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Barbiturate-induced increase of biocatalytic hydroxylation and *N*-dealkylation of *N*,*N*-diethylaniline by whole-cell incubation with *Bacillus megaterium*

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Abstract

Bacillus megaterium was used in biotransformation studies to test the influence of several barbiturates towards the recently observed *N*-dealkylation and arylic hydroxylation of *N*,*N*-diethylaniline. Certain barbiturates (phenobarbital and amobarbital) induced increased enzyme activity in whole-cell incubations with *B. megaterium* resulting in an increasing amount of *N*-deethylated and hydroxylated arylic (*ortho*- and *para*-hydroxylation) products, whereas *ortho*-hydroxylation was slightly favored. Phenobarbital did not show any effects towards hydroxylation activity, but *N*-dealkylation activity was highly induced. Finally, metyrapone highly reduced hydroxylation and *N*-dealkylation activity.

Keywords: *Bacillus megaterium*; Hydroxylation; *N*-dealkylation; Barbiturates

1. Introduction

Microorganisms are able to biotransform a broad range of inorganic and organic substrates with high efficiency, selectivity and substrate specifity. As many microbes are still not culturable under laboratory conditions (only up to 1%) (Schloss and Handelsman, 2005), many biocatalysts for whole-cell incubations are not available but will be discovered during the next years as new culturing methods will be established. Especially the field of metagenomics will also offer great possibilities to bridge the cultivation gap and therefore will become a valuable tool for the biotechnological industry, e.g. production of new antibiotics or bioremediation of contaminated sites (Gillespie et al., 2002; Singh, 2006). In a developed substrate-selected screening described previously (Taupp et al., 2006a), we recently isolated a *Bacillus megaterium* strain able to biotransform unpolar *N*,*N*-dialkylarylamines to the corresponding polar arylic hydroxylated (*ortho*- and *para*-hydroxylation) and *N*-dealkylated products (Taupp et al., 2005, 2006a, b) (Fig. 1).

This strain was also successfully used to produce the food flavor methyl-anthranilate by *N*-demethylation of natural *N*-methyl methylanthranilate (Taupp et al., 2005). Those observed reactions again point out that *B. megaterium* is as a very versatile microorganism and many biotransformations are carried out by several cytochrome P450 enzymes (CYP-450) for several isolated and characterized *B. megaterium* strains which were identified and well studied (Vary, 1994): CYP-450 BM-3 (CYP 102A1) (ATCC 14581) and P-450 BM-1 (CYP 106A1) which are both fatty acid hydroxylases and P-450meg (CYP 106A2) (ATCC 13368) known to be a steroid hydroxylase (Munro and Lindsay, 1996). Especially P-450 BM-3 was well studied and characterized and catalyzes the hydroxylation of saturated long chain-fatty acids at subterminal positions

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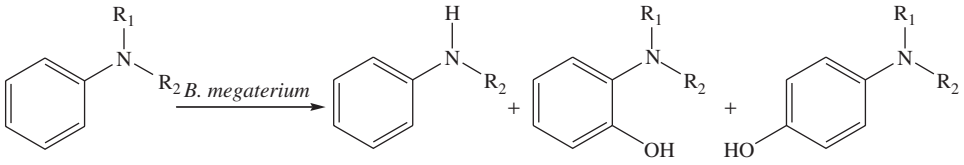

Fig. 1. Biotransformation of various *N*,*N*-dialkylarylmines to the corresponding hydroxylated and *N*-dealkylated products by *Bacillus megaterium* ($R_1 = R_2 = CH_3$: *N*,*N*-dimethylaniline; products: $R_2 = CH_3$ = *N*-methylaniline, $R_1 = R_2 = CH_3$ = *N*,*N*-dimethyl-2-aminophenol/*N*,*N*-dimethyl-4-aminophenol; $R_1 = R_2 = C_2H_5$: *N*,*N*-diethylaniline; products: $R_2 = C_2H_5$ = *N*-ethylaniline and $R_1 = R_2 = C_2H_5$ = *N*,*N*-diethyl-2-aminophenol/*N*,*N*-diethyl-4-aminophenol; $R_1 = CH_3/R_2 = C_2H_5$: *N*-ethyl-*N*-methylaniline; products: $R_2 = C_2H_5$ = *N*-ethylaniline and $R_1 = CH_3/R_2 = C_2H_5$ = *N*-ethyl-*N*-methyl-2-aminophenol/*N*-ethyl-*N*-methyl-4-aminophenol).

(ω-1 to ω-3), fatty amides, and alcohols and the hydroxylation and epoxidation of mono-unsaturated fatty acids (Narhi and Fulco, 1987; Yun et al., 2007). Phenobarbital (I) and many other barbiturates act as nonsubstrate inducers of both fatty acid monoxygenase activity and cytochrome P-450 content of *B. megaterium* ATCC 14581 (English et al., 1997; Narhi and Fulco, 1986). Recently, CYP 102A1 substrate specificity was modified and engineered to include, among others, polycyclic aromatic hydrocarbons, short- and medium-chain fatty acids and alkanes as well as to function with human P450-like activity, e.g. *N*-dealkylation and *O*-deethylation (Kubo et al., 2006; Lussenburg et al., 2005; Urlacher et al., 2006). Another hydroxylase, named P450meg (CYP 106A2) from *B. megaterium* (ATCC 13368), showed distinct substrate activity toward steroids and hydroxylates aniline leading to *para*-aminophenol (Berg and Rafter, 1979; Berg et al., 1976). Enantioselective hydroxylation of unfunctionalized arylalkanes by an isolated *B. megaterium* was reported by Adam et al. (2000), although in this case no arylic hydroxylation was observed. Phenobarbital (I) changed the regioselectivity of the observed microbial hydroxylation and increased the amounts of biotransformation products, without an effect on the enantioselectivity. As the hydroxylation activity of *B. megaterium* towards fatty acids was inducible by barbiturates, the observed hydroxylation activity towards *N*,*N*-diethylaniline of our topsoil isolate was further investigated. Main goal was to induce the hydroxylation activity resulting in higher amounts of a more polar molecule which could then be more feasible for further biodegradation. Investigations were started to influence and induce enzymes being possibly responsible for the observed hydroxylation and *N*-dealkylation of *N*,*N*-diethylaniline. In this contribution, we present our results for the induction and inhibition of possible enzymes being involved in the observed biotransformation reactions.

2. Materials and methods

2.1. Chemicals

N,*N*-Diethylaniline (⩾99.0% purity) was purchased from Merck (Darmstadt, Germany). Used barbiturates phenobarbital (I) and amobarbital (II) (purity ⩾99.0%) were a kind gift of the Department of Pharmacy and Chemistry, Institute of Pharmacy, University of Würzburg, Germany. Organic solvents used for extraction were distilled before use. Metyrapone (⩾99.0% purity) was purchased from Fluka (Taufkirchen, Germany).

2.2. Microorganisms

B. megaterium, used in substrate-incubation studies, was isolated from topsoil (Karlstadt, Germany) in a selective screening process and characterized by 16S rDNA and DNA/DNA hybridization against the *B. megaterium* type strain ATCC 14581 as recently described (Taupp et al., 2005, 2006a). The isolated *B. megaterium* strain was deposited in the BCCM™/LMG, Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent (RUG), B-9000 Gent, Belgium, under the LMG number 23147.

2.3. Gas chromatography–mass spectrometry (GC–MS)

GC–MS analysis was performed using an Agilent 6890 gas chromatograph with split injector (1:20) coupled to an Agilent 5973 mass selective detector and an Agilent 7683 automatic liquid sampler. The system was equipped with the Chem Station software. A J&W DB Wax fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) was employed. The temperature program was 3 min isothermal at 50 °C and then increased with 4 °C/min to 220 °C using 1.0 ml/min helium constant flow. The MS operating values were as follows: ionization voltage 70 eV (electron impact ionization), and ion source and interface temperatures 230 °C. Bacterial metabolites were identified by comparison with authentic reference compounds regarding retention times and mass spectra as described before (Taupp et al., 2006a). Quantification was done by adding up all peak areas of volatile compounds; yields were calculated in percent as GC-detectable amounts. A two-tailed Student's *t*-test was used for statistical analysis. *P* values of 0.05 were considered statistically significant.

2.4. Substrate acceptability tests

Liquid minimal media (75 ml) was prepared according to Dworkin and Foster (1958), a *Pseudomonas*-trace element solution was added to a final concentration of 5 ml/l and autoclaved at 121 °C for 16 min (Atlas, 1993). The culture was maintained under sterile conditions during the addition of 750 μl of sterile glucose solution (50%). For inoculation of the liquid media, a single colony was taken from freshly grown agar plates (on standard methods agar plates; Bio Merieux′, Nürtingen, Germany). After inoculation, the culture was pregrown overnight and then shaken in the presence of 100 μmol of the substrate *N*,*N*-diethylaniline for 18 h. To verify the stability and authenticity of the used substrate, control experiments without bacteria were carried out; no by-products were observed under these conditions. After incubation, the culture was worked up by sonication of the bacterial broth for 10 min, and centrifugation for 20 min at 3000 × *g*. The supernatant was extracted two times with 50 ml of diethylether and two times with 50 ml dichloromethane. The combined organic layers were dried over Na_2SO_4 and filtered. The organic solvent was evaporated under reduced pressure and the residue was redissolved in dichloromethane and 1 μl was submitted to GC–MS analysis.