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ORIGINAL PAPER

Efficient biotransformation of herbicide diuron by bacterial strain *Micrococcus* sp. PS-1

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Abstract A Gram-positive, *Micrococcus* sp. strain PS-1 capable of utilizing phenylurea herbicide diuron as a sole carbon source at a high concentration (up to 250 ppm) was isolated from diuron storage site by selective enrichment study. The taxonomic characterization with 16S rRNA gene sequencing (1,477 bp) identified PS-1 as a member of *Micrococcus* sp. It was studied for the degradation of diuron and a range of its analogues (monuron, linuron, monolinuron, chlortoluron and fenuron). The shake flasks experiments demonstrated fast degradation of diuron (up to 96% at 250 ppm within 30 h incubation) with the addition of small quantity (0.01%) of non-ionic detergent. The relative degradation profile by the isolate was in the order of fenuron > monuron > diuron > linuron > monolinuron > chlortoluron. Further, the biochemical characterization of catabolic pathway by spectroscopic and chromatographic techniques demonstrated that the degradation proceeded via formation of dealkylated metabolites to form 3,4-dichloroaniline (3,4-DCA). It was the major metabolite formed, associated with profound increase in degradation kinetics in presence of appropriate additive.

Keywords *Micrococcus* sp. · Degradation · Phenylurea herbicides · Diuron · 3,4-DCA

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Introduction

Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) is a substituted phenylurea herbicide used as a broad-spectrum pre-emergence weed control in a wide variety of crops (Tomlin 1997). It is easily taken up from the soil by the root system of plants and rapidly translocated into their stems and leaves via xylem by transpiration. It functions primarily by inhibiting the Hill reaction in photosynthesis by limiting the production of high-energy compounds such as adenosine triphosphate (ATP) used for various metabolic processes (Turnbull et al. 2001). Besides its use as herbicide it is also employed as an active ingredient in antifouling boat paints and in algaecide formulations used in fountains and aquaculture (Sorensen et al. 2008). Due to the extensive use of diuron and other phenylurea herbicides, their residues are detected in ground and surface water in concentrations exceeding their permissible limits (Lapworth and Gooddy 2006; Eriksson et al. 2007). These herbicides have been shown to be endocrine disruptors, showing ecotoxic effects, and are known to make adverse effects on human health (Tixier et al. 2001). These are relatively persistent in soil with a half-life of 90–180 days, which varies from several weeks to years in different types of soil environment affected by pH, temperature,

moisture content and soil matrix (Giacomazzi and Cochet 2004; Louchart and Voltz 2007).

Microbial degradation is considered to be the primary mechanism for the dissipation of these herbicides from the environment (Moncada 2004). It has been demonstrated that diuron is biodegraded aerobically by two successive N-demethylation reactions followed by the cleavage of amide bond leading to the formation of 3,4-dichloroaniline (3,4-DCA), a highly toxic metabolite affecting the cell growth (Tixier et al. 2002). The bacterial strains capable of mineralizing diuron and other phenylurea herbicides have been isolated from soils that have been treated with these herbicides for prolonged periods (Sorensen et al. 2005; Bazot et al. 2007). Badawi et al. reported the degradation of chlortoluron, diuron, isoproturon, and linuron by the soil fungus *Mortierella* sp. Gr4 demonstrating rapid degradation of linuron resulting in the formation of successive dealkylated metabolites and 3,4-DCA (Badawi et al. 2009).

However, enhanced biodegradation of phenylurea herbicides by bacterial pure cultures has not been previously presented. The present study was aimed to screen some novel microorganisms from diuron storage site, capable of rapid degradation of diuron and other phenylurea herbicides to non toxic metabolites. The biochemical characterization of catabolic pathway and the metabolites formed were studied by high performance liquid chromatography (HPLC), infrared (IR), matrix-assisted laser desorption/ionization (MALDI) and nuclear magnetic resonance (NMR) techniques. To the best of our knowledge, this is the first report showing the rapid degradation of diuron and its analogues by *Micrococcus lylae* species.

Materials and methods

Chemicals and media

Phenylurea herbicides and their putative intermediates (analytical standards) diuron, fenuron, monuron, linuron, monolinuron, chlortoluron, 1,2-dichlorobenzene (DCB), 4,5-dichlorobenzene-1,2-diol (4,5-DBD) and 1(3,4-dichlorophenyl) methyl urea (DCPMU) were purchased from Sigma Chemical Co. (USA). 3,4-dichloroaniline (3,4-DCA), methanol, ethyl acetate and chloroform-d_1 (deuterated $CDCl_3$) and Triton X-100 were purchased from Merck, India. For enrichment and degradation studies, media devoid of carbon (MSM) or/and nitrogen sources (MMK) were prepared (Supporting Information ST).

Enrichment and isolation of diuron degrading bacterial strain

Soil samples were collected from the storage area of diuron manufacturing unit (Ankhleshwar, India) and brought to the laboratory in a sealed polyethylene bags under aseptic conditions. Soil samples were further enriched by adopting selective enrichment approach (Singh et al. 2004) to screen out diuron degrading bacterial strains from the herbicide contaminated soil. For the enrichment of bacterial isolates, capable of degrading phenylurea herbicides, a glass column (21 cm × 1.5 cm) partially sealed from both ends was added with 10 g of soil. Diuron solution (10 ppm) prepared in 10% methanol in deionized water was added daily into the column and the solution was kept circulating slowly under a mild flow through condition at constant flow rate (0.5 ml/min). The enrichment was done for approximately 8 weeks by keeping the initial diuron concentration constant (10 ppm) for 2 weeks and gradually increased up to 250 ppm at the incremental step 50 ppm per week. At each step control was also taken, having no diuron as a carbon source. Extensive screening was done before and after each enrichment cycle as to allow only diuron degrading microorganisms to be enriched. For screening, soil samples were taken out aseptically and serial dilutions up to 10^{-6} were made in normal saline (0.85%) and plated on MSM agar plates having diuron (10–250 ppm). The plates were incubated at 37°C and the growth was monitored for 5 days. The isolated strains were tested to check their ability to degrade diuron in liquid 25 ml minimal medium (MSM) using 250 ml flask at 37°C on a shaker at 200 rpm. The strain PS-1 was selected for degradation experiments as it was showing the fastest growth in liquid MSM medium (supplementary information S1). It was harvested in the late exponential growth phase and washed twice in a sterile phosphate buffer before the initiation of the experiments. Growth was monitored by measuring OD_{600} using Shimadzu UV Spectrophotometer (1800) at different concentrations (10–250 ppm) of diuron and its major analogues (fenuron, monuron, diuron, linuron, monolinuron and chlortoluron) in liquid MSM medium supplemented with 0.5% glucose and a small

quantity (0.01%) of non-ionic detergent (Triton X-100).

The initial taxonomic characterization of the strain PS-1 strain was done by different biochemical tests using the Bergeys Manual of Determinative Bacteriology (Holt et al. 1994) (Supporting Information ST). The strain was grown on MSM agar plates at 37°C (pH 7.5) for 24 h and gram reaction was determined by using the HiMedia Gram Staining kit according to the manufacturer's instructions. Cells morphology was analyzed by scanning electron microscopy [SEM-JSM 6100 (JEOL)]. For SEM sample preparation, 1 ml of media was taken out after 24 h and was centrifuged at 4,000 rpm for 10 min to form a loose pellet followed by three consecutive washings with deionised water. The pellet was resuspended in 500 µl water and a drop of the sample was placed on SEM stub. The sample was sputtered with gold for the analysis. Further characterization of isolate was done by 16S rRNA gene analysis. The 16S rRNA sequencing was carried out by M/s Bangalore Genei, India according to standard procedure and sequence was deposited in the Genebank nucleotide sequence databases (Accession no. GQ 284587). The sequences of closely related validly published taxa were retrieved from the GenBank database and aligned and the distances between the sequences were calculated.

Transformation studies of diuron by the strain PS-1

The degradation of phenylurea herbicides by bacterial isolate PS-1 was studied by growing the cells in 100 ml liquid MSM medium. The cells were harvested in mid-exponential phase (OD_{600} ~0.6), washed and resuspended in 5 ml of the same media and inoculated in 45 ml MSM in 250 ml flasks. 250 ppm solution of each herbicide (diuron, fenuron, monuron, linuron, monolinuron and chlortoluron) was prepared in methanol and added into flasks containing media. The flasks were incubated at 37°C for 48 h at 200 rpm. Growth profile of the strain was further checked with the addition of 0.5% glucose supplemented with small quantity (0.01%) of non-ionic detergent along with herbicides. The identification of different compounds present in the culture supernatant was established by comparing HPLC retention times and mass spectra with those of respective authentic standards. For the characterization of different metabolites in the degradation pathway, samples were extracted at regular intervals by taking 5 ml media from the broth, and centrifuged at 6000 rpm for 10 min. The supernatant was extracted using agitation with 5 ml of ethyl acetate and the upper organic layer was separated. This was repeated three times followed by a final washing with ethyl acetate. The organic layer was collected and pooled in a round bottom flask and the solvent was evaporated in a rotavapour (Buchi, Switzerland). The residue left was reconstituted in 3 ml methanol and filtered through 0.2 μ syringe filter (Microdevice, Ambala, India) and was stored at −20°C before further characterization. Quantitative analysis of diuron and its metabolites was performed by Shimadzu High Pressure Liquid chromatography (HPLC) system equipped with a UV–Vis detector at 252 nm using Phenomenex C18 column (250 × 4.6 mm) (Luna 5 μ 100R). Samples were extracted at different time intervals and injected into HPLC with a constant injection volume of 20 µl. Methanol: Water (60:40) was used as a mobile phase at a flow rate of 0.5 ml/min. Quantification of diuron degradation was performed with the help of the standard curves generated with the known concentrations of standards of diuron and formed intermediates. For mass determination, 10 µl of sample dissolved in methanol was introduced into MALDI-TOF mass spectrometer (Applied Biosystems, USA). For NMR analysis, samples were dried completely and dissolved in 600 µl of deuterated $CDCl_3$. ^{1}H-spectra were recorded for all samples on JEOL (ACX 300 MHz) NMR spectrometer. Chemical shifts in the spectra were referenced to solvent $CDCl_3$. FTIR spectra were recorded in the spectral region of 4,400–400 cm^{-1} using Perkin-Elmer (RX1) equipped with AgCl windows. A drop of sample was placed between KBr plates and an emaciated film of the sample was made for the analysis.

Results and discussion

Isolation of diuron degrading bacterial strain and its characterization

Nineteen bacterial strains were isolated after 8 weeks enrichment treatment, from the soil obtained from pesticide (diuron) storage site in Gujarat state, India. It was attributed to the cause that the soil with prolonged treatment of pesticides for many years at

storage site with further enrichment in laboratory might eventually enhanced the microbial adaptation to diuron. During enrichment treatment, the concentration of diuron was increased gradually from 10 to 250 ppm. Seven isolates were screened out on the basis of their ability to grow on MSM agar plates having diuron (Supporting Information, T1). The selected seven isolates were tested to check their ability to degrade diuron by growing them in liquid MSM medium. The strain PS-1 showed the rapid degradation of diuron at higher concentration (up to 250 ppm) and was selected for further degradation studies (Supporting Information; Fig. S1).

The strain was a gram-positive cocci occurring in tetrads. It formed yellow colored, opaque, and entire margin colonies when grown on nutrient agar. This isolate was found to be capable of showing best growth at 37°C and at pH inclined towards the alkaline side (pH 7–8). Figure 1 shows the growth profile of the strain PS-1, as monitored in different concentrations of diuron as a sole carbon source (a), diuron with 0.5% glucose and a small quantity (0.01%) of non-ionic detergent (Triton X) (b) and diuron with 0.5% glucose (without non-ionic detergent) (c). It was established that the use of detergent (surfactant) has the potential to increase the rate of remediation of non-polar pollutants by the strain PS-1. This is explained because at aqueous concentrations above critical micelle concentrations (CMC) surfactants can increase the apparent water solubility of organic pollutants (Smith et al. 1997). Also, these agents help in increasing the bacterial cell wall permeability due to their reaction with membrane lipids and proteins by the formation of channels (holes) through the membrane (Burgoyne et al. 1995; Guha et al. 1998; Brown et al. 1999; Wong et al. 2004). By adding a small concentration (0.01%) of Triton X-100, we observed significant enhancement of pesticide degradation by bacteria (up to 96% at 250 ppm within 30 h incubation). Further increase in the concentration of diuron (>250 ppm), media became more hydrophobic due to low solubility of diuron in water (42 mg/l) and hence, aggregation of pesticide was observed. The cell growth under such condition was inhibited as diuron at higher concentration (beyond 250 ppm) tends to form aggregates in media and thus affecting the cell growth.

16S rRNA gene sequence analysis of the strain PS-1 (1,477 bp) was done for its identification. NCBI Genebank and RDP database search showed that the strain PS-1 has maximum sequence similarity with *Micrococcus lylae* (99%). The phylogenetic analysis of strain PS-1 with its closely related strains was performed with distance estimation approach employing neighbor joining method by using Mega 4 software and the optimal tree with the sum of branch length = 0.28575764 is shown in Fig. 2. The statistical significance and confidence limit of the phylogenetic analysis were determined by calculating the bootstrap values over 1,000 replications. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option) with a total of 1,323 positions in the final dataset.

To the best of our knowledge, this is the first report showing the rapid degradation of diuron by the strain *Micrococcus lylae*. Degradation of diuron is usually slow and follows first order kinetics until sometime between 29 and 43 days incubation, when a dramatic increase in rate occurred (Cullington and Walker 1999). However, the strain *Micrococcus lylae*, isolated from the diuron storage site could show rapid degradation of diuron within 36 h of incubation.

Identification and characterization of the metabolites produced in diuron transformation

Strain PS-1 was examined for its ability to degrade phenylurea herbicides by harvesting the strain in its late exponential growth phase followed by washing in a sterile phosphate buffer (pH 7.2). The initial cell density of strain PS-1 in the degradation experiments was approximately 10^6 cells/ml, which was reconstituted in double distilled water (50 ml) containing 250-ppm diuron. It was found that cells of strain PS-1 accumulated 3,4-DCA in its initial phase in the medium when grown on diuron, suggesting the involvement of an initial hydrolysis step of the amide bond in the degradation of diuron after de-alkylation as revealed by FTIR data. In FTIR spectra, the characteristic band at 2,930 cm^{-1} corresponding to $-CH_3$ gradually disappeared with increase in incubation time. This confirmed that the degradation started with demethylation of diuron which subsequently transformed to 3,4-DCA. The formation of diuron intermediates were identified by comparing the HPLC retention times with standards of diuron and degraded

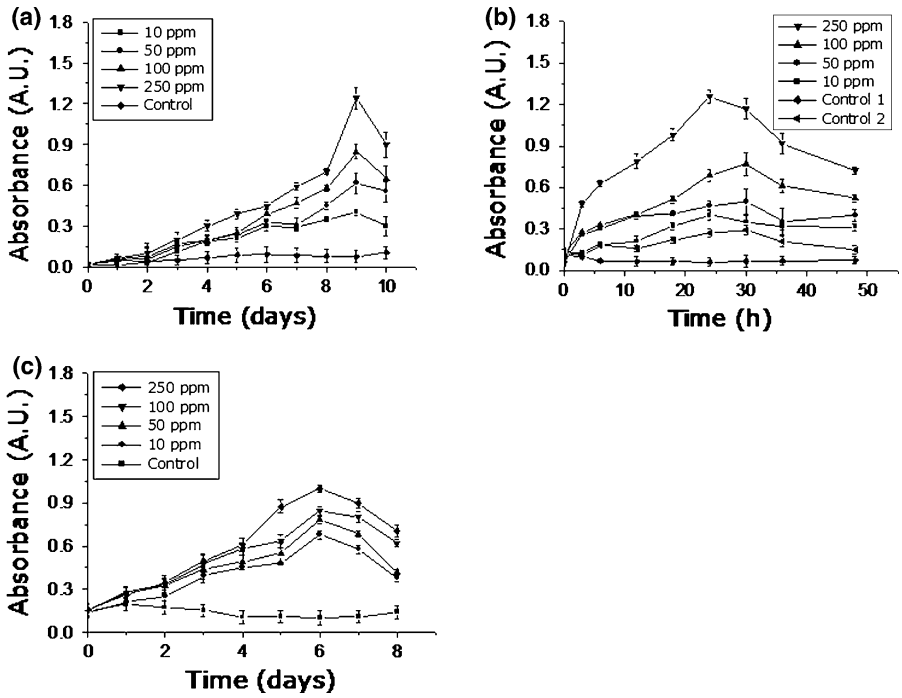


Fig. 1 Growth characteristics of *Micrococcus* sp. strain PS-1. **a** Diuron as a sole carbon source in MSM (control : uninoculated). **b** MSM with diuron and 0.5% Glucose 0.01% Triton (control 1—in presence of glucose only and control 2—uninoculated media). **c** MSM with diuron and 0.5% glucose (without Triton X) (control: uninoculated). The data is mean values ($n = 3$). The *bars* indicate the standard deviation

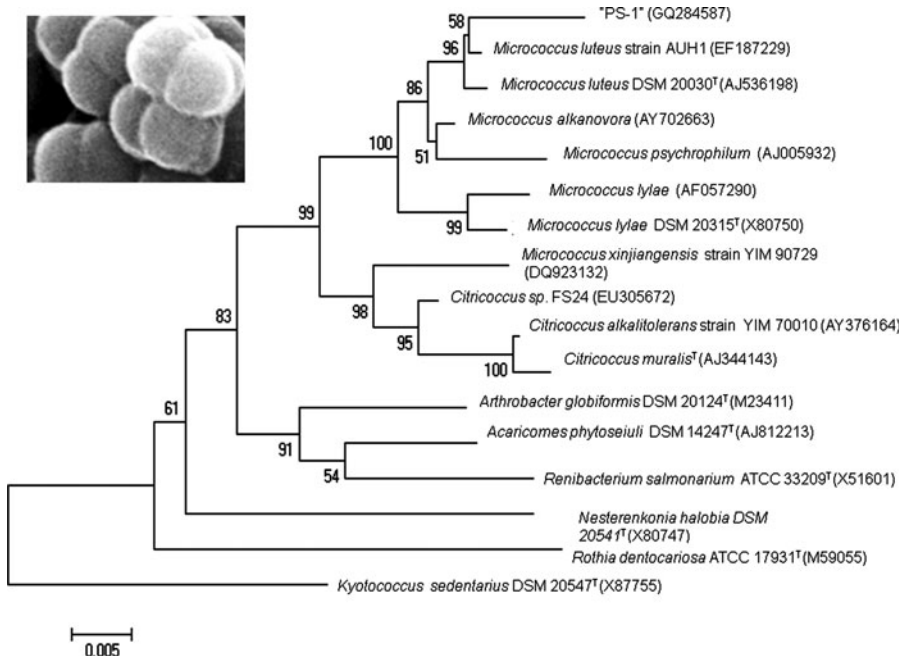


Fig. 2 Phylogenetic neighbour-joining tree made in MEGA 4 software showing the relationship of strain PS-1 with its closely related strains. *Kyotococcus sedentarius* DSM 20547^T(X87755) was used as an out-group. Number of nodes indicate level of bootstrap support >50%, based on the analysis of 1,000 replicates. GenBank accession numbers are given in parentheses. *Inset* of the figure shows the SEM micrograph of strain PS-1 (scale bar: 15 mm = 1.5 μm)

intermediates. Figure 3 shows the transformation of diuron into 3,4-DCA and formation of other intermediates in the degradation pathway (a–d). Quantification of diuron degradation and the formation of its metabolites were performed with the help of standard curve generated with the known concentrations of standards. After 6 h of incubation, the major intermediate was 3,4-DCA, which started converting after 24 h into 4,5-DBD, as depicted in Fig. 4. Earlier reports also suggested that diuron was degraded into 3,4-DCA, as a major metabolite, by diverse bacterial isolates (Travkin et al. 2003). The presence of other metabolites

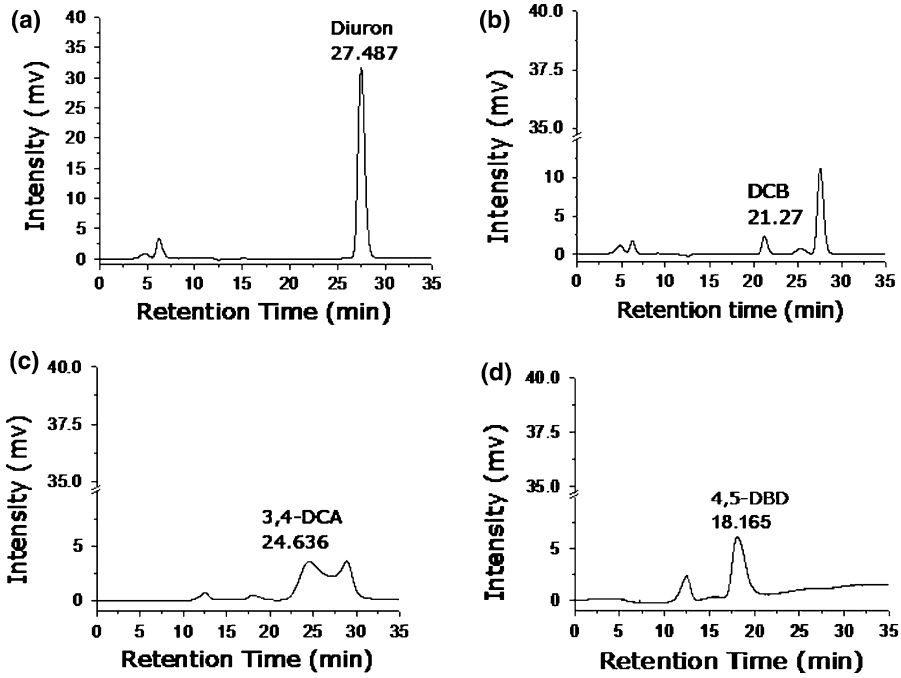


Fig. 3 Identification of diuron and its putative degradation intermediates at different time intervals **a** 0 h, **b** 12 h, **c** 24 h and **d** 36 h. The formation of diuron intermediates were identified by comparing the HPLC retention times with authentic standards

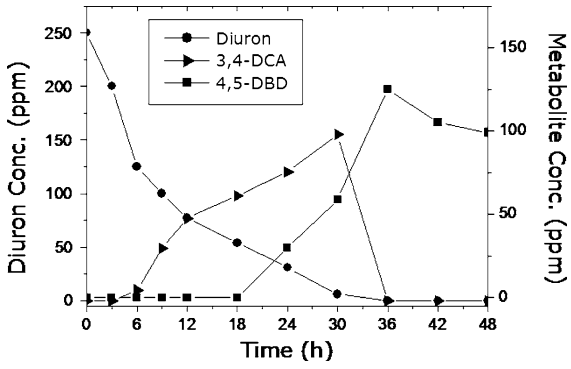


Fig. 4 Quantification of diuron degradation and the formation of its intermediates. The concentrations of diuron, 3,4-DCA and 4,5-DBD were determined with the help of standard curve generated with known concentrations of their standards after HPLC analysis

were identified by mass spectroscopy. In the mass studies, the new peaks formed during degradation of diuron at 216, 163, 146, 178 and 190 correspond to 1-(3,4-dichlorophenyl)-3-methyl urea (DCPMU), 3,4-DCA, DCB, 4,5-DBD and 3-chloro-4-oxohexanedioicacid (3-COHDA), respectively (Fig. 5b–f). Similarly, in the NMR spectra, no methyl group peaks were observed after incubation for 12 h suggesting dealkylation of diuron. By comparing the ^{1}H NMR spectra ($CDCl_3$ $-d_1$: $\delta = 7.25$) of diuron and its metabolites at 0 h: δ [7.8 (1H; CH)], δ [7.6 (1H; CH)], δ [7.23 (1H; CH)], δ [6.8 (IH; NH)], δ [2.97 (6H; $2CH_3$)] with 24 h: δ [6.76 (1H; CH)], δ [6.52 (1H; CH)], δ [6.43 (1H; CH)], δ [4.0 (2H; NH)], it was observed that the methyl peak at 2.97 ppm was completely disappeared which subsequently transformed to 3,4-DCA within 24 h. Phenyl ring breakage due to the formation of 4,5-DBD was confirmed by the disappearance of peaks in the aromatic region given by the ring protons (7, 8, 7.6, 7.23 ppm) in 36 h. With the available spectroscopic and chromatographic results, proposed degradation pathway by the strain PS-1 is presented (Fig. 6).

The degradation capability of strain PS-1 for diuron and other phenylurea herbicides (Fig. 7a) suggested that the relative degradation profile by the isolate was in the order of fenuron > monuron > diuron > monolinuron > linuron > chlortoluron as depicted in Fig. 7b. The light and dark blocks in the figure represent % of degraded and undegraded pesticide, respectively. The strain PS-1 was able to degrade methyl phenylurea herbicides (fenuron, monouron and diuron) with almost equal efficiency. However, the degradation was slow for methoxy phenylurea herbicides (linuron and monolinuron), and very low reactivity was observed against chlortoluron. In contrast, some earlier reports into bacterial degradation of

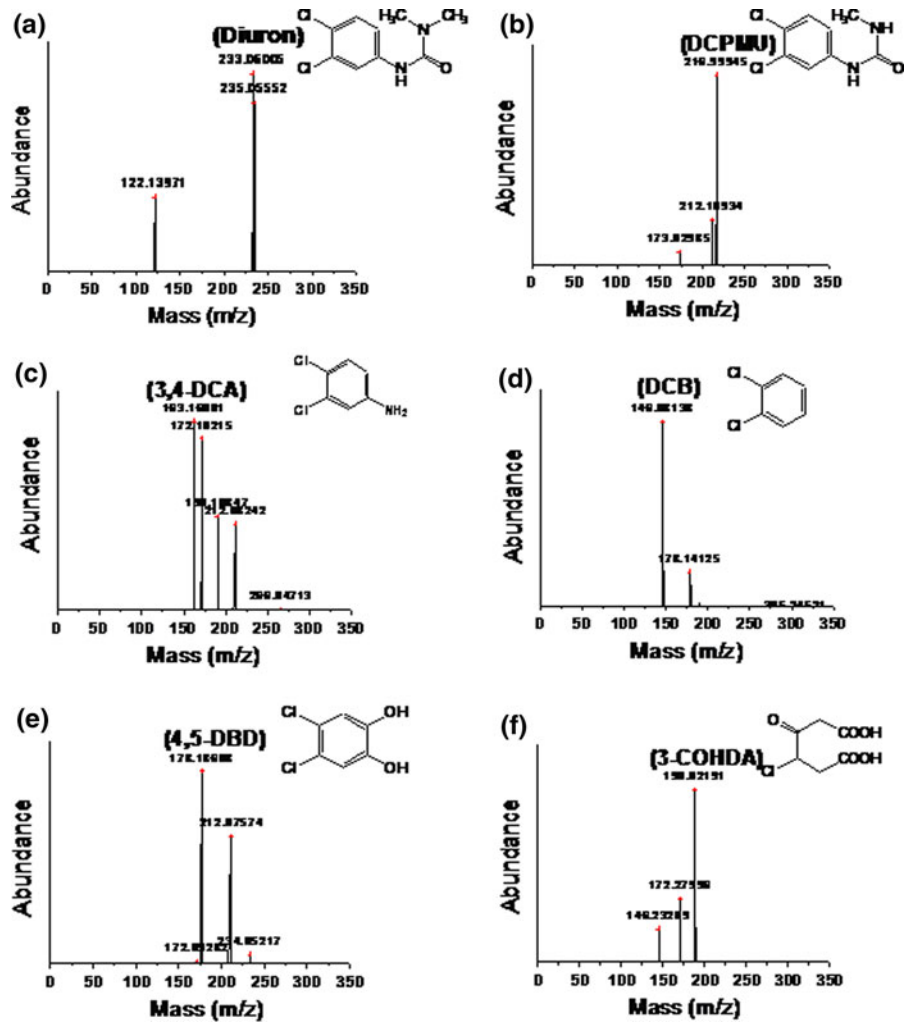


Fig. 5 Mass spectra of diuron and its intermediates formed during its degradation

phenylurea herbicides demonstrated the activity of bacterial isolates against methoxy substituted compounds only (Roberts et al. 1993) with exception, where a bacterial strain was capable of degrading both dimethyl substituted compounds (diuron and isoproturon) and also the methoxy substituted compound linuron (Roberts et al. 1998). However, in studies with fungi degrading phenylurea herbicides, it has been shown that soil fungus is capable of degrading both dimethyl- and methoxy-substituted urea herbicides (Shelton et al. 1996). In our study, degradation of these herbicides and their metabolites were attributed to several structural features of the molecule. Fenuron, monuron and diuron with dimethyl groups on the phenyl ring were degraded more rapidly than monolinuron and linuron having methoxy groups in place of methyl groups. Removal of 3-chlorine substituent from diuron also reduced the rate of degradation, as observed in the case of chlorotoluron. This suggests that the size and the nature of the urea N′-substituents and also ring chlorination, particularly at the 4-position of phenyl ring, is a determining factor in the occurrence of degradation of phenylurea herbicides.

The key enzymatic step in the mineralization of diuron was the hydrolysis of the amide bond, leading to the production of 3,4-DCA. Strain PS-1 performs this hydrolysis step very efficiently in the presence of 0.5% glucose and 0.01% Triton X. The reason for the increase in degradation kinetics in the presence of Triton X can be attributed that various non-ionic detergents such as surfactants effectively enhanced

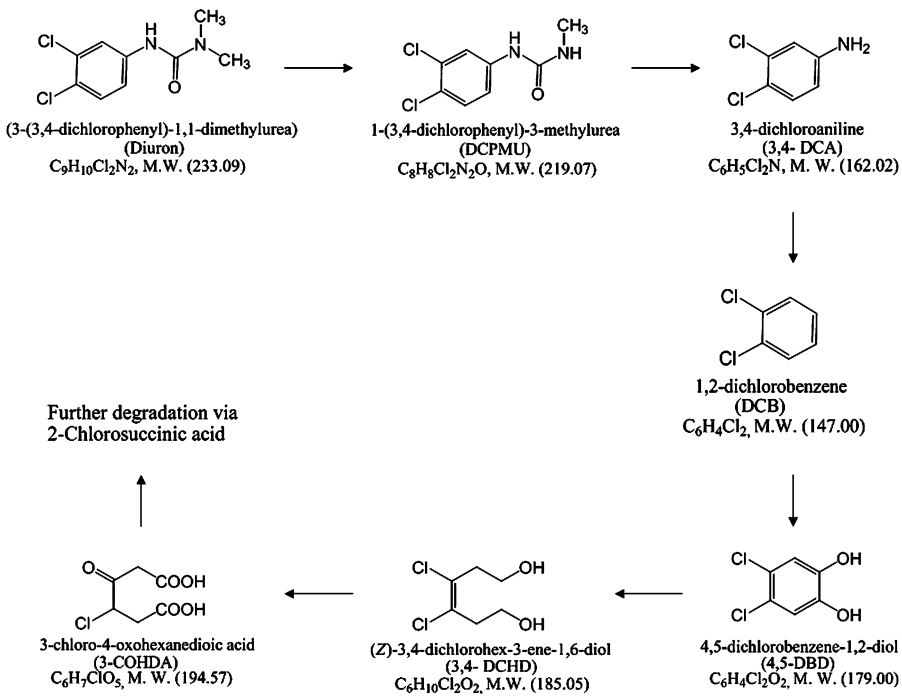
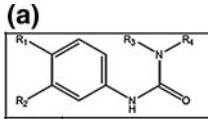


Fig. 6 The proposed degradation pathway of diuron by the strain PS-1. Metabolites were identified by different spectroscopy and chromatography techniques

(a)



		R_1	R_2	R_3	R_4
1	Fenuron	-	-	CH_3	CH_3
2	Monuron	Cl	-	CH_3	CH_3
3	Diuron	Cl	Cl	CH_3	CH_3
4	Monolinuron	Cl	-	CH_3	OCH_3
5	Linuron	Cl	Cl	CH_3	OCH_3
6	Chlortoluron	CH_3	Cl	CH_3	CH_3

(b)

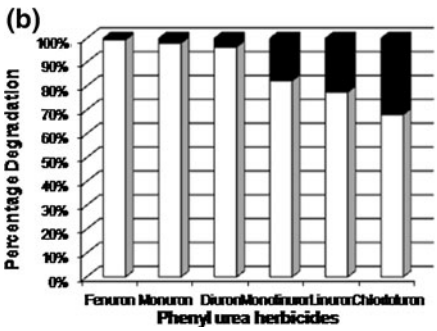


Fig. 7 **a** General structure of diuron and its analogues. **b** Percentage degradation of diuron and its analogues by strain PS-1 after 24 h. Light and dark blocks represent % of degraded and undegraded pesticide, respectively. The data is mean values of three successive experiments ($n = 3$)

the solubility of organic compounds, which, probably increase the direct contact between bacterial cells and compounds. This significantly improves the substrate utilization rates of microorganisms (Pang et al. 2005).

In conclusion, the strain PS-1 isolated from diuron storage site appeared to be highly efficient in degrading diuron concentrations. The isolate was capable of degrading other phenyl urea herbicides, with the rate of degradation in the order of fenuron > monuron > diuron > linuron > monolinuron > chlortoluron. The degradation of these herbicides by the bacterial strain *Micrococcus* sp. (PS-1) suggests that this culture could be ideal for remediation of soil and water affected by these herbicides, as well as their shared metabolite 3,4-DCA. This is the first described bacterium capable of degrading diuron rapidly at higher concentration (up to 250 ppm). Further research is needed into the degradation of diuron by PS-1 before it can be used to remediate contaminated soils.

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