The Pharma Innovation Journal 2018; 7(4): 508-512

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2018; 7(4): 508-512

www.thepharmajournal.com
Received: 08-02-2018
Accepted: 11-03-2018

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Exopolysaccharides of bacterial endophytes from medicinal plant of forest origin show antibacterial and biosurfactant properties

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Abstract
Forests are a dynamic entity harboring different life forms and ecological niches inside them. Medicinal plants form an important component of benefits we derive from forests and has been part of traditional medicine since ages. Recently, endophytes from such medicinal plants have gained attention owing to discovery of numerous bioactive compounds secreted by them and its diverse biotechnological applications. We aimed to isolate bioactive compounds from bacterial endophytes derived from medicinal plants of forest origin.
We searched forest region of Haryana for a medicinal plant (*Withania sominfera*) having significance in traditional Indian medicine-Ayurveda. We isolated and identified bacterial endophytes from this medicinal plant and found an isolate producing exopolysaccharides. The production of exopolysaccharides was optimized and checked for presence of anti-bacterial and biosurfactant properties.
We could find many isolates from the stem and leaf explants from *W. somnifera* plant. A distinct colony which was identified as *Bacillus subtilis* from stem explant was found to produce exopolysaccharides in liquid culture. This exopolysaccharide was found to have antibacterial activity against pathogenic *E. coli* and *S. typhimurium* strains. This exopolysaccharide also showed biosurfactant properties.
We isolated and identified a bacterial endophyte from wild medicinal plant. This isolate was found to produce exopolysaccharide which had antibacterial and biosurfactant properties. This isolate has clear medicinal values and might be of use to industry both for production and bioremediation purposes.

Keywords: bacterial endophytes, forest medicinal plants, exopolysaccharides, antibacterial compounds, bioremediation.

Introduction
Forests are a rich source of biodiversity sustaining all forms of life including plants, animals and microbes. Although, predominant forest cover has shrunken worldwide, but still a vast majority of life forms are yet to be identified. Withania somnifera, fam. Solanaceae (aka Ashwagandha) also called as “Indian Ginseng” is an important herb of Indian Ayurvedic medicine and shows many health benefits ranging from inducing happiness, physical well-being, brain relaxation, maintaining reproductive balance and protection from many diseases. It is routinely given as tonic to children, stimulant for adults and as anti-stress for mature age groups [1]. Remarkable differences in its quality has been observed depending upon its biogeographical location and have been biochemically characterized. Numerous scientific studies on this plant has been done and it has been found to have anti-tumor properties, Alzheimer's disease and many more [2]. But, the diversity of microbial endophytes of this plant has not been explored. Few studies have focused on fungal endophytes and that too have explored only a few properties [3]. We wished to explore the bacterial endophytes from this plant and isolate bioactive compounds from it.
Microbes inhabit almost all spheres of life both individually or in association with larger life forms such as animals and plants. The association of microbes with plants could be both negative and positive interactions, useful or deleterious to other life forms, including humans. Microbes which colonize intercellular spaces within plants without manifesting any disease are called as Endophytes [4]. Research on endophytic microbes inhabiting medicinal plants gained momentum after discovery and commercial exploitation of Taxol from an endophytic fungi [5]. More than 2000 reports of fungal and bacterial endophytes have been made and many remain unexplored. Many of these endophytic plant symbionts possess important biological functions such as antibiosis, anti-cancerous, production of exopolysaccharides and many more including

Bioremediation [6]. These beneficial attributes are otherwise absent in the microbe when found in natural environment and is postulated to have been acquired from the source plant.

Exopolysaccharides (EPS) are complex extracellular polymers of biological origin having high molecular weight carbohydrate monomers and are produced from a variety of organisms ranging from aquatic animals, microbes and has been particularly useful in emulsification reaction for hydrophobic substrates [7]. At low pH, they may increase viscosity of constituent solutions and can emulsify diverse types of hydrocarbon compounds, which makes them amenable to rapid biodegradation. EPS from few Lactobacillus strains have gained attention owing to potential health benefits due to them and have been reported to have antioxidant and antibacterial activities [8, 9]. EPS from bacterial endophytes can be of novel applications especially due to production of many novel bioactive compounds by endophytes from medicinal plants of forest origin. Thus, we aimed to identify EPS producing bacterial endophytes which might have novel bioactive properties and added benefit of fast generation time. We isolated endophytes and identified it sequentially to ascertain its classification. We then estimated the yield of EPS production and bioactive potential in the EPS produced from the isolates.

Materials and methods

Collection and preparation of plant material

Withania somnifera (Ashwagandha plant) was identified and authenticated based on its botanical characteristics (leaf morphology and orange fruiting body; Figure 1). Leaf and stem samples were collected from forest area near Deer Park, Panchukula, Haryana (Coordinates: 30.719741, 76.861456). The samples were transported to lab in sterile plastic sampling bags and surface-sterilized for isolation of bacterial endophytes as follows: The leaf and stem samples were washed in running tap water to remove surface dirt, epiphytes, etc and washed three times with autoclaved distilled water. The samples were then dipped sequentially in 70% Ethanol for 5 minutes followed by 0.15% Mercuric chloride solution for 5 minutes. The samples were again washed three times with autoclaved distilled water and then surface dried using sterile tissue paper. Using sterile scalpel and forceps, the stem samples were peeled off, bifurcated laterally into half and cut into 1 cm long explants while intact leaf samples were cut in to 1 cm size explants. To access proper surface sterilization, last washing solution was spread-plated on a separate LB agar plate.

Fig 1: Morphological features of *Withania somnifera* plant.

Isolation of endophytic microbes

These explants were put on Luria-Bertani (LB) agar petriplates for 1-2 weeks for appearance of bacterial colonies. The appeared colonies were picked and streaked on fresh LB agar plates for isolating pure cultures. The pure colonies were then picked again in test tubes containing 5ml LB broth and kept for shaking overnight at 150 rpm and 37 ºC in a shaking incubator. These cultures were then identified as Gram positive or negative based on Gram staining.

DNA extraction

Purified cultures were sub-cultured twice in 5 ml LB broths and the overnight grown cultures were centrifuged in microcentrifuge tubes as a cell pellet. The cell pellet was washed twice by 50 mM Tris-EDTA (TE) buffer and then resuspended in 250 µl TE buffer + 50 µl lysozyme (50 mg/µl concentration). It was then kept on ice for 30 minutes, followed by bead beating for 10 minutes with intermittent incubation on ice. Then, the mixture was incubated at 65C for 30 minutes to degrade RNA contaminants. 50 µl of 10% SDS was added to the mixture and incubated for 30 minutes at 37 ˚C. The mixture was then centrifuged at 10,000 x g for 5 minutes and the upper aqueous phase was taken in fresh microcentrifuge tubes. 500 µl of Phenol: Chloroform: Isoamylol (25:24:1) was added to the mixture and shaken for 20 minutes at room temperature. The mixture was then centrifuged at 10,000 x g for 5 minutes and upper aqueous phase was taken in fresh microcentrifuge tubes. The DNA was precipitated using 500 µl of 100% ethanol, centrifuged at 12,000 x g for 10 minutes and the cell pellet was left to air-dry. The dried cell pellet was resuspended in 30 µl of autoclaved deionized water and ran on 0.8% agarose gel to check its integrity and yield of DNA.

Bacterial identification

To amplify the 16S rRNA gene sequence, gene-specific PCR was done using the universal primers (Forward: 5'-TGAGATTGA TCGTCGCTCTG-3'; Reverse: 5'-TAGCAGGTGATGCAGGCGCT-3'). The PCR amplification protocol was as follows: Initial denaturation at 95 ˚C for 3 min; followed by 35 cycles of (95 ˚C for 45 sec, 54 ˚C for 45 sec and 72 ˚C for 90 sec), final extension step at 72 ˚C for 5 minutes and incubation at 4 ˚C for 10 minutes. The amplified PCR product was ran on agarose gel and specific band at 1500 bp size was cut and purified using Mdi (Ambala, India) gel purification kit, as per manufacturer's conditions. The purified product was sent for DNA sequencing to Eurofins (Bangalore, India) and the DNA sequences were analyzed for finding the identity of isolates using BLAST [10]. A phylogenetic tree was constructed using MEGA6 software [11].

Extraction of exopolysaccharides from endophytic bacterial isolates

Few bacteria were found to show mucoid morphology and were hypothesized to produce exopolysaccharides. Chosen isolate was inoculated at 1% initial concentration in 200 ml culture inside a 1 liter flask and left for 36 hours at 37 ºC and boiling for 10 minutes to deactivate intracellular enzymes. The cells were then centrifuged at 10,000 x g for 10 minutes and the cell pellet was washed twice with 50 mM Tris buffer. The proteins were removed from the cell mass using mild thermal denaturation. The mixture was centrifuged at 10,000 x g for 10 minutes and the final supernatant was mixed with 2 volumes of Ethanol and evaporated till it got concentrated to 200 µl volume. The mixture was cooled down and kept overnight at 4 ºC, followed by dialysis with double distilled water, changing the water thrice during dialysis. The amount of polysaccharides was estimated colorimetrically at 490 nm using modified phenol-sulfuric acid method [12], taking dextrose as standard for comparisons.

Antibacterial activity of EPS and statistical analysis

Overnight grown cultures of *E. coli* O157 and *S. typhimurium* ATCC 13311 were diluted to $O.D._{600}$ = 1.2 (corresponding to 10^7 CFU/ml), spread on LB petriplates in triplicates. Three holes of 5 mm diameter and 15 mm depth were punched inside this petriplate and 50 µl of EPS isolated was carefully pipetted into it and kept overnight at 37 ºC. The zone of clearance was measured and expressed in Diameter± S. D. All the measurements have been expressed as Mean± standard deviation and were estimated for accuracy with $p<0.05$. The analysis was performed using MS-Excel software and data shown are derived from at least three independent experiments.

Results and discussion

Isolation of endophytic microbes and their identification

We did not observed any contamination from the last wash of the explants washing steps. This shows that the isolates were indeed of endophytic nature and our experimental workflow is reliable. Many outgrowths from the stem explant were observed and which were further streaked to get pure colonies (Figure 2A). A gram-positive isolate, K_AS21, was observed with mucoid colony morphology from the stem explant of the aswagandha plant (Figure 2B). No mucoid colonies were observed from leaf explants and hence were kept separately. Medicinal plants are reported to host many endophytic microbes which are considered to be linked with production of diverse pharmaceutically important bioproducts. Numerous bacterial endophytes have been isolated from medicinal plants of Indian origin. Ashwagandha (*W. somnifera*) is a wild solanaceous medicinal plant used in many ayurvedic preparations and has been called as Indian *ginseng* owing to its numerous medicinal properties such as for their antibacterial and antifungal applications [13, 14]. Only few studies have been done on Ashwagandha plant for exploring their bacterial endophytes and have been focused only on fungal endophytes[3]. Although endophytes mostly colonize the roots, but their diversity is much different from stem endophytes and thus endophytes of stem origin are also physiologically distinct[15]. Thus, our work on stem explants yielded many endophytes, and we focused on colonies which showed mucoid morphology. These mucoid colonies are usually considered as an indication for production of exopolysaccharides.

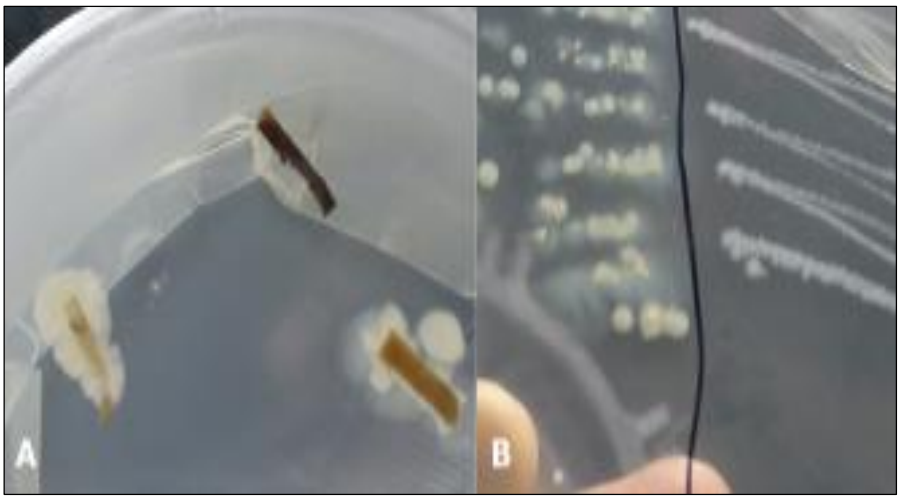

Fig 2: Endophytic colonies observed from A) Stem explants B) Pure colonies obtained by streaking.

Genomic DNA of K_AS21 was isolated without any shear and no contamination by RNA was observed (Figure 3A). DNA sequencing for 16 rRNA gene amplified by PCR (Figure 3B) and BLAST analysis and phylogenetic tree analysis showed its closest identity (98% similarity) with *Bacillus subtilis* subsp. *subtilis* DSM10. The bacterial endophytes have been shown to produce many bioactive products with antibacterial, antifungal and exopolysaccharides

production showing biosurfactant properties [16]. Earlier report for medicinal effects from the secreted products of endophytic Bacillus isolates was reported for antioxidative properties owing to their extracellular enzymes, such as cellulose, pectinase, xylanase and amylase [17]. Apart from applications in biological control and plant benefits, endophytes and their products have been increasingly used in medicinal products. The *Bacillus subtilis* group isolated by us has been previously reported to produce novel bioactive molecules [18] and we also observed production of exopolysaccharides by our strain.

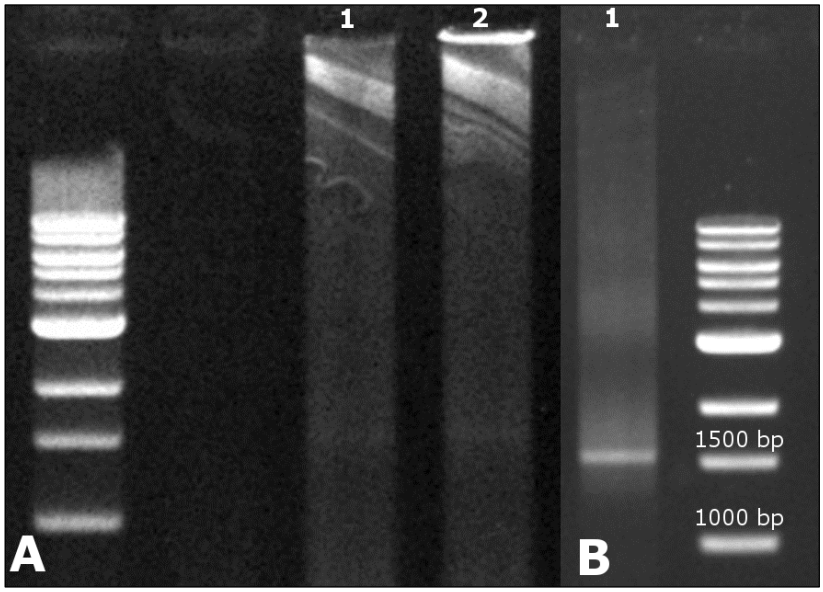

Fig 3: A) Genomic DNA isolated from K_AS21 strain, Lane 2 shows the yield and integrity of DNA **B)** Lane 1 shows the PCR product for 16 rRNA gene at desired size range (approx. 1500 bp).

Production of exopolysaccharides from identified bacterial isolates

The mucoid colony morphology on the plates was indicative of production of exopolysaccharides and we found that our isolate produced EPS at high levels (500 mg and with concentration =0.19 mg/ml). Many EPS molecules are a part of a larger group of water-soluble compounds called as soluble microbial products (SMP) that are interesting due to their broad range of actions and low toxicity to the host. EPS are associated with many biological actions including immune-modulation, antiviral, antioxidant, anti-tumor and biosurfactant properties [19]. Also, the host plants are also found to produce similar products as the endophytes.

Antibacterial activity in EPS from K_AS21 isolate

The EPS from strain K_AS21 isolate showed high antibacterial activity against *E. coli* O157 and *S. typhimurium* strains (Figure 4). This shows that EPS from novel endophytes from plants of wild origin have diverse applications especially as therapeutic agents against bacteria. Our results are in conformance with similar reports from other studies in which bacterial EPS have been shown to inhibit growth of other pathogenic strains [20]. Antibacterial properties in EPS isolated from bacterial strains have been previously reported and considered as a potent strategy due to fast growth and high production from bacterial strains. We also observed biosurfactant property in the EPS, detailed analysis of which is yet to be done. This biosurfactant property could be very useful in oil spills and management of oil and oil products during and after manufacture. This is usually brought by amphiphilic EPS molecules by increasing the solubilization of oil molecules and facilitating their degradation [21].

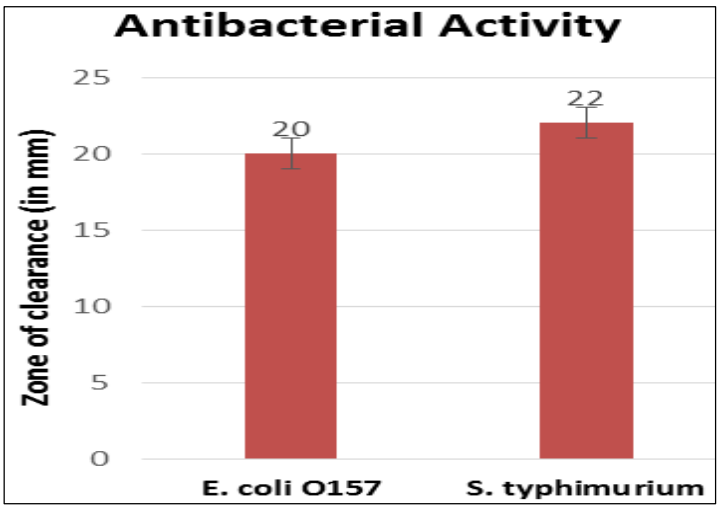

Fig 4: Antibacterial activity of EPS produced from K_AS21 isolate.

Conclusions

Endophytes from wild plants of forest origin are valuable in bioprospecting owing to rich repertoire of novel biological molecules produced by them. We isolated many endophytic bacteria from leaf and stem from a wild medicinal plant (*W. somnifera*) having a prominent place in ayurvedic medicine. Out of many isolates, one isolate was found to have distinct morphology and was found to produce exopolysaccharides. The isolate was characterized by polyphasic approach and molecular identification led to its final classification. The isolate was grown and production of exopolysaccharides was scaled up to obtain high amount of exopolysaccharides per unit volume of the bacterial culture. The exopolysaccharides from our isolate showed high antibacterial activity against two model pathogens *E. coli* and *S. typhimurium*. Altogether, we have identified a high exopolysaccharide producing bacterial endophyte which shows anti-pathogenic attributes and might have enormous applications in pharmaceuticals, industry and oil bioremediation.

Acknowledgements

We acknowledge the administrative and infrastructure support by Uttaranchal University, Dehradun; ICAR-NDRI, Karnal and Lovely Professional University, Phagwara, Punjab. We also extend our gratefulness to Dr. Jibananda Misra, Lovely Professional University, Phagwara, Punjab for critical comments and discussion throughout the work.

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