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# Production of 3-nitrocatechol by oxygenase-containing bacteria: optimization of the nitrobenzene biotransformation by *Nocardia* S3

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**Abstract** Twenty-one microorganisms were screened for their ability to convert nitroaromatics into 3-nitrocatechol as a result of the action of an oxygenase. Cultures containing toluene dioxygenases and phenol monooxygenases accumulated 3-nitrocatechol during incubation with nitrobenzene and nitrophenol, respectively. *Nocardia* S3 was selected and studied in more detail. Toluene-pregrown cultures were able to degrade nitrobenzene with a concomitant formation of 3-nitrocatechol. The rates of nitrobenzene utilization decreased throughout the biotransformation period and finally the accumulation ceased. The gradual deterioration of the biotransformation rates was not a consequence of depletion of the NADH pool, but was due to the accumulation of 3-nitrocatechol. The inhibition of nitrobenzene biotransformation by 3-nitrocatechol greatly impacts 3-nitrocatechol production processes.

## Introduction

Catechol derivatives are important precursors for the large-scale synthesis of pharmaceuticals, and in several instances nitrosubstituted catechols have been found to be very useful in multistep syntheses (Hartog and Wouters 1988; Scharrenburg and Frankena 1996). However, chemical synthetic routes for 3-nitrosubstituted catechols are not efficient because of low yields and expensive multistep processes. Consequently, a biological process for the production of nitrosubstituted catechols may be desirable for the economical synthesis of these compounds.

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Formation of 3-alkyl-, 3-phenyl- and 3-halocatechols by intact cells of *Pseudomonas* and *Rhodococcus* species containing oxygenases has been studied (Johnston and Renganathan 1987; Gibson et al. 1990; Warhurst et al. 1994; Held et al. 1998). However, the production of 3-nitrocatechol has not been investigated in detail. Accumulation of 3-nitrocatechol from nitrobenzene in *Pseudomonas putida* F1 and *Pseudomonas* sp. JS150 is due to a toluene dioxygenase that forms 3-nitro-1,2-dihydrodiol (Haigler and Spain 1991). This compound is further metabolized via a $NAD^+$-dependent dihydrodiol dehydrogenase to 3-nitrocatechol. Alternatively, 3-nitrocatechol could be formed by the oxidation of 3-nitrophenol due to a non-specific toluene dioxygenase (Spain and Gibson 1988; Haigler and Spain 1991).

In this report, we have screened oxygenase-containing bacteria for their ability to produce 3-nitrocatechol. As potential substrates, we considered nitrobenzene, nitrobenzoate and nitrophenol. Producing strains should, except when nitrophenol is used as substrate, contain not only the oxygenase but also a dihydrodiol dehydrogenase. Moreover, the resulting 3-nitrocatechol should not be degraded. On the basis of whole-cell studies, we selected *Nocardia* S3, which harbored the required dioxygenase and dehydrogenase.

Two options to optimize 3-nitrocatechol production were available. In a molecular approach, it is possible to overexpress relevant genes in a suitable host. In this work, we concentrated instead on optimization of 3-nitrocatechol production by the wild-type strain in order to define the scope and limitations of 3-nitrocatechol production.

## Materials and methods

### Organisms and culture conditions

Microorganisms tested (Table 1) were obtained from the culture collection of the Division of Industrial Microbiology and the Department of Microbiology of the Wageningen Agricultural University (Wageningen, The Netherlands), the National Collections of

**Table 1** Production of 3-nitrocatechol by bacteria harboring oxygenases

| Strain | Growth Substrate | Test substrate (1 mM) | 3-Nitrocatechol production[a] | Reference |
|---|---|---|---|---|
| *Pseudomonas cepacia* JHR22 | Biphenyl | Nitrobenzene | – | Havel and Reineke (1993) |
| *Pseudomonas* sp. NCIMB 10643 | Biphenyl | Nitrobenzene | – | Smith and Ratledge (1989) |
| *Nocardia* sp. NCIMB 10503 | Biphenyl | Nitrobenzene | – | Smith and Ratledge (1989) |
| *Alcaligenes eutrophus* H850 | Biphenyl | Nitrobenzene | – | Bedard et al. (1987) |
| *Sphingomonas* sp. B1 | Biphenyl | Nitrobenzene | – | Gibson et al. (1973) |
| *Alcaligenes* JB1 | Biphenyl | Nitrobenzene | + | Parsons et al. (1988) |
| *P. putida* PaW701 | Naphthalene | Nitrobenzene | – | Cane and Williams (1982) |
| *P. fluorescens* DSM 6506 | Naphthalene | Nitrobenzene | – | Barnsley et al. (1976) |
| *P. putida* PpG7 | Naphthalene | Nitrobenzene | – | Barnsley et al. (1976) |
| *P. putida* F1 | Toluene | Nitrobenzene | ++ | Gibson et al. (1968) |
| *Pseudomonas* sp. JS150 | Toluene | Nitrobenzene | ++ | Haigler and Spain (1991) |
| *Nocardia* S3 | Toluene | Nitrobenzene | +++ | Hartmans et al. (1990) |
| *Corynebacterium* C125 | Toluene | Nitrobenzene | ++ | Schraa et al. (1987) |
| *Xanthobacter* 124X | Toluene | Nitrobenzene | ++ | Tweel et al. (1986) |
| *P. putida* Idaho | Toluene | Nitrobenzene | – | Cruden et al. (1992) |
| *P. putida* mt-2 | Toluene | 2-Nitrobenzoate | – | Worsey and Williams (1975) |
| | | 3-Nitrobenzoate | – | |
| *P. putida* BG1 | Toluene | 2-Nitrobenzoate | – | Whited et al. (1986) |
| | | 3-Nitrobenzoate | – | |
| *P. putida* DSM 548 | Phenol | 2-Nitrophenol | – | Bayley and Wigmore (1973) |
| | | 3-Nitrophenol | – | |
| *P. stutzeri* DSM 6538 | Phenol | 2-Nitrophenol | + | Baggi et al. (1987) |
| | | 3-Nitrophenol | ++[b] | |
| *P. putida* DSM 6521 | Phenol | 2-Nitrophenol | + | Molin and Nilson (1985) |
| | | 3-Nitrophenol | ++ | |
| *Pseudomonas* sp. JS6 | Phenol | 2-Nitrophenol | + | Spain and Gibson (1988) |
| | | 3-Nitrophenol | ++ | |

[a] 3-Nitrocatechol production with active cultures ($OD_{660}$=4) was measured by HPLC after 2 h of incubation. (+ <0.05 mM, ++ 0.05–0.4 mM, +++ >0.8 mM)

[b] Trace amount of 4-nitrocatechol was detected in the reaction mixture with 3-nitrophenol

Industrial and Marine Bacteria (Aberdeen, Scotland), the Deutsche Sammlung von Mikro-organismen (Braunschweig, Germany), and the Northern Regional Research Center (US Department of Agriculture, Peoria, Ill.). *Pseudomonas* sp. JS150 and *Pseudomonas cepacia* JHR22 were kindly donated by J.C. Spain and W. Reineke, respectively.

*P. putida* strains mt-2 and BG1 were kept on slants of a mineral salts medium containing 5 mM 3-methylbenzoate and 15 g agar/l. All other strains were kept on slants of a mineral salt medium containing 5 g glucose, 3.5 g yeast extract and 15 g agar/l. Mineral salts medium was prepared as described by Hartmans et al. (1986). Cultures of *P. putida* strain PaW701, PpG7 and mt-2 were supplemented with 40 mg tryptophan/l and cultures of *Pseudomonas* sp. JS6 were supplemented with 50 mg yeast extract/l. Naphthalene (0.64 g/l) and biphenyl (0.77 g/l) were supplied as acetone solutions to the sterile Erlenmeyer flasks. Mineral salt medium was added after evaporation of the acetone. Toluene and phenol were added to a final concentration of 1 mM and 4 mM, respectively.

*Nocardia* S3 was routinely grown in a carbon-limited chemostat culture (Applikon, The Netherlands) with a working volume of 1 l. The impeller speed was 450 $min^{-1}$, the temperature was 30 °C, and the pH was kept constant at 7.0 by titration with 2 mM NaOH. Mineral salts medium was supplied to the chemostat culture, resulting in a dilution rate of 0.04 $h^{-1}$±0.001. For the experiments with toluene/ethanol-grown cells, the mineral salts medium was supplemented with 1 g ethanol/l. Air and toluene-saturated air were supplied via the headspace at a rate of, respectively, 200 and 14 ml/min using thermal mass flow controllers (Brooks Instruments B.V., Veenendaal, The Netherlands).

## Biotransformation of nitroaromatics

Batch or chemostat cultures were harvested by centrifugation (16,000 *g* for 10 min at 4 °C) and washed twice with 50 mM potassium phosphate buffer, pH 7. Incubations were performed with freshly harvested cells in 50 mM potassium phosphate buffer, pH 7. These cells (10 ml) were incubated with 1 mM substrate in 250-ml bottles sealed with Teflon-lined Mininert septa (Alltech, Deerfield, Ill.). Cells were incubated at 30 °C with vigorous shaking. At intervals, 750-µl samples were removed for analysis. Dry weight was determined by centrifuging 30-ml samples, washing with demineralized water (twice), and drying at 108 °C.

## Enzyme assays

Cell extracts of chemostat cultures were prepared by sonification for 10 min, after which the crude extract was centrifuged for 45 min (40,000 *g*, 4 °C). Protein was determined according to the method of Bradford (1976). All enzyme assays were performed at 30 °C, using extracts in 50 mM potassium phosphate buffer at pH 7.0. Dihydrodiol dehydrogenase activity was measured spectrophotometrically as described previously (de Bont et al. 1986). Catechol-1,2-dioxygenase and catechol-2,3-dioxygenase activities were determined as described by Gibson (1971). The reaction was monitored spectrophotometrically by measuring the accumulation of cleavage products (Dorn and Knackmuss 1978; Wallis and Chapman 1990).

## Oxygen consumption experiments

Oxygen consumption by washed cells in 50 mM potassium phosphate buffer(total volume 4 ml), pH 7, was measured with a Clark-

type oxygen electrode (Yellow Springs Instruments, Yellow Spring, Ohio). Endogenous oxygen uptake was measured for 4 min at 30 °C; subsequently, 50 µl of 4.0 mM substrate in 50 mM potassium phosphate buffer, pH 7, was added. Results were corrected for endogenous oxygen consumption in the absence of substrate.

### Analytical methods

Nitrobenzene, nitrobenzoate, nitrophenol and 3-nitrocatechol (donated by Solvay Farmaceuticals B.V., Weesp, The Netherlands) were analyzed by HPLC. Samples were diluted with an equal volume of methanol containing 0.2% (v/v) trifluoroacetic acid to stop the reaction and were clarified by centrifugation. HPLC was performed on a Chrompack RP $C_8$ column (4.6 mm, 25 cm) at 30 °C with methanol-water-trifluoroacetic acid (500:500:1) as mobile phase at a flow rate of 1.5 ml/min. Compounds were detected by their UV $A_{210}$ with a UV-detector and quantitated by comparison to authentic standards. The identities of the nitroaromatics were confirmed by GC-MS analysis.

## Results

Twenty-one strains containing an oxygenase (Table 1) were tested for their ability to produce 3-nitrocatechol either from nitrobenzene, nitrobenzoates or nitrophenols. Strains known to contain a biphenyl dioxygenase did not produce 3-nitrocatechol from nitrobenzene with the exception of *Alcaligenes* JB1, which produced the catechol to a very limited extent. Strains harboring a naphthalene dioxygenase did not oxidize nitrobenzene. Toluene-dioxygenase-containing strains, such as *P. putida* F1, *Nocardia* S3, *Pseudomonas* JS150, *Corynebacterium* C125, and, to a lesser extent, *Xanthobacter* 124X, rapidly transformed nitrobenzene into 3-nitrocatechol. Toluene-grown cultures of *P. putida* strain mt-2 and BG1, containing a toluene monooxygenase (TOL-plasmid), were tested for their ability to transform 2-nitrobenzoate and 3-nitrobenzoate. These strains did not degrade these compounds and consequently no nitrocatechol was formed. Strains containing phenol monooxygenase were tested for the degradation of 2-nitrophenol and 3-nitrophenol. *P. stutzeri* DSM 6538, *P. putida* DSM 6521, and *Pseudomonas sp.* JS6 degraded 3-nitrophenol with the accumulation of 3-nitrocatechol. Moreover, a trace amount of 4-nitrocatechol was detected in the reaction mixture of *Pseudomonas stutzeri* DSM 6538.

The above experiments show that several strains are able to accumulate 3-nitrocatechol. Based on its relative rapid conversion rate of nitrobenzene, *Nocardia* S3 was examined in greater detail.

### Biotransformation of nitrobenzene by *Nocardia* S3

Toluene-grown *Nocardia* S3 rapidly converted nitrobenzene into a major product detected by HPLC. The HPLC retention time of 5.18 min and spectral properties of the metabolite were identical to those of 3-nitrocatechol. The identity of 3-nitrocatechol was confirmed by GC-MS analysis of culture fluids. Nitrobenzene was converted to 3-nitrocatechol (87% conversion) with an initial rate of product formation of 7.8 U/g cell dry weight (Fig. 1A). No degradation of the 3-nitrocatechol was observed. Moreover, the addition of 1 mM 3-fluorocatechol, as inhibitor of the catechol 2,3-dioxygenase (Bartels et al. 1984), or 1 mM 3-methylcatechol, as competitor for both catechol dioygenases, did not result in improved 3-nitrocatechol accumulation from nitrobenzene (data not shown).

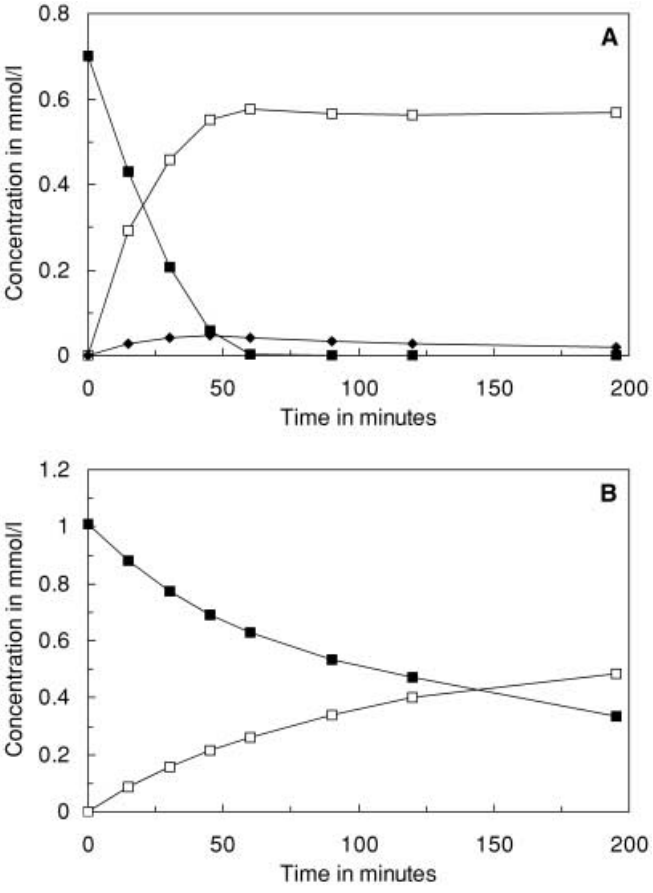


**Fig. 1** Biotransformation of nitrobenzene (**A**) and 3-nitrophenol (**B**) by toluene-grown cells of *Nocardia* S3 (2.51 mg cells/ml). Experimental details are described in Materials and methods. The conversion of either nitrobenzene (□) or 3-nitrophenol (×) to 3-nitrocatechol (●) was followed by HPLC

One other metabolite was detected by HPLC; its retention time (6.78 min) and spectral properties were identical to those of 3-nitrophenol. The identity of 3-nitrophenol was confirmed by GC-MS analysis. 3-Nitrophenol accumulated slowly during incubation with nitrobenzene (maximal concentration of about 0.05 mM), with an initial velocity of 0.7 U/g cell dry weight. After the complete bioconversion of nitrobenzene into 3-nitrocatechol, the concentration of 3-nitrophenol decreased slowly in time (Fig. 1A). To determine whether *Nocardia* S3 converted 3-nitrophenol, cells were incubated with 3-nitrophenol (Fig. 1B). Toluene-pregrown cells were able to convert 3-nitrophenol to 3-nitrocatechol as detected by HPLC. The rate of 3-nitrophenol conversion de-

**Table 2** Specific enzyme activities (nmol/min mg protein) in *Nocardia* S3

| Enzyme | Assay substrate | Growth substrate | |
|---|---|---|---|
| | | Toluene | Toluene and ethanol |
| Dehydrogenase | 1,2-Dihydrodiol | 112±6 | 48±1 |
| | 3-Methyl-1,2-dihydrodiol | 145±1 | 42±1 |
| 1,2-Dioxygenase | Catechol | 199±5 | 51±3 |
| | 3-Methylcatechol | 767±18 | 325±20 |
| | 4-Methylcatechol | 45±2 | 24±1 |
| 2,3-Dioxygenase | Catechol | 744±17 | 92±8 |
| | 3-Methylcatechol | 1372±84 | 163±15 |
| | 4-Methylcatechol | 53±1 | 7±1 |

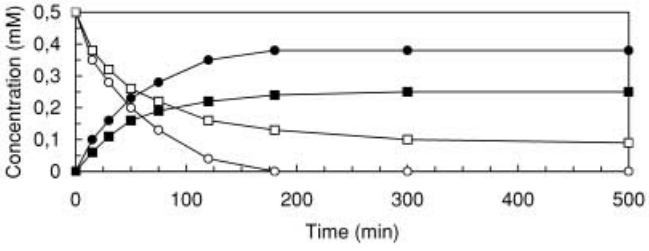


**Fig. 2** Biotransformation of nitrobenzene (*open symbols*) to 3-nitrocatechol (*solid symbols*) by washed cell suspensions of *Nocardia* S3. Cells were grown on toluene (0.96 mg cells/ml) (*circles*), and toluene with 2.5 mM ethanol (1.11 mg cells/ml) (*squares*). The conversion nitrobenzene to 3-nitrocatechol was followed by HPLC

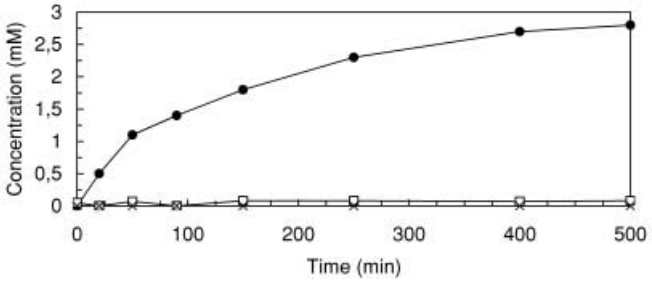


**Fig. 3** Biotransformation of nitrobenzene by toluene-grown cells of *Nocardia* S3 (11.5 mg cells/ml) with 25% dibutylphthalate as a separate solvent phase. Experimental details are described in the text. The aqueous concentration of nitrobenzene (□) to 3-nitrocatechol (●) and 3-nitrophenol (×) was followed by HPLC

creased slowly in time. The initial rate of 3-nitrocatechol formation from 3-nitrophenol was 2.3 U/g cell dry weight, and the conversion from 3-nitrophenol was 72%.

## Addition of energy-regenerating substrates

In order to determine if depletion of the NADH pool in *Nocardia* S3 is of importance during the biotransformation of nitrobenzene, toluene/ethanol-grown cells were incubated either in the presence or absence of ethanol as the energy-generating substrate. We observed no enhancement of 3-nitrocatechol production due to ethanol addition (data not shown). However, compared to toluene-grown cells we observed lower transformation rates and yields (Fig. 2). The initial conversion velocity for toluene-grown cells was 5.4 U/g cell dry weight, whereas toluene/ethanol-grown cells converted nitrobenzene with an initial velocity of 4.1 U/g cell dry weight. The nitrobenzene conversion with toluene-grown cells was 88% compared to 61% with toluene/ethanol-grown cells. Enzyme activities were determined to explain these results (Table 2). We were unable to measure the specific activity of the toluene dioxygenase due to its instability in cell-free extract. For the other toluene-degrading enzymes, it was observed that the specific activity of 1,2-dihydrodiol dehydrogenase was two to three times lower in toluene/ethanol-pregrown cells. Two catechol dioxygenases were detected, and the specific activity of both enzymes was four to eight lower in toluene/ethanol-pregrown cells.

## Biotransformation of nitrobenzene using an organic phase of dibutylphthalate

To determine whether nitrobenzene caused the gradual deterioration of the 3-nitrocatechol production rates, high cell densities, in combination with a permanently low concentration of nitrobenzene, were used. The relatively low concentration of nitrobenzene was achieved by including 25% (v/v) dibutylphthalate (containing 20 mM nitrobenzene) as a separate solvent phase. This resulted in a nitrobenzene concentration of less than 0.1 mM in the water phase. 3-Nitrocatechol was produced, without the accumulation of 3-nitrophenol, at an initial rate of 2.2 U/g cell dry weight, and this rate decreased slowly in time (Fig. 3). The addition of molecular oxygen to vigorously shaken cultures did not increase production rates (data not shown).

Most of the nitrocatechol was dissolved in the water phase (calculated log $P_{ow}$ of 3-nitrocatechol is –0.74), while nitrobenzene preferentially dissolved in the organic phase (calculated log $P_{ow}$ of nitrobenzene is 1.81). The production of 3-nitrocatechol in the system with dibutylphthalate was 0.24 mmol/g cell dry weight (Fig. 3) compared to 0.23 mmol/g cell dry weight produced without dibutylphthalate (Fig. 1A). In both experiments, the conversion rates decreased in time. However, by adding dibutylphthalate the concentration of nitrobenzene was kept low. These results indicate that the accumulation of the product 3-nitrocatechol, and not the accumulation of the by-product 3-nitrophenol, inhibited nitrobenzene conversion.

**Table 3** Effect of 3-nitrocatechol on the oxygen consumption rate in *Nocardia* S3

| 3-Nitrocatechol (μM) | Oxygen consumption rate (nmol/min mg protein): | | | |
|---|---|---|---|---|
| | Endogenous | Toluene | 3-Methyl-1,2-dihydrodiol | 3-Methylcatechol |
| 0 | 33.5±3 | 541±17 | 431±13 | 1438±54 |
| 10 | 38.1±4 | 423±28 | 383±26 | 856±49 |
| 50 | 37.4±2 | 228±15 | 242±17 | 370±7 |
| 100 | 37.6±2 | 161±28 | 143±20 | 266±17 |
| 500 | 40.1±4 | 27±7 | 68±9 | 96±6 |

Effect of 3-nitrocatechol on oxygen consumption rates in *Nocardia* S3

The hypothesis that 3-nitrocatechol inhibits the toluene-degrading enzymes in *Nocardia* S3 was tested. Toluene-grown *Nocardia* S3 was tested for oxygen consumption rates in the presence of 3-nitrocatechol. The oxygen consumption rates of *Nocardia* S3 with toluene, 3-methyl-1,2-dihydrodiol, and 3-methylcatechol as substrate drastically decreased due to the addition of 3-nitrocatechol, whereas the endogenous oxygen consumption rates did not change significantly (Table 3). Increasing the amount of added substrate did not increase the oxygen consumption rates. Preincubation with 3-nitrocatechol did not change the endogenous oxygen consumption rates, indicating a direct inhibitory effect of 3-nitrocatechol on the enzymes involved in toluene metabolism.

## Discussion

In the present work, the production of 3-nitrocatechol by bacteria harboring oxygenases was investigated. Several strains were tested for their ability to produce 3-nitrocatechol, and many organisms were able to carry out this reaction, as previously shown for *Pseudomonas sp.* JS150 and *P. putida* F1 (Haigler and Spain 1991; Spain and Gibson 1988). We also observed that phenol monooxygenases were able to produce 3-nitrocatechol from 3-nitrophenol. Surprisingly, none of the naphthalene-dioxygenase-containing strains was able to produce 3-nitrocatechol, and only one biphenyl dioxygenases-containing organism was able to produce a trace amount of 3-nitrocatechol.

Of all the strains tested, toluene-grown *Nocardia* S3 was able to convert nitrobenzene to 3-nitrocatechol most rapidly. It appears that the nitrobenzene biotransformation is analogous to the reaction pathway of toluene in *P. putida* F1 (Zylstra and Gibson 1989). This is indicated by the oxygen consumption rates (Table 3) and the enzyme activities (Table 2). 3-Nitrophenol was previously shown to be formed by the spontaneous decomposition of the intermediate 3-nitro-1,2-dihydrodiol (Haigler and Spain 1991). The 3-nitrophenol accumulated during the conversion of nitrobenzene was transformed to 3-nitrocatechol (Fig. 1B). This was also demonstrated for the toluene dioxygenase in *P. putida* F1, which was non-specific and responsible for the oxidation of several substituted phenols (Spain and Gibson 1988).

It has previously been described that nitrobenzene serves as a potent inhibitor of catechol formation in *Pseudomonas* T-12 (Johnston and Renganathan 1987). The gradual deterioration of the biotransformation rates in *Nocardia* S3 could therefore be due either to inhibition by nitrobenzene or to product inhibition by 3-nitrocatechol. The inhibition by 3-nitrocatechol was also demonstrated with oxygen consumption experiments. The toluene oxygen consumption rate of *Nocardia* S3 decreases to 5% of its initial value due to the addition of 500 μM 3-nitrocatechol (Table 3), whereas the endogenous oxygen consumption rate was not affected by 3-nitrocatechol. This clearly indicates the inhibitory effect of 3-nitrocatechol on toluene-degrading enzymes. Irrespective of the presently unknown nature of inhibition, this observation has great impact on 3-nitrocatechol production by bacteria that contain toluene dioxygenases.

In conclusion, the results presented here demonstrate that product inhibition of 3-nitrocatechol severely limits its production by *Nocardia* S3. In situ removal of the compound is therefore important; this option has been studied in the bioformation of other catechols. Continuous removal of 3-phenylcatechol was achieved by adding solid resins (Held et al. 1999), and activated charcoal was employed in the production of 3-methylcatechol (Robinson et al. 1991). It may prove difficult to arrive at an economically attractive production process unless ways are found to resynthesize the inactivated enzymes at a high rate in the production strain. Thus, the regulation of expression of these enzymes should be studied in detail, also under 3-nitrocatechol-producing conditions. A more direct way would be a recombinant-DNA approach, involving the overproduction and continued induction of these enzymes in a suitable host. In the case of rather hydrophobic catechols, such as 3-phenylcatechol, organic-solvent-resistant strains might be very appropriate, because the added advantage of partitioning of the product to an organic phase may assist in combating toxicity (de Bont 1998). But in the case of the hydrophilic 3-nitrocatechol, it seems more reasonable to exploit recombinant strains in the presence of solid adsorbing compounds.

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