

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. ~~The core objective revolves around the molecular identification of elite cultures and pivotal genes, responsible for EPS production namely dex (dextransucrase). Soil samples were collected from diverse drought affected areas, and A total of 72 bacterial cultures were isolated from 67 soil samples bacterial cultures that are capable of secreting EPS were isolated using specific medium. A total of 72 cultures were isolated from 67 soil samples. The core objective revolves around the molecular identification of elite cultures and pivotal genes, responsible for EPS production namely dex(dextranucrase).~~

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Comment [THS2]:
*Describe the method briefly and
*Highlight the findings of study

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 (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.

Comment [THS3]: Mention the rationale behind the objective and the hypothesis in brief.

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.

Comment [THS4]: Provide characters of soils in table and mention the place of research

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} dilutions were spread over the TSA (Trypticase soy agar) plate. ~~Inoculated plates were~~ and incubated at 30°C for 3-5 days. After incubation the mucoid colonies were picked up.

Comment [THS5]: Give the media composition and citation

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 ~~and separation of cells followed was done~~ by centrifugation at 10000 rpm for 20 minutes ~~was done to separate these cells from 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.

Comment [THS6]: Inoculum load and Quantity of broth, recovery

Comment [THS7]: Citation

2.5 Quantification of the exopolysaccharides (EPS) of bacterial isolates

The recovered EPS was ~~then subjected to an estimation procedure~~ 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°C in a water bath and then cooled to 35°C . 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} (Deka *et al.*, 2019) [5].

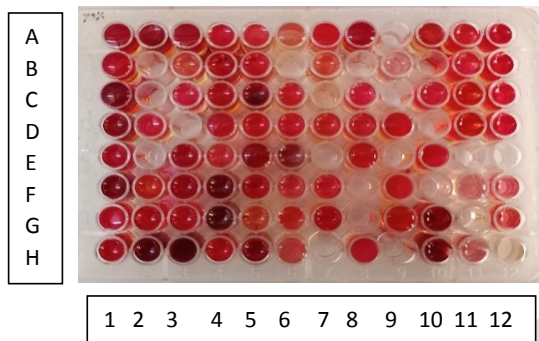


Fig.1. Quantification of Exopolysaccharide in ELISA plate reader

2.6 Isolation of genomic DNA from bacterial isolates

In order to amplify the specific gene for EPS production. Genomic DNA was isolated from 22 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 Molecular confirmation of genes required for EPS 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 50 μ L was prepared. This mixture included 5 μ L of both forward and reverse primers, 5 μ L of the DNA sample, 10 μ L of molecular-grade water, and 25 μ L 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 sucrose 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

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(Jahan et al., 2015)[6]. wrong

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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 μ L for the PCR mixture. This mixture included 5 μ L each of the forward and reverse primers, along with 5 μ L of the sample DNA. Additionally, 10 μ L of molecular grade water and 25 μ L 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 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 selected cultures 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 *Bacillus sp* (Venterino 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 64.19 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 40.08 mg ml⁻¹ (values not shown). The isolates that produced higher EPS levels were selected for further study and the values were given in table 1. The isolate A1 registered significantly higher production of EPS (64.19 mg ml⁻¹) than any other culture. This was followed by the

Comment [THS10]: Characterization and the names not mentioned

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 (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].

Comment [THS11]: Characterization ?

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

SINo	Source of the sample	Location	Designation 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
1	PODAMPATTI	9.52° N, 78.09°E	202	49.92 ± 0.18 ^f
1	ANNIYENTHAL	9.74° N, 78.38° E	21	49.3 ± 0.02 ^f
1	PULLIYANGULAM	9.57° N, 78.27° E	422	49.08 ± 0.13 ^f
1	THURUPATHUR	10.10° N, 78.59° E	T2	48.24 ± 0.18 ^f
1	PODAMPATTI	9.52° N, 78.09° E	211	45.43 ± 0.14 ^f
1	PULIYANGULAM	9.57° N, 78.27° E	44(1)	44.79 ± 0.14 ^f
1	MARANDAPATTI	12.39° N, 78.00° E	673	43.98 ± 0.05 ^f
1	THIRUPACHETHI	9.77° N, 78.34° E	276	41.48 ± 0.13 ^f
1	POOMALAI PATTI	9.58° N, 78.31° E	121	40.90 ± 0.15 ^f

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Comment [THS12]: Reference culture ?

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1	PODAMPATTI	9.52° N, 78.09° E	212	40.84 ± 0.11 ^f
2	ARASAKULAM	9.67° N, 78.11° E	193	40.1 ± 0.16 ^f
2	PADI	12.18° N, 78.09° E	694	40.08 ± 0.07 ^f
CD			4.10	
S.ED			2.02	

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

Through the utilization of the primer pair *WConDexfw* and *WConDexrev*, 22 isolates were subjected to molecular testing for the presence of the EPS gene *dex* (dextranucrase) 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 dextranucrase 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*.

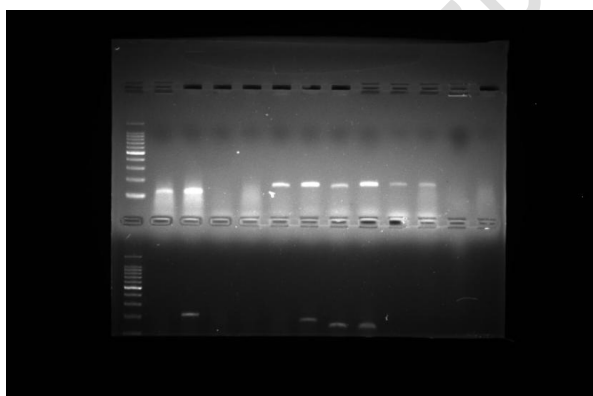


Fig 2. Agarose gels (1.2% w/v) showing the amplicons of dextranucrase gene (*dex*) using primer pairs *WconDexfw* and *WconDexrev*

Lane no	Isolate No	<i>dex</i>	Lane no	Isolate no	<i>dex</i>	Lane no	Isolate no	<i>dex</i>	Lane no	Isolate no	<i>dex</i>
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	+			

FigTable-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 A1 exhibited 100% identity to *Bacillus altitudinis*

Comment [THS14]: Give the phylogenetic tree

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.

Comment [THS15]: Refine the findings

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Comment [THS16]: Follow the journal requirements

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