



Exploration of Exopolysaccharide Producing Bacterial Cultures from Various Drought Affected Region

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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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

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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 [1] and aggregation [2]. 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) [3] 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°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 separation 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 [5]. The recovery of the EPS was about $1.5 \mu\text{l ml}^{-1}$.

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° 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 'y' indicates OD and 'x' values indicate the amount of EPS production. Estimated EPS was expressed in mg ml^{-1} [6].

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 [7]. 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 500 μL of 1X TE buffer, 30 μL of 10% SDS and incubated in water bath for 1hour at 37°C. Then 100 μL of 5M NaCl and 80 μL of CTAB was added and incubated for 10 minutes at 65°C later equal volume of chloroform: iso amylalcohol (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°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°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 *WConDexrev* primer (3'-GGTTCAATCACGGCTAACG-5') for dextransucrase. To initiate the PCR, a combined solution of 50 μL 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 MgCl_2 . 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 [8]. The PCR products were separated and visualized using agarose gel electrophoresis [9]. The resulting gel was then analysed and documented using the Alpha imager TM 1200 gel documentation device.

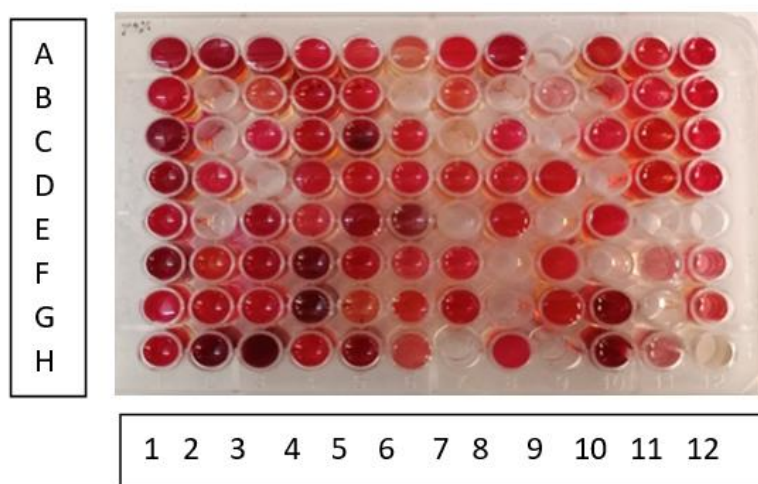


Fig. 1. Quantification of exopolysaccharide in ELISA plate reader

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 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 [10]. Subsequently, the PCR products were separated using agarose gel electrophoresis, [9]. 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 [11].

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 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 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 [12]. 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 [13].

3.4 Molecular Identification of the Dextranucrase 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* (dextranucrase) at amplification size of 1037 bp [8]. 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. [14]. 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* [15].

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

| SI 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) EDUKATTUR | 10.23° N, 77.49° E | A1 | 64.19 ± 0.17 ^a |
| 2. | BARIJUM | 9.69° N, 78.45°E | 183 | 62.41 ± 0.03 ^b |
| 3. | ATTUPANNAI III | 10.24° N, 77.49° E | BJ5 III | 58.47 ± 0.17 ^c |
| 4. | ANNIYENTHAL | 10.23° N, 77.49° E | AP III | 53.93 ± 0.21 ^{bc} |
| 5. | NARIKUDI | 9.74° N, 78.38° E | 2110 | 52.91 ± 0.10 ^{bcd} |
| 6. | SOWLOOR | 9.58° N, 78.31° E | 102 | 52.85 ± 0.20 ^{cd} |
| 7. | ARUPUKOTTAI | 12.16° N, 78.15° E | 653 | 52.20 ± 0.14 ^d |
| 8. | PODAMPATTI | 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, 78.27° E | 422 | 49.08 ± 0.13 ^f |
| 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. | POOMALAI PATTI | 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 | |

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

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

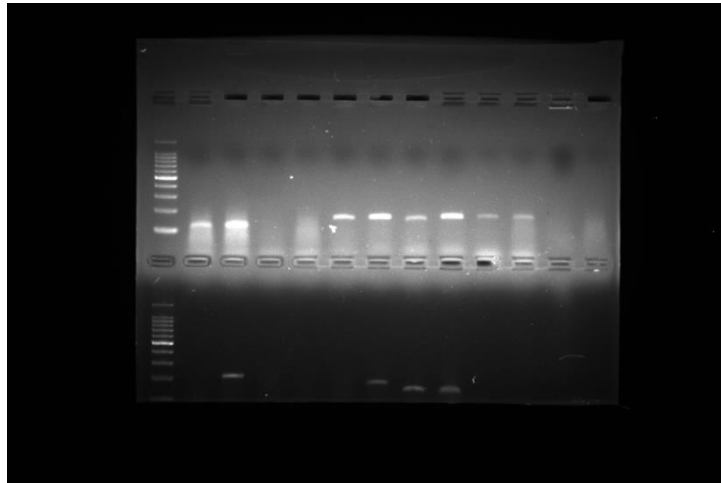


Fig. 2. Agarose gels (1.2% w/v) showing the amplicons of dextranucrase gene (*dex*) using primer pairs *WconDex fw* and *WconDex rev*

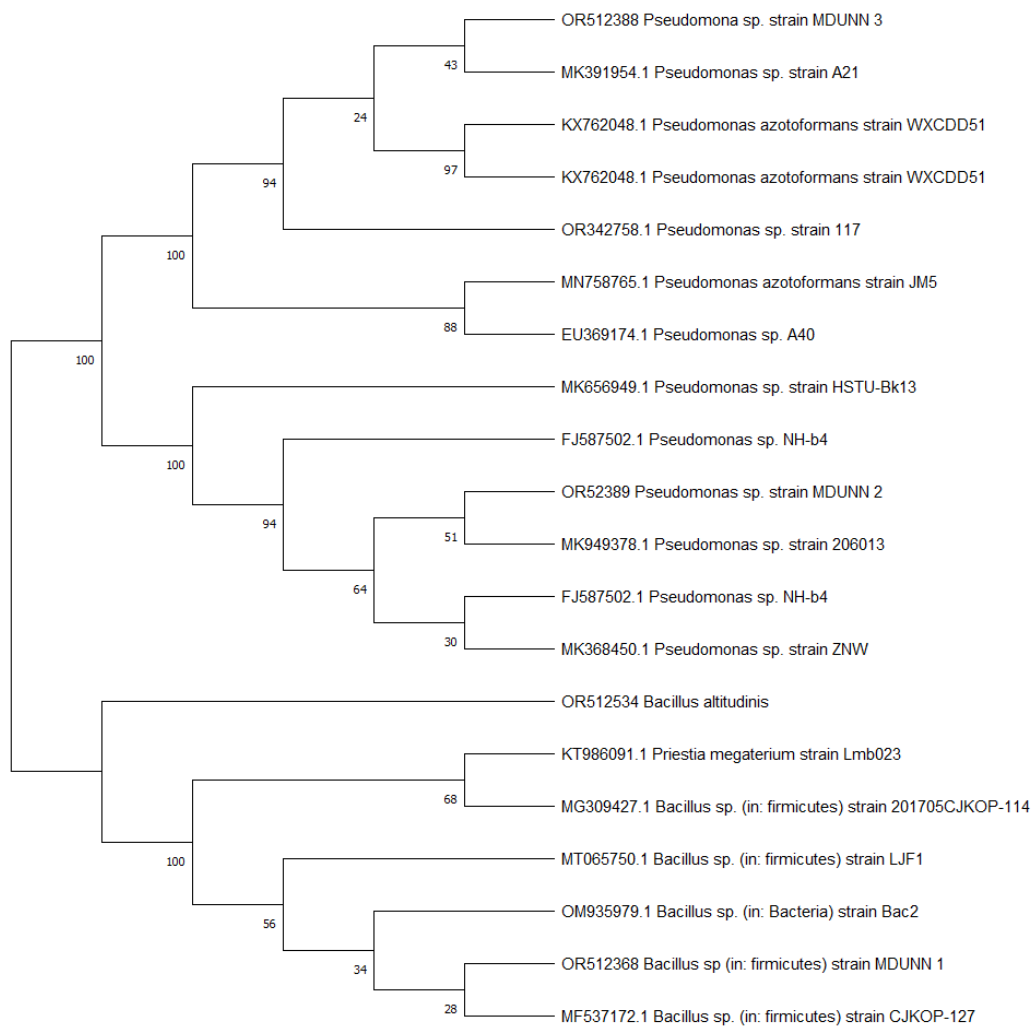


Fig. 3. Phylogenetic tree based on 16S rDNA sequence of elite EPS producing bacterial isolates from drought affected regions

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* (Fig. 3.).

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.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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