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Biodegradation of isoproturon using a novel *Pseudomonas aeruginosa* strain JS-11 as a multi-functional bioinoculant of environmental significance

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ABSTRACT

Biodegradation of phenylurea herbicide isoproturon was studied in soil microcosm bioaugmented with a novel bacterial strain JS-11 isolated from wheat rhizosphere. The molecular characterization based on 16SrDNA sequence homology confirmed its identity as *Pseudomonas aeruginosa* strain JS-11. The herbicide was completely degraded within 20 days at ambient temperature with the rate constant of 0.08 day^{-1}, following the first-order rate kinetics. In stationary phase, at a cell density of 6.5×10^9 CFU mL^{-1}, the bacteria produced substantially increased amounts of indole acetic acid (IAA) in the presence of tryptophan as compared with the control. Also, the bacteria exhibited a time-dependent increase in the amount of tri-calcium phosphate solubilization in Pikovskaya's medium. Further screening of the strain JS-11 for auxiliary activities revealed its remarkable capability of producing the siderophores and hydrogen cyanide (HCN), besides antifungal activity against a common phytopathogen *Fusarium oxysporum*. Thus, the versatile *P. aeruginosa* strain JS-11 with innate potential for multifarious biological activities is envisaged as a super-bioinoculant for exploitation in the integrated bioremediation, plant growth and disease management (IBPDM) in contaminated agricultural soils.

1. Introduction

The agrochemical isoproturon (N,N-dimethyl-N′-[4-(1-methylethyl)phenyl]urea) is a systemic phenylurea herbicide, which is mainly used for pre- and post-emergence control of annual grasses and broad-leaf weeds in cereals [1]. Due to its intensive and repeated usage as well as its persistence in the environment, it is frequently detected in the surface and groundwater bodies at concentrations exceeding the European Union drinking water limit of 0.1 $\mu g\,L^{-1}$ [2]. It is reported to be directly or indirectly toxic to a wide variety of organisms including aquatic invertebrates [3], fresh water algae [4], microbial communities [5], plants [6], and is also reported to be carcinogenic to animals and humans [7,8]. Therefore, understanding the fate of isoproturon in soil is important for developing strategies to minimize its environmental impact. Indeed, microbial degradation is the primary mechanism for mineralization of isoproturon [9]. Several bacterial strains such as *Sphingomonas* sp. SRS2 [10], *Arthrobacter* sp. N2 [11], *Sphingomonas* sp. F35 [12], *Methylopila* sp. TES [13], and *Sphingobium* strains [14] have been reported to degrade this herbicide. Sørensen et al. [15] have demonstrated the enhancement of isoproturon degrading capability of *Sphingomonas* sp. SRS2, when grown in co-culture with strain SRS1, not able to degrade isoproturon. Similarly, several bacterial consortia have been reportedly involved in the degradation of pesticides [16], however, the studies related to isoproturon degradation using pure bacterial isolates from agricultural soil are limited. It has also been observed that one or more previous applications of the same pesticide or another pesticide with a similar chemical structure result in its increased rate of microbial degradation in soil. Even some of them failed to provide adequate control against their target pests and pathogens [17]. This accelerated or enhanced degradation may cause economic losses to farmers as it forces them to increase the pesticide use by several-fold with only a nominal increase in crop output [17]. Furthermore, the indiscriminate use of pesticides for a longer time may also pose another serious problem of pesticide residue accumulation in soil, which could be detrimental to both the soil and human health, and demand effective remedies. Therefore, in order to overcome these problems, a more functional and eco-friendly approach involving microbes with multi-functional activities is essentially needed in order to compensate the loss of biocontrol activity due to accelerated pesticide degradation in contaminated soils.

As a prominent group of rhizospheric microorganisms, the pseudomonads have substantially contributed to bioremediation, plant growth promotion and biocontrol of pathogens. Members of

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this versatile genus have demonstrated a catabolic potential with inherent capacity for xenobiotics degradation [18]. Mechanisms that make pseudomonads an effective biocontrol agent against pathogens involve the production of siderophores [19], antibiotics [20], and extracellular enzymes [21]. Therefore, the use of such rhizospheric bacteria in bioremediation of pollutants in soils has been proposed for disseminating the desired bioinoculants in contaminated soils. To the best of our understanding, very few reports are available on the microorganisms with multifaceted characteristics that confer concurrent plant growth promoting and biocontrol abilities besides their role in pesticide degradation [22,23]. Thus, the prime objective of the present study was to screen the isoproturon resistant bacterial isolates from wheat rhizospheric soil for selection of an efficient strain with inherent isoproturon catabolizing ability, and multiple auxiliary plant beneficial traits. The most promising isoproturon degrader strain JS-11 has exhibited inorganic phosphate solubilizing activity, indole acetic acid (IAA), siderophores and hydrogen cyanide production in addition to antifungal activity. This novel bacteria could be exploited as a natural super-bioinoculant for providing an effective and eco-friendly alternative solution to the herbicide contamination, plant growth and pathogen control problems, based on its innate bioremediation, plant growth and disease management (IBPDM) potential.

2. Materials and methods

2.1. Chemicals and reagents

Technical grade (99% purity) isoproturon (CAS No. 34123-59-6) was obtained from Sigma Chemical Company, St. Louis, MO, USA. Isoproturon (75% WP) was obtained from Modern Insecticide Ltd., India. All other chemicals, solvents and nutrient media were purchased from Sigma Chemical Company (USA), Sisco Research Laboratory (India) and Hi Media (India), respectively.

2.2. Isolation and characterization of IPU degrading bacteria

Bacteria capable of degrading isoproturon were isolated from wheat rhizosphere of isoproturon contaminated soil by the enrichment culture technique. The soil homogenate was inoculated in 50 mL nutrient broth (yeast extract, 1.5; beef extract, 1.5; peptone, 5.0; sodium chloride, 5.0; g L^{-1}, pH 7.0 ± 0.2) supplemented with 100 μg mL^{-1} isoproturon and incubated at 28 ± 2 °C. Subsequently, 0.2 mL of this culture was plated on M9 mineral salt agar (ammonium sulphate, 1.0; dipotassium hydrogen orthophosphate, 1.0; disodium hydrogen orthophosphate, 2.1; magnesium sulphate, 0.01; calcium chloride, 0.10; ferric chloride, 0.001; copper sulphate, 0.040; sodium molybdate, 0.002; g L^{-1}, pH 7.2 ± 0.2) supplemented with 200 μg mL^{-1} of isoproturon. The resulting colonies were repeatedly sub-cultured in M9 medium containing 500 μg mL^{-1} of isoproturon to confirm their catabolizing ability. A total of 185 colonies were picked up from M9 agar plates and the isolates were initially screened for isoproturon tolerance up to 3 mg mL^{-1} using minimum inhibitory concentration (MIC) technique. Identification and characterization of the isolate JS-11 was done on the basis of the colony morphology, biochemical characteristics following Bergey's manual of systematic bacteriology [24]. The catalase and oxidase tests were performed using standard procedures. In brief, nutrient agar slants inoculated with strain JS-11 were incubated at 28 °C for 48 h. Appearance of O_2 bubbles upon addition of 3% H_2O_2 was considered as positive test for catalase. The oxidase activity was carried out using disc assay (Hi-Media, India), where the change in color of disc to deep purple was regarded as a positive test. The nitrate reduction was performed by the traditional colorimetric procedure [25]. Nitrate reduction was observed in the culture medium supplemented with nitrate of sulfanilic acid and N,N-dimethyl-1-naphthylamine, which react with nitrite to produce a pink/red coloration indicative of nitrate reduction. Also, the metabolic profile of the strain including the carbohydrate and amino acid utilization was determined using BIOLOG GN plates following the recommendations of the producer (Biolog Inc., Hayward, CA, USA). The phylogenetic analysis was based on 16SrDNA sequence homology [26].

2.3. PCR amplification, cloning and sequencing of bacterial 16SrDNA

The isolate JS-11 was grown in nutrient broth at 28 ± 2 °C. Cells were harvested after 24 h and processed immediately for DNA isolation by standard procedure [27]. Using the purified genomic DNA as target, the gene coding for 16SrRNA was amplified employing primers, fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAG CC-3′) [28] complementary to the 5′ and 3′ regions of eubacterial 16SrRNA genes, respectively. The PCR amplification was carried out in a final volume of 50 μL. Briefly, the amplification reaction containing 50 ng template DNA, 30 pmole each of universal primers primer, 0.2 mM dNTPs, and 2.5 U Taq polymerase (Gibco-BRL, USA) in 1 μL PCR buffer (Sigma, USA) was run on an Biometra personal cycler. The amplification reaction was cycled as follows: 94 °C for 1 min, 55 °C for 1 min; 72 °C for 1 min and a post-dwell at 72 °C for 3 min for 30 cycles. The amplicon was analyzed on 1.5% agarose gel in 1× TAE, run at 50 V for 2 h and purified using QIA quick PCR purification kit, Qiagen, USA. The purified amplicon (1.5 kb) was sub-cloned with in *lac*Z gene in pGEM-T easy vector (3.015 kb) (Promega, USA). The selected clone was subjected to sequencing of 16SrRNA gene fragment with SP6 and T7 sequencing primers using ABI prism 3730 sequencer. The sequence of JS-11 was submitted to GenBank NCBI database and accession no. EF378653 obtained. The homologous 16SrRNA gene sequences were subjected to the multiple sequence alignments by ClustalW multiple alignment method using BioEdit (version 5.0.9). Phylogenetic tree was constructed by the neighbour-joining (NJ) method with nucleotide pair-wise genetic distances corrected by Kimura two-parameter method [29] using TreeCon tool. The reliability of tree topology was subjected to a bootstrap test and numbers at nodes indicate bootstrap support values as a percentage of 1000 replications.

2.4. Growth kinetics of isoproturon degrading isolates

The bacterial isolate was freshly grown in M9 medium (pH 7.2) containing 200 μg mL^{-1} isoproturon as a sole source of carbon. For determination of growth kinetics, 0.1 mL culture of the isolate JS-11 was inoculated into 20 mL each of the nutrient broth and mineral salt medium without and with 1 mg mL^{-1} isoproturon in 100 mL Erlenmeyer flask. The culture was incubated at 28 ± 2 °C in a shaker water bath. At regular time intervals, the optical density at 600 nm was measured and the viable bacterial counts in terms of colony forming units (CFU mL^{-1}) were determined by plating the serially diluted cultures on agar plates. The growth curves were obtained by plotting the log CFU mL^{-1} as a function of time.

2.5. Kinetics of biodegradation

The biodegradation of isoproturon in soil microcosm was performed as described earlier [22,23,30]. The soil (sandy loam) microcosms in triplicate consists of 1 kg each of sterilized rhizospheric soil (control), unsterilized rhizospheric soil, and bioaugmented unsterilized rhizospheric soil were prepared in autoclaved earthen pots. The soil samples were amended with isoproturon (EC 75%) at a loading of 15 kg a.i. ha^{-1} (10× of recommended dose) equivalent to a.i. 10 mg kg^{-1} of soil. The