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Biodegradation of 2-Nitrotoluene by *Micrococcus* sp. strain SMN-1

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Abstract A bacterial consortium capable of degrading nitroaromatic compounds was isolated from pesticide-contaminated soil samples by selective enrichment on 2-nitrotoluene as a sole source of carbon and energy. The three different bacterial isolates obtained from bacterial consortium were identified as *Bacillus* sp. (A and C), *Bacillus flexus* (B) and *Micrococcus* sp. (D) on the basis of their morphological and biochemical characteristics and by phylogenetic analysis based on 16S rRNA gene sequences. The pathway for the degradation of 2-nitrotoluene by *Micrococcus* sp. strain SMN-1 was elucidated by the isolation and identification of metabolites, growth and enzymatic studies. The organism degraded 2-nitrotoluene through 3-methylcatechol by a *meta*-cleavage pathway, with release of nitrite.

Keywords Bacterial consortium · Nitroaromatics · *Micrococcus* sp. strain SMN-1 · 2-Nitrotoluene

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Introduction

Nitroaromatic compounds are the major group of environmental pollutants because of their extensive use and toxicity. Nitroaromatics such as nitrobenzene, nitrotoluenes, nitrophenols, nitrobenzoates and nitroanilines are extensively used in industry for the manufacture of pesticides, explosives, dyes, drugs and plastics (Nishino and Spain 2004; Zylstra et al. 2000). There are reports of widespread contamination of water, soil and atmosphere by nitroaromatic compounds (Golab et al. 1979; Leuenberger et al. 1988). Nitrotoluenes are used in the large scale production of explosives such as 2,4,6-trinitrotoluene (TNT), which has led to the widespread contamination of soil by explosives. These chemicals are highly toxic, mutagenic and carcinogenic to humans and animals (Bruning et al. 1999; Nipper et al. 2001; Rieger and Knackmuss 1995; Yen et al. 2002). Nitrotoluenes are classified as priority pollutants by the U.S. Environmental Protection Agency (EPA). It is therefore of considerable interest to investigate the metabolitic fate of nitroaromatic compounds in the environment. Microbial removal of the nitro group from nitroaromatics has been reported to occur either by reductive or oxidative mechanisms (Boopathy and Manning 1996; Hughes and Williams 2001; James et al. 2000; Nadeau and Spain 1995; Nishino and Spain 2006; Walia et al. 2002). The oxidative degradation of nitroaromatic compounds have been reported to involve formation of methylcatechols

with release of nitrite (Haigler et al. 1994; Nishino et al. 2000). Whereas the reductive degradation of nitroaromatics have been shown to involve the reduction of nitro group into amino group and subsequent release of ammonia (Nishino and Spain 1993; Schenzle et al. 1997; Yabannavar and Zylstra 1995). There are reports of degradation of 4-nitrotoluene by *Pseudomonas* species involving oxidation of methyl group followed by reduction of nitro group and subsequent removal of ammonia (Haigler and Spain 1993). However, much less information is available on the bacterial degradation of 2-nitrotoluene except that studied in *Pseudomonas* sp. strain JS42, which involves formation of 3-methylcatechol with release of nitrite (Haigler et al. 1994) and the biotransformation of 2-nitrotoluene to 2-aminotoluene by *Pseudomonas putida* OU83 (Walia et al. 2003). In this paper, we report the isolation and characterization of an indigenous bacterial consortium capable of degrading nitroaromatic compounds and the pathway for the degradation of 2-nitrotoluene by *Micrococcus* sp. strain SMN-1.

Materials and methods

Chemicals

2-Nitrotoluene, 3-nitrotoluene, 4-nitrotoluene, 2-nitrobenzoic acid, 3-nitrobenzoic acid, 4-nitrobenzoic acid, 2-nitro *m*-xylene, 1,3-dinitrobenzene, nitrobenzene, 4-nitrophenol, 4-nitroaniline, 3-nitroaniline, 2,4-dinitrotoluene, 2,6-dinitrotoluene, catechol, 3-methylcatechol and protocatechuic acid with 99% purity were obtained from Merck, Fluka, Sigma-Aldrich. All other chemicals were of analytical grade obtained from commercial sources.

Isolation of 2-nitrotoluene-degrading bacteria

A mixed bacterial culture capable of degrading 2-nitrotoluene was isolated from pesticide-contaminated soil samples after repeated enrichments in mineral salts medium containing 2-nitrotoluene (15 mM) as sole source of carbon and energy as reported by Tallur et al. (2008). The cultures obtained after enrichment were incubated on rotary shaker (150 rpm) at 30°C. Growth was determined turbidometrically by measuring absorbance at 600 nm. The bacterial cultures were purified by repeated streaking on 2-nitrotoluene-mineral salts agar plates. The bacterial colonies appeared on these plates were morphologically characterized and then identified biochemically (Holding and Collee 1971).

Identification of bacterial isolates by 16S rRNA gene analysis

The bacterial 16S rRNA gene was amplified from the total genomic DNA using universal eubacteria-specific primers 16F27 (5′ CCA GAT TTT GAT CMT GGC TCA G 3′) and 16R1525XP (5′ TTC TGC AGT CTA GAA GGA GGT GWT CCA GCC 3′), which yielded a product of approximately 1,500 base pairs. The polymerase chain reaction (PCR) conditions were 35 cycles of 95°C denaturation for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and an additional cycle of extension at 72°C for 10 min. The PCR product was purified by PEG-NaCl precipitation (Sambrook et al. 1989). Briefly, the PCR product was mixed with 0.6 volumes of PEG-NaCl solution (20% PEG 6000, 2.5 M NaCl) and incubated for 10 min at 37°C. The precipitate was collected by centrifugation at 12,000 rpm for 10 min. The pellet was washed twice with 70% ethanol and dried under vacuum, which was then resuspended in glass-distilled water at a concentration >0.1 pmol/ml. The purified product was directly sequenced using a Big Dye terminator kit (Applied Biosystems, Inc., Foster City, CA) as described earlier (Pidiyar et al. 2004). The primers used to obtain the complete sequence of 16S rRNA gene of the isolates were the same as for PCR amplification (16F27N and 16R1625XP). An internal primer (16F536, 5′ GTG CCA GCA GCC GCG GTR ATA 3′) was also used in addition to the other primers. The sequencing reactions were run on AB1-PR1SM automated sequencer (ABI-3730 DNA analyzer). The nucleotide sequence analysis was done at BLASTn site at NCBI server (www.ncbi.nlm.nih.gov/BLAST). The alignment of the sequences were done using ClustalW program VI.82 at the European Bioinformatics site (www.ebi.ac.uk/clustalw). The analysis of 16S rRNA gene sequence was done at Ribosomal Database Project (RDP) II (http://rdp.cme.msu.edu). The sequence was refined manually after cross-checking with the raw data to remove ambiguities. The phylogenetic tree was constructed using the aligned sequences by the neighbor-joining (NJ) method using

Nucleotide: Maximum Composite Likelihood distances in MEGA4.1 software (Tamura et al. 2007). The sequences of the 16S rRNA gene of the isolated strains of *Bacillus* sp. "A" and "C", *Bacillus flexus* strain "B" and *Micrococcus* sp. "D" are available under the Genbank accession numbers EF491963.1, EF157301.1 and AF218240.1.

Utilization of nitroaromatic compounds by bacterial isolates

The ability of the isolated bacterial consortium to utilize various nitroaromatic compounds as sole carbon source was determined by measurement of growth in mineral salts medium containing 0.2% (wt/vol) of the compounds. The rate of utilization of 2-nitrotoluene during growth of *Micrococcus* sp. strain SMN-1 was determined spectrophotometrically by measurement of decrease in UV absorbance of 2-nitrotoluene at 255 nm. The uninoculated controls were used to determine any transformation of 2-nitrotoluene affected by the physical factors.

Isolation and identification of metabolites

The metabolites were isolated from culture filtrate of the organism grown on 2-nitrotoluene by extraction with diethyl ether. The metabolites were analyzed by thin layer chromatography (TLC) on silica gel G plates using the following solvent systems: (A) chloroform: acetic acid (95:5, vol/vol), (B) toluene–ethylacetate–acetic acid (60:30:5, vol/vol), (C) benzene–methanol–acetic acid (45:8:4, vol/vol) and (D) benzene–dioxan–acetic acid (74:2:2, vol/vol). The metabolites were visualized under ultraviolet (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. Phenolic compounds gave their characteristic color on spraying with diazotized *p*-nitroaniline or with Gibbs reagent (2% solution of 2,6-dichloroquinone-4-chlorimide in methanol). *o*-Dihydroxy compounds were detected by spraying with Arnow's reagent (1937). Aldehydes were detected by spraying with solution of 2, 4-dinitrophenylhydrazine (0.1%) in 2 M HCl. The metabolites were also analyzed by HPLC (Shimadzu, Japan) equipped with SPD-10AVP UV-Detector using Silica gel-packed C_{18} column (4.6 × 250 mm) of particle size (5 µm) (Phenomenex) and solvent 50 mM H_3PO_4–methanol (60:40, vol/vol) as mobile phase at the flow rate of 1 ml min^{-1}.

GC-MS analysis was performed using a Shimadzu QP2010 Plus. GC-MS apparatus equipped with quadruple mass filter Rtx-5MS capillary column (30 m × 0.25 mm), scan interval 0.5 s and mass range 40–500 *m*/*z*. The temperature programme was held at 50°C for 1 min with 20°C increase min^{-1} to a final temperature 280°C for 14.5 min and the injector temperature was kept at 250°C. The injection volume was 1 µl and the carrier gas was helium. The mass spectrometer was operated at electron ionization energy of 70 eV. Nuclear magnetic resonance (NMR) spectra were recorded using 300 MHz spectrometer (Bruker AMX) of metabolites with tetramethylsilane (TMS) as the internal standard. UV–visible spectra were obtained by using spectrophotometer (Hitachi, 3100). The IR (Infra red) spectra were recorded with Nicolet Impact 410 FT-IR.

Nitrite released in the culture supernatant during degradation of 2-nitrotoluene was determined spectrophotometrically as described by Afkhami et al. (2004) using sodium nitrite as a standard. A pellet of sodium hydroxide and a small amount of chloroform were added to standard sodium nitrite to prevent the liberation of nitrous acid and to inhibit the bacterial growth. Ammonia was estimated by phenate method (APHA 1999).

Enzyme assays

Cell-free extracts were prepared from washed cells suspended in 50 mM phosphate buffer pH 7.0 by sonication (Ultrasonic processor, model XL 2010) for 5 min and centrifugation at 12,000 × g for 45 min at 4°C. The supernatant was used for enzyme assays. Catechol 1,2-dioxygenase activity was assayed spectrophotometrically by measuring the increase in absorbance at 260 nm due to the formation of *cis*,*cis*-muconic acid according to Hayaishi et al. (1957), Catechol 2,3-dioxygenase activity was assayed spectrophotometrically by measuring the increase in absorbance at 375 nm due to the formation 2-hydroxymuconic semialdehyde according to Kim et al. (1992). Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as the standard. One unit of enzyme activity was defined as the amount required to catalyze the

Table 1 Morphological and biochemical characteristics of the bacterial isolates

Characteristics	Observations Bacterial isolates			
	A	B	C	D
Morphological				
Cell shape	Rod	Rod	Rod	Coccus
Gram reaction	+ve	+ve	+ve	+ve
Motility	Motile	Motile	Motile	Nonmotile
Endospores	Present	Present	Present	Absent
Pigment formation	Absent	Absent	Absent	Absent
Biochemical				
Catalase	+	+	+	+
Urease	−	−	−	+
Arginine dihydrolase	+	+	+	+
Starch hydrolysis	+	+	+	+
Gelatin hydrolysis	+	+	+	+
H_2S production	−	−	−	−
Nitrate reduction	+	+	+	+
Growth on 5% NaCl	+	+	+	+
MR reaction	+	+	+	−
Oxidation/fermentation of glucose	Oxidation	Oxidation	Oxidation	Oxidation
Citrate utilization	+	+	+	+

+ Present; − absent

formation or consumption of 1 μmol of product or substrate per minute.

Statistical analysis

Experiments were carried out in triplicate. All the results are presented as mean ± standard deviation (SD). Data obtained were analyzed statistically by using one way ANOVA (SPSS. 7.5 version) followed by Duncan multiple range test (Duncan 1955).

Results and discussion

Characterization of organisms

An indigenous bacterial consortium was isolated from soil samples by enrichment with 2-nitrotoluene as sole source of carbon and energy. The three different bacterial isolates obtained from a bacterial consortium were characterized by their cultural, morphological and biochemical properties as shown in Table 1. All the bacterial isolates were aerobic and Gram-positive. The optimum temperature and pH for the growth of isolates were 32°C and 7.0 respectively. According to Bergey's Manual of Determinative Bacteriology (Holt et al. 1994) these isolates were identified to be belonging to genus *Bacillus* species and *Micrococcus* sp. The isolates were identified by a phylogenetic analysis based on 16S rRNA gene sequences (Fig. 1). The complete sequence of 16S ribosomal RNA gene of the bacterial isolates (A, B, C and D) were determined. Analysis of sequences were done at RDP II and NCBI, where relevant sequences from these data bases were downloaded for further analysis (Cole et al. 2005). The isolate A and C were identified as *Bacillus* sp., B as *B. flexus* strain XJU-4 and D as *Micrococcus* sp. *Micrococcus* sp. was designated as *Micrococcus* sp. strain SMN-1 and used for further studies.

Growth on various nitroaromatic compounds

The ability of the bacterial consortium to utilize various nitroaromatic compounds (0.2%, wt/vol) as sole source of carbon and energy is given in the

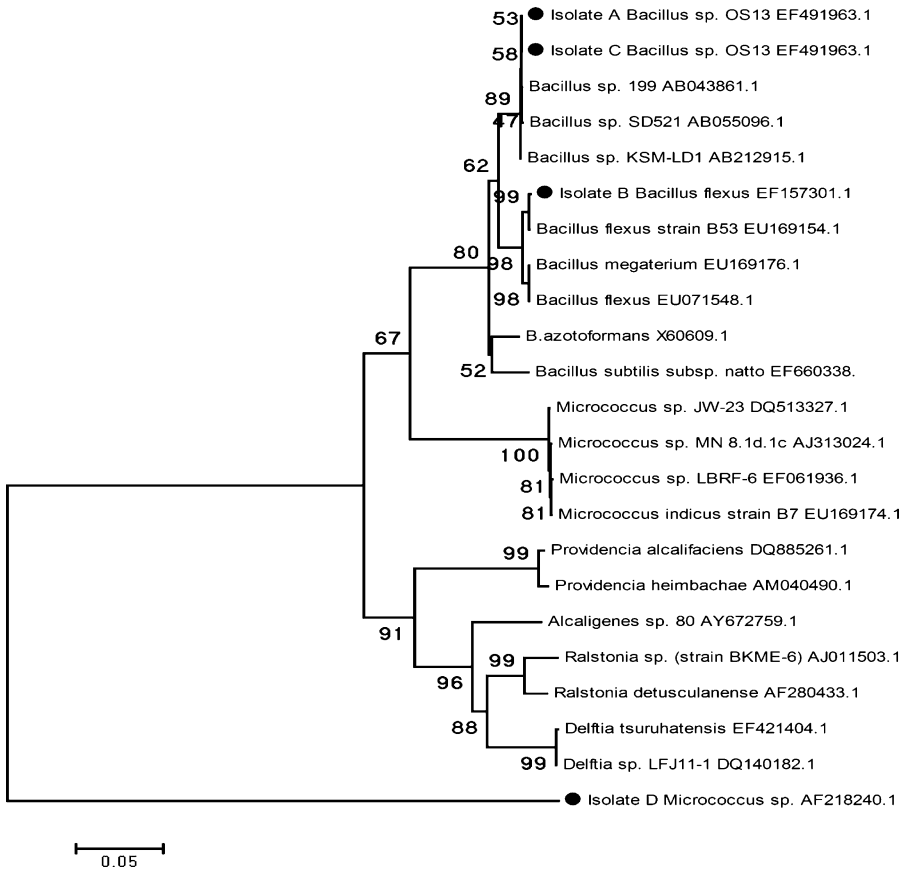

Fig. 1 Phylogenetic relationships established by the neighbour-joining method (using MEGA4.1 software) based on 16S rRNA gene sequences (1,348 bp) of isolated bacterial strains (A, B, C and D). Scale bar, no. of nucleotide changes/ sequence position. The *number* at nodes shows the bootstrap values obtained with 1,000 resampling analyses

Table 2 Utilization of various nitroaromatic compounds by bacterial consortium

Compounds	Growth
2-Nitrotoluene	++
3-Nitrobenzoic acid	++
4-Nitrobenzoic acid	++
3-Nitrotoluene	++
Nitrobenzene	++
2-Nitro *m*-xylene	++
1,3-Dinitrobenzene	−
4-Nitrophenol	+
4-Nitroaniline	+
3-Nitroaniline	+
2,4-Dinitrotoluene	+
2,6-Dinitrotoluene	+
2-Nitrobenzoic acid	−
4-Nitrotoluene	+

++ Good growth; + moderate growth; − no growth

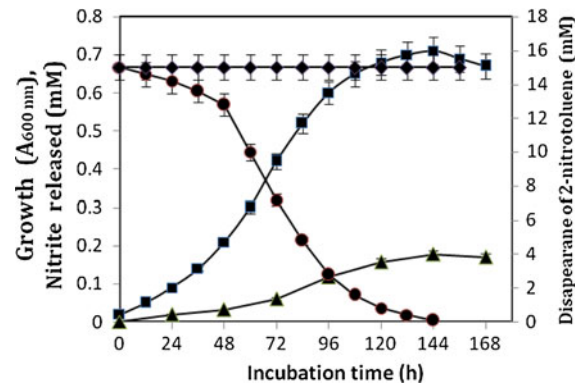

Fig. 2 Utilization of 2-nitrotoluene (*filled circles*) during growth (*filled squares*) of *Micrococcus* sp. strain SMN-1 and the release of nitrite (*filled triangles*) in the culture supernatant. Uninoculated controls (*filled diamonds*) in the mineral salts medium containing 15 mM 2-nitrotoluene. Data values represent averages of three replicate determinations

Table 3 Chromatographic and spectral characteristics of metabolites of 2-nitrotoluene

Characteristics	Isolated compound	Authentic
TLC: Rf values in different solvents		
A	0.49	0.49
B	0.74	0.75
C	0.78	0.77
D	0.56	0.57
Melting point (°C)	66	66
HPLC (retention time in min)	11.40	11.40
UV absorption λ_{max} in methanol (nm)	270	270
GC-MS molecular peak M^+ at *m/z*	51, 78, 106, 124	51, 78, 106, 124

Solvent systems A, B, C and D as described in "Material and methods" section

HPLC high-performance liquid chromatography

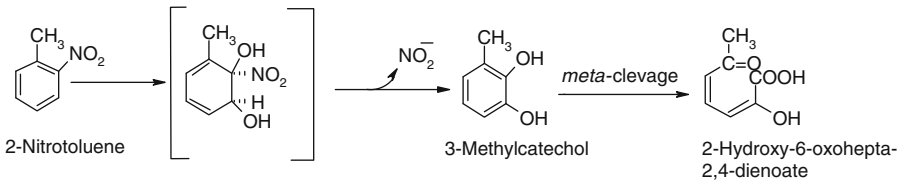

Fig. 3 Proposed pathway for the degradation of 2-nitrotoluene by *Micrococcus* sp. strain SMN-1

Table 2. The bacterial consortium utilized most of the nitroaromatic compounds as growth substrates except 1, 3-dinitrobenzene and 2-nitrobenzoic acid. *Micrococcus* sp. strain SMN-1 utilized 2-nitrotoluene, 3-nitrotoluene, 3-nitrobenzoic acid, 4-nitrobenzoic acid, 4-nitrophenol and nitrobenzene for its growth. The growth of *Micrococcus* sp. strain SMN-1 on 2-nitrotoluene (15 mM) as sole source of carbon is shown in Fig. 2. There was complete utilization of 2-nitrotoluene at 15 mM by the organism within 6 days of growth. The growth of the organism and depletion of concentration of 2-nitrotoluene was found to be significant ($\alpha = 0.05$) with respect to duration according to Duncan multiple range test. There was a release of nitrite during growth of the organism on 2-nitrotoluene (Fig. 2). The cells of *Micrococcus* sp. strain SMN-1 grown on 2-nitrotoluene utilized catechol and 3-methylcatechol as carbon sources.

Identification of metabolites of 2-nitrotoluene

The analysis of the culture extracts of *Micrococcus* sp. strain SMN-1 grown on 2-nitrotoluene by TLC revealed presence of a compound whose R_f value corresponded well with that of authentic 3-methylcatechol (Table 3). HPLC analysis of isolated metabolite showed the retention time of 11.40 min identical to that of authentic 3-methylcatechol. The GC-MS of isolated compound showed molecular peak M^+ at *m/z* 124, is in good agreement with empirical formula $C_7H_8O_2$. The main mass fragments observed at *m/z* 51, 78, 106 and 124 were in good agreement with that of authentic 3-methylcatechol (Table 3). The IR spectrum showed characteristic absorption bands of –OH stretching at 3,049 cm^{-1}, C=O stretching at 1,674 cm^{-1}, aromatic CH stretching at 2,923 cm^{-1}, C–O stretching at 1,281 cm^{-1} and C=C stretching at 1,625 cm^{-1}. The proton magnetic resonance spectrum of the isolated metabolite showed aromatic protons appeared as multiple ranging from δ 6.4 to δ 6.8 ppm, phenolic hydroxyl protons appeared at δ 5.2 and δ 5.1 ppm and methyl protons appeared as multiple ranging from δ 2.0 to δ 2.4 ppm.

Enzyme activities in the cell-free extracts

The cell-free extracts of *Micrococcus* sp. strain SMN-1 grown on 2-nitrotoluene contained the activity of catechol 2,3-dioxygenase but not catechol 1,2-dioxygenase. The specific activities of catechol 2,3-dioxygenase for catechol and 3-methylcatechol were determined to be 0.386 and 0.459 respectively. The cell-free extracts of glucose-grown cells did not contain any of these enzyme activities. These results suggest that the enzymes involved in the degradation of 2-nitrotoluene were induced by growth of the organism on 2-nitrotoluene.

It is evident from above results that the culture supernatants of *Micrococcus* sp. strain SMN-1 grown on 2-nitrotoluene as sole carbon source contained 3-methylcatechol as metabolite with the release of nitrite. Ammonia was not detected in the culture supernatants of organism grown on 2-nitrotoluene. Thus the organism degraded 2-nitrotoluene by oxidative instead of reductive mechanism. Growth and enzymatic studies have confirmed that 2-nitrotoluene was degraded through 3-methylcatechol by *Micrococcus* sp. strain SMN-1. The cells of *Micrococcus* sp. strain SMN-1 grown on 2-nitrotoluene utilized 3-methylcatechol as carbon source. The presence of high activities of catechol 2,3-dioxygenase in the cell-free extract of *Micrococcus* sp. strain SMN-1 grown on 2-nitrotoluene suggested that 3-methylcatechol was further oxidized through *meta*-cleavage pathway as shown in Fig. 3. The pathway for degradation of 2-nitrotoluene in *Micrococcus* sp. strain SMN-1 appears to be similar to that described in *Pseudomonas* sp. strain JS42 (Haigler et al. 1994) but differs from that described in *P. putida* strain OU83, which involves reduction of 2-nitrotoluene to 2-aminotoluene (Walia et al. 2003).

Thus the present studies have revealed that the isolated bacterial consortium was versatile in degrading a variety of nitroaromatic compounds that enter the environment and there was a complete degradation of 2-nitrotoluene by *Micrococcus* sp. strain SMN-1. Such bacterial strains could be useful in the bioremediation of soils and industrial effluents contaminated with toxic nitroaromatic compounds.

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