Exploration of exopolysaccharide producing bacterial cultures from various drought affected region

ABSTRACT

Exopolysaccharides (EPS) are extracellular macromolecules secreted by microorganisms as a firmly bonded capsule or a loosely connected slime layer. (Angelin *et al.*, 2020)[1].These substances 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.Soil samples were collected from diverse drought affected areas, and bacterialcultures that are capable of secreting EPS were isolated using specific medium. A total of 72 cultures were isolated from 67soil samples. The core objective revolves around the molecular identification of elite cultures and pivotal genes, responsible for EPS production namely *dex*(dextransucrase).

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 toimprovements in soil structure (Bensalim*et al.*, 1998) [2] and aggregation. (Naseem *et al.*, 2014) [3].This study aims to isolate the bacterial cultures having the potential of EPS production.

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.

UNDER PEER REVIEW

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}. From this 0.1ml of sample from 10^{-5} and 10^{-6} dilution were spread over the TSA (Trypticase soy agar) plate. Inoculated plates were incubated at 30°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(Ventorino*et al.*, 2019)[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

The bacterial isolates in the log phase were introduced into 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 followed by centrifugation at 10000 rpm for 20 minutes was done to separate these cells form the broth. 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.

2.5 Quantification of the exopolysaccharides (EPS) of bacterial isolates

The recovered EPS was then subjected to an estimation procedure 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° Celsius in a water bath and then cooled to 35 ° 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 yindicates OD and x values indicate the amount of EPS production. Estimated EPS was expressed in mg ml^{-1}(Deka *et al.*, 2019)[5]..

UNDER PEER REVIEW

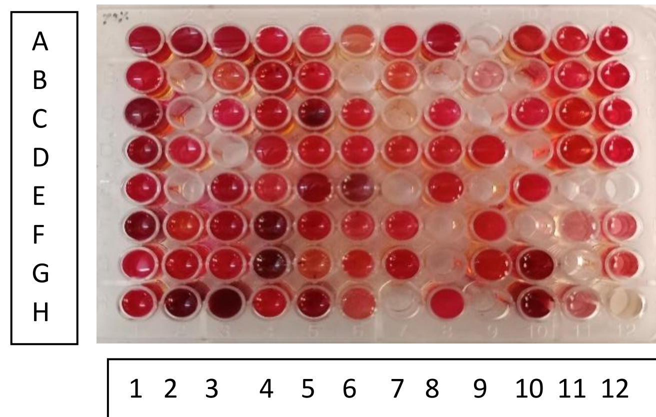

Fig.1. Quantification of Exopolysaccharide in ELISA plate reader

2.6 Isolation of genomic DNA from bacterial isolates

In order to amplify the specific gene for EPS production. Genomic DNA was isolated from selected bacterial isolates. Based on the quantity of EPS produced the top 22 isolates were subjected to the isolation of genomic DNA in order to amplify the specific gene. The selected bacterial isolates were grown in NA broth overnight. Subsequently, they were utilized for the purpose of DNA extraction, employing the acetyl trimethyl ammonium bromide (CTAB) method (Jahan *et al*., 2015)[6]..

2.7 Molecularconfirmation ofgenesrequiredforEPS biosynthesis

The polymerase chain reaction (PCR) was employed to detect the presence of specific genes, namely *dex* (dextransucrase), responsible for encoding proteins essential in EPS biosynthesis. This process involved using specific primers, such as *WConDexfw* (5'-TGTGGATTCAGGACACCGTA-3') and *WConDex* rev primer (3'-GGTTCAATCACGGCTAACG-5') for dextransucrase. To initiate the PCR, a combined solution of 50µL was prepared. This mixture included 5 µL of both forward and reverse primers, 5 µL 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 MgCl2. The PCR reaction consisted of specific temperature and time cycles: an initial denaturation at 94°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 (Malang *et al., 2015*)[7]. The PCR products were separated and visualized using agarose gel electrophoresis (Green and Sambrook in 2019)[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

UNDER PEER REVIEW

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 (Noble and Weisberg in 2005)[9]. Subsequently, the PCR products were separated using agarose gel electrophoresis, (Green and Sam brook in 2019)[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 (Yahia *et al*., 2005)[10]. Totally 92 bacterial isolates were obtained.

3.2 Screening of isolates for EPS production

All the isolates were subjected to screening onsalt 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 selected cultures were sub cultured and pure cultures were maintained for further study and thesame was used earlier for screening of EPS producing bacterial cultures such as *Pseudomonas sp*and*Basillussp(*Ventorino*et al.,*2019)[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, 22 isolates exhibited the highest EPS levels, ranging from 64.19mg ml^{-1} to 53.93 mg ml^{-1}while the remaining isolates recorded EPS quantities within the range of 52.91mg $ml^{-1}$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

UNDER PEER REVIEW

isolate 183 (62.41 mg ml^{-1}) and BJ5 III (58.47mg 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 (*Hunter et al., 2005*)[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 lactic acid bacteria typically ranges from 10 mg L^{-1} to 400 mg L^{-1} (*Jurášková et al., 2022*)[12].

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

Sl no	Source of the sample	Location	Desination of the bacterial cultures	Quantity of exopolysaccharides (mg ml^{-1})
1.	MADURAI (COLLECTED FROM DEPARTMENT)	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° N, 78.09°E	202	49.92 ± 0.18[f]
11.	ANNIYENTHAL	9.74° N, 78.38° E	21	49.3 ± 0.02[f]
12.	PULLIYANGULAM	9.57° N,	422	49.08 ± 0.13[f]

UNDER PEER REVIEW

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

UNDER PEER REVIEW

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

Through the utilization of the primer pair *WConDexfw* and *WConDex* rev, 22 isolates were subjected to molecular testing for the presence of the EPS gene *dex*(dextransucrase) at amplification size of 1037bp (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*.

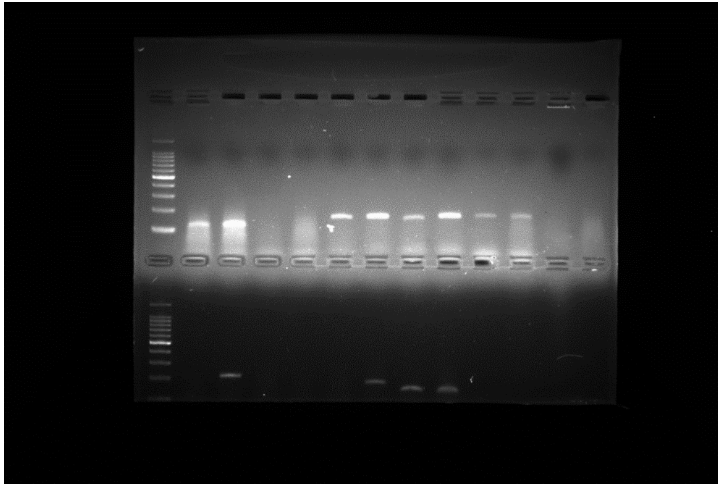

Fig 2Agarose gels (1.2% w/v) showing the amplicons of dextransucrase gene (*dex*) using primer pairs *WconDexfw* and *WconDexrev*

Lane no	Isolate No	*dex*	Lane no	Isolate r	*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.	AP III 4	-	9.	201	+	15.	211	-	21.	193	+
4.	BJ5 III	+	10.	202	+	16.	44(1)	-	22.	694	-
5.	2110	+	11.	141	+	17.	673	-			
6.	102	+	12.	A1	+	18.	276	+			

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

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*

4. Conclusion

Considering the outcomes outlined above, *Bacillus altitudinis*A1, *Bacillus*sp. *(firmicutes)*, 183out 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

UNDER PEER REVIEW

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.

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. Genomic DNA extraction methods: a comparative case study with gram–negative organisms. Banats J 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.