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Biodegradation of pendimethalin by *Bacillus circulans*

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A bacterium capable of degrading herbicide, pendimethalin, was isolated from the contaminated soil by enrichment culture technique and identified as *Bacillus circulans*. The organism grew on pendimethalin (1 g/L) as the sole source of carbon and accumulated 6-aminopendimethalin and 3,4-dimethyl 2,6-dinitroaniline as metabolites in the culture medium. The cell-free extract of *B. circulans* grown on pendimethalin contained the activities of pendimethalin nitroreductase and pendimethalin mixed function oxidase. The results suggest that the bacterium degraded pendimethalin by nitroreduction to 6-aminopendimethalin and by oxidative dealkylation to 3,4-dimethyl 2,6-dinitroaniline and pentane, which was utilized as the source of carbon and energy for growth.

Keywords: Biodegradation, pendimethalin, *Bacillus circulans*

Introduction

Pendimethalin [N-(1-ethyl propyl) 2,6-dinitro-3,4-xylidine, a selective preemergent herbicide of dinitroaniline group, is used extensively for weed control in cotton, rice, soyabean and tobacco[1-3]. It is also used to control broad leaved weeds and grassy weed species in a number of crop and non-crop areas and on residential lawns. The US Environmental Protection Agency (EPA) has classified pendimethalin as a persistent bioaccumulative toxic (PBT); it is of low acute toxicity, but causes thyroid follicular cell adenomas in male and female rats and has been classified as Group C, a possible human carcinogen. Pendimethalin is also highly toxic to fish and aquatic invertebrates. It is moderately persistent in aerobic soil environments. However, its use may adversely affect endangered species of terrestrial and semi aquatic plants and invertebrates including mollusks, fishes and birds[4]. It is, therefore, essential to investigate the metabolic fate of such a toxic chemical in the environment. Soil microorganisms that are repeatedly exposed to pesticides may develop new capabilities to degrade such chemicals. There are very few reports on the degradation of pendimethalin by soil microorganisms[5-7]. In this paper, we describe the isolation and characterization of *B. circulans,* which degrades pendimethalin.

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Materials and Methods

Chemicals

Pendimethalin and trifluralin were generous gift from Rallis Agrochemicals India Ltd., Mumbai, India. N-(1-Ethylpropyl)-3,4-dimethyl-2-nitrobenzene-1,6-diamine and 3,4-dimethyl-2,6-dinitroaniline were synthesized following the procedure of Singh and Kulshrestha[7]. All other chemicals used were of analytical grade.

Organism and Growth Conditions

The organism was isolated from herbicide-contaminated soil by selective enrichment on pendimethalin as the sole source of carbon. It was grown on mineral salt medium[8] containing pendimethalin (0.1% w/v) in Erlenmeyer flasks on a rotary shaker (150 rpm) at room temperature. Growth was measured turbidometrically at 660 nm. The identification of the pendimethalin-degrading organism was done on the basis of its morphological, cultural and biochemical characteristics. The biochemical tests were done as per the procedure of Holding and Collee[9]. DNA isolation and determination of its G+C content was done as described by Marmur[10] and Mandel and Marmur[11].

Growth of the isolated organism on various organic compounds such as 4-nitroaniline, trifluralin, anthracine, o-nitrophenol, o-phthalic acid, 1-naphthol, pentane, protocatechuic acid, 6-aminopendimethalin and 3,4-dimethyl-2,6-dinitroaniline was determined in the mineral salts medium supplemented with these compounds (0.1% w/v) as the sole source of carbon.

Utilization of pendimethalin during the growth of *B. circulans* was measured at different incubation periods by extraction of residual pendimethalin with ethylacetate, followed by HPLC analysis (retention time for pendimethalin 2.15 min).

Oxygen Uptake Studies

The oxygen uptake by *B. circulans* grown on pendimethalin was measured in an Oxygraph fitted with Clark oxygen electrode (Hansatech, Germany). The cells were harvested in the mid-logarithmic phase by centrifugation at 10,000×g for 20 min and washed twice with 50 m*M* phosphate buffer (*p*H 7.0). Oxygen uptake rates are expressed as nmol of O_2 consumed/min/mg of dry cells. The values were corrected for endogenous respiration.

Isolation and Identification of Metabolites

The metabolites were extracted from the culture filtrate of *B. circulans* grown on pendimethalin (0.1% w/v) using ethyl acetate and the residue obtained was dissolved in methanol. It was analyzed for metabolites by TLC on silica gel G plates using the following solvent systems.(A) Hexane-ethyl acetate (1:1 v/v), (B) Toluene-methanol (9:1 v/v) and (C) Toluene-dioxan-acetic acid (90:20:4 v/v). The metabolites were visualized under UV light at 254 nm or by exposure to iodine vapours and also by spraying with 1% $FeCl_3$-$K_3Fe(CN)_6$ solution in water. The pendimethalin and its metabolites being deeply coloured were visible on the chromatograms. Metabolites were analyzed by reversed-phase HPLC with a 5 µ spherisorb-ODS (C_{18}) column (25 cm × 4.6 mm) using acetonitrile-phosphate buffer (50 m*M*, *p*H 7.0) in the ratio of 70:30 (v/v) as the mobile phase at a flow rate of 1 mL/min. Peaks were detected at 254 nm. UV-visible absorbance spectra were recorded with 'Hitachi' 150-120 spectrophotometer. The mass spectra were recorded with Jeol, MS-DS 303 operated at 70 eV.

Enzyme Assays

The cell-free extracts were prepared from the washed cells suspended in three volumes of 50 m*M* phosphate buffer (*p*H 7.0) by sonication (Ultrasonic processor XL 2010) for 5 min and centrifugation at 10,000× g for 40 min at 4ºC. The clear supernatant was used for enzyme assays. Pendimethalin nitroreductase activity was assayed spectrophotometrically by measuring the decrease in absorbance at 340 nm due to the oxidation of NADPH[12]. Pendimethalin N-dealkylase activity was assayed in the cell-free extracts prepared according to Salokhe and Govindwar[13] by colorimetric measurement of 3,4-dimethyl-2,6-dinitroaniline formation as follows: The reaction mixture containing 10 m*M* phosphate buffer (*p*H 7.5), NADPH generating system (2.65 m*M* $NADP^+$, 15.2 m*M* glucose-6-phosphate and glucose-6-phosphate dehydrogenase), cell-free extract and 1 µ*M* pendimethalin was incubated at 28ºC for 5 min. The reaction was stopped by the addition of 0.2 mL of 50% TCA and centrifuged. To the supernatant, 0.1 mL of 1% $NaNO_2$ and 0.1 mL of 0.1N HCl were added. After 1 min, 0.3 mL of 0.1% β-naphthol in 0.1N NaOH was added. The red colour formed was measured at 490 nm. The protein was determined by the method of Lowry *et al*[14]. One unit of enzyme activity was defined as the amount required to catalyze the formation or consumption of 1 µmol of product or substrate per min. Specific activities were expressed as units per mg protein.

Results and Discussion

Characterization of Organism

The morphological, cultural and biochemical characteristics of pendimethalin-degrading organism are given in Table 1. It was an aerobic, Gram-positive, motile, spore forming rod shaped bacterium. The G+C content of DNA from the bacterial strain was found to be 36.2 moles%. The strain was identified as *Bacillus circulans* based on standard literature[15].

Growth of Organism on Various Aromatic Compounds

B. circulans utilized herbicides, pendimethalin and trifluralin, 4-nitroaniline, protocatechuic acid, anthracene, o-nitrophenol, o-phthalic acid, 1-naphthol and pentane as growth substrates (Table 2). However,

Table 1—Taxonomic characteristics of the organism

Characteristics	Observation
Morphological	Rods, occur in short chains, Gram-positive, motile, endospores present
Cultural	Circular, semi-transparent whitish colonies on nutrient agar plates, turbid with sediment in nutrient broth
Physiological	Oxidase and catalase positive. Hydrolysed casein, starch and gelatin. Indole and H_2S not produced. Acid but no gas from glucose and maltose. Nitrate reduced to nitrite. No growth in 7.5% NaCl and at 41ºC. Utilized acetate, succinate, lactate and citrate

6-aminopendimethaline and 3,4-dimethyl-2,6-dinitroaniline did not support the growth of the organism. *B. circulans* grew on pendimethalin (0.1% w/v) as the sole source of carbon as shown in Fig. 1.

Table 2— Growth of *B. circulans* on various organic compounds

Compound	Growth
Pendimethalin	+
Trifluralin	+
4-Nitroaniline	+
Anthracene	+
o-Nitrophenol	+
o-Phthalic acid	+
1-Naphthol	+
Pentane	+
Protocatechuic acid	+
6-Aminopendimethalin	-
2,4-Dimethyl-2,6-dinitroaniline	-

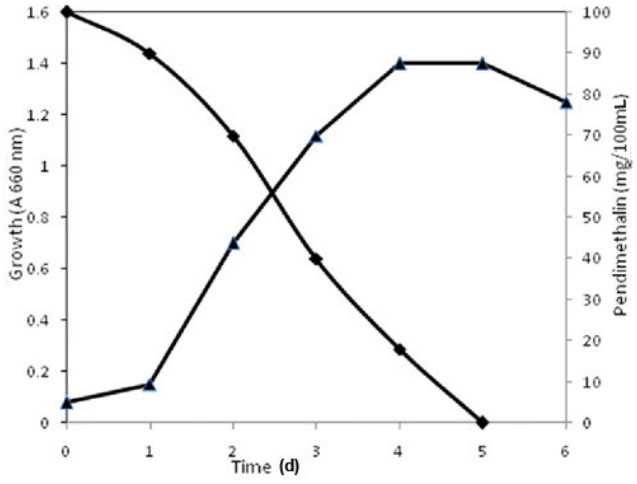

Fig. 1—Utilization of pendimethalin (■-■) during growth (▲-▲) of *B. circulans*.

Identification of Metabolites

The analysis of culture extracts of *B. circulans* grown on pendimethalin by TLC revealed the presence of compounds I and II, whose Rf values corresponded with those of authentic 6-aminopendimethalin [N-(1-ethylpropyl)-3,4-dimethyl-2-nitrobenzene-1,6-diamine)] and 3,4-dimethyl 2,6-dinitroaniline, respectively (Table 3). The purified compounds were analyzed by HPLC. Mass spectra of isolated compound I (Fig. 2) showed molecular peak M^+ at m/z 251 in agreement with the molecular formula $C_{13}H_{26}N_3O_2$. The fragmentation pattern showed the base peak at m/z 236 (M^+-CH_3), 220 (M^+-C_2H_6) and 191 (M^+-C_4H_{12}). Mass spectra of isolated compound II (Fig. 3) showed molecular peak M^+ at m/z 211 in agreement with the molecular formula $C_8H_{14}N_3O_4$. The fragmentation pattern showed the ion peaks M^+ at m/z 181 (M^+-NO), 121 (M^+-$2NO_2$) and the base ion peak at m/z 55. These spectral data corresponded well with that of authentic 6-aminopendimethalin and 3,4-dimethyl-2,6-dinitroaniline.

Oxidation of Metabolites by Whole Cells

The whole cells of *B. circulans* grown on pendimethalin readily oxidized pendimethalin and pentane but not 6-aminopendimethalin and 3, 4-dimethyl 2, 6-dinitroaniline. Glucose-grown cells failed to oxidize any of these compounds (Table 4). These results suggest that the metabolites, 6-aminopendimethalin and 3,4-dimethyl-2,6-dinitroaniline, were not further oxidized by the organism. But the pentane released by N-dealkylation of pendimethalin was oxidized and utilized as the source of carbon and energy.

Enzyme Activities in Cell-free Extracts

The cell-free extracts of *B. circulans* grown on pendimethalin contained the activities of pendimethalin

Table 3—Chromatographic and spectral properties of metabolites of pendimethalin by *B. circulans*

Property	Isolated compound I	Authentic 6-amino pendimethalin	Isolated compound II	Authentic 3,4-dimethyl 2,6-dinitroaniline
1. TLC: R_f values				
A	0.87	0.87	0.74	0.74
B	0.80	0.80	0.54	0.54
C	0.94	0.94	0.83	0.83
2. Melting point (ºC)	80ºC	80ºC	136ºC	136ºC
3. HPLC: Retention time (min)	7.63	7.63	1.40	1.40
4. UV- visible absorption λ_{max} in methanol (nm)	229, 433	229, 433	234, 424	234, 424

Solvent systems A, B & C are described in materials and methods

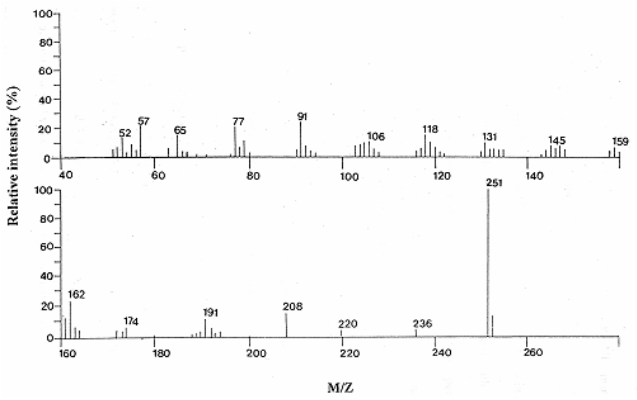

Fig. 2 — Mass spectrum of isolated compound I

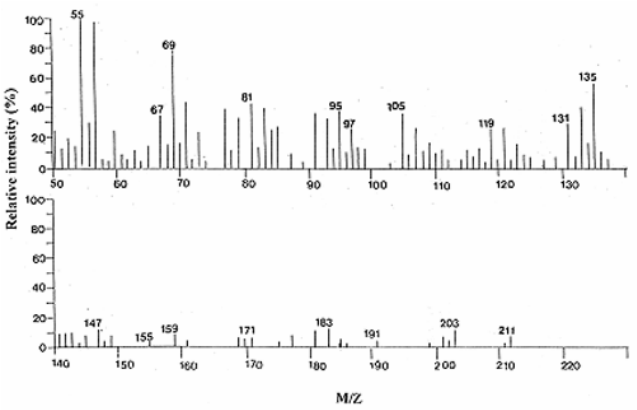

Fig. 3—Mass spectrum of isolated compound II

nitroreductase (0.07 Units/mg of protein) and pendimethalin N-dealkylase (0.05 Units/mg of protein). The cell-free extracts of glucose-grown cells did not contain any of these enzyme activities. The results have indicated that these enzymes of pendimethalin degradation were induced by the growth of the organism on pendimethalin.

The *Bacillus* species are known to be versatile in degrading a wide variety of aromatic compounds in the natural environment. It is evident from the results that the isolated *B. circulans* degraded the herbicide, pendimethalin, by nitroreduction to yield 6-aminopendimethalin. Pendimethalin was also degraded by oxidative N-dealkylation to yield 3,4-dimethyl-2,6-dinitroaniline and pentane. However, 6-aminopend-imethalin and 3,4-dimethyl-2,6-dinitroaniline were not further metabolized because they neither supported growth of organism nor stimulated oxygen uptake. But the pentane, released by oxidative N-dealkylation of pendimethalin, was utilized as the sole source of carbon and energy for the growth of the organism. The acetylation, aryl

Table 4—Oxygen uptake rates with various compounds by washed cell suspensions of *B. circulans* grown on pendimethalin

Substrate (1 µmol)	Oxygen uptake* (nmol/min/mg of dry cells) by cells grown on	
	Pendimethalin	Glucose
Pendimethalin	45	00
6-Aminopendimethalin	00	00
3,4-Dimethyl-2,6-dinitroaniline	00	00
Pentane	42	00

*The values are corrected for endogenous respiration rates.

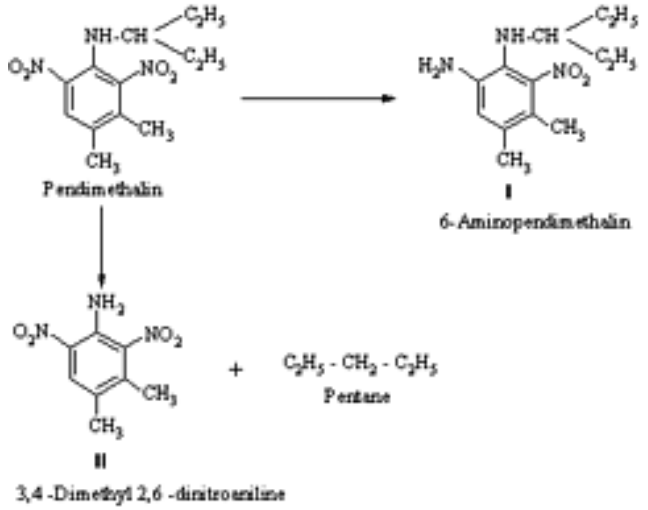

Fig. 4—Pathway proposed for the degradation of pendimethalin by *B. circulans*

methyl oxidation and cyclization products of pendimethalin, as reported in *Azotobacter chroococcum*[9] were not detected in culture filtrates of *B. circulans*[5].

The presence of activities of pendimethalin nitroreductase and pendimethalin N-dealkylase in the pendimethalin-grown cells but not in glucose-grown cells have confirmed that the organism degraded pendimethalin by nitroreduction and oxidative N-dealkylation as shown in Fig. 4.

The microbial degradation of dinitroaniline herbicides, pendimethalin and trifluralin, has been reported to occur most often by oxidative N-dealkylation and nitroreduction[5-7,16]. The present studies have shown that pendimethalin was degraded by *B. circulans* through oxidative N-dealkylation and nitroreduction pathways. The nitro group reduction and oxidative N-dealkylation destroys the herbicidal activity of pendimethalin, leading to its detoxification[5].

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