 nm using an ELISA plate reader and the OD was recorded. EPS was calculated using the formula; y = 0.0608x, wherein ‘y’ indicates OD and ‘x’ values indicate the amount of EPS production. Estimated EPS was expressed in mg ml $^{-1}$ [5].

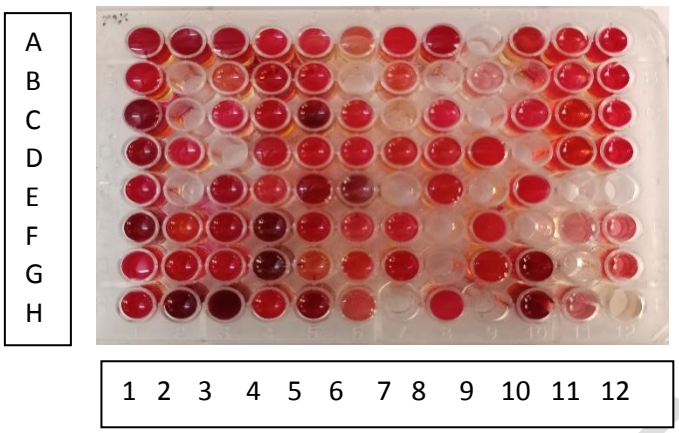

Fig.1. Quantification of Exopolysaccharide in ELISA plate reader

2.6 Isolation of genomic DNA from bacterial isolates

In order to amplify the specific gene for EPS production. Genomic DNA was isolated from 21 selected bacterial isolates were subsequently, utilized for the purpose of DNA extraction, employing the cetyl trimethyl ammonium bromide (CTAB) method [6]. The selected bacterial isolates were grown in Nutrient broth and kept in a shaker at 150 rpm for 3 days. The bacterial suspension was centrifuged at 8000rpm for 10 minutes. Discard the supernatant and suspend the pellet with 500µl of 1X TE buffer, 30 µl of 10% SDS and incubated in water bath for 1hour at 37^{o}C. Then 100µl of 5M Nacl and 80µl of CTAB was added and incubated for 10 minutes at 65^{o}C later equal volume of chloroform: iso amylalchohol (24:1) and centrifuged at 10000rpm for 15 minutes. To the top clear aqueous layer equal volume of isopropanol was added and kept overnight at - 20^{o}C and centrifuged at 10000rpm for 15 minutes, the pellets were collected. The pellets was washed with 70% ethanol and air dried for 30 minutes. The pellets 50 µl of TE buffer or milliQ water was added and stored at -20^{o}C.

2.7 Molecular confirmation of genes required for EPS biosynthesis

The polymerase chain reaction (PCR) was employed to detect the presence of specific gene, *dex* (dextransucrase), which is responsible for encoding proteins essential in EPS biosynthesis. This process involved using specific primers, such as *WConDex fw* (5'-TGTGGATTCAGGACACCGTA-3') and *WCon Dexrev* primer (3'-GGTTCAATCACGGCTAACG-5') for dextransucrase. To initiate the PCR, a combined solution of 50µL was prepared. This mixture included 5µL of both forward and reverse primers, 5µL of the DNA sample, 10 µL of molecular-grade water, and 25 µL of the master mixture containing components such as dNTPs, Taq DNA polymerase, and $MgCl_2$. The PCR reaction consisted of specific temperature and time cycles: an initial denaturation at 94^{o}C for 4 minutes, followed by 30

cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, extension at 72°C for 100 seconds, and a final extension at 72°C for 7 minutes for detecting the dextran sucrase gene [7]. The PCR products were separated and visualized using agarose gel electrophoresis [8]. The resulting gel was then analysed and documented using the Alpha imager TM 1200 gel documentation device.

2.8 Molecular characterization of bacterial isolates through 16S rRNA sequencing

The isolates which showed the amplification of the specific genes were identified as per the following specific protocol. The technique of Polymerase Chain Reaction (PCR) was employed to amplify the 16S rRNA gene that is present within the isolated genomic DNA. This amplification was achieved through the use of universal bacterial primers: the 27F forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') and the 1492R reverse primer (3'-AAGGAGGTGATCCAGCCGCA-5). The Polymerase Chain Reaction (PCR) was performed using a total volume of 50 µL for the PCR mixture. This mixture included 5 µL each of the forward and reverse primers, along with 5 µL of the sample DNA. Additionally, 10 µL of molecular grade water and 25 µL of a master mixture containing dNTPs, Taq DNA polymerase, and MgCl2 were added. The PCR reaction was carried out under standard conditions, including initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 40 seconds, and extension at 72°C for 90 seconds. A final extension step was performed at 72°C for 7 minutes [9]. Subsequently, the PCR products were separated using agarose gel electrophoresis, [8]. Finally, the gel was observed and captured using the Alpha Imager TM 1200 gel documentation system followed by submission of the sequence in the NCBI blast to identify the bacterial culture.

3. Results and Discussion

3.1 Isolation of bacteria from drought soil

A total of 92 bacterial cultures were successfully isolated from the soil samples exposed to drought conditions by following the method of spread plate technique on TSA (Tryptic soy agar) plates. TSA medium served the purpose of refining the colonies that displayed the capability to produce EPS. The isolates capable of EPS production were effectively singled out and isolated for further study [10].

3.2 Screening of isolates for EPS production

All the isolates were subjected to screening on salt agar base (SAB). Results showed that out of 92 bacterial isolates, 72 were able to grow on SAB medium, indicating that these culture were able to produce EPS. The 72 cultures screened were based on the slimy and mucoid nature and were sub cultured and pure cultures were maintained for further study and the same was used earlier for screening of EPS producing bacterial cultures such as *Pseudomonas sp* and *Basillus sp* [4].

3.3 Quantification of cultures for Exopolysaccharide (EPS) production

The cultures exhibited growth in SAB medium were further selected for quantification of EPS. EPS was quantified using phenol sulfuric acid assay. Notably, 21 isolates exhibited the highest EPS levels, ranging from 64.19 mg ml^{-1} to 53.93 mg ml^{-1} while the remaining isolates recorded EPS quantities within the range of 52.91 mg ml^{-1} to 40.08mg ml^{-1} (values not shown). The isolates that produced higher EPS levels were selected for further study and the values were given in table1. The isolate A1 registered significantly higher production of EPS (64.19 mg ml^{-1}) than any other culture. This was followed by the isolate 183 (62.41 mg ml^{-1}) and BJ5 III (58.47 mg ml^{-1}) and AP III (53.93 mg ml^{-1}). Rest of the isolates did not show any significant variation in EPS production. EPS in bacteria are hydrophilic compounds containing a significant water component, constituting around 97% of their polymer matrix. This polymer structure serves as a protective barrier against desiccation during periods of water scarcity [11]. EPS also function as reservoirs, effectively capturing and retaining water as well as essential nutrients within their complex structures. This retention mechanism prevents nutrient leaching and ensures a consistent supply of vital elements to nourish plants, even during drought episodes. Moreover, EPS act as catalysts, intensifying the activity of diverse soil enzymes. This enzymatic stimulation drives critical biochemical reactions that facilitate nutrient cycling and the decomposition of organic matter, ultimately enhancing soil vitality. The production of EPS by white coloured smooth lactic acid bacteria typically ranged from 10 mg L^{-1} to 400 mg L^{-1} [12].

Table 1. Estimation of the Exopolysaccharides production by the bacterial isolates

Sl No	Source of the sample	Location	Designation of the bacterial cultures	Quantity of exopolysaccharides (mg ml^{-1})
1.	(COLLECTED FROM DEPARTMENT OF AGRICULTURAL COLLAGE AND RESEARCH INSTUTUTE)	10.23° N, 77.49 ° E	A1	64.19 ± 0.17^{a}
2.	EDUKATTUR	9.69 ° N, 78.45 °E	183	62.41 ± 0.03^{b}
3.	BARIJUM	10.24 ° N, 77.49 ° E	BJ5 III	58.47 ± 0.17^{c}
4.	ATTUPANNAI III	10.23 ° N, 77.49 ° E	AP III	53.93 ± 0.21^{bc}
5.	ANNIYENTHAL	9.74 ° N, 78.38 ° E	2110	52.91 ± 0.10^{bcd}
6.	NARIKUDI	9.58 ° N, 78.31 ° E	102	52.85 ± 0.20^{cd}
7.	SOWLOOR	12.16 ° N, 78.15 ° E	653	52.20 ± 0.14^{d}
8.	ARUPUKOTTAI	9.42 ° N, 78.07 ° E	401	50.92 ± 0.24^{e}
9.	PODAMPATTI	9.52 ° N, 78.09 ° E	201	50.12 ± 0.09^{f}

10.	PODAMPATTI	9.52 ^{o} N, 78.09 ^{o} E	202	49.92 ± 0.18^{f}
11.	ANNIYENTHAL	9.74^{0} N, 78.38^{0} E	21	49.3 ± 0.02^{f}
12.	PULLIYANGULAM	9.57 ^{o} N, 78.27 ^{o} E	422	49.08 ± 0.13^{f}
13.	THURUPATHUR	10.10 ^{o} N, 78.59 ^{o} E	T2	48.24 ± 0.18^{f}
14.	PODAMPATTI	9.52 ^{o} N, 78.09 ^{o} E	211	45.43 ± 0.14^{f}
15.	PULIYANGULAM	9.57 ^{o} N, 78.27 ^{o} E	44(1)	44.79 ± 0.14^{f}
16.	MARANDAPATTI	12.39 ^{o} N, 78.00 ^{o} E	673	43.98 ± 0.05^{f}
17.	THIRUPACHETHI	9.77 ^{o} N, 78.34 ^{o} E	276	41.48 ± 0.13^{f}
18.	POOMALAIPATTI	9.58 ^{o} N, 78.31 ^{o} E	121	40.90 ± 0.15^{f}
19.	PODAMPATTI	9.52 ^{o} N, 78.09 ^{o} E	212	40.84 ± 0.11^{f}
20.	ARASAKULAM	9.67 ^{o} N, 78.11 ^{o} E	193	40.1 ± 0.16^{f}
21.	PADI	12.18 ^{o} N, 78.09 ^{o} E	694	40.08 ± 0.07^{f}
CD				4.10
S.ED				2.02

3.4 Molecular identification of the Dextransucrase gene *(dex)*

Through the utilization of the primer pair *WConDexfw* and *WConDex* rev, 21 isolates were subjected to molecular testing for the presence of the EPS gene *dex* (dextransucrase) at amplification size of 1037 bp (Malang *et al*., 2015)[7]. The results revealed that among the tested isolates, 12 of them harboured the genes responsible for encoding the dextransucrase protein, a key component in EPS biosynthesis. Conversely, the rest of the isolates lacked the specific gene associated with the protein responsible for EPS biosynthesis. Similarly, Chenu *et al*., (1993) [13]. used the gene *dex* to screen the bacterial cultures for EPS production and showed that out of 15 cultures, 8 were observed positive for the presence of the gene *dex*.

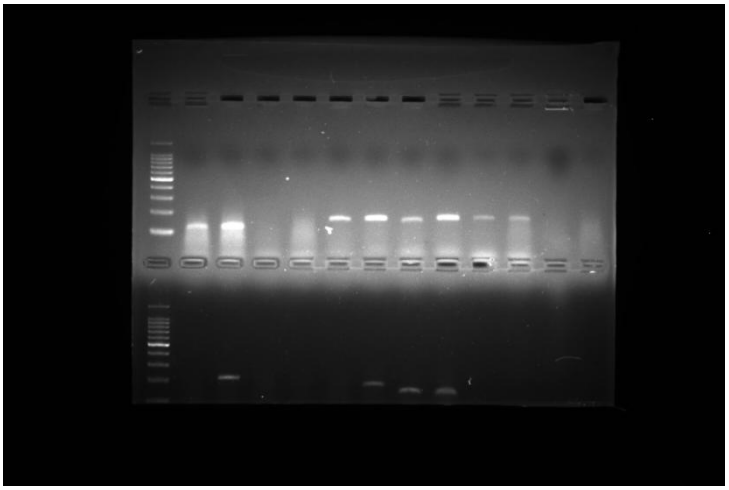

Fig 2. Agarose gels (1.2% w/v) showing the amplicons of dextransucrase gene (*dex*) using primer pairs *WconDex fw* and *WconDex rev*

Lane no	Isolate No	*dex*	Lane no	Isolate n	*dex*	Lane no	Isolate no	*dex*	Lane no	Isolate	*dex*
1.	AP III	+	7.	653	+	13.	422	-	19.	121	+
2.	183	+	8.	401	+	14.	T2	+	20.	212	+
3.	694	-	9.	201	+	15.	211	-	21.	193	+
4.	BJ5 III	+	10.	202	+	16.	44(1)	-			
5.	2110	+	11.	141	+	17.	673	-			
6.	102	+	12.	A1	+	18.	276	+			

Table 2. Agarose gels (1.2% w/v) showing the amplicons of dextransucrase gene (*dex*) using primer pairs *WconDex fw* and *WconDex rev*

3.5 PCR amplification of 16S rRNA

The molecular characterization through 16S rRNA sequencing was carried out for the four promising, maximum exopolysaccharides producing isolates. Further the BLAST analysis of 16S rRNA sequence revealed that isolate 183 exhibited 99.24% identity to *Bacillus sp. (firmicutes)*, isolate BJ5 III exhibited 98.22% identity to *Pseudomonas azotoformans* while isolate AP 3 exhibited 98.94% identity to *Pseudomonas sp* and isolate A1exhibited 100% identity to *Bacillus altitudinis* (fig 3.).

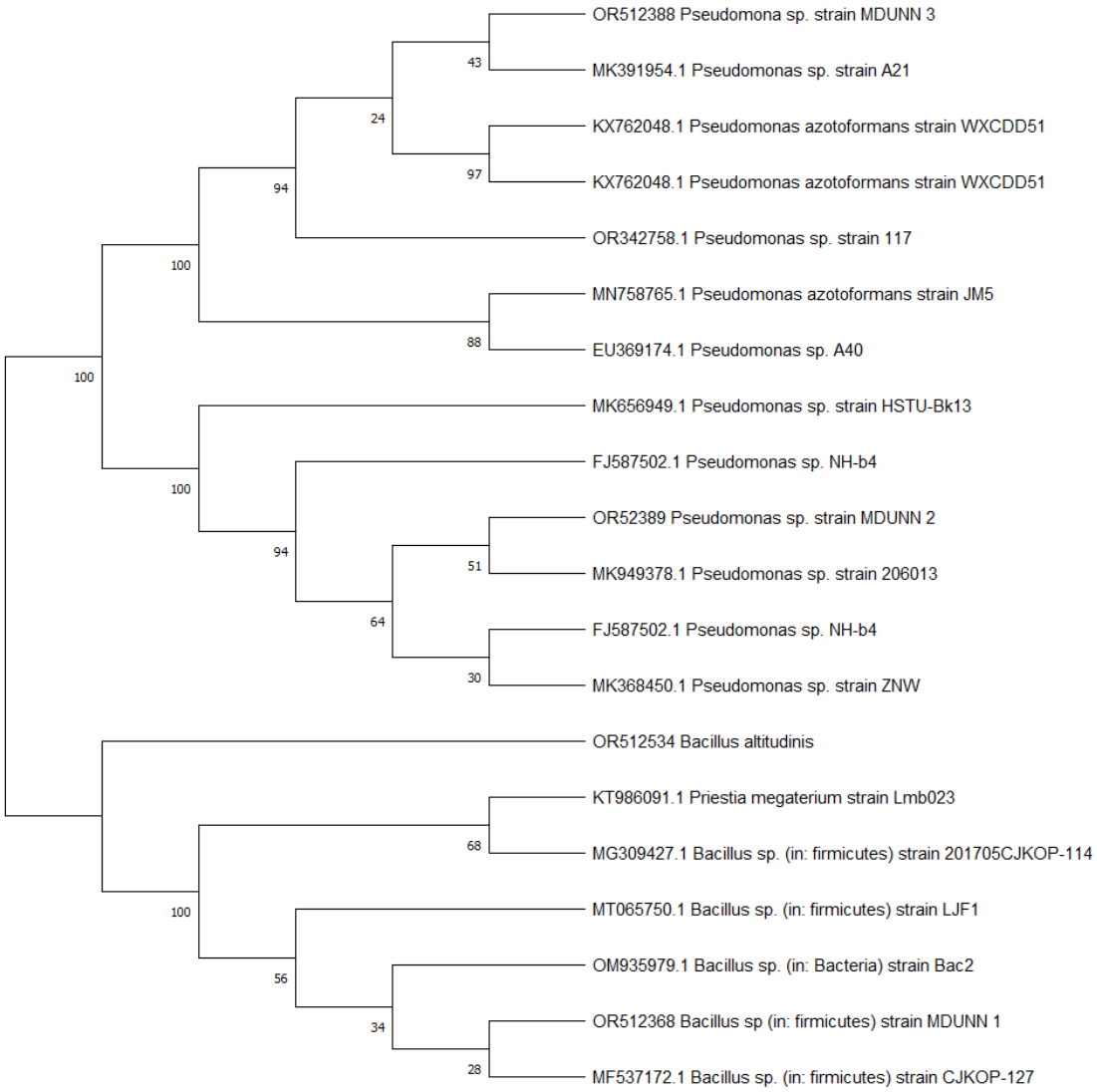

Fig 3. Phylogenetic tree based on 16S rDNA sequence of elite EPS producing bacterial isolates from drought affected regions.

4. Conclusion

Considering the outcomes outlined above, *Bacillus altitudinis* A1, *Bacillus* sp. *(firmicutes)* 183 out performed for EPS production, followed by *Pseudomonas azotoformans* BJ5 III and *Pseudomonas* sp., AP III are the best EPS producers than the other evaluated isolates. These identified cultures exhibited noteworthy exopolysaccharide secretion, which constitutes the exclusive focus of this study. Their remarkable exopolysaccharide production positions them as the primary culture for further analysis and potential application as a alternative technology for enhanced root development and easy access of soil moisture and nutrients by its role of aggregation of soil particles there by employing EPS producing bacterial cultures for improving soil health and aid in drought alleviation.

REFERENCE

1. Angelin J, Kavitha M. Exopolysaccharides from probiotic bacteria and their health potential. International Journal of Biological Macromolecules. 2020 Nov 1;162:853-65.

2. Bensalim S, Nowak J, Asiedu SK. A plant growth promoting rhizobacterium and temperature effects on performance of 18 clones of potato. American Journal of Potato Research. 1998 May;75:145-52.

3. Naseem H, Bano A. Role of plant growth-promoting rhizobacteria and their exopolysaccharide in drought tolerance of maize. Journal of Plant Interactions. 2014 Jan 2;9(1):689-701.

4. Ventorino V, Nicolaus B, Di Donato P, Pagliano G, Poli A, Robertiello A, Iavarone V, Pepe O. Bioprospecting of exopolysaccharide-producing bacteria from different natural ecosystems for biopolymer synthesis from vinasse. Chemical and Biological Technologies in Agriculture. 2019 Dec;6:1-9.

5. Deka P, Goswami G, Das P, Gautom T, Chowdhury N, Boro RC, Barooah M. Bacterial exopolysaccharide promotes acid tolerance in Bacillus amyloliquefaciens and improves soil aggregation. Molecular Biology Reports. 2019 Feb 1;46:1079-91.

6. Jahan S, Chowdhury SF, Mitu SA, Shahriar M, Bhuiyan MA, Banats J. Genomic DNA extraction methods: a comparative case study with gram–negative organisms. Biotechnol. 2015 Jan 1;6(11):61-8.

7. Malang SK, Maina NH, Schwab C, Tenkanen M, Lacroix C. Characterization of exopolysaccharide and ropy capsular polysaccharide formation by Weissella. Food microbiology. 2015 Apr 1;46:418-27.

8. Green MR, Sambrook J. Polymerase chain reaction. Cold Spring Harbor Protocols. 2019 Jun 1;2019 (6):pdb-top095109.

9. Noble RT, Weisberg SB. A review of technologies for rapid detection of bacteria in recreational waters. Journal of water and health. 2005 Dec 1;3(4):381-92.

10. Kaci Y, Heyraud A, Barakat M, Heulin T. Isolation and identification of an EPS-producing Rhizobium strain from arid soil (Algeria): characterization of its EPS and the effect of inoculation on wheat rhizosphere soil structure. Research in Microbiology. 2005 May 1;156(4):522-31.

11. Hunter RC, Beveridge TJ. High-resolution visualization of Pseudomonas aeruginosa PAO1 biofilms by freeze-substitution transmission electron microscopy. Journal of bacteriology. 2005 Nov 15;187(22):7619-30.

12. Jurášková D, Ribeiro SC, Silva CC. Exopolysaccharides produced by lactic acid bacteria: From biosynthesis to health-promoting properties. Foods. 2022 Jan 8;11(2):156.

13. Chenu C. Clay—or sand—polysaccharide associations as models for the interface between micro-organisms and soil: water related properties and microstructure. Soil Structure/Soil Biota Interrelationships. 1993 Jan 1:143-56.

14. Andhare, P., Goswami, D., Delattre, Cedric Delattre, Guillaume Pierre, Phillipe Michaud, Hilor Pathak. Edifying the strategy for the finest extraction of succinoglycan from *Rhizobium radiobacter* strain CAS. *Appl Biol Chem* **60**, 339–348 (2017). https://doi.org/10.1007/s13765-017-0286-8

15. Timmusk S, Abd El-Daim IA, Copolovici L, Tanilas T, Kännaste A, Behers L, Nevo E, Seisenbaeva G, Stenström E, Niinemets Ü. Drought-tolerance of wheat improved by rhizosphere bacteria from harsh environments: enhanced biomass production and reduced emissions of stress

volatiles. PLoS One. 2014 May 8;9(5):e96086. doi: 10.1371/journal.pone.0096086. PMID: 24811199; PMCID: PMC4014485.