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Pendimethalin Degradation by Multi-stress Tolerant Plant Growth Promoting *Pseudomonas aeruginosa* Strains Isolated from Soybean Rhizosphere

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Abstract

The demand for pendimethalin (PND), a pre- and early post-emergence dinitroaniline herbicide is increasing after the recent ban of the most widely used glyphosate to prevent cultivation of herbicide-tolerant crops. PND is a poorly water-soluble, recalcitrant, carcinogenic nitroaromatic compound that modify soil microbiota and often leach into soil, water bodies and crop produce. The present study aimed to explore multi-stress tolerant plant growth promoting PND degrading microbes from contaminated soyabean rhizosphere. A multi-step selective enrichment strategy resulted in isolation of 14 drought resistant bacteria in mineral salt medium. The high-performance liquid chromatography evidenced 97% PND degradation (100 mg L^{-1}) by isolate PND3 and PND4. All the isolates produced nitrite and mineralised PND as a sole source of C, N, and energy over a period of 7 days. The TLC analysis suggested that the PND degradation product could be 3,4-dimethyl 2,6-dinitroaniline for C utilization, while concomitant nitrite formation suggested nitrogenase-dependent PND degradation pathway. The polyphasic characterization based on morphological, VITEK-2 biochemical, 16S ribotyping and phylogenetic analysis revealed the similarity of the isolates to *Pseudomonas aeruginosa* (~99.5%). Thus, these new strains demonstrated relatively high efficiency of PND mineralisation and therefore a potent bioremediation and plant growth promoting agent at pesticide contaminated fields.

***Key words*:** Pesticide-tolerance, Rhizosphere, Nitroaromatics, Bioremediation, Plant growth promotion

Pendimethalin (PND) (CAS registry number 40487-42–1); [$C_{13}H_{19}N_3O_4$; N-(1-ethylpropyl)–2,6-dinitro-3,–4 xylidine; Molecular mass 281.312 Da] is a dinitroaniline herbicide with nitrated aromatic ring structure consisting of hydroxyl (-OH) and nitro (-NO_2) groups. It is hydrophobic and sparingly soluble in 0.275 ppm water [1]. PND is widely applied to soil as a selective pre-plant, pre-emergence, and sometimes post-emergence herbicide in variety of crop plants including cotton, soybean, maize, wheat, rice, peas, and vegetable crops to control annual grasses, certain broad leaf weeds of dryland crops and non-crop areas. It is indirectly accountable for plant growth promotion under tropical, subtropical, as well as temperate conditions [2-3]. PND is also recommended for use on fruit, grapes, vegetable, oil seeds, cereals, tobacco, and ornamental plants at 2 kg ha^{-1} in the European Union (EU) and at 6.7 kg ha^{-1} in the USA [4]. It is the third most frequently used selective herbicide throughout the world and has been on the market for almost 35 years [5]. The demand for PND in crop protection raised from 9 to 114.3 tons, with more than 12-fold increase in the northern part of India alone and utilized almost 11.8 tons of PND per year for protection of the cotton crop [6]. It has given widespread excellent weed control at 0.66–1.68 kg/ha in both leaf and fruit vegetable crops [7]. PND application shows acceptable levels of crop reaction, viz., transient slight initial growth inhibition, necrosis, leaf deformation and slight injury to flowers [8]. PND has relatively (i) low volatility due to vapor pressure of 3×10^{-3} mm Hg at 25°C; (ii) hydrophobic nature; (iii) longer persistent time in soil due to low leaching potential (half-life of 76–98 days in agriculturally relevant soils and sediment water under aerobic and anaerobic condition, respectively); and (iv) strong inhibitory action on mitotic cell division in developing root shoot system [9].

A very meagre amount of PND reaches the target and the remaining accumulates into the environment, where it adversely affects crop, animal, and public health [10]. PND enters in the surface water mainly as runoff due to heavy rains, which results in 2–134 µg h^{-1} residue to water sediments [11]. PND persist by physical adhesion to soil, organic fraction, sediment, and clay particles to exert phytotoxicity to non-target plant crops. PND (i) affects symbiosis between legume and

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Rhizobium; (ii) reduce nodulation by >25%; (iii) suppress rhizosphere nutrient cycling by microbes; (v) reduces soil nematode by 35–36%; and (vi) inhibits roots and shoots in seedlings. It has shown toxicity effects onto (i) onion and maize roots up to 1 mg kg^{-1} [12-13], (ii) the growth of funnel plants by inhibiting the tubulin production during mitosis (iii) root knot nematode and (iv) inhibit mitotic cell division in growing root system [14-15].

PND is listed as a persistent bio accumulative toxin and a possible human carcinogen (group C) by US EPA and can easily escape and enter the food chain with meagre possibility for degradation [16]. PND is (i) slight acute toxic compound (toxicity class III) (ii) slightly toxic by skin exposure, with dermal LD_{50} of ≥ 2000 mg kg^{-1} in rats; and (iii) mildly irritant to the eye of rabbits (iv) exerts toxicity to plants, microbes, fish with LC_{50} (96 h) for rainbow trout and blue gill sunfish of 0.14 and 0.2 mg L^{-1} and also rats with oral LD_{50} of 1.05–5.0 g kg^{-1} [17-18]. PND exposure in the agriculture health study has shown: (i) increased incidences of lung, rectal, and pancreatic cancers [16]; (ii) genotoxic effects on the fish species *Oreochromis niloticus* and aquatic invertebrates [19-20]; (iii) mild hemotoxic effect in female rats after administration of dosage for 90 days [21] and classified as a persistent bio accumulative toxic agent [22]. Extensive exposure of PND for prolonged time can induce cytotoxicity to living CHO cells, neuro behavioural disorders, disrupt the endocrine, reproductive, and immune system and cause thyroid follicular cell adenoma [23-26].

Overall, this scenario suggests the necessity for immediate removal of PND from the contaminated soil to prevent environment and health hazard. Until now, various abiotic avenues have been employed for removal of PND, but they have either lacked specificity or have not proved to be efficient and reliable. A variety of chemical and environmental parameters, such as complex structure, volatility, water solubility, pH, and dissolved oxygen matter, etc. determine the fate of PND degradation in the contaminated soil. Further, the abiotic mode of degradation (i) causes decomposition to toxic fumes of NO_x; (ii) separates unwanted compounds without destruction; (iii) generates toxic intermediates; and (iv) poses several issues for on-site or off-site treatment system. Hence, abiotic degradation route is obsolete and less preferred alternative in the present era. Microbes can (i) transform complex compound to simple form; (ii) bind, thrive, colonize, and metabolically utilize the compound as an energy source; (iii) convert PND into simple and non-toxic compound due to their involvement in nutrient cycling [27]; (iii) help to incorporate the recalcitrant PND into biogeochemical cycle; (iv) generate non-toxic side degradation product and support plant growth; (v) interact in the microbiome to warrant complete mineralization of the toxic chemical and make it bio-available to plants. Hence, applications of microorganisms are the most preferred strategy to degrade the nitroaromatic PND. Many bacterial and fungal species capable of degrading PND have been isolated and the metabolic pathways have been characterized [28]. However, knowledge of the physiological, biochemical, and genetic mechanisms of PND biodegradation is still limited. Of the biodegrading microbes, majority have showed (i) less tolerance, (ii) produced intermediate of unknown toxicity, (iii) not evaluated in the field for multi-stress tolerance like drought, pH, salinity, crop cycles, etc. The use of microorganisms with plant growth promoting traits in addition to removing herbicides from polluted environments through biodegradation techniques is a promising approach and deserves more emphasis [29]. Thus, the aim of the present study was to explore plant growth promoting multi-stress tolerant PND degrading microbes from contaminated rhizospheric soil for its bioremediation application.

MATERIALS AND METHODS

Chemicals and microbiological media

All the chemicals and solvents were AR grade, except methanol and acetonitrile (HPLC grade) and procured from Sigma-Aldrich (USA), Merck (Germany) or Thermo-Fisher (India). Pendimethalin (PND) was technical grade (97% purity) for routine assays or high purity reference (Sigma, USA) for spectrophotometric and chromatographic analysis. Microbiological culture media and ingredients were acquired from Hi-media (Mumbai). All the glassware was cleaned with 6N HCl and rinsed thrice with double distilled water before use. PND was first dissolved in methanol and then filtered through a 0.22 µm Millipore membrane filter before use**.** Mineral salts medium (MSM**)** comprised of (g L^{-1}): K_2HPO_4, 0.630; KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.2; $FeSO_4$, 0.01; and $CaCl_2$, 0.1 to support initial growth and supplemented with PND as a sole C, N and energy source. Agar was added at 30.0 g L^{-1} for preparation of solid medium. Microbiological media was sterilized at 121°C for 20 min prior to addition of filter sterilized PND.

Soil collection and analysis

Approximately 100 g soyabean rhizospheric soil samples were collected at random from contaminated site near a manufacturing industry (Lat-Lon: 20.69-77.06). Samples were drawn by detaching root and rhizosphere soil at a surface depth of 2–20 cm. Samples were collected in a sterile plastic bag and processed within 6-12 hours. Selected moist samples were shed dried before the experiments and composite soil sample was analysed for various physical and chemical parameters as per standard protocol [30].

Isolation and selective enrichment of pendimethalin-degrading microbes

A series of standardized selective enrichment stages was carried out for isolation of PND degrading bacteria [31-33]. Soil aliquot (10 g) was added to a 500 ml capacity Erlenmeyer flask with 100 ml of sterile Luria-Bertani (LB) broth and incubated on a rotary shaker at 120 rpm for 5 days at 30°C in dark. The modified LB broth was prepared by sterilization, followed by supplementation with filter sterile PND (100 mg L^{-1}) and used for further enrichment. About 20 ml of the enriched culture broth was added into a 500 ml capacity Erlenmeyer flask containing 80 ml of modified LB broth. The flasks were incubated on a rotary shaker (120 rpm) at 30°C in the dark for 5 days. Further, each enrichment cycle (4 times) involved reinoculation of the culture medium (20 ml) into 80 ml of fresh modified LB medium containing PEG 6000 (4% w/v) and PND at an interval of 5 days under shaking at 30°C in dark. After each enrichment cycle, microscopic cell counting using crystal violet monochrome staining was performed to observe for increase in cell number. After four rounds of enrichment, 0.1 ml aliquots of serial dilutions (tenfold) were spread onto modified LB agar containing PND (100 mg L^{-1}) and incubated at 30°C for 5 days. The morphologically distinct bacterial colonies were purified by streaking on the modified LB agar and maintained on agar slopes or glycerol stock solutions. These morphologically distinct cultures were designated as PND1, PND2, …., etc. and further evaluated for PND degrading potential.

Identification of pendimethalin degrading isolates

a) *Phenetic characteristics*

The selected isolates were characterized based on morphological traits such as cell and colony morphology, Gram character, motility, etc. using conventional methods. Detailed biochemical characteristics of potent PND degrading strains (PND3 and PND4) were analyzed by VITEK 2 system in compliance to the Bergey's Manual of Systematic Bacteriology. A sterile applicator stick was used to transfer microbial colonies of the pure culture and suspended in 3 ml of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12 × 75 mm clear polystyrene test tube. Identification cards were inoculated with microorganism suspensions using an integrated vacuum apparatus. Each test tube containing the microorganism suspension was placed into a cassette and the identification card was placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube. The filled cassette is placed automatically into applied vacuum chamber station. Air was re-introduced into the station and the organism suspension was forced through the transfer tube into micro-channels that filled all the test wells. After incubation, turbidity measurement was performed using Densichek. Calculations were performed on raw data and compared to thresholds to determine reactions for each test.

b) *16S ribotyping*

The genomic DNA of microbial cultures was isolated using single colony lysis method and its quality was evaluated on 1% agarose gel [31]. The 16S rRNA gene was PCR amplified using 0.5 µM each of 27F and 1492R primers (5`-AGA GTT TGA TCC TGG CTC AG and 5`-TAC GGT TAC CTT GTT ACG ACT T, respectively) in a 25 µl reaction mixture containing template (40 ng), *Taq* buffer (1X) with *Taq* polymerase, $MgCl_2$ (1.5 mM) and dNTPs (500 µM). The PCR conditions were 94ºC: 3 min followed by 35 cycles of 94ºC: 30 sec; 50ºC: 30 sec; 72ºC: 90 sec and final extension of 72ºC for 10 min. The PCR products were purified using Exonuclease I - Shrimp Alkaline Phosphatase (Exo-SAP) and visualized on 0.7% agarose gel. Deoxyribonucleic acid (DNA) cycle sequencing reaction for PCR amplicon was carried out using forward and reverse primers using Big Dye Terminator ver. 3.1 cycle sequencing kit using POP7 polymer on ABI 3730XL Genetic Analyzer (Life Technologies, USA) as per manufacturer instructions. The base called nucleotide sequences were used for contig construction using Chromaslite ver. 1.5 and comprised of 1489 bp non-ambiguous nucleotides. Phylogenetic analysis was performed through multiple alignments of homologous Deoxyribonucleic acid (DNA) sequences downloaded from NCBI GenBank database using the BLASTN program [34] in MEGA11 using Maximum Likelihood algorithm with 1000 bootstraps [35].

Assessment of pendimethalin degrading trait

a) *Primary screening of pendimethalin-degrading bacteria using plate assay*

Each isolated strain was rejuvenated into 20 ml of sterile LB broth containing 100 mg L^{-1} PND and incubated under aerobic conditions (120 rpm) in dark for 48 hours at 30ºC before use. Aliquots (2 ml) of each culture broth were transferred into a sterile 2 ml microfuge tube and centrifuged (10,000 x g). Supernatant were discarded and the pellets were dissolved in 100 µl of sterile saline. Then, a loopful of each cell suspension was spot inoculated on a modified LB agar plate with 100 mg L^{-1} PND and incubated for 7 days at 30ºC. Growth accompanied by a clear halo around the colony was recorded as putative PND degradation potential. Experiments were conducted in triplicates.

b) *Screening for utilization of pendimethalin as sole C, N and energy source*

Each isolate was screened separately for utilization of PND as a sole C, N and energy source by transfer of 48 h old inoculum prepared from modified LB broth (20 ml) to Mineral Salt Medium (MSM) containing 100 mg L^{-1} PND as sole carbon source. The flasks were incubated for 15 days under dark and aerobic conditions (120 rpm) at 30ºC. Growth was occasionally monitored by observing turbidity and re-inoculation in fresh MSM for at least 3 consecutive rounds. Isolates were recovered on pesticide containing MSM agar medium. The isolates showing growth on MSM medium were used for thin layer chromatography (TLC) and then, for quantitative degradation assay using high-performance liquid chromatography (HPLC).

c) *Chromatographic assessment of pendimethalin degradation*

For each degradation assay, seed culture was prepared by pre-incubating the isolates separately in MSM broth spiked with 100 mg L^{-1} PND and incubation at 30ºC on a rotary shaker at 120 rpm for 48 h. Cells were collected at the mid-exponential phase by centrifugation at 8,000 x *g*. The supernatant was discarded, and the cell pellets were washed twice with fresh sterile MSM broth before re-suspension in the same fresh medium to 100 ml volume. The initial cell density was adjusted to McFarland standard A_{600} = 0.5 using a spectrophotometer (Shimadzu UV mini-1280, Japan). All the cultures were developed aerobically by incubation on a rotary shaker at 120 rpm at 30ºC for 6 days. After incubation, the culture supernatant was collected by centrifugation (8,000 x *g*, 20 min) and metabolites were recovered by ethyl acetate extraction in a separating funnel. For this, equal volume of ethyl acetate was mixed with the culture supernatant and mixed vigorously before collecting the polar aqueous phase. The solvent extraction procedure was repeated thrice, and the excess of ethyl acetate was evaporated using rotavapor at 80 °C (Rotavapor R-124, Buchi, Germany). After complete drying to crystals, 1 ml of HPLC-grade methanol was added to a rotaflask. The filter sterilized (0.22 µm) aliquots were used for detection of intermediates by TLC. For this, a microliter aliquot was spotted onto a TLC plate (silica gel 60 F_{254}, Merck, Germany) and separation was performed using solvent system Hexane: Ethyl acetate (1:1) before observation under UV and visible illumination. The retardation factor (*Rf*) was calculated as ratio of distance migrated by the solute to the distance migrated by the solvent system.

HPLC analysis was performed using an isocratic setup on Shimadzu UPLC 2020 (Singapore) equipped with PDA detector (SPD-M20A) monitored at A_{254}. The stationary phase was C18 octadecyl silane column (internal diameter, 4.6 mm; length, 250 mm) filled with Kromasil 100-5 (particle size 5 µm with 100 Å pore size) maintained at 40ºC using a column oven, while the mobile phase was UPLC-grade acetonitrile: ultrapure water (75:25, v/v). Each sample (20 µl) was injected using an autosampler and eluted at a flow rate of 0.8 ml min^{-1}. Calibration curve was prepared using a series of increasing concentrations of reference PND (Sigma, USA). The PND concentrations were plotted against the peak area to determine the slope and deducing unknown concentrations.

Estimation of nitrite

Aliquot (1 ml) of suitable dilution (10 times) of the supernatant obtained from MSM culture medium was mixed with 2.5 ml of sulfanilic acid and incubated at room temperature for 10 min. Then, 2.5 ml of N-(1-napthyl)ethylenediamine hydrochloride solution (0.04%) was added and reincubated at room temperature for 20 min before re-dilution (50x) and

measurement of A_{543} nm. The nitrite concentration was inferred from a reference calibration curve prepared using various dilutions of sodium nitrite.

RESULTS AND DISCUSSION

Isolation of pendimethalin-degrading microbes

Agricultural soil, especially rhizospheric soil is well-known for presence of plant growth supportive microbes [36]. When the rhizosphere is consistently exposed to a xenobiotic such as PND, it can be anticipated for presence of tolerant and degradative microbes. The detailed characteristics of the composite soil sample of the soyabean rhizosphere supported this assertion and is summarized in (Table 1).

Table 1 Physic-chemical and particle size characterization of contaminated soil sample

Properties	Unit	Result	Max-Min limit	Inference
pH	--	7.67	6.5-7.5	Slight alkaline
Conductivity	mS cm^{-1}	0.274	0-1	Ok
Sulphur	ppm	39.65	10-20	Sufficient
Potassium	Kg ha^{-1}	829.5	130-336	Abundant
Exchangeable calcium	%	0.14	0.05-0.41	Saltier
Sand	%	47.43	-	-
Clay	%	4.61	2.5-5.0	-
Organic carbon	mS cm^{1}	0.94	0.40-0.60	More
Calcium	mEq	0.41	2.5-5.0	High
Magnesium	mEq	0.14	2.5-5.0	High
Available nitrogen	Kg/ha	206.98	280-560	Sufficient

The soil was observed to be humid and slightly alkaline pH of 7.67 with a composition of 47.43%, 47.96% and 4.61%, sand, silt, and clay, respectively which was supportive for holding of moisture and aerobic growth. There were ample macronutrients viz. organic carbon (0.94%), nitrogen (206.98 Kg ha^{-1}), phosphorus (91.95 Kg ha^{-1}) and exchangeable potassium (829.5 Kg ha^{-1}) along with micronutrients i.e., sodium, calcium, magnesium, zinc, copper (as 352.94, 0.41, 0.14, 3.30, 1.94, 39.35) and elements (sulphur and magnesium of 39.65 ppm and 0.14 mEq, respectively) which could encourage microbial diversity and plant-microbe interaction. Similarly, high poyta (829.5 kg ha^{-1}) was also supportive to the resident microflora and possible PND degradation by multi-stress tolerant microbes. In a similar approach, PND degrading yeasts were isolated from soils with long-term exposure [37]. Similarly, plant growth promoting and multiple pesticide tolerant Pseudomonas Sp. Were isolated from *Vigna radiata* (L.) rhizosphere [38].

Table 2 Morphological and IMViC characteristics of the isolates

Characteristic	Strain	
	PND3	PND4
Cell shape	Rod	Rod
Motility	Motile	Motile
Colony characteristics on LB agar		
Shape	Circular	Circular
Colour	Off white	Faint yellow
Elevation	Elevated	Elevated
Margin	Smooth	Smooth
Surface	Smooth	Smooth
Opacity	Opaque	Opaque
Biochemical tests		
Indole	-	-
Methyl red	-	-
Vogus-Proskauer	-	-
Citrate utilization	+	+

NR = not reported; + = positive; - = negative

Bacteria have inherent ability to resist complex environmental stress and establish xenobiotic degradation abilities [39]. The selective enrichment emerged as a useful strategy in the present work as the amended PND served as abiotic stress to possibly trigger adaptive mutation in microbes. After four rounds of enrichment in modified LB medium containing PND under dark aerobic conditions, a total of 14 purified bacterial cultures with distinct biochemical characteristics were obtained and designated as PND1, PND2, PND3, …, PND14. The efficiency of the enrichment strategy was estimated using microscopic bacterial count which remained incremental at each round of enrichment and indicated adaptive efficiency of the isolates (data not shown). Later, the use of PND as sole C and N source was evaluated by transfer of each culture from LB broth to synthetic MSM nutrient medium devoid of any other carbon or nitrogen source for successive sub-culturing rounds. All the strains showed growth but varied cell multiplication rates in the synthetic MSM medium. After multiple rounds of sub-culture, all the strains were further maintained on the MSM medium.

Identification of pendimethalin degrading isolates

a) *Phenetic characteristics (VITEK 2)*

All the strains were characterized based on morphological and IMViC traits (Table 2) and the most potent PND degradative strains viz. PND3 and PND4 were analysed using VITEK 2 system (Table 3).

Both the strains utilized D-glucose, D-galactose, L-malate, D-mannitol and produced L-lactate, malonate, succinate as well as citrate which decrease soil pH, increase microbial activity, alter microbial community composition and has a role as a defensive chemical in roots and nodules [40]. The arylamidase activities for proline, tyrosine, alanine and glycine with assimilation of L-histidine amino acids is an index of nitrogen mineralization to NH_4^+ ions for availability to plants in soil [41]. The strain PND4 was distinct in production of gamma- glutamyl- transferase, utilization of mannose but could not utilize the plant origin coumarate phenylpropanoid. As plants activate and accumulate various phenolic compounds under abiotic stress conditions [42], the non-utilization of phenylpropanoid is a useful trait of the selected isolate. Based on the biochemical characteristics deduced through VITEK, both the strains closely related to *Pseudomonas* genus. As per Bergey's classification of bacteria, *Pseudomonas* genus is conspicuous for vast diversity of species, therefore, further DNA based investigation to identify the strains was undertaken.

Table 3 Biochemical characteristics of isolates PND3 and PND4 examined by VITEK-2 analysis

Test substrate	Abbreviation	Concentration (mg)	Isolate PND3	Isolate PND4
Ala- Phe-Pro-Arylamidase	APPA	0.0384	-	-
Adonitol	ADO	0.1875	-	-
L-Pyrrolydonyl- Arylamidase	PyrA	0.018	-	-
L-Arbitol	IARL	0.3	-	-
D-Cellobiose	dCEL	0.3	-	-
β-Galactosidase	BGAL	0.036	-	-
H_2S Production	H2S	0.0024	-	-
β-N-acetyl-E glucosaminidase	BNAG	0.0408	-	-
Glutamyl- aryalamidase pNA	AGLTp	0.0324	-	-
D-Glucose	dGLU	0.3	+	+
α- Galactosidase	AGAL	0.036	+	+
Ornithine decarboxylase Base	ODC	0.3	-	-
B-Glucosidase	BGLU	0.036	-	-
L-Malate assimilation	IMLTa	0.042	+	+
L-Proline araminidase	ProA	0.0234	+	+
α- Glucosidase	AGLU	0.036	-	-
Tyrosine arylamidase	TyrA	0.0276	+	+
Urease	URE	0.15	-	-
D- Sorbitol	dSOR	0.1875	-	-
Saccharose/ Sucrose	SAC	0.3	-	-
D- Tagatose	dTAG	0.3	-	-
Glu-Gly-Arg- Aryamidase	GGAA	0.0576	-	+
L-Lactate assimilation	lLATa	0.186	+	+
D- Trehalose	dTRE	0.3	-	-
Gamma- Glutamyl- Transferase	GGT	0.0228	-	+
Fermentation/ Glucose	OFF	0.45	-	-
β- Glucosidase	BGLU	0.036	-	-
D-Maltose	dMAL	0.3	-	-
D-Mannitol	dMAN	0.1875	+	+
D-Mannose	dMNE	0.3	-	+
β- Xylosidase	BXYL	0.0324	-	-
β-Alanine- arylamidase	BAIap	0.0174	+	+
Lipase	LIP	0.0192	-	-
Palatinose	PLE	0.3	-	-
Phosphatase	PHOS	0.0504	-	-
L-Histidine assimilation	IHISa	0.087	+	+
O/129 Resistance (COMP.Vibrio)	O129R	0.0105	+	+
Ellman	ELLM	0.03	-	-
N -Acetyl glucosamine assimilation	NAGA	0.0306	-	-
Lysine decarboxylase	LDC	0.15	-	-
Malonate	MNT	0.15	+	+
5-Keto D-Gluconate	5KG	0.3	-	-
L-Lactate alkanisation	ILATk	0.15	+	+
Glycine- arylamidase	GlyA	0.012	+	+
Coumarate	CMT	0.126	+	-
Succinate alkalinisation	SUCT	0.15	+	+
Citrate (Sodium)	CIT	0.054	+	+

+ positive; - negative

Table 4 Comparative 16S rDNA sequence similarity profile of the isolated strains PND3 and PND4 (GenBank Accession No. MN581674 and MN581675, respectively) as observed from NCBI BLAST analysis

Accession	GenBank Accession	Query coverage (%)	Alignment Gaps	E-value	Percent identity (%)
Pseudomonas aeruginosa ATCC 10145	NR114471	99	4	0.0	99.60
Pseudomonas aeruginosa DSM 50071	NR117678	100	4	0.0	99.53
Pseudomonas aeruginosa NBRC 12689	NR113599	98	4	0.0	99.59
Pseudomonas otitidis MCC10330	NR043289	100	4	0.0	98.18
Pseudomonas lalkuanensis PE08	NR179771	100	6	0.0	97.72

b) *16S ribotyping*

The 16S rRNA gene sequence is an important molecular chronometer for species identification along with biochemical characteristics. The 16 rRNA gene sequence was successfully amplified using the 27F and 1492R primer pair for both the isolates and approximately 1.4 kb amplicon was obtained by Sanger's cycle sequencing reaction for both strains. The NCBI BLAST similarity search analysis with the 16S ribosomal RNA

sequence database and type strain filters showed similarity of both the strains PND3 and PND4 with *Pseudomonas aeruginosa* ATCC 10145 (99.6% similarity and 99% query coverage) and with *P. aeruginosa* DSM 50071 (99.53% similarity with 100% query coverage) followed by *P. otitidis* MCC 10330 (98% match), thus showing closest homology to genus *Pseudomonas* and species *aeruginosa* (Table 4). Further, the 16S rDNA sequences of the strains were used for phylogenetic analysis through multiple alignments of homologous DNA sequences downloaded from NCBI GenBank database in MEGA11 software. The results are depicted in (Fig 1).

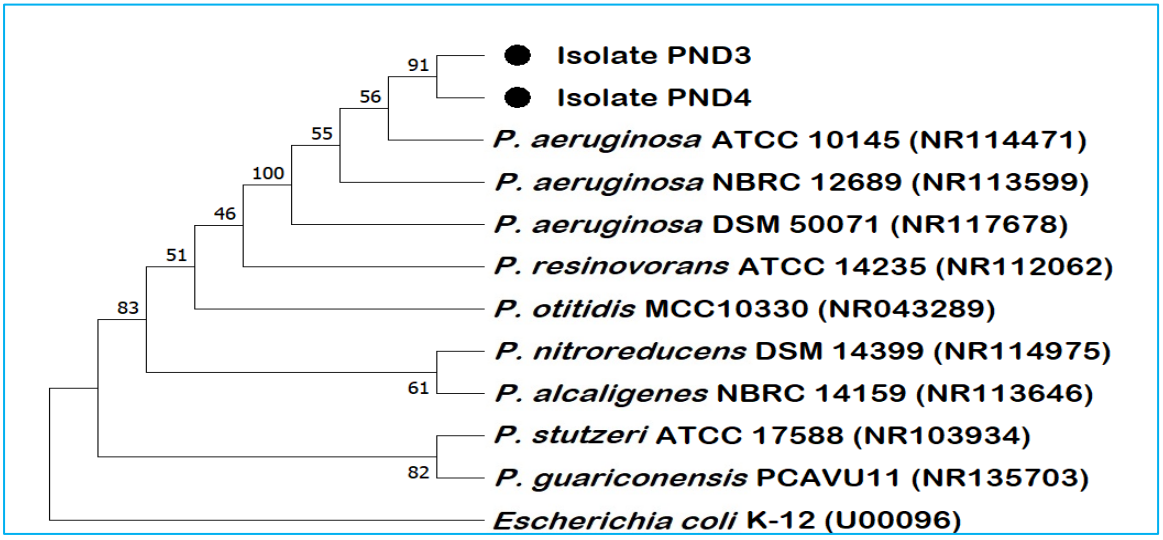

Fig 1 The evolutionary history of isolates PND3 and PND4 was inferred using the Neighbor-Joining method [43], while the evolutionary distances were computed using the Maximum Composite Likelihood method. The bootstrap consensus tree inferred from 1000 replicates is represented in percentage as units of the number of base substitutions per site are shown next to the branches and computed using MEGA11 [35]

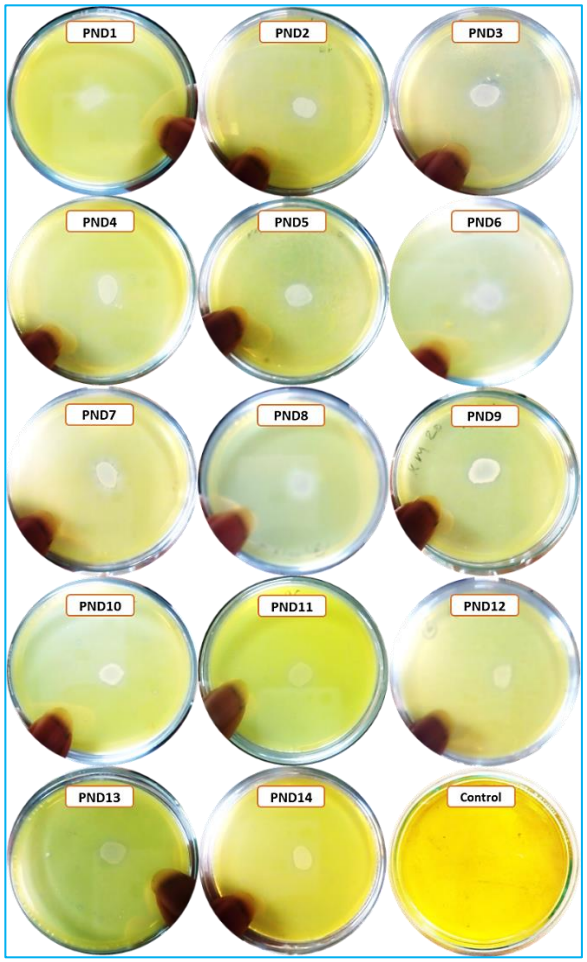

Fig 2 Primary screening of pendimethalin (PND) degrading bacteria using plate assay. The observations are recorded after 9 days of spot inoculation of each strain PND1, PND2, …, PND14 and control (uninoculated) on modified LB medium (with 100 mg L^{-1} PND) showing growth and clear radial halo around the colony

The 16S rRNA gene sequence of both the strains closely clustered with *Pseudomonas aeruginosa* ATCC 10145 and *P. aeruginosa* strain NBRC 12689 with a 56% and 55% bootstrap support, respectively. The mutual 16S rDNA sequence similarity among the strains by pairwise alignment was 100%, indicating that both the strains were identical. However, the biochemical characteristics and PND degradation rate significantly differed among strain PND3 and PND4. It is known that identification of strains of *Pseudomonas* genus cannot be merely based on 16S rDNA, but additionally requires to include *rpoB, rpoD* and *gyrB* housekeeping genes [44-45]. The results therefore necessitate sequencing the additional housekeeping genes or adopting a whole genome comparison phylogenomic and polyphasic approach for detailed species characterization, which was out of scope for our study. The curated DNA sequences were deposited to NCBI GenBank with accession no. MN581674 and MN581675, respectively.

Assessment of pendimethalin degrading traits

The selective enrichment strategy conducted for 4 rounds in modified LB medium containing PND resulted in screening of 14 isolates designated as PND1, PND2, …, PND14. The pure cultures of these isolates were screened by spot assay on modified LB agar medium containing 100 mg L^{-1} PND and observed for halo around the colonial growth. The results are depicted in (Fig 2-3).

Growth was observed, however at variable rate for all the isolates on the modified LB medium containing 100 mg L^{-1} PND reflecting tolerance to the presence of PND. However, a clear halo was recorded for most of the isolates except PND9, PND11 and PND14, and hence, abandoned in further experiments. Similarly, isolates PND10 and PND12 showed a halo only after 6 days of incubation thus indicting that pendimethalin (PND) was not preferred source of C or N for co-metabolism. The largest zone of clearance was observed for the isolates PND4 (15 mm) followed by PND3 (14.3 mm) with a colony diameter approximately same as the rest of the isolates.

Thus, even though the growth rates were same for all the isolates, the pendimethalin (PND) utilization capacity and preference greatly varied. It was therefore imperative to screen the isolates based on growth and pendimethalin (PND) utilization potential as sole source of C, N, or both in synthetic culture medium.

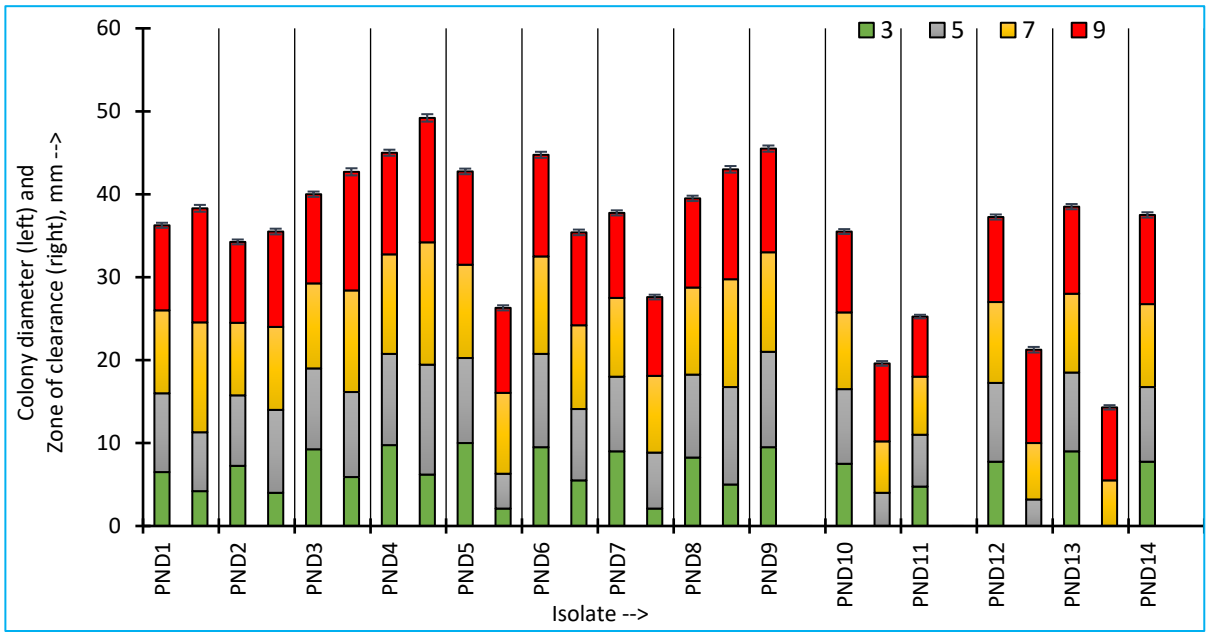

Fig 3 Colony diameter and corresponding diameter of zone of clearance (halo) around the colony of each isolate PND1, PND2, …, PND14 observed on modified LB medium (containing 100 mg L^{-1} pendimethalin) recorded at 3 (green), 5 (grey), 7 (orange) and 9 (red) days. Error bars represent percent error calculated for observations of experiment conducted in triplicate

Further subsequent transfer of the screened isolates in MSM nutrient medium was undertaken for evaluation with PND as sole source of C, N and energy for 3 consecutive rounds for 15 days. The residual PND was extracted using ethyl acetate and detected using TLC. The results are shown in (Fig 4).

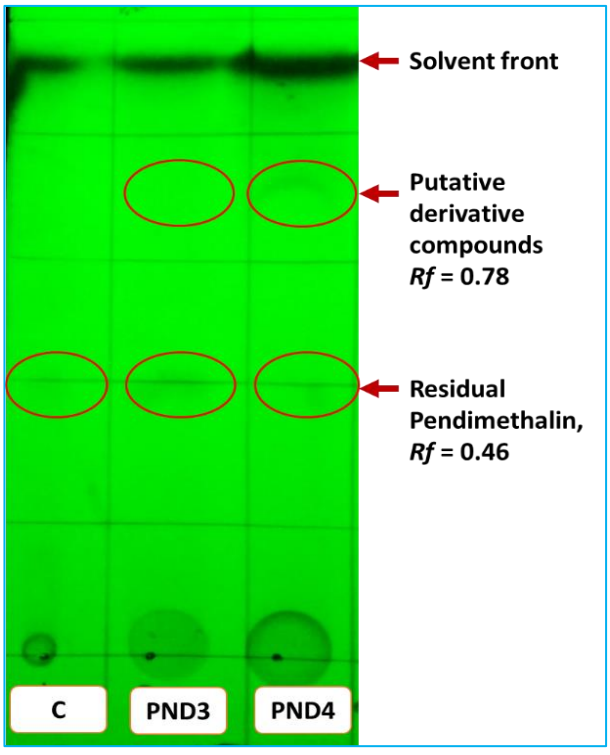

Fig 4 Thin layer chromatography (TLC) profile observed under UV illumination showing migration of solvent extract of 5-day old MSM medium containing 100 mg L^{-1} pendimethalin (PND) inoculated with the isolates PND3 and PND4. Keys: C: control (uninoculated MSM broth with 100 mg L^{-1} PND); *Rf*: Retardation factor

The TLC profile of the MSM media inoculated with the strains PND3 and PND4 showed residual PND at *Rf* = 0.46 which corroborated with the control (uninoculated MSM amended with PND). Additionally, an unidentified spot with *Rf* = 0.78 was observed. In a similar analysis, previously, pendimethalin degradative *Bacillus circulans* was characterized to produce 6-aminopendimethalin and 3,4-dimethyl 2,6-dinitroaniline metabolic end products (*Rf* = 0.87 and 0.74, respectively on hexane: ethyl acetate TLC system) as end products of nitroreductase and pendimethalin N-dealkylase enzymatic reaction under aerobic conditions [46]. Although these compounds were further not oxidized by *B. circulans*, the pentane released by the N-dealkylation of PND was utilized as sole source of C and energy. In the present study, the unidentified compound with *Rf* = 0.78 coincides with the *Rf* value of 3,4-dimethyl 2,6-dinitroaniline and hence, is probably the same metabolic intermediate/ end product indicating possible nitroreductase enzyme mediated nitroreduction of PND. Besides, in the present study, PND is utilized as sole source of N in addition to C, indicating possible mineralisation of PND unlike *B. circulans*. Thus, a detailed mass spectrometric analysis can confirm the identity of the derivative compounds in the present study to confirm the PND degradation pathway.

For quantitative analysis of PND degradation by the isolates, HPLC analysis was performed. (Table 5) summarizes the PND degradative performance after 7 days of incubation, while the chromatograph of the calibration curve, reference PND (Sigma, USA) and residual PND in MSM media is shown in (Fig 5).

The HPLC chromatograms revealed a retention time (RT) of 9.4 min for reference PND that coincided to the residual PND in the samples. Additionally, a major peak at RT = 7.5 min was also observed for all the samples corresponding to strains PND1, PND3, PND4, PND7 and PND8 indicating presence of a common intermediate or metabolic end product. The chromatograms corresponding to strains PND2, PND5, PND6, PND10 and PND12 showed only the residual PND, while

PND13 showed multiple minor residual peaks indicating possible mineralization of PND. The qualitative estimation revealed a significant reduction of the initial PND concentration (100 mg L^{-1}) in the range of 21.2% to 96.01% by all the strains under aerobic conditions. Evidently, strains PND3 and PND4 showed 97% and 92% degradation efficiency after 7 days indicating almost complete PND metabolic utilization as sole source of C, N and energy.

Table 5 Quantitative pendimethalin degradation performance of *Pseudomonas aeruginosa* strains after 6 days of incubation at 30°C under aerobic conditions in the synthetic MSM medium devoid of any C and N source but amended with 100 mg L^{-1} of the herbicide

P. aeruginosa strain	PND1	PND2	PND3	PND4	PND5	PND6	PND7	PND8	PND10	PND12	PND13
Residual PND (ppm)	48.63	36.84	3.09	7.24	25.03	78.80	37.91	27.84	26.62	14.81	12.50
PND degradation (%)	51.37	63.16	96.91	92.76	74.97	21.20	62.09	72.16	73.38	85.19	87.50

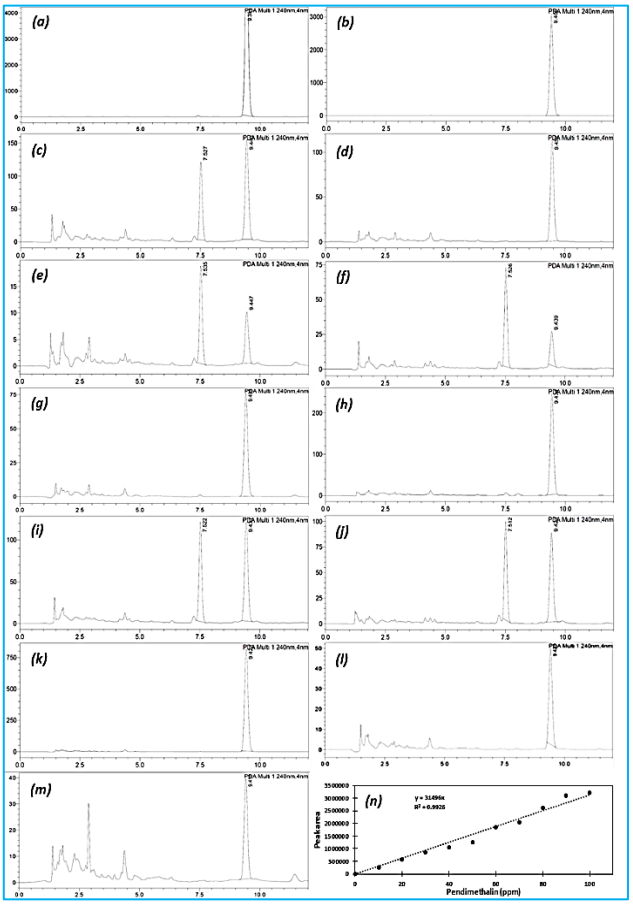

Fig 5 HPLC chromatogram of
(a) Reference pendimethalin (Sigma, USA)
(b) PND extract obtained from uninoculated MSM medium as control
(c-m) PND extract obtained from MSM medium inoculated with PND1, PND2, PND3, PND4, PND5, PND6, PND7, PND8, PND10, PND12 and PND13, respectively after 6 days of incubation; X-axis = time (min) and Y-axis = response (mAu) and
(n) calibration curve of reference pendimethalin

Notably, there has been only a single report of *P. aeruginosa* (identified by routine biochemical test) to utilize 30.75% of 400 ppm initial PND concentration in 7 days and detected *N*-(1-ethylpropyl)-3-methyl-2,6-diaminobenzine as metabolic end product by gas chromatography mass spectrometry (GC-MS) analysis. The strain is reported to mineralize PND after 30 days incubation with a 77 ppm residual PND [47]. The study also suggested that the end product could be formed by reduction of NO_2 groups into NH_2 followed by demethylation. The present study also reasserts a possible similar degradation pathway. In *P. aeruginosa*, the rate of degradation is suggested to be biphasic and faster PND degradation is observed in initial 7 days. In the present study, the strains PND3 and PND4 seems more efficient with high rate of PND degradation than the previous reports to reach 97% degradation. Many of the previous studies have reported that a concentration of 100 mg L^{-1} PND was much higher than its residue concentration in the soil environment and it has typically been used in biodegradation studies. *Paracoccus* sp. P13 was observed to degrade 100 mg L^{-1} PND within 2 days and 200 mg L^{-1} PND within 5 days. Similarly, *A. chroococcum* is demonstrated to degrade 45 and 55% of 25 µg ml^{-1} PND within 10 and 20 days, respectively [48]. Earlier, *Lecanicillium saksenae* from a loamy sand soil degraded 99.5% of 25 mg L^{-1} PND within 10 days [49]. Similarly, of the diverse *Bacillus* genus, *B. mycoides* and *B. cereus* could degrade 71.75% and 80.25% of an initial 400 mg L^{-1} PND, respectively within 30 days [47]. *Bacillus* sp. Y3 degraded 99.5% of 100 mg L^{-1} PND within 2.5 days [3]. However, the plant growth promoting traits of these isolates have not been studies. Similar to the members of Bacillus genus, *Pseudomonas* Spp. is well-known for its xenobiotic degradation as well as for its plant growth promotion activity [32-33]. A majority of *Pseudomonas* Spp. are herbicide tolerant attributed to its antioxidative enzymes and metabolic plasticity [38], [50]. These include *P. putida* [51-52], *P. pickettii* [53], *P. aeruginosa* [47], *P. fluorescens* [54] and *P. plecoglossicida* PD1 [55]. The degradation ability, environmental condition and time taken by these microorganisms varied differently. Compared to these isolates, the strain PND3 and PND4 can possibly be better candidates in eliminating PND residue pollution because of their plant growth promotion traits.

Nitroreduction is the key step in biodegradation of nitroaromatic compounds including pendimethalin in many of the reported genera of microorganisms [28]. The enzyme responsible for nitroreduction of PND is not described except for *Bacillus subtilis* Y3 for which PND nitroreductase was cloned [2]. Recently, Fourier Transform Infrared (FTIR) spectroscopy was undertaken to elucidate PND degradation by *Planococcus* and proposed dehydrogenases as first regulatory enzyme of PND-degradation pathway by molecular docking [56]. The enzyme was demonstrated to reduce the C-6 nitro group of the aromatic ring of PND to yield 2-nitro-6-amino-N-(1-ethylpropyl)-3,4-xylidine. Thus, in the present study, concentration of nitrite formation was monitored along with the residual PND that would indicate PND degradation. The results are illustrated in (Fig 6).

All isolates degraded PND with concomitant release of nitrite in the range of 0.5- 4.5 µg ml^{-1} for 7 days. Although high concentration (~120 ppm) of nitrite is toxic to plants, a moderate concentration promotes plant growth [57]. Besides, nitrite is an intermediary compound formed during nitrification and readily converted to nitrate and gaseous N compounds (NO, NO_2, N_2O and CH_3ONO) in a pH dependent manner by soil microorganisms to increase its organic nitrogen content [58]. It is known that PND contamination (4–12 mg L^{-1}) in soil negatively affects microbial growth and inhabiting microbes viz. N assimilation and uptake via nitrate reductase, nitrite

reductase, and glutamine synthetase [59]. Thus, the presence of PND degrading microbe with nitrite formation can be considered as beneficial trait of the isolated *P. aeruginosa* strains to promote plant growth.

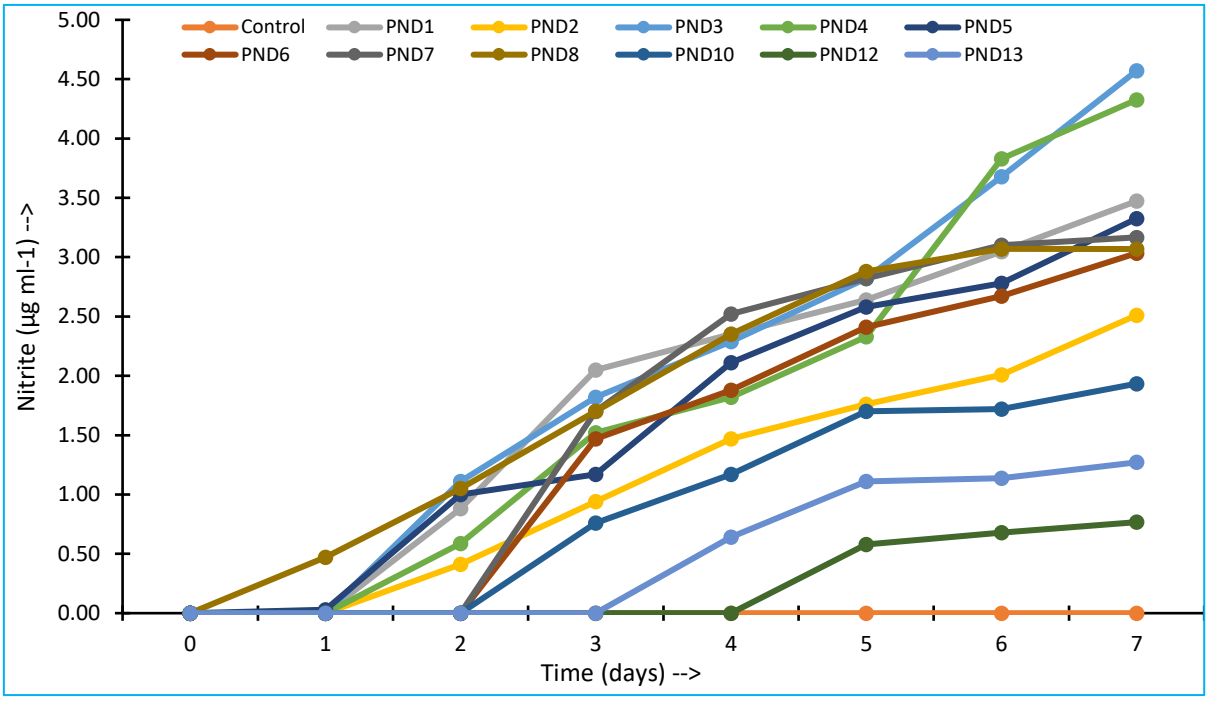

Fig 6 Production of nitrite in the MSM media inoculated with the isolated strains of *Pseudomonas aeruginosa* for a period of 7 days

CONCLUSION

The strains PND3 and PND4 isolated from contaminated soil using selective enrichment strategy highly efficient to degrade ~97% of PND and identified using polyphasic approach as *P. aeruginosa* (~99.5%). The TLC analysis suggested that the PND degradation product could be 3,4-dimethyl 2,6-dinitroaniline for C source, while previous reports of PND degradation by *Pseudomonas* Spp. has suggested *N*-(1-ethylpropyl)-3-methyl-2,6-diaminobenzine as end product where PND is used as both C, N and energy source to produce nitrite. The results suggest further metabolic investigation to confirm if the strains use nitrogenase-dependent PND degradation pathway.

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