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EXOPOLYSACCHARIDE PRODUCING BACTERIA ASSOCIATED WITH THE GARDEN SLUG *LAEVICAULIS ALTE* (FERUSSAC)

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Abstract:

Exopolysaccharides are a class of biopolymers that posses significant biological activities. They are being prospected for several diverse applications such as food additives, nutraceuticals, biomaterials etc. Here we report exopolysaccharide producing enterobacteria isolated from slime and gut contents of the garden slug *Laevicaulis alte* (Férussac). These bacteria were isolated on yeast extract mannitol medium and identified by 16S rRNA gene sequencing. The isolates were screened for β-glucan production on yeast extract mannitol medium supplemented with aniline blue. Four bacteria were isolated and all isolates were identified as members of *Enterobacteriaceae* family. These four isolates tested positive for β-glucan production. Since these isolates produce a considerable amount of β-glucan type exopolysaccharides; they present as appealing candidates for the aforementioned prospecting purposes.

Key Words: Garden Slug**,** *Enterobacteriaceae*, Biopolymers, Exopolysaccharides, β-Glucans & Bioactives

1. Introduction:

Exopolysaccharides (EPS) are an important class of carbohydrate polymers that are produced by bacteria, fungi, and algae. In these organisms; the EPS play important physiological roles such as buffering toxins, protecting from extreme environmental conditions, and assisting in biofilm formation and surface attachment (Colvin et al., 2011; Elifantz et al., 2005; Roberson and Firestone, 1995; Upadhyay et al., 2011).

The compositional and structural diversity of these EPS and their physiological activities have encouraged researchers to prospect them for an array of applications. Such studies have brought out diverse biological activities of EPS that range from antioxidant activities (Ye et al., 2012) and modulation of the immune system (Chang et al., 2011) to antitumor action (Ruiz-Ruiz et al., 2011). These activities have made EPS a potent candidate for applications in pharmaceuticals and food industries (Roca et al., 2015; Ryan and Ross, 2015). Among the various classes of EPS, the β-glucans produced by bacteria including the gut bacteria are reported to display profound beneficial health effects (Guleria et al., 2015; Schlörmann and Glei, 2017). They display biological activities such as anti-cancer (Choromanska et al., 2017; Zhu et al., 2015), antioxidant (Alp et al., 2012; Błaszczyk et al., 2015), immune modulation (Stier et al., 2014), and protection against nephrotoxicity of certain chemicals (Koc et al., 2011; Nagaraj et al., 2016).

The bacteria that live in association with plants and animals have a higher capacity to produce EPS. In this paper; we report EPS producing bacteria isolated from a garden slug *Laevicaulis alte* (Férussac).

2. Materials and Methods:

2.1 Sample Collection, Dissection, and Isolation of Bacteria: The slugs *Laevicaulis alte* (Férussac) were collected from a garden in the Yenepoya University campus (Mangalore, India). The slugs were washed with sterile distilled water and the slime and the gut contents obtained by dissection were suspended in sterile saline and homogenized. This suspension was serially diluted and plated on Yeast Extract Mannitol (YEM) agar medium and the plates were incubated at 32°C for 24-48 h. The EPS producing isolates were identified by their mucoid colonies. Pure cultures of such mucoid colony forming bacteria were obtained and were grown in YEM broth for 48 h and preserved at −80° with 30% (v/v) glycerol for further use.

2.2 Identification of Isolates by 16S rRNA Gene Sequencing: Genomic DNA of isolates was extracted from the 48 h cultures in YEM broth using genomic DNA extraction kit (Mobio Inc). The PCR amplification of the 16S rRNA gene was carried out using 3F/9R universal primer pair: 9R (5-AAGGAGGTGATCCAACCGCA-3) 3F (5-CCTACGGGAGGCAGCAG-3). BigDye terminator cycle sequencing kit was used for sequencing. Nucleotide sequence determination of the PCR product was performed by an automatic DNA sequencer (ABI PRISM 310, Applied Biosystem, USA) (217) Sequence data were aligned and compared with available standard sequences of bacterial lineage in the GenBank of National Center for Biotechnology Information using Basic Local Alignment Search Tool.

2.3 Screening for β-Glucan Production: The isolates were streaked on YEM agar medium supplemented with 0.05 g/L aniline blue incubated at 32°C for 24 h. The β-glucan producers were identified based on the formation of blue-coloured colonies on this medium.

2.4 EPS Production, Extraction, Purification, and Yield of EPS: For the extraction of EPS, all isolates were cultured in YEM broth at 32 °C in a shaking incubator (120 rpm). After incubation, the broth was centrifuged (7000 rpm, 10 min) and the cell-free supernatant was mixed with ice-cold ethanol (in 1:3 ratio) to precipitate EPS. The precipitated EPS was collected by centrifugation (9000 rpm, 10 min). The EPS pellet was washed with distilled water and reprecipitated with ice-cold ethanol. For purification, the EPS was dissolved in distilled water and dialyzed against distilled water for 24 h in a 12-kDa cut-off dialysis membrane (HiMedia, India). The dialyzed EPS was lyophilized, weighed to estimate the yield, and stored for further use.

3. Results and Discussions:

3.1 Bacterial Isolates and Their Taxonomic Identity: Four bacteria were isolated from the garden slug *L. alte* (two from the slime and two from the gut contents). The taxonomic identity of the isolates was verified by 16S rRNA gene sequencing. All the isolates were found to belong to the *Enterobacteriaceae*; a family of Gram-negative, facultatively anaerobic, and non-spore-forming rods (Table 1). Many members of this family including the genus *Klebsiella*, *Enterobacter*, and *Pantoea* have been reported to produce EPS (Marques et al., 2017; Silvi et al., 2013; Sivakumar et al., 2016).

Table 1: Isolates obtained from *L. alte* gut contents and slime and their taxonomic identity based on 16S rRNA gene sequencing

No	Isolate Designation	Source of Isolation	Taxonomic Identity	Similarity to Type Strain (%)
1	AS1	*L. alte* gut contents	*Klebsiella* sp.	99.3
2	YG1		*Enterobacter* sp.	99.2
3	YS2	*L. alte* slime	*Enterobacter* sp.	100
4	YM1		*Pantoea* sp.	99.7

3.2 β-Glucan Production by Isolates and their EPS Yield: All 4 isolates screened for production on β-glucan by aniline blue method tested positive (Table 2) as they formed blue coloured colonies on YEM medium supplemented with 0.05% aniline blue (Figure 1). The β-glucan producers form blue coloured colonies on medium supplemented with aniline blue due to the complex formation between aniline blue and the triple helix of β-1, 3 glucan units; the backbone of β-glucans (Nakanishi et al., 1976; Wood and Fulcher 1984).

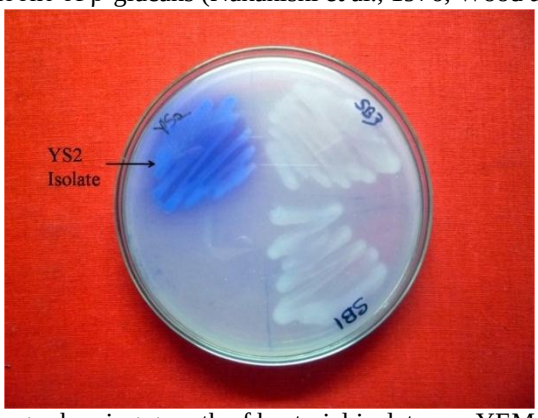

Figure 1: Representative image showing growth of bacterial isolates on YEM medium supplemented with 0.05g/L aniline blue. The β-glucan producing YS2 isolate forms blue coloured colonies compared to the colourless colonies of non β-glucan producers.

The EPS yield of isolates is presented in Table 2. All the isolates except *Klebsiella* sp. AS1 were incubated for 96 h in YEM. The *Klebsiella* sp. AS1 isolate was incubated for only 36 h in YEM. At that time point, the broth culture viscosity increased to an extent that interfered with further incubation and downstream processing. Despite the shorter incubation period; the *Klebsiella* sp. AS1 isolate produced the maximum amount of EPS at 4.5 g/L. Among the isolates; *Enterobacter* sp. YG1 was found to be the lowest EPS producer with 2.2 g/L EPS produced at 96 h incubation.

Table 2**:** Screening of isolates for β-1, 3 glucan production and the yield of EPS produced by them in YEM medium at 32°C temperature incubation. Data presented as mean ± SD.

Isolate	β-1, 3 Glucan Production	Incubation Time (h)	EPS Yield (g/L)
Klebsiella sp. AS1	Positive	36	4.50 ± 0.30
Enterobacter sp.YG1	Positive	96	2.20 ± 0.50
Enterobacter sp.YS2	Positive		3.45 ± 0.45
Pantoea sp.YM1	Positive		3.10 ± 0.00

4. Conclusions:

A total of 4 EPS producing bacteria associated with *L.alte* slug were isolated in this study. All the isolates belong to the *Enterobacteriaceae* family. Many genera of this family have been reported to be associated with Molluscans including slugs (Müller et al., 1995; Stalder et al., 2014). However, reports on such bacteria producing EPS are scarce. Among the isolates; *Klebsiella* sp. AS1 produces the highest amount of EPS.

All isolates were identified as β-glucan producers. These results showcase the potential of non-conventional sources for obtaining EPS producing bacteria. Since these isolates produce β-glucan type EPS; their EPS can be considered as attractive agents for bioprospecting in areas such as pharmaceuticals, biomedical applications, and industrial applications.

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