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Original article

Utilization of endophytic strain *Bacillus* sp. SBER3 for biodegradation of polyaromatic hydrocarbons (PAH) in soil model system

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ABSTRACT

Total eight endophytic bacteria were isolated from the roots of *Populus deltoides* growing in non-contaminated sites at natural vegetation of Garhwal Himalayas, Uttarakhand, India. Among these eight, only SBER3 isolate was able to metabolize wide range of polyaromatic hydrocarbons (PAH) and other hydrocarbon used in the study i.e. anthracene, naphthalene, benzene, toluene and xylene on minimal salt basal medium (MSB) as sole source of carbon and energy. It was identified as *Bacillus* sp. on the basis of 16S rDNA sequence. Furthermore, quantitatively *Bacillus* sp. SBER3 was able to produced 22 μg ml^{-1} of IAA after 4 days and solubilized 0.96 μg ml^{-1} of available phosphorus respectively after 120 h. In addition, *Bacillus* sp. SBER3 also produced siderophore and 1 aminocyclopropane-1-carboxylate (ACC) deaminase. Along with these traits, SBER3 under *in vitro* condition inhibited the phytopathogenic fungi *Rhizoctonia solani*, *Macrophomina phaseolina*, *Fusarium oxysporum* and *Fusarium solani* with (percent growth inhibition) PGI of 60%, 61.5%, 64.3% and 12%, respectively. Microscopic examination under the influence of *Bacillus* sp. SBER3 revealed abnormalities of mycelia structure in case of *R. solani*, *F. oxysporum* and *M. phaseolina*. Although, mean growth rate and survival under varying osmotic stress regime were also evaluated under *in vitro* condition. Interestingly, in liquid culture medium *Bacillus* sp. SBER3 reduced appreciable amount i.e. 83.4% and 75.1% of anthracene and naphthalene, respectively after 6 days of incubation. Notwithstandingly, isolate SBER3 proved to be a competent rhizobacteria in rhizosphere niche in treatments T_1 (Sterile soil + plant cuttings + bacterization) and T_2 (sterile soil + anthracene + plant cuttings + bacterization). Rhizoremediation potential of *Bacillus* sp. SBER3 was demonstrated in polyaromatic hydrocarbon contaminated soil model system. Significant enhancement in shoot, root length, root and shoot biomass including stem girth of *P. deltoides* with respective to control was also recorded and concurrently endophytic isolate *Bacillus* sp. SBER3 degraded 45.6% of PAH in soil model system after 120 days as determined by HPLC analysis.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are hazardous environmental pollutants that possess carcinogenic and mutagenic properties [1,2]. The common sources of PAHs in environment include natural sources like forest and rangeland fires, oil seeps, volcanic eruptions and exudates from trees also burning of fossil fuel, coal tar, wood, garbage, refuse, used lubricating oil, municipal solid waste incineration and petroleum spills [3]. Due to the PAHs high toxicity and wide distribution in the environment including air, water, soils, and sediments, some PAHs have been listed as priority pollutants by the United States Environmental Protection Agency [4]. PAHs do not degrade easily under natural conditions due to their high molecular weight. Therefore, they gathered

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significant concern because of their presence in all components of environment especially, in soil due to their resistant towards biodegradation and potential to bio-accumulate [5].

Remedial options using physico-chemical treatments are expensive and are environmentally invasive while, their high cost sometime makes them prohibitive for the treatment of large contaminated sites [6]. In many of such applications, used for removal of contaminant, bioremediation is good alternative [7,8,76]. Now a days, comparison to bioaugmentation, microbe-assisted phytoremediation i.e. rhizoremediation, appears to be potentially effective for removal and/or degradation of organic contaminants from impacted soils, especially when used in conjunction with appropriate agronomic techniques [75]. Plant-microbial interactions in rhizosphere offer very useful means for remediating environments contaminated with recalcitrant PAH compounds [74]. Bacteria that can produce indole acetic acid (IAA), siderophores and solubilize inorganic phosphate, HCN, ACC deaminase activity are capable of stimulating plant growth; help plants for optimal growth [9–11]. Endophytic bacteria are found in all plant species and span a wide range of bacterial phyla [12,13], while certain endophytic bacteria have been shown to enhance plant growth, increase plant resistance to pathogens, salt stress, such that their commercial potential has received much study [14]. Moreover, in order to improve the efficacy and the consistency of the bacteria, the use of rhizosphere-competent strain is required [15].

Endophytic bacteria can be defined as bacteria colonizing the internal tissues of plants (an intimate niche) without causing symptoms of infection or negative effects on their host [16]. Endophytic bacteria have been isolated from a variety of healthy plant species ranging from herbaceous crop plants [17] and different grass species [18] to woody tree species [19]. In general, Pseudomondaceae, Burkholderiaceae and Enterobacteriaceae are among the most common genera of cultivable endophytic species found from the contaminated sites [20]. Additionally to their beneficial effects on plant growth, endophytes have considerable biotechnological potential to improve the applicability and efficiency of remediation of pollutants [21]. Certain endophytic bacteria have been shown to metabolize polyaromatic hydrocarbons (PAHs) pollutants, enhance plant growth, increase plant resistance to pathogens, drought and even herbivores, such that their commercial potential has received much study [22]. The possible advantages of using endophytic microorganisms to improve xenobiotic remediation were summarized by Newman and Reynolds [23]. Along with the production of novel chemicals, many endophytes have shown a natural capacity for xenobiotic degradation or may act as vectors to introduce degradative traits. The ability of some endophytes to show resistance to heavy metals/antimicrobials and degrade organic compounds probably stems from their exposure to diverse compounds in the plant/soil niche. This natural ability to degrade these xenobiotics is being investigated with regard to improving phytoremediation [19,24,25].

Earlier, Bisht et al. [26] have reported the four PAHs degrading from the rhizosphere of *Populus deltoides* growing in non-contaminated sites while Heinonsalo et al. [27] also isolated petroleum hydrocarbon degrading bacteria from non-contaminated lignin rich forest humus soils. Earlier, extensive geographical survey of several contaminated soils by Mueller et al. [28], resulted in recovery of several species of PAHs degrading bacteria and endophytic bacteria were also isolated which were found to be resistant to high concentrations of heavy metals, BTEX (benzene, toluene, ethyl-benzene and xylene), TCE or PAHs have been identified [8,19].

We have tried to take advantage of the plant–bacterium relationship for degrading PAHs compounds, in present study, eight endophytic bacteria were isolated from the rhizosphere of *P. deltoides* growing in non-contaminated sites of Himalayan regions of Uttrakhand state, India. Out of these isolates, SBER3 had broad substrate exploitation range as it was able to utilize various hydrocarbons. The plant *P. deltoides* was chosen for rhizoremediation, due to its highly branched deep root system that can be used as a vector/bio-injection of root-colonizing bacteria. In view of the above facts, the present study was attempted to isolate and screen endophytes for biodegradation of PAHs for effective rhizoremediation in a soil-model system.

2. Materials and methods

2.1. Isolation of endophytes

Endophytic bacteria were isolated from roots of poplar trees (*P. deltoides*) growing at natural vegetation sites in Uttarakhand Himalayas. For this, poplar roots (approximately 9 cm long and 4 mm in diameter) were surface sterilized by 95% ethanol for 5 min followed by treatment with 10% calcium hypochlorite (containing 0.1% Sodium Dodecyl Sulfate) for 10 min and 0.1% mercuric chloride for 5 min and then rinsed it 5–6 times with sterile distilled water (SDW). The effectiveness of the surface sterilization procedure was verified by plating uncut plant tissue and samples of the final rinsed water on suitable medium. Absence of growth after incubation on these control plates confirmed sterilization. The explants of root material was macerated in 0.8% saline solution, the homogenate was serially diluted and plated on Luria Burtanii (LB) medium [29] and incubated at 27 °C. Colonies of endophytes appeared on surface were picked carefully and stored on LB slants for further studies.

2.2. Screening of endophytes for PAHs metabolization

Endophytic bacteria were screened by spray plate technique [30,31] for PAHs viz. anthracene, naphthalene, benzene, toluene, xylene biodegradation using minimal salt basal medium (MSB). Prior to spreading of soil dilutions, anthracene hydrocarbon solutions (0.1% w/v ethyl acetate) were sprayed onto the surface of MSB agar medium. However, other solid hydrocarbon substrates were also experimented with same method as described above and liquid hydrocarbon substrates were provided in vapor phase in wax sealed desiccators [32].

2.3. Screening of endophytic bacteria for secondary metabolites production

Endophytic bacteria were further checked for plant growth promoting activities viz. indole-3-acetic acid (IAA) production, phosphate solubilization, siderophore and HCN (Hydrogen cyanic acid) production and ACC deaminase. IAA production was determined by Salkowski's method [33]. The potential to solubilize phosphate was checked by the method of [34], amount of free phosphorus liberated by the isolate was estimated by chlorostannous reduced molybdo-phosphoric acid blue color method and the absorbance of blue color produced was measured at 680 nm (OD_{680}) in a UV–Vis spectrophotometer [35]. HCN production was observed by Miller and Higgins [36], siderophore production was detected qualitatively as described by Schwyn and Neilands [37] and for ACC deaminase activity determination, Honma and Shimomura [38] method was applied.

2.4. In vitro antifungal activity of endophytic isolates

The test fungal strains *Fusarium oxysporum* and *Fusarium solani* were procured from MTCC, Chandigarh, India with accession No-MTCC 284 and 350 while the *Macrophomina phaseolina*, *Rhizoctonia*