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Rhizoremediation prospects of Polyaromatic hydrocarbon degrading rhizobacteria, that facilitate glutathione and glutathione-S-transferase mediated stress response, and enhance growth of rice plants in pyrene contaminated soil

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ABSTRACT

Rhizoremediation is a strategy where pollutant degrading bacteria are augmented through plant roots using plant-microbe interaction. Therefore, for effective rhizoremediation of pyrene contaminated soil, bacterial strains were experimented for amelioration of stress response in host plant along with biodegradation ability. A total of 28 bacteria, having ability to degrade polycyclic aromatic hydrocarbons were isolated from contaminated sites and checked for their plant growth promoting attributes, such as indole acetic acid (IAA) production, phosphate solubilization, atmospheric nitrogen fixation and siderophore release. Among these isolates, *Klebsiella pneumoniae* AWD5 was found to degrade 60% of pyrene. While other isolates, i.e. *Alcaligenes faecalis* BDB4, *Pseudomonas fragi* DBC, *Pseudomonas aeruginosa* PDB1, *Acinetobactor* sp. PDB4 degraded 48.5%, 50.29%, 31.3% and 36% of pyrene, respectively, after 6 days of incubation. *K. pneumoniae* AWD5 produced 94.2 µg/ml IAA and 3.1 mM/mg/h unit of ACC deaminase, which was best among eighteen indole acetic acid producers and five of the 1-aminocyclopropane-1-carboxylate (ACC) deaminase producing isolates. *P. aeruginosa* PDB1 resulted in highest phosphate solubilization activity of 875.26 ng/ml of soluble phosphate among seven phosphate solubilizers. The isolates AWD5 and PDB1 both have shown a good amount of siderophore release (56.3% and 84.3% unit). There was 19.1% increase in shoot length of rice seedlings treated with PDB1 in presence of pyrene. Similarly, 26.5% increase in the root length of AWD5 treated rice was recorded in pyrene contaminated soil. Bacterial inoculation also induced and improved the stress response in host plant, in presence of pyrene, as suggested by the superoxide dismutase, glutathione and glutathione-S-transferase activities in rice.

1. Introduction

Pyrene is one of the most abundant high molecular weight (HMW) polycyclic aromatic hydrocarbons (PAH) with four aromatic rings and also considered as a priority pollutants among 16 PAHs listed by The United States Environmental Protection Agency (USEPA) (Agrawal et al., 2018). It has very slow rate of biodegradation due to molecular stability and high hydrophobicity. Pyrene and other HMW-PAH are accumulating in soil due to excessive industrial production, transportation, refuse burning, gasification and incineration of chemical polymers (Mrozik et al., 2003). Also, incomplete combustion of coal, oil, petrol, wood are contributing towards PAH contamination (Breivik et al., 2004; Kadri et al., 2017). Pyrene is a highly toxic compound and affects several organisms, e.g. neurodevelopmental defects in fishes (He et al., 2012), growth and reproductive stress in earthworm (Wu et al., 2012), oxidative stress in young children (Singh et al., 2008), and also known to causes DNA damage and decreased cell viability (Park et al., 2006). In some plants, the PAH are accumulated in tissues after uptake which further leads to cytotoxicity and mutagenic effects on host (Flowers-Geary et al., 1996).

Reclamation of PAH polluted soils is one of the major challenge in agroforestry. Therefore, potential bacterial isolates with excellent PAH-degradation ability and soil fertility improvement attributes are required for application as a sustainable strategy of bioremediation of polluted sites (Bello-Akinosho et al., 2016). Previous studies have shown that many microorganisms associated with plants, and endophytic bacteria, have enormous potential to degrade PAH (Vinas et al., 2005) and some of these microorganisms can even reduce PAH concentrations in plants (Dai et al., 2010; Prabhu et al., 2017). The rhizosphere is a nutrient rich environment, where microbial activity is

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high, as compared to bulk soils (Hartmann et al., 2008). Siciliano et al. (2001) reported that plants living in contaminated environments select the population of contaminant-degrading bacteria in their rhizosphere. Further, plant-growth promoting rhizobacteria (PGPR), that may degrade and eliminate contaminants benefit the plant by removing negative effects of contaminants on growth of the plant (Kuiper et al., 2004). The growth of plants has been reported to impede due to stress induced by contaminant in soil environment (Amrani et al., 2015). Although PAH-degrading bacteria have been traditionally isolated from various environments, but considering the benefits of bio-augmentation of bacteria through plant roots, several studies are now focused on the isolation and identification of rhizospheric bacteria with PAH degrading abilities (Kuiper et al., 2001; Toussaint et al., 2012). In fact, *P. putida* have been reported to effectively degrade naphthalene in the rhizosphere when applied via root system as a vehicle to penetrate the soil levels, which normally are difficult for bacteria to reach otherwise, and it also protected grass seeds and seedlings against high naphthalene concentrations (Kuiper et al., 2001). In fact, the root exudates of plants provide a nutrient rich habitat for the microorganisms, while these microbes act as biocatalysts to remove the pollutants from the rhizosphere and soil (Oberai and Khanna, 2018; Kotoky et al., 2018).

Therefore, for the successful application of rhizoremediation strategy, the plants should be able to grow in the polluted sites, supported by bacteria colonizing the roots zone. Also, the bacteria should be able to express their plant growth promoting (PGP) attributes in polluted environment and enhance the plant growth in the contaminated sites by reducing the stress induced by contaminant. In present study, pyrene degrading bacteria was studied for PGP characteristic (IAA, Siderophore, phosphate, ACC deaminase activity) in pyrene amended medium, and its effect was monitored on the growth and stress response of black rice under the exposure in pyrene contaminated environment.

2. Materials and methods

2.1. PAH degrading bacteria

PAH degrading bacteria were isolated by enrichment technique. The soil samples (rhizosphere and non-rhizosphere) were collected from several sites of industrial waste, and were enriched in half strength nutrient broth amended with 0.005% pyrene and incubated for 24 h at 30 °C at 140 rpm (Singha and Pandey 2017).

2.2. PAH degradation

PAH degradation efficiency of isolates was checked by growing them in minimal medium BH broth containing 0.01% pyrene, followed by incubation at 30 °C, 140 rpm for 144 h (Singha and Pandey, 2017). The residual amount of pyrene in the flasks were extracted by liquid-liquid extraction through proper mixing of the broth cultures with equal volume of ethyl acetate, then acidified the extract to pH 2–3 using concentrated HNO_3, and repeated the extraction thrice (Hesham et al., 2014). The residual amount of pyrene was detected by GC–MS using Clarus 680 Gas Chromatograph with Clarus 600C MS mass spectrometer system. The column used was 5MS Capillary Column: Length: 60 m, I.D: 0.25 mm; (Max. Program Temperature 350 °C) Phase Reference: 5% diphenyl 95% dimethyl polysiloxane (low bleed) Helium was the carrier gas, used at 1 ml/min constant flow. The column temperature was detained at 70 °C for 5 min, increased by 4 °C/min to 290 °C, and detained for 10 min. The analysis was completed with a ramp of 20 °C/min to 320 °C held for 20 min to remove any remaining compounds. The mass spectrometer was operated in electron impact (EI) mode at 70 eV (EV) in the full scan mode from 50 to 450 *m/z* over 8 −55 min. Injector and detector temperatures were 270 °C and 280 °C respectively.

2.3. Screening of plant growth promoting attributes

2.3.1. IAA production

IAA production was checked in two different conditions – either nutrient broth or minimal medium supplemented with pyrene as described by Singha and Pandey (2017). The isolates were freshly grown in both the medium supplemented with 0.02% L-tryptophan. The supernatant was collected by centrifugation of culture (8000 rpm, 4 °C) after every 24 h interval and mixed with Salkowski reagent (35% perchloric acid with 0.5 M $FeCl_3$). After 30 min incubation, optical density (OD) of resulting solution was measured at 530 nm. (Gordon and Weber, 1951).

2.3.2. Siderophore production

Siderophore production was confirmed by formation of orange halos around bacterial colonies on Chrome Azurol S (CAS) agar plates, incubated at 30 °C for 48 h (Schwyn and Neilands, 1987). The cultures were inoculated in Guass medium, an iron deficient medium containing (gl^{-1}): $MgSO_4{\cdot}7H_2O$, 0.2; $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 3.0; K_2HPO_4, 6.0; Succinic acid, 4.0 at 30^0C on a rotary shaker (120 rpm). The siderophore production was estimated by CAS-shuttle assay (Payne, 1994).

2.3.3. Phosphate solubilization

The phosphate (P) solubilization activity for the isolates were checked on modified Pikovskaya agar medium (gl^{-1}): agar, 20; glucose, 10; $(Ca_3)_2PO_4$, 5; $(NH_4)_2SO_4$, 0.5; yeast extract, 0.5; NaCl, 0.2; KCl, 0.2; $MgSO_4{\cdot}7H_2O$, 0.1; $MnSO_4{\cdot}2H_2O$, 0.002, $FeSO_4{\cdot}7H_2O$, 0.002, at pH-7 (Nautiyal, 1999). The quantitative estimation of P content in the supernatant was estimated using the vanado molybdate colorimetric method (Koenig and Johnson 1942) which was conducted in two different culture conditions (either glucose or pyrene used as carbon and energy source).

2.3.4. Nitrogenase activity and nif H amplification-nitrogen-fixation assay

The Burks's nitrogen-free culture medium was used to screen isolates for atmospheric nitrogen-fixing ability. The appearance of appreciable growth within 7 days of aerobic incubation at 28 °C was indicative of the isolates' nitrogen-fixing ability. Nitrogen fixation ability of the isolates was confirmed by Acetylene Reduction Assay (ARA) as described by Hardy et al. (1968). The bacterial isolates were inoculated in a 12 ml vials containing 6 ml semisolid nitrogen free medium and incubated at 30 °C for 36 h. Then the vials were sealed with rubber septa and the gas phase of the vial was replaced with acetylene (10% v/v) and re-incubated at 30 °C for 24 h. The ethylene produced by acetylene reduction was detected using Gas-Chromatograph fitted with a flame ionization detector. After completion of ARA, the cells were collected and estimation of protein was done. The amount of ethylene produced was calculated by formula, ARA = (f × c × 60)/ 360 × Xnmole C_2H_4/μg/pr/h.

For PCR analysis of the *nifH*gene, a set primer nifH F: 5′-CGTTTT ACGGCAAGGGCGGTATCGGCA-3′ and nifH R: 5′- TCCTCCAGCTCCTC CATGGTGATCGG −3′ were used Bhaganagare et al. (2013); PCR reaction conditions for the amplification of *nifH*gene fragment was as follows: initial step of denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 51–57 °C for 30 s, and elongation at 72 °C for 1 min followed by a final step of extension at 72 °C for 5 min. Amplified PCR products were fractionated on 1.5% agarose gel.

2.3.5. ACC deaminase activity-

ACC deaminase activity was determined in bacterial isolates by measuring the production of α-ketobutyrate from the ACC (1-aminocyclopropance-1-carboxylicacid) cleavage by ACC deaminase (Penrose and Glick, 2003). The freshly grown bacterial cultures were re-suspended in 20 ml of DF salts minimal medium supplemented with either $(NH_4)_2SO_4$ or ACC (3.0 mM) as a sole nitrogen source. Another