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Exopolysaccharide Producing Endophytic Bacteria Isolated From The Plant *Derris elliptica.*

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ABSTRACT

This study was aimed to isolate, identify and growth optimization of exopolysaccharide (EPS) producing endophytic bacteria .Surface sterilized root nodules of *Derris elliptica* was used to isolate exopolysaccharide producing endophytic bacteria. Prominent mucoid colonies on Yeast extract mannitol media were screened and identified using 16S rRNA gene sequencing and phylogenetic was constructed with related reference strains. EPS production was optimised using YEM. The presence of carbohydrates in the EPS was confirmed by phenol-sulphuric acid method. Optimum EPS producing strains were designated as YU16-RN3, YU16-RN5 and identified as *Burkholderia* sp. and *Enterobacter* sp. respectively. The isolates YU16-RN3 and *YU16*-RN5 could produce 1.8g/L and 2.8g/L of EPS in YEM broth respectively. The carbohydrate content of the EPS-RN3 and EPS-RN5 was found to be 25.13 ± 0.17% and 40.34 ± 0.12 % respectively. The isolated genera have various beneficial applications in both agricultural and biotechnological fields. Future studies on the characterization of their metabolites are required to explore more about these isolates.

Keywords: *Derris elliptica*, *Burkholderia* sp., *Enterobacter* sp., exopolysaccharides, 16S rRNA gene sequencing, phylogenetic tree.

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INTRODUCTION

Endophytic bacteria are beneficial microbes that ubiquitously reside in living plant tissues with symbiotic associations [1]. In majority of plants, endophytes inhabit roots more easily than the shoot tissues [2]. Endophytes are capable of synthesizing bioactive compounds that are used by plants for defense against pathogens and some of these compounds have proven to be useful for novel drug discovery. Endophytic bacteria are one chief source of natural anticancer products, that are known to produce numerous bioactive anticancer compounds, such as anthracyclines, glycopeptides, polysaccharides, aureolic acids, anthraquinones, enediynes, antimetabolites, carzinophilin , mitomycins [3,4,5]including alkaloids, terpenoids, flavonoids, and steroids [6]. Exopolysaccharides gained attention as new source for cancer treatment due to its unique physico-chemical properties. Polysaccharides containing sulfur and uronic acids exhibits antioxidant activity, inhibitis cell proliferation and cancer formation. Most bacterial EPS show higher water solubility and exhibit better viscosifying, thickening, stabilizing, gelling and emulsifying activities [7]. EPS produced by different bacterial species have been shown to have stable rheological and emulsifying properties over wide ranges of temperature, pH and ionic strength, which ensure their applications in various food products under different conditions [8]. Biosorption of toxic heavy metals by soil bacteria has been extensively studied by several researchers [9]. The alginate (EPS) produced by *Azotobacter* in soil helped in the remediation of toxic metals and maintained soil ecology [10]. Derris plants are used as insecticides and also for stunning fish. D. elliptica is used as a curative for scabies and toothache. It has been reported as molluscidal , antimycotic against plants fungi and inhibitory activity against *Escherichia coli* [11].Antioxidant activity and larvicidal activity of the phytochemical compounds from *Derris elliptica* have been reported [12]. Exopolysaccharides are important quorum sensing molecules during plant–bacteria association as they are involved in both plant pathogenesis and symbiotic association. The versatile physico-chemical and biological properties exhibited by these exopolysaccharides,made them potential substances in different industrial fields [13].Hence a preliminary study was carried out to isolate and identify the bacteria from the root nodules of *Derris* elliptica. The isolates were also explored for the production of exopolysaccharides.

MATERIALS AND METHODS

Bacterial isolation and Screening of exopolysaccharide producing endophytic bacteria

The root nodules of the sand dune legume *Derris elliptica* from the coastal regions of Mangalore (12 °47'3524"N74 °51'12.4344"E) was collected and used for the isolation of EPS producing bacteria. The root nodules were separated from the main roots of the plant and washed with sterile water thoroughly to clean off the mud and sand particles, were surface-sterilized by immersing in 70% ethanol for 30 s and washed twice with sterile distilled water [14]. Later were macreated and the suspensions obtained were dilluted to 10^{-6} in a sterile solution of NaCl (0.9%). One hundred microliters of the diluted suspensions were inoculated into yeast extract mannitol (YEM) agar (Himedia, India) plates [15]. The plates were incubated at 32°C up to 5 days, and those which showed mucoidal growth were streaked onto plates containing the same media for isolation and identification of isolates. Based on the mucoid phenotypic appearance of the colonies, strains were considered as exopolysaccharide producing bacteria [16] and preserved in 30% (*V*/*V*) glycerol at -80^0 C.

Molecular characterization of bacterial Isolates

Sequencing and phylogenetic analysis

Bacteria were grown in YEM broth for 24 hrs at 32^0 C. Genomic DNA was extracted and purified using a commercial kit (QIAGEN KIT). Electrophoresis on 0.8 % Agarose gel of extracted DNA was done and the bands formed were visualized on a Gel Documentation System. After the purity check of DNA (Colibri, Nanodrop spectrophotometer), the 16S rRNA gene was amplified by PCR using universal primers 27F and 1492R. An aliquot of PCR product of isolates was directly sequenced using an ABI PRISM 310 instrument using the same primers mentioned above. Sequence data was aligned and compared with available standard sequence. The acquired 16S rRNA gene sequences were submitted to National Center of Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov/). By using sequenced data a phylogenetic tree was constructed using the Jukes–Cantor model and the neighbor-joining method using Clustal W software, in Molecular Evolutionary Genetics Analysis Tool (MEGA X) [17].

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Growth optimization of the isolates

The screened isolates were further evaluated for EPS production. The inoculum was prepared by transferring bacterial colony into 250 ml conical flask containing 50 ml of YEM broth. Inoculated flasks were incubated on shaker at 100 rpm for 120 h at 32^0 C. At regular intervals (24, 48, 72, 96, 120h) culture broth was harvested and the yield of the EPS was noted. The strains that yielded maximum EPS were selected for the further study. The broth was made cell free by centrifugation (8000×g) for 15 minutes. To the cell free supernatant, chilled Isopropyl alcohol was added to in ratio 1:3 (v/v) and kept at 4^0 C for 24 hours [18] to precipitate EPS. The precipitated EPS were recovered by centrifugation (8,000×g) and purified by wither-solubilising Milli Q water and re-precipitation with isopropyl alcohol in ratio1:3. And finally purified by dialysis (MWCO 12,000, HiMedia, India) against distilled water for 48 h. The dialysed EPS was lyophilized and subjected for the determination of carbohydrate content by Phenol sulphuric acid method [19] to confirm the presence of EPS.

Statistical analysis

Data was reported as the mean of the experiments standard errors (SEM). Each experimental condition was performed in triplicate (n=3). One way analysis of variance (ANOVA) was used to analyze data. p<0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

Overall, 12 mucoid colonies were isolated from the root nodules of the plant *Derris elliptica*. Out of those only two colonies showed prominent mucoidal growth were selected based on their morphological differences observed on YEM solid medium to study further. The strains were named as YU16-RN3 (Figure 1) and YU16-RN5 (Figure 2) and were used to evaluate EPS production in YEM broth.

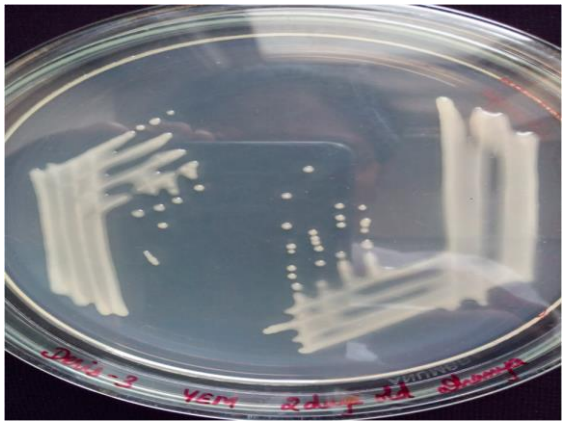

Figure 1: *Burkholderia* sp. YU16-RN3

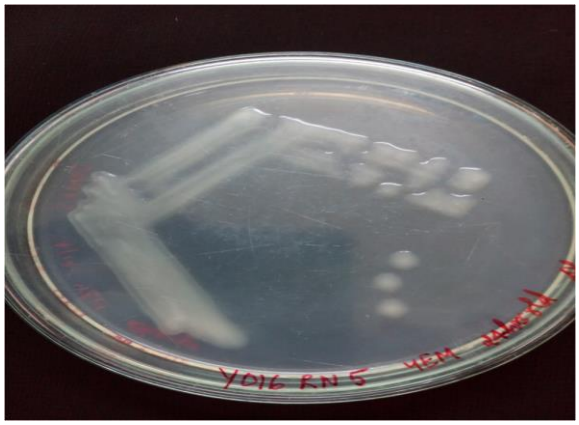

Figure 2: *Enterobacter* sp.YU16-RN5

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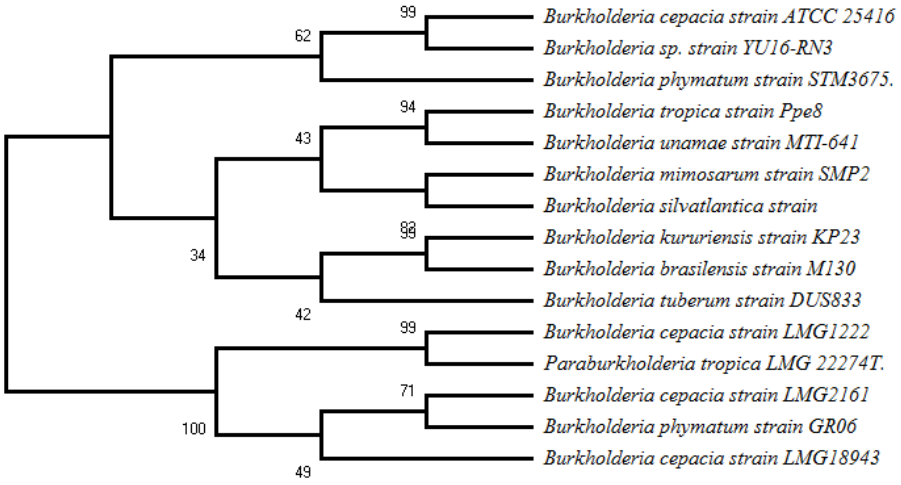

Figure 3: Phylogenetic dendrogram based on 16S rRNA gene sequence comparisons indicating the position of the strain YU16-RN3 within the genus *Burkholderia*. Analysis was performed by the neighbour-joining method using software MEGA X. Boostrap values based on 1000 replications are indicated at branching points, and bar 0.01 substitutions per nucleotide position.

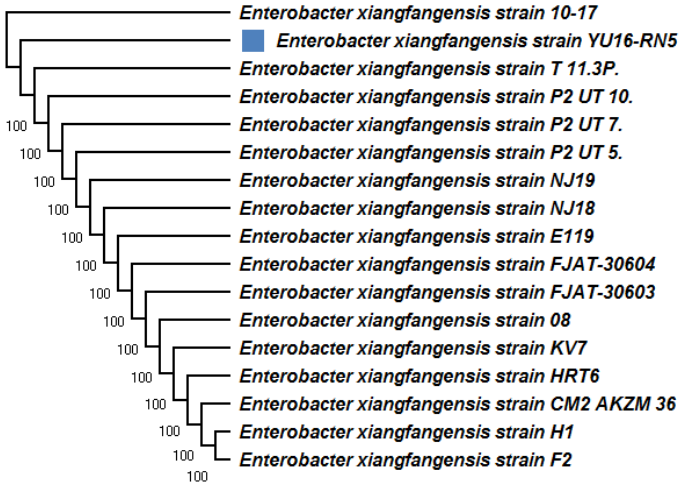

Figure 4: Phylogenetic dendrogram based on 16S rRNA gene sequence comparisons indicating the position of the strain YU16-RN5 within the genus *Enterobacter*. Analysis was performed by the neighbour-joining method using software MEGA X. Boostrap values based on 1000 replications are indicated at branching points, and bar 0.01 substitutions per nucleotide position.

The analysis of the 16S rRNA gene sequences revealed that the isolates YU16-RN3 and YU16-RN5 belonged to two different taxonomic genera *Burkholderia* and *Enterobacter* respectively with 100% similarity to *Burkholderia cepacia* and *Enterobacter xiangfangensis.* The partial nucleotide sequences of 16S rRNA gene of the isolates have been deposited in Gen bank under accession number MH191374 and MH191375 respectively. The phylogenetic tree shows the relationships between the isolates with their related reference species (Figure 3,4). Previous studies found that the genera belonging to *Rhizobium, Sinorhizobium* and *Bradyrhizobium* could nodulate the roots of the *Derris elliptica* plant [20].This result suggests that a high level of genetic diversity could occur among the microsymbionts of *Derris elliptica* which may be due to the adaptation strategy acquired by them to the environmental conditions influenced by the root exudates which contains a wide variety of metabolites and protein molecules [21].

Optimization of growth media for production of EPS by the isolates was done using YEM broth. Yeast extract (that contains copious amount of protein, amino acid and vitamin) is the most suitable nitrogen source for EPS production. Nitrogen sources can support cell growth by boosting up proteins, nucleic acids synthesis and enzymes production. The optimal temperature for EPS production by most endophytes ranges from 24 to 30 ◦C [13].However, the isolates YU16-RN3 and YU16-RN5 produced maximum amount of EPS at 32 ◦C after 96 h of incubation period. The isolates *Enterobacter* sp. YU16-RN5 and *Burkholderia* sp.YU16-RN3 could produce 1.8g/L and 2.8g/L of EPS respectively in YEM broth. The presence of carbohydrates in the EPS was confirmed by the amount of sugars quantified in the test. The standard spectrophotometric assay carried out for the determination of carbohydrate content in the extracted EPS from the two isolates showed positive results. The carbohydrate content of the EPS-RN3 and EPS-RN5 was found to be 25.13 ± 0.17% and 40.34 ± 0.12 % respectively.

The family *Enterobacteriaceae* contains a large number of genera that are found in different environmental niches. Strains belonging to the genus *Enterobacter* have been isolated from the plant roots growing in desert soil and arid lands. Several researchers reported that members of *Enterobacter* sp. produce molecules with plant growth promoting (*Enterobacter* sp. EnB1) and biopesticide properties (*Enterobacter* sp. B6), act as a capable nitrogen- fixers (*E. oryzae* Ola 51T) with multi-stress tolerance properties [22] *Enterobacter* sp.ETH-2 and *Enterobacter cloacae* WD7 produced EPS with flocculating activity [23].The EPS of *Enterobacter* sp.MS16 was reported as a biosurfactant with antifungal activity [24].

The genus *Burkholderia* comprises more than 62 species isolated from a wide range of environmental niches. Although most studies have focused on the pathogenic species of *Burkholderia* due to their clinical importance, the subsequent characterization of non-pathogenic plant-associated species has led to beneficial ecological perception on *Burkholderia* species. Since then several species are reported as atmospheric nitrogen fixers that exhibited a considerable potential for bioinoculation (*Burkholderia brasilensis* "M 130 and *Burkholderia kururiensis*), that promote plant growth, while others are proposed for biotechnological uses, such as bioremediation of chemical pollutants and aromatic compounds (Strains of *B. xenovorans*, *B. unamae* , *B. sartisoli* , and *Burkholderia phenoliruptrix*) and biocontrol agents *(B. phytofirmans* PsJN) [25].

CONCLUSION

The genus *Enterobacter* and *Burkholderia* has myriad applications in terms of both agriculture and biotechnological fields. Therefore studies required to characterize the EPS by the said strains in bioremediation process and to determine the potentiality of these isolates in sustainable agricultural practices as biofertilizers finds prospect.

Conflict of interest statement: We declare that we have no conflict of interest.

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