Recombinant Chlorobenzene Dioxygenase from *Pseudomonas* sp. P51: A Biocatalyst for Regioselective Oxidation of Aromatic Nitriles

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Abstract: An efficient biocatalyst was developed for the *cis*-dihydroxylation of aromatic nitriles. The chlorobenzene dioxygenase (CDO) genes of *Pseudomonas* sp. strain P51 were cloned under the strict control of the P*alk* promoter of *Pseudomonas putida* GPo1. *Escherichia coli* JM101 cells carrying the resulting plasmid pTEZ30 were used for the biotransformation of benzonitrile in a 2-L stirred tank bioreactor. Use of a stable expression system resulted in an average specific activity and an average volumetric productivity of 1.47 U/g cdw and 120 mg of product/h/L, respectively. The values represent a three-fold increase compared to the results of the similar biotransformations with *E. coli* JM101 (pTCB144) where the genes of CDO were expressed under the control of *lac* promoter. The productivity of the *cis*-dihydroxylation process was limited by product toxicity. Removal of the products at toxic concentrations by means of an external charcoal column resulted in an additional increase in product concentration by 43%. *E. coli* JM101 (pTEZ30) was further used for the regio- and stereospecific dihydroxylations of various monosubstituted benzonitriles, benzyl cyanide, and cinnamonitrile. Biotransformations resulted in products with 42.9–97.1% enantiomeric excess. Initial enzymatic activities and isolated yields were obtained in the range of 1.7–4.7 U/g cdw and of 3–62%, respectively.

Keywords: aromatic nitriles; asymmetric synthesis; biocatalysis; biotransformations; dihydroxylation; *in situ* product removal

Introduction

The use of enzymes as catalyst in organic synthesis provides new routes to synthesize previously undescribed compounds.[1–3] Asymmetric dihydroxylation of aromatic compounds by bacterial dioxygenases[4,5] (Figure 1) is such a reaction, where the product *cis*-dihydrodiols are valuable chiral synthons used in organic syntheses.[6–8] Next to catalyst selectivity, process efficiency depends on turnover frequency, total turnover number and volumetric productivity.[9] The rates of *cis*-dihydroxylation of non-natural (non-physiological) substrates are generally relatively low, compared to the rates achieved with natural substrates such as toluene for TDO, naphthalene for NDO and chlorobenzene for CDO.[10,11] Recombinant biocatalysts circumvent such limitations of (mutated) wild-type cells. Volumetric productivities can be increased and total reaction times can

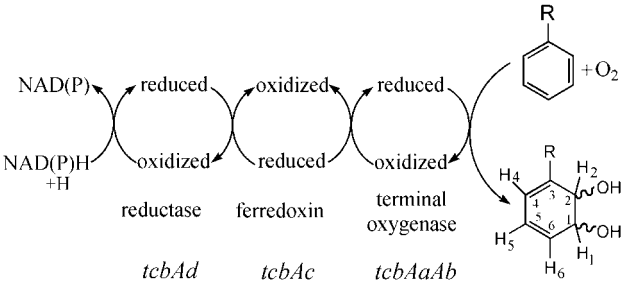

Figure 1. Structural organization of chlorobenzene dioxygenase and designation of the protons and carbons of *cis*-dihydrodiols formed by chlorobenzene dioxygenase. Gene products; *tcbAa*: large subunit of terminal oxygenase, *tcbAb*: small subunit of terminal oxygenase, *tcbAc*: ferredoxin, *tcbAd*: reductase.

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be prolonged on preparative scales by expressing the genes from an efficient and controllable promoter.[12,13]

We developed a new biocatalyst by cloning the genes of the chlorobenzene dioxygenase (CDO) of *Pseudomonas* sp. strain P51[14] behind the *alk* promoter *alkBp* of *Pseudomonas putida* Gpo1[15] resulting in pTEZ30. We previously showed that *E. coli* JM101 (pTEZ30) can be used for efficient *cis*-dihydroxylation of cinnamonitrile up to technical scales.[16] Here, we describe the construction of the expression plasmid pTEZ30 and evaluate the efficiency of the biocatalyst *E. coli* JM101 (pTEZ30) based on specific activity of the cells and the volumetric productivity of the biotransformation of benzonitrile in a reactor on a 2-L scale. The results are compared to the results of similar biotransformations with *E. coli* JM101 (pTCB144)[14] as biocatalyst where the CDO genes were cloned under the control of *lac* regulatory system.[17] Subsequently the new biocatalyst was used for the stereo- and regiospecific dihydroxylations of various *ortho*-, *meta*- and *para*-substituted benzonitriles, benzyl cyanide, and cinnamonitrile. Dihydroxylation of aromatic nitriles is especially interesting, since it offers the possibility for one-pot multistep catalysis with a further engineered catalyst carrying, e.g., nitrilase or nitrile hydratase. All reaction products were characterized as thoroughly as possible and the reaction parameters such as specific activity of the cells and the yields of the reactions were determined.

Results

Effect of Induction on the Growth of *E. coli* (JM101) (pTEZ30) and Identification of Dioxygenase Activity

The growth rate of whole-cell biocatalysts has a significant effect on biotransformation efficiency[16] and generally decreases with the enzyme production. Therefore the effects of the presence of inducer, induction of *alk* promoter, and the production of enzymes on cell growth were examined. The growth of *E. coli* JM101 (−), *E. coli* JM101 (pRS), and *E. coli* JM101 (pTEZ30) (Table 1) was followed for 5 hours in the absence and in the presence of the inducer [0.05% dicyclopropyl ketone (DCPK)]. The specific growth rates of all three strains were identical (0.56 h^{-1}) in the absence of the inducer (Figure 2). The presence of the inducer caused a slight decrease in specific growth rates of *E. coli* JM101 (−), and *E. coli* JM101 (pRS) cells but decreased the specific growth rate of *E. coli* JM101 (pTEZ30) cells significantly to 0.36 h^{-1}. This shows that the presence of 0.05% DCPK, and expression of *alk*Bp genes has just a minor effect on cell growth, whereas the enzyme production decreases the specific growth rate by 36%.

The specific activity of *E. coli* JM101 (pTEZ30) was tested in shake-flasks with naphthalene as a substrate, since CDO showed the highest activity towards this compound in a previous work[18] and the corresponding product is commercially available. The cells showed an initial specific activity of 2.86 units per gram cell dry

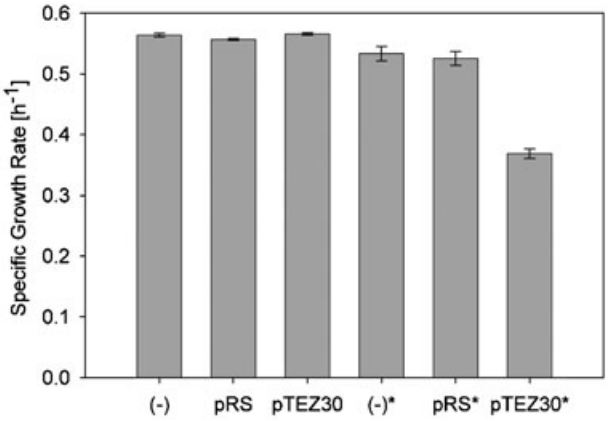

Figure 2. Specific growth rates of induced and non-induced *E. coli* JM101 (−), *E. coli* JM101 (pRS), and *E. coli* JM101 (pTEZ30) cells in 20 mL M9 medium in shaking flasks. * Induced.

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristics	Source or reference
Strains of *E. coli*		
DH5α	*supE44*, Δ*lacU169* (Φ80*lacZ*ΔM15) *hsdR17 recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*	[62]
JM101	*supE thi-1* Δ*(lac-proAB)* F′ [*traD26 pro AB*$^+$ *lacI*^{q} *lacZ*ΔM15]	[58]
Plasmids		
pTCB144	*lac, tcbAa, tcbAb, tcbAc, tcbAd genes* Ap^r	[14]
pSPZ2MA	Source of *alkBp and AlkS,* Ap^r	[12]
pTEZ10	pSPZ2MA with *tcbAa*	This work
pTEZ20	pTEZ10 with *tcbAa*,[a] *tcbAb, tcbAc, and tcbAd*	This work
PBRNSKmΔ	Source of Km^r	[12]
pTEZ30	*AlkBp, AlkS, Km^r, tcbAa, tcbAb, tcbAc, and tcbAd*	This work
PRS	*AlkBp, alkS, Km^r*	[30]

[a] The plasmid contains only a part of the *tcbAa* genes.

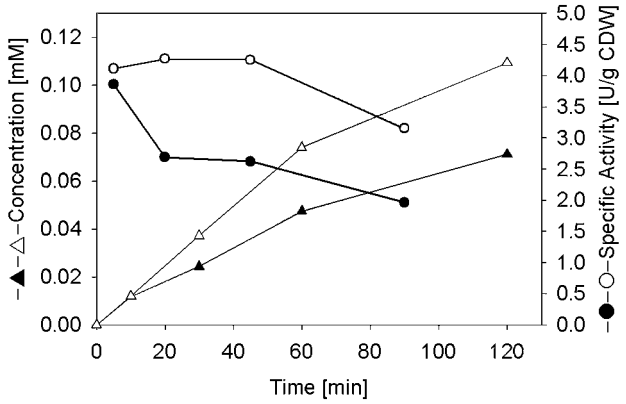

Figure 3. Product formation and specific activities of the biotransformation of naphthalene to *cis*-1,2-dihydro-1,2-naphthalenediol in 20-mL shaking flasks with *E. coli* JM101 (pTCB144) (open symbols) and with *E. coli* JM101 (pTEZ30) (closed symbols). The assay was performed as described in Materials and Methods.

weight (U/g cdw) during the biotransformation of naphthalene to *cis*-1,2-dihydro-1,2-naphthalenediol. One unit is defined as 1 μmol of product formed in one minute. During the biotransformation, the specific activity decreased to 1.96 U/g cdw and 0.075 mM of product was formed. Specific activities of the cells harboring pTCB144 were slightly higher than the activities of the cells harboring pTEZ30 (Figure 3).

Biotransformation of Benzonitrile on 2-L Scale

To evaluate the stability of the developed biocatalyst in the bioreactor and the efficiency of the biotransformation with it, we performed the biotransformation of benzonitrile to *cis*-1,2-dihydroxy-3-cyanocyclohexa-3,5-diene (BNDD) in a bioreactor on a 2-L scale with *E. coli* JM101 (pTEZ30) (Figure 4). The results were compared with the results of the biotransformation with *E. coli* JM101 (pTCB144) cells. Both *E. coli* JM101 (pTEZ30) and *E. coli* JM101 (pTCB144) cells showed similar growth properties in the reactor during the biotransformations (Figure 4A). Although the cell growth behaviors were very similar (Figure 4B), the product formation rates differed significantly. With *E. coli* JM101 (pTCB144), the initial activity and the volumetric productivity were 0.67 U/g cdw and 0.07 g of product/h/L, respectively, and both decreased significantly during the biotransformation as presented in Figure 4C. Analysis of colony forming unit by plating *E. coli* JM101 (pTCB144) cells taken at the end of the biotransformation on LB agar plates with and without ampicillin showed that only 13% of the cells contained the plasmid and confirmed the instability of antibiotic resistance (data not shown). During the biotransformation the product concentration was increased to a maximum of only 2.37 mM in 8.7 hours corresponding to an average volumetric productivity of 40 mg of product/h/L.

The initial volumetric productivity of *E. coli* JM101 (pTEZ30) cells was similar to that of *E. coli* JM101 (pTCB144) cells. In contrast, *E. coli* JM101 (pTEZ30) which confers kanamycin resistance was quite stable during the biotransformation even at the high cell densities. This resulted in an increase of the volumetric productivity to a maximum of 0.26 g of product/h/L after 5 hours. In 8.5 hours 7.5 mM product were formed, yielding an average volumetric productivity of 120 mg product/h/L, which is 3-fold the average volumetric productivity achieved with *E. coli* JM101 (pTCB144) cells. The volumetric productivity decreased after five hours with a significant decrease in specific growth rate, which is probably due to the toxic effect of the product. We diluted the cells taken at this time point and plated them on LB agar plates with and without kanamycin. Equal numbers of colonies were obtained in both cases, which shows high stability of the plasmid. However, the comparison of viable cell counts to the spectrometrically determined total number of cells in the reactor indicated that only 60% of the total number of cells measured in the reactor (data not shown) were living cells. The correlation between specific growth rate and volumetric productivity indicates that, when the dioxygenase genes are efficiently expressed, productivity of the biotransformations becomes limited by product toxicity. This indicates that use of the expression system *E. coli* JM101 (pTEZ30) in biotransformations of benzonitrile may result even in higher volumetric productivities when the toxic product is removed from the reaction mixture. At the end of the biotransformation, product concentration reached 10.74 mM, which is 4.5-times higher than the maximum concentration achieved in biotransformations with *E. coli* JM101 (pTCB144). A summary of the comparison of the process parameters is given in Table 2.

Biotransformation of Aromatic Nitriles

We used the expression system *E. coli* JM101 (pTEZ30) to investigate the dihydroxylation of various aromatic nitriles. For that purpose we performed the biotransformations of the substrates listed in Table 3 in a reactor on a 300-mL scale. The dihydrodiol metabolites were isolated and identified by GC-MS of their respective *n*-butylboronate derivatives (BB derivatives) and by H NMR spectroscopy (Table 3). BB derivatives of *cis*-dihydrodiol isomers were separated on a GC column with an achiral stationary phase and could be distinguished from those of *trans*-dihydrodiols because of the significant mass difference between their molecular ions.[19] Enantiomers of *cis*-dihydrodiols were separated as their BB derivatives on a GC column with a chiral stationary phase and quantified. NMR spectroscopy was used to

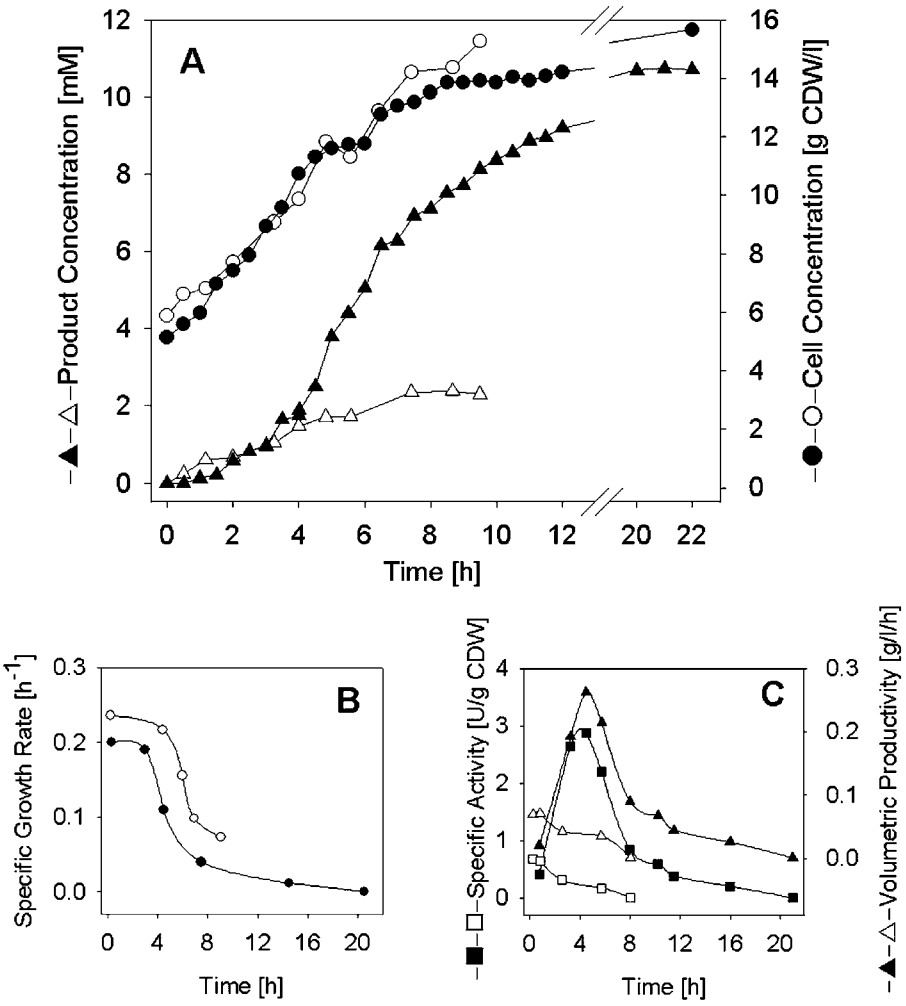

Figure 4. Biotransformation of benzonitrile to *cis*-1,2-dihydroxy-3-cyanocyclohexa-3,5-diene in a reactor on a 2-L scale. Open and closed symbols represent the values obtained with *E. coli* JM101 (pTCB144) and *E. coli* JM101 (pTEZ30), respectively. *A*. Cell growth and product formation. *B.* Growth rate profile during biotransformation. *C.* Activity and productivity profiles.

Table 2. Process parameters of biotransformation of benzonitrile using *E. coli* JM101 (pTCB144) and *E. coli* JM101 (pTEZ30) in a reactor on a 2-L scale.

	E. coli JM101 (pTCB144)	*E. coli* JM101 (pTEZ30)[a]
Total amount of product produced (g)	0.65	2.12
Bioconversion yield (%)	72	73.5
Maximum growth rate (h^{-1})	0.24	0.20
Maximum specific activity (U/g cdw)	0.67	2.87
Average specific activity (U/g cdw)	0.43	1.47
Maximum volumetric activity (U/L)	10.5	43.0
Average volumetric activity (U/L)	4.56	14.3
Maximum volumetric productivity (g prod/h/L)	0.07	0.26
Average volumetric productivity (g prod/h/L)	0.04	0.12

[a] Values were calculated for the first 9 hours of the biotransformation for a better comparison to the biotransformation using *E. coli* JM101 (pTCB144).

determine the structure and the position of the hydroxy groups. Figure 1 shows our nomenclature to specify the protons of the dihydrodiol compounds. GC-MS and NMR measurements of the products showed that the *cis*-dihydroxylation occurred on the unsubstituted ring at the 1,2 positions with all substrates tested.

Table 3. Products of *cis*-dihydroxylation of aromatic nitriles by *E. coli* JM101 (pTEZ30).

Substrate	Ads. max. [nm]	*m/z* of prominent ions (relative abundance)[a]	Chemical shifts (δ; ppm) and coupling constants (*J*, Hz)	ee [%]	Suggested structure
Benzonitrile	285	203, M^+ (25); 146, (19); 119 (100); 103 (41); 91 (86); 76 (28)	4.16 (1H, dd, $J_{2,6}$, 0.6, $J_{2,1}$ 6.06, 2-H), 4.28 (1 H, m, 1-H), 6.10 (1 H, m, 5-H), 6.28 (1 H, m, 6-H), 6.84 (1 H, dd $J_{4.5}$, 6.2, $J_{4,6}$ 0.8, 4-H)	88.4	*cis*-1,2-Dihydroxy-3-cyanocyclohexa-3,5-diene
2-Chlorobenzonitrile	291	71% 237, M+(9); 180, (14); 153, (100); 137, (58); 125, (45); 102, (17); 75, (16)	4.24 (2 H, m, 1-H, 2-H), 5.0–6.0 (2 H, 2 broad s, OH), 6.08 (1 H, dd, $J_{5,1}$ 2.0, $J_{5,6}$ 10.1, 5-H), 6.31 (1 H, dd, $J_{6,1}$ 3.2, $J_{6,5}$ 10.1, 6-H)	42.9	*cis*-1,2-Dihydroxy-3-cyanocyclohexa-4-chloro-3,5-diene
2-Bromobenzonitrile	295	281, M^+ (26); 267, (6); 253, (43); 239, (3); 226, 6); 198, (100); 182, (57); 171, (48)	4.14 (1 H, d, $J_{2,1}$ 6.06, 2-H), 4.24 (1 H, dd, $J_{1,5}$ 2.0, $J_{1,2}$ 6.06, 1-H), 5.2–6.0 (2 H, 2 broad s, OH), 6.27 (2 H, m, 5-H, 6-H)	ND	*cis*-1,2-Dihydroxy-3-cyanocyclohexa-4-bromo-3,5-diene
2-Fluorobenzonitrile	282	221, M^+ (8); 194, (9); 164, (15); 137, (100); 121, (64); 109, (48); 94, (13)	4.16 (1 H, dd, $J_{2,1}$ 6.06, J 12.11, 2-H), 4.28 (1 H, m, 1-H), 5.39 (1 H, d, *J* 6.86, OH), 5.65 (1 H, d, *J* 6.45, OH), 6.08 (1 H, m, 5-H), 6.38 (1 H, m, 6-H)	89.6	*cis*-1,2-Dihydroxy-3-cyanocyclohexa-4-fluoro-3,5-diene
3-Fluorobenzonitrile	285	221, M^+ (7), 192, (5); 164, (20); 137, (25); 121, (100); 109, (34); 94, (14)	4.09 (1 H, dd, $J_{2,1}$ 6.06 J 11.71 2-H), 4.22 (1 H, m, 1-H), 5.16 (1 H, d, J 6.05, OH)	ND	*cis*-1,2-Dihydroxy-3-cyanocyclohexa-5-fluoro-3,5-diene
4-Chlorobenzonitrile	296	237, M^+ (9); 202, (64); 180, (19); 170, (7); 153, (21); 146, (86); 137, (100); 125, (29);	4.08 (1 H, m, 2-H), 4.47 (1 H, m, 1-H), 5.87 (2 H, m, OH, OH), 6.38 (1 H, d, $J_{5,4}$ 6.06, 5-H), 6.85 (1 H, dd, $J_{4,2}$ 2.02, $J_{4,5}$ 6.46 4-H)	97.1	*cis*-1,2-Dihydroxy-3 -cyanocyclohexa-6-chloro-3,5-diene
4-Fluorobenzonitrile	285	221, M^+ (6); 192, (4); 164, (34); 137, (100); 121, (67); 109, (48); 94, (14)	4.17 (1 H, m, 2-H), 4.49 (1 H, s, 1-H), 5.89 (3 H, m, OH, OH, 5-H), 6.89 (1 H, m, 4-H)	ND	*cis*-1,2-Dihydroxy-3-cyanocyclohexa-6-fluoro-3,5-diene
4-Methylbenzonitrile	295	217, M^+ (69); 202, (100); 190, (8); 160, (21); 146, (21); 133, (46), 116, (54); 105, (51); 89, (34); 78, (30)	2.00 (3 H, s, Me-H), 4.08 (1 H, d, $J_{2,1}$ 6.06, 2-H), 4.28 (1 H, d, $J_{1,2}$ 6.06, 1-H), 4.4–5.2 (2 H, 2 broad s, OH), 5.83 (1 H, d, $J_{5,4}$ 5.65, 5-H), 6.65 (1 H, d, $J_{4,5}$ 5.65, 4-H)	ND	*cis*-1,2-Dihydroxy-3-cyanocyclohexa-6-methyl-3,5-diene
Cinnamonitrile	319	228, M^+ (92); 172, (46); 145, (73); 129, (39); 117, (100); 102, (28); 90, (45); 77, (25)	3.34 (2 H, s,) 4.10 (1 H, m, 2-H), 4.23 (1 H, m, 1-H), 4.81 (1 H, d, *J* 5.62, OH), 5.09 (1 H, d, *J* 7.27, OH), 5.81 (1 H, d, *J* 16.55 5-H), 6,01 (2 H, m, 6-H) 6.36 (1 H, m, 4-H)	95.1	*trans*-3-[(5*S*,6*R*)-5,6-Dihydroxycyclo-hexa-1,3-dienyl]-acrylonitrile
Benzyl cyanide	262	217, M^+ (38); 177, (100); 160, (10); 133, (94); 121, (35); 117, (26); 105, (61); 89, (26); 78, (51)	3.47 (2 H, s, H-CH_2) 3.9 (1 H, d, $J_{2,1}$ 6.06 2-H), 4.04 (1 H, m, 1-H), 4.83 (2 H, broad s, OH), 5.89 (3 H, m, 4-H, 5-H, 6-H)	66.4	*cis*-1,2-Dihydroxy-3,5-cyclohexadienyl)-acetonitrile

[a] The *m/z* values of the prominent ions of the mass spectra of the BB derivatives are given.
ND, not determined.

Table 4. Biotransformation of aromatic nitriles with *E. coli* JM101 (pTEZ30) containing CDO.

Substrate	Conversion[a] [%]									Activity[b] [U/g cdw]	Yield[c] [%]	Yield[d] [%]
	15 min	30 min	1 h	2 h	3 h	4 h	5 h	6 h	7 h			
Benzonitrile	4	7	14	26	33	40	47	51	55	3.9	82	62
2-Chlorobenzonitrile	3	8	10	15	18	21	24	26	27	4.0	7	3
2-Bromobenzonitrile	4	9	11	16	19	21	23	25	26	4.4	27	12
2-Fluorobenzonitrile	6	11	19	25	28	31	35	36	37	4.7	15	4
3-Fluorobenzonitrile	1	3	5	12	17	21	26	29	33	1.7	36	21
4-Chlorobenzonitrile	2	5	7	18	29	37	46	53	58	2.5	47	17
4-Fluorobenzonitrile	3	5	8	15	19	21	24	25	27	2.9	79	11
4-Methylbenzonitrile	3	6	9	21	31	41	51	59	67	3.3	85	52
Cinnamonitrile	3	6	12	27	36	43	49	51	52	3.1	56	33
Benzyl cyanide	2	6	10	20	24	28	33	35	38	3.0	61	10

[a] Calculated according to the product formation in 20-mL shake flask experiments.
[b] Activity of the cells in the first 30 minutes of the biotransformations in 20-mL shake flask experiments.
[c] Yields of the biotransformations on 300-mL scale
[d] Yield of the biotransformations (on 300-mL scale) after purification of the products.

Specific activity of the cells and the rate of conversion are important factors for a scale-up of such biotransformations. Therefore we performed the biotransformations in 20-mL shake flasks and determined the reaction rates and the specific activity of the biocatalyst *E. coli* JM101 (pTEZ30). The results are presented in Table 4. Biotransformations with *ortho*- substituted benzonitriles showed higher activities than with *meta*- or *para*-substituted benzonitriles. The highest activity was obtained with *ortho*-fluorobenzonitrile. Biotransformation of *p*-methylbenzonitrile resulted in the highest bioconversion (67%) after 7 hours.

The yields of the biotransformations performed on a 300-mL scale were in the range of 4–62% (Table 4). The non-optimized biotransformation of 4-methylbenzonitrile resulted in a significantly higher yield 52%, compared to the yield of biotransformation of other substituted aromatic nitriles.

Partitioning in Aqueous Two-Phase Systems

The results of the biotransformation of benzonitrile showed the effect of product toxicity on cell growth and volumetric productivity. To examine this effect further, we aimed at removing the product from the reaction medium to non-toxic concentrations during the biotransformation. This would allow us to examine if the volumetric productivity of the process could be recovered by reducing the toxic effects of the product. For this purpose we tested the well established ISPR concepts[20–25] to remove the product from the reaction medium to non-toxic concentrations. First, *in situ* product extraction using aqueous two-phase systems (ATPS) was tested. Amongst the aqueous two-phase systems (ATPS) investigated [polyethylene glycol (PEG)/phosphate, PEG/ammonium sulfate, PEG/magnesium sulfate and PEG/dextran], PEG/$MgSO_4$ was the least toxic system to the cells, and allowed cell growth and substrate conversion (data not shown). Concentrations as high as 10 to 15% of monophasic PEG8000 allowed cell growth identical to the control experiments on plain culture media. 2.5% to 4.5% $MgSO_4$ reduced the cell growth up to 30%. The partition coefficients of the substrate and product in PEG4000/$MgSO_4$ and PEG8000/$MgSO_4$ systems, were elucidated (Table 5). However, the partition coefficients of benzonitrile and BNDD were not high enough to keep cells well separated from the substrate and product, so the option of using ATPS was discarded, despite the compatibility with the cells. As an alternative to extractive reactions, adsorptive reactions were studied. This has been used, amongst others, for ISPR of fluorocatechol, a compound resembling BNDD, during conversion of fluorobenzene by *Pseudomonas putida*,[23] and in pilot-plant processes for microbial biotransformations.[25–28] We determined preliminary adsorption isotherms of benzonitrile and BNDD for various adsorbents and plotted them double-logarithmically to facilitate comparison (Figure 5). Sepabeads did not adsorb either benzonitrile or BNDD significantly. Further-

Table 5. Partition coefficients of benzonitrile and benzonitrile dihydrodiol (BNDD).

	16% PEG4000-5.6% $MgSO_4$	15% PEG8000-4% $MgSO_4$	15% PEG8000-6% $MgSO_4$
K_{BN}	2.7	1.9	3.9
K_{BNDD}	1.6	1.4	1.5

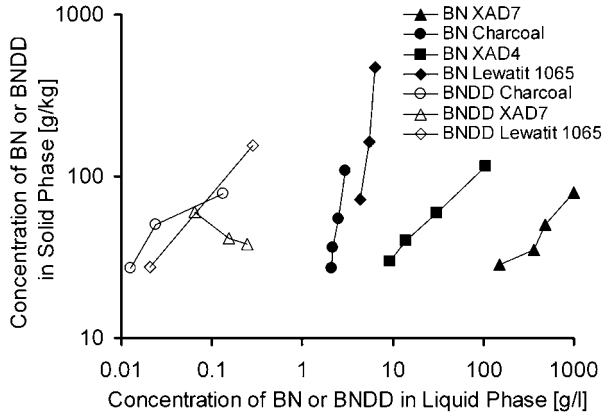

Figure 5. Adsorption isotherms of benzonitrile (BN) and *cis*-2-dihydroxy-3-cyanocyclohexa-3,5-diene (BNDD) using various adsorbents.

more, the adsorption of BNDD by XAD4 was not significant. When Charcoal, XAD7 and Lewatit 1065 were used as adsorbents, the concentrations of BNDD remaining in the liquid phase were quite low. With the same adsorbents, the concentrations of benzonitrile in the liquid phase were low as well but a few orders of magnitudes higher (Figure 5). Therefore these adsorbents were found suitable for extraction of the BNDD.

After determining the adsorption isotherms of the products and substrates, we tested the effects of the adsorbents on biomass accumulation in the absence and in the presence of benzonitrile. The results are presented in Figure 6. Cell amount in the presence of Lewatit 1065 reached only 36% of the cell amount without any adsorbent. XAD7, charcoal, and Sepabeads did not have a significant effect on cell growth. Addition of benzonitrile in amounts that would be normally quite toxic to the cells did not have a significant effect on growth in the presence of these adsorbents. In the presence of charcoal, one of the best adsorbents for benzonitrile (BN) and BNDD, biomass (even in the presence of 15 mM BN) reached the same density as in experiments without

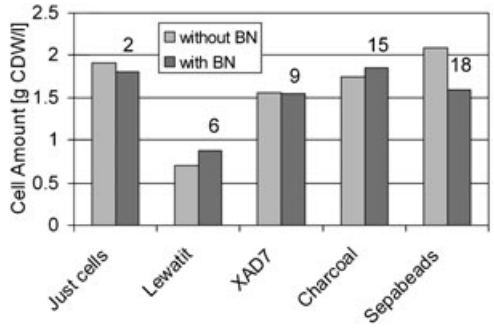

Figure 6. Effects of adsorbents and benzonitrile (BN) on biomass accumulation. Numbers above the bars show the concentration (mM) of BN.

any adsorbents and BN. Therefore, charcoal was used for the *in situ* product adsorption for biotransformation experiments.

Biotransformation of Benzonitrile with Integrated Product Recovery

To examine the toxic effect of the product on cell growth and volumetric productivity, we aimed to remove the product from the reaction medium to non-toxic concentrations during the biotransformation. For that purpose, we performed a biotransformation of benzonitrile in a 2-L bioreactor equipped with an adsorption column containing 35 g charcoal. To exclude high substrate concentrations that could cause toxic effects, benzonitrile was added repeatedly. The product concentration increased in the first 7.5 hours of the biotransformation with an average specific activity of 1.3 U/g cdw. At the end of this period, the specific growth rate decreased to zero due to the toxic effect of the product, which decreased the specific activity to 0.21 U/g cdw (Figure 7). At this time point the cell-containing broth was circulated between the reactor and the loop. After the adsorption cycle, the product concentration decreased from 7.4 to 1.9 mM and all the substrate in the reaction medium was adsorbed. After the addition of new substrate, product formation started again and the specific activity reached 46% of that observed in the first period. The product concentration reached again the toxicity threshold and a decrease in specific growth rate and specific activity was observed. Therefore, another cycle of adsorption was performed and product concentration decreased to 2.4 mM. After this cycle, only 30% of the activity was recovered. At the end of the reaction, the total product formation increased up to 15.4 mM giving a bioconversion yield of 61.6% and a product concentration of 2.1 g/L. The cells were still active and the product formation was continuing. Toxic concentrations of products (7–8 mM) before the adsorption cycle resulted in a long lag phase of the cells afterwards, which decreased the specific activity after the adsorption.

Discussion

Recombinant Whole Cells as Biocatalyst

Use of recombinant cells to express dioxygenase genes for whole-cell *cis*-dihydroxylations has several advantages. Recombinant cells are well defined and therefore easy to handle.[29] Expression of genes can be controlled by inducible promoters.[30,31] This makes it possible to regulate the expression to optimize enzymatic activities and to allow the cells to grow to high densities before the induction phase. Generally, inducers are not substrates

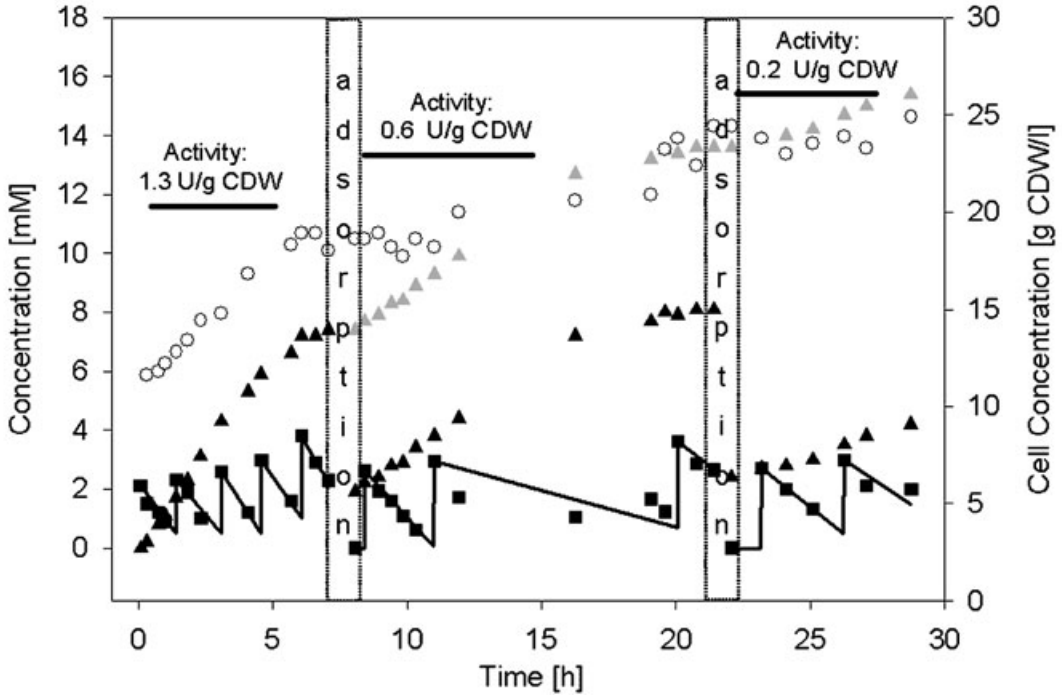

Figure 7. Biotransformation of benzonitrile to *cis*-1,2-dihydroxy-3-cyanocyclohexa-3,5-diene using *E. coli* JM101 (pTEZ30) cells in a reactor with integrated *in situ* product removal. Substrate was added repeatedly. After 7.5 and 21.5 hours, bioreactor medium was passed over an external loop packed with activated charcoal. ○, cell amount; ▲, product concentration; ▲, total amount of product formed; &ablock, substrate concentration.

for the dioxygenases and can easily be separated from the dihydrodiols at the end of the reaction. Background activities can easily be evaluated with control experiments whereas the effects of the other enzymes on stereochemistry of the product in mutant strains are difficult to interpret.[32,33] Therefore, it is generally accepted that recombinant cells will be more efficient in whole-cell *cis*-dihydroxylation processes especially for the non-natural substrates such as aromatic nitriles. Here we showed that the use of efficient and stable expression systems for dioxygenases can result in high volumetric productivities for *cis*-dihydroxylation processes. The development of efficient and stable recombinant cell expression systems will also have a high impact on the synthetic applicability of engineered dioxygenases, which are steadily increasing in number.[34–37]

The *alk* Regulatory System as an Expression System for CDO.

Recombinant cells have been used for dihydroxylation processes based on whole cells to produce several different *cis*-cyclohexadienediols.[29,38,39] However, in most expression systems used so far, such as pDTG601,[40] pKST11,[41] pDTG141,[42] p1/1,[39] pLAC365, pTAC365,[43] and pTCB144,[14] the dioxygenase genes were cloned downstream of promoters such as the *lac* or *tac* promoter. Such promoters are known to be not tightly regulated and the leakiness of these regulatory systems have been shown before.[43] Tightly controllable promoters enable the separation of the growth and enzyme production phases and therefore both phases can be optimized to yield a maximum volumetric productivity. This will be very useful for *cis*-dihydroxylations of non-natural substrates like aromatic nitriles. The non-induced cells harboring pTCB144, where the CDO genes were cloned downstream of *lac* promoter resulted in 26% of the activity of the cells that were induced for 4 hours (data not shown). On the other hand, the *alk* promoter of the *alk* regulatory system of *Pseudomonas putida* GPo1, *alkBp,* allows a tightly regulated gene expression in *E. coli*.[31] Recombinant cells containing expression systems based on the *alk* regulatory system have been successfully used as a biocatalyst for whole cell biotransformations on a preparative scale by several groups.[31,44–48] In our experiments, CDO gene expression was tightly controlled by *alkBp* in pTEZ30 and the non-induced *E. coli* JM101 (pTEZ30) cells did not show any activity.

Selection Markers for a Stable Expression

A drawback of the plasmid encoded production systems conferring resistance to β-lactam antibiotics like ampi-

cillin, penicillin G, and amoxicillin, is the need for addition of antibiotics to maintain the stable production.[49] In high cell density cultures, the β-lactamase produced by cells cleaves one bond of the four-membered ring of β-lactam antibiotics. This results in the disappearance of the antibiotic resistance and leads to the low plasmid stability. During the biotransformation of benzonitrile, the low stability of the plasmid pTCB144, conferring ampicillin resistance, resulted in decreasing specific activities and volumetric productivities. This shortened the biotransformation time and resulted in low product concentrations. On the other hand, the expression plasmid pTEZ30 conferring kanamycin resistance was stable and enabled the specific activity to increase during the biotransformation. Other antibiotics functioning as a protein synthesis inhibitor like tetracycline or neomycin can also be used as a stable selection marker.

During the biotransformation of benzonitrile, *E. coli* JM101 (pTEZ30) efficiently expressed the dioxygenase genes resulting in significant increases in specific activities and volumetric productivities. We showed, that once the efficient expression system was achieved, the volumetric productivity of the *cis*-dihydroxylations became limited by product toxicity. The toxic effects of the aromatic substrates and the product *cis*-diols have been well examined and have been solved by the use of different feeding strategies,[50] regulated substrate addition,[51] two-liquid phase reaction media,[52] and *in situ* product recovery.[22,23] 46% of the specific activity could be recovered by removing the product from the reaction medium by adsorption. The decrease in the specific growth rate and specific activity after the adsorption might be due to adsorption of some nutrients that are essential to the cells for the activity and/or because of the stressed or partially permeabilized as a result of high product concentrations. Therefore adsorption cycles can be performed earlier (before the amount of product reaches the toxic concentration) which may lead to a further increase and high recovery of the specific activity. The results showed clearly that the volumetric productivity of the biotransformations using the expression system *E. coli* JM101 (pTEZ30) could be further increased and the reaction time can be prolonged if the product can be removed from the reaction medium to non-toxic concentrations during the reaction.

Substrate Range

To date there are very few reactions described where aromatic nitriles were converted to the corresponding *cis*-dihydrodiols. Toluene dioxygenase (TDO) catalyzes the biotransformation of benzonitrile to *cis*-(1S,2R)-1,2-dihydroxy-3-cyanocyclohexane-3,5-diene,[53] (2-cyanoethyl)benzene to (5*S*,6*R*)-1-(2-cyanoethyl)-5,6-dihydroxycyclohexa-1,3-diene[54] and *trans*-cinnamonitrile to (*trans*-cinnamonitrile)-*cis*-2,3-dihydrodiol.[55] CDO catalyzes the transformation of benzonitrile to *cis*-dihydroxy-cyclohexadienecarbonitrile and benzyl cyanide to 1-(*cis*-1,2-dihydroxy-3,5-cyclohexadienyl)-acetonitrile (main product 98%), 2-(*cis*-2,3-dihydroxy-4,6-cyclohexadienyl)-acetonitrile, and 1-(*cis*-3,4-dihydroxy-1,5-cyclohexadienyl)-acetonitrile.[18] In our previous work we described the *cis*-dihydroxylation of cinnamonitrile to *trans*-3-[(5*S*,6*R*)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile.[16] Here we showed that *E. coli* JM101 (pTEZ30) carrying CDO was able to dihydroxylate various substituted benzonitriles as well as cinnamonitrile and benzyl cyanide to the corresponding *cis*-dihydrodiols. Regiospecific dihydroxylation occurred at the 1,2 positions. Enantiomeric excesses of the products are in the range of 42.9–97.1%. Differently, the toluene dioxygenase-catalyzed dihydroxylation of benzonitrile and (2-cyanoethyl)benzene results in *cis*-diols with very high enantiomeric excess.[53,54] It was also shown before that the enantiomeric excess of the products from CDO-catalyzed reactions can be significantly different from those of TDO-catalyzed reactions.[18] Our results can be a basis for future work to learn about the differences in enantiomeric excess. Our ee values were determined by analyzing the BB derivatives of the products with GC-MS. When a single peak was obtained, we could not determine whether the peak was the result of a pure enantiomer with a very high ee or enantiomers that could not be separated under the analytical conditions.

In our previous work the absolute stereochemistry of cinnamonitrile has been determined to be *trans*-3-[(5*S*,6*R*)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile.[16] The absolute stereochemistry of the other products could not be determined because the single enantiomers of the products were not available to us as standards.

The specific activity of *E. coli* JM101 (pTEZ30) cells towards the aromatic nitriles tested (1.7–4.7 U/g cdw) were in the same range as the specific activity of the *E. coli* DH5α (pTCB144) carrying the same enzyme towards other substrates like benzene (4.3 U/g protein), toluene (9.1 U/g protein), and chlorobenzene (8.7 U/g protein).[18] The isolated yields of the biotransformation of substrates (other than benzonitrile) in reactors were between 3–52%. One way to increase the yields of these reactions is to optimize the reaction conditions. In our set-up, substrate and product concentrations were not measured continuously during the reaction and substrate was added repeatedly every hour. To counter the substrate and product toxicity, controlled addition of the substrate[16,51] and *in situ* product removal can be integrated.[20;23] Another way to increase the yields is to improve the isolation and purifications steps. Continuous phase extraction with ethyl acetate can be used as an alternative to the evaporation of the aqueous phase[29] to improve the yields. The yields after the reactions are significantly higher than those after purification (Table 4). This shows that purification yields can also be improved significantly.

Conclusion

Several improvements in process design for *cis*-dihydroxylations have been achieved based on the use of different feeding strategies,[50] regulated substrate addition,[51,56,57] two-liquid phase reaction media,[52] and *in situ* product recovery.[22,23] However, these advances need to be complemented with biocatalyst design where the tailored whole cells are used as biocatalyst. Here, we showed that the volumetric productivity of the *cis*-dihydroxylation processes could be increased significantly by use of an efficient and stable recombinant expression system as biocatalyst. Once the expression is stable, the volumetric productivity becomes limited by product toxicity. Well-established ISPR approaches such as adsorption on a solid matrix[20,22,23] can be applied to remove the toxic effect of the product. This may enable one to further increase the efficiency of the overall process based on the recombinant biocatalyst.

We applied the biocatalyst *E. coli* JM101 (pTEZ30) carrying CDO for the regio- and stereoselective dihydroxylation of benzonitrile, *ortho*-, *meta*- and *para*-substituted benzonitriles, as well as cinnamonitrile and benzyl cyanide. *cis*-Dihydroxylations were at the 1,2 positions and the products have 42.9 to 97.1% enantiomeric excess. Further work aimed at screening for a nitrilase for the selective hydrolysis of the nitrile group is under way. This may enable the production of new chiral synthons in significant yields and amounts.

Experimental Section

Strains, Plasmids, Materials, and Chemicals

The strains and plasmids used and constructed are listed in Table 1. Restriction and DNA modification enzymes were obtained from Roche Molecular Biochemicals (Rotkreuz, Switzerland) and New England Biolabs (Schwalbach, Germany). The High Pure Plasmid Isolation Kit of Roche Diagnostics GmbH (Indianapolis, IN, USA) was used for small-scale plasmid DNA isolations following the supplier's protocol. Amberlite resins XAD4 and XAD7 were obtained from Rohm & Haas (Frankfurt, Germany), activated charcoal (TYP: DGF 0.5–2.5) was a gift from CarboTech Aktivkohlen GmbH (Essen, Germany), Lewatit 1065 and Sepabeads were obtained from Bayer AG (Leverkusen, Germany), and Mitsubishi Chemicals (New York, USA), respectively. 3-Fluorobenzonitrile, and dicyclopropyl ketone were obtained from Aldrich and other chemicals were obtained from Fluka AG (Buchs, Switzerland).

Media and Cultivation Conditions

We used Luria-Bertani (LB) broth (Difco, Detroit, MI.) complex or M9 minimal medium[58] supplemented per liter with 1 mL of trace elements solution US*, the composition of which is described elsewhere.[59] Solid media contained 1.5% (wt/vol) agarose (Difco). M9 medium contained glucose as a carbon source 0.5% (wt/vol), thiamine 0.001% (wt/vol), and antibiotic: ampicillin (100 mg/L) for cells containing pTCB144; kanamycin (50 mg/L) for cells containing pTEZ30. Cultivations for cloning procedures were carried out at 37 °C. Other precultures and cultures of the *E. coli* strains were grown at 30 °C on a horizontal shakers at 200 rpm. The cells containing pTCB144 and pTEZ30 were induced with 0.5 mM isopropyl-β-D-thiogalactosidase (IPTG) or 0.05% (vol/vol) dicyclopropyl ketone (DCPK), respectively.

Construction of the Expression Plasmid pTEZ30

The cloning and DNA modification protocols used have been described elsewhere.[58] 0.97 kb of the *tcbAa* genes of pTCB144 were amplified by polymerase chain reaction (PCR) using the two primers (P1: 5′ ACGTGAGGAG*CATATG*AATCACACCGAC 3′) and (P2: 5′ CGTTC**GGCGCGCC**T*GGATCC*CATGTCCGAACC 3′). Three cloning sites were introduced with these primers: an *NdeI* site in the forward primer (in italics), and a *BamHI,* and *AscI* site in the reverse primer (in italics and bold, respectively). The PCR product and the plasmid pSPZ2MA (carrying *alkS* and *alkBp*) were digested with *NdeI* and *AscI* followed by insertion of the PCR product into pSPZ2MA (Figure 8). The resulting plasmid was called pTEZ10. The plasmid pTCB144 was digested with *ApaI* and *BamHI* and the 3.17 kb fragment was inserted into the plasmid pTEZ10 after digestion with *ApaI* and *BamHI*. The resulting plasmid carrying *alkS, alkBp, tcbAa*, *tcbAb*, *tcbAc*, *and tcbAd* and Ap^r genes was called pTEZ20. Then pTEZ20 was partially digested with *NotI* and the resulting 8.27 kb fragment was isolated. The unique *NotI* site of pBRNSKMΔ (carrying a Km^r gene) was cut and ligated with the 8.27 kb fragment of the partial digestion. The final plasmid carrying *alkS, alkBp, tcbAa*, *tcbAb*, *tcbAc*, *tcbAd*, and Km^r was called pTEZ30.

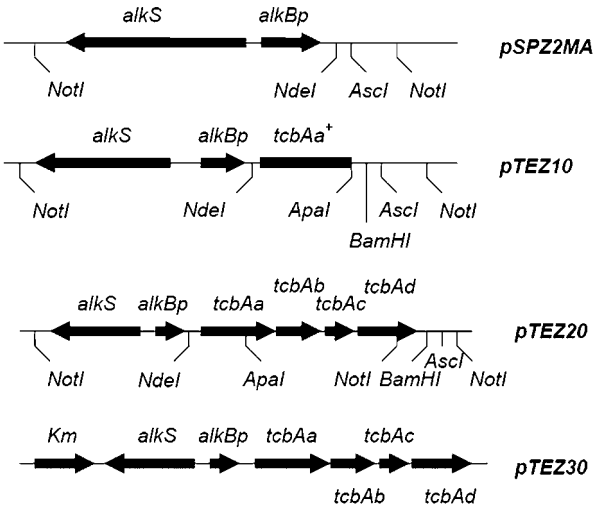

Figure 8. Structure of the genetic elements constructed in this study. *tcbAa*$^+$: The first 0.97 kb of the tcbAa gene.

Cell Growth and Biotransformations in Shake-Flasks

A single colony was transferred into 5 mL LB medium and incubated for 10 hours. 100 mL M9 medium were inoculated with 1 mL of the LB preculture and incubated overnight. Two times 40 mL M9 medium were inoculated to an A_{450} of 0.1 using M9 preculture and incubated. Cells were grown to an A_{450} of $\sim$0.3 and one of the two cultures was induced. Cell growth of both cultures was followed spectrophotometrically. For biotransformation experiments in shake- flasks, cultures were centrifuged at 4 °C after 4 hours of induction, washed with an equal volume of M9 medium without carbon source, and resuspended in 20 mL M9 medium to a cell concentration of 0.29 g cdw/L and 1.3 g cdw/L for the biotransformation of naphthalene, and aromatic nitriles, respectively. Naphthalene and solid aromatic nitriles were added from stock solutions dissolved in methanol to a final concentration of 1 mM and 2 mM, respectively. Liquid aromatic nitriles were added directly to a concentration of 2 mM. Samples were taken and centrifuged at 4 °C and at 20,000 × *g* for 5 minutes. Supernatants were frozen at −20 °C until the measurements. All assays were performed twice.

Biotransformation of Benzonitrile on 2-L Scale

Biotransformation of benzonitrile in a reactor on 2-L scale was performed as described elsewhere.[16] The cells were induced with the addition of 0.5 mM IPTG or 0.05% DCPK to the reactor. Benzonitrile was added to a final concentration of 2 mM and then added repeatedly to keep the substrate concentration between 0.5 and 2 mM. Samples were taken at different time points, diluted 10 times with ice-cold acetonitrile and centrifuged at 4 °C. Supernatants were filtered through a cellulose membrane filter 0.2 μm (Spartan, Schleicher & Schuell GmbH, Germany) and analyzed. Biotransformations were carried out twice and nearly identical results were obtained.

Biotransformation of Aromatic Nitriles in Reactors on 300-mL Scale

E. coli JM101 (pTEZ30) cells were grown and induced in a bioreactor on a 2-L scale as described above. Four hours after induction, cells were transferred into six sterile Sixfors bioreactors) Infors, Bottmingen, Switzerland) (300 mL cell suspension to each). The temperature was kept at 30 °C and the pH was adjusted to 7.2 with 25% NH_4OH and 25% phosphoric acid. The reactors were aerated at a rate of 2 liters per minute and stirred at 300 rpm. Biotransformation was started by addition of substrate. An additional amount of 0.3 mmol substrate was added repeatedly every hour from a 100 mM stock solution in MeOH. Liquid substrates were added directly to the same concentration. The reaction was continued for 7 hours. At the end of the biotransformations, samples were taken from each reactor, treated as described above and analyzed *via* HPLC.

Product Recovery and Purification

The medium in the reactor was centrifuged at 500 rpm and at 4 °C for 15 minutes to separate the cells from the medium. To decrease the volume, the supernatant was evaporated in a rotary evaporator at room temperature to a volume of approximately 50 mL. Then it was diluted to 1 liter with ice-cold acetonitrile for the precipitation of salts and proteins. The supernatant was then evaporated to approximately 3 mL and the product was purified on a 1.9 cm i.d. × 30 cm silica gel (60) column with 80% ethyl acetate and 20% hexane as the eluent.

Analytical Procedures

HPLC Analysis: The formation of metabolites was measured by a Hewlett Packard HP 1050 Ti reversed phase HPLC with a Nucleosil C18 RP column (pore size, 100 Å; particle size 5 μm; inner diameter, 12.5 cm × 2 mm) (Macherey-Nagel AG, Oensingen, Switzerland). The UV detector (HP 1040M Series II Diode-Array) was set at a wavelength range of 210–600 nm. Product formation from the naphthalene biotransformation was quantified with the help of an authentic standard. Products from biotransformations of aromatic nitriles were purified, identified, and used as standards for quantifications.

GC-MS Analysis: After purification of the products, their *n*-butylboronate derivative (BB derivative) was prepared as described elsewhere[18] and 1 μL of the diluted samples was injected at 50 °C into a series 8000 GC, combined with an MD800 mass spectrometer (Fisons Instruments, Beverly, MA, USA), on a SPB-1 column (SUPELCO, length, 30 m; inner diameter, 0.32 mm; film thickness, 0.25 mm fused silica). The column temperature was increased to 250 °C at 10 °C/min. Electron impact (EI) spectra were obtained at −70 eV. GC-MS raw data were analyzed using the software package MassLab (Fisons).

The enantiomers were separated and quantified by GC-MS. The enantiomers were separated as their BB derivatives on a cyclodextrin column BGB-172 (length, 30 m; inner diameter 0.25 mm; film thickness, 0,25 μm), obtained from BGB Analytik AG (Anwil, Switzerland). Samples of the BB derivatives were diluted with cyclohexane and 1 μL of the diluted samples was injected on column at 120 °C. The column temperature was programmed as follows: 2 °C/min to 230 °C, and 10 °C/min to 250 °C. All samples were analyzed by electron ionization (EI, −70 eV) using full scan monitoring (m/z = 50 to 400). The enantiomeric excess (ee) was defined as $(A_1 - A_2)/(A_1 + A_2) \times 100$, where A_1 and A_2 are the peak areas of the BB derivatives of the two *cis*-dihydrodiol enantiomers, and A_1 was the larger peak area.

^{1}H NMR Spectroscopy: NMR spectra were recorded at 300 MHz using the Bruker Avance 300-MHz instrument with QNP probehead and autosampler at ambient temperature. Tetramethylsilane was used as an internal reference and fully deuterated DMSO was used as solvent, unless stated otherwise. Chemical shifts are given in parts per million relative to tetramethylsilane (at 0 ppm).

Partitioning in Aqueous Two-Phase Systems

Aqueous two-phase systems were composed according to standard procedures.[60] 30 mg benzonitrile (BN) or *cis*-1,2-dihydroxy-3-cyanocyclohexa-3,5-diene (BNDD) were added to a vial containing about 5 mL upper phase and 5 mL lower phase. The vial was sealed and shaken at 22 °C. After 90 minutes both phases were separated by centrifugation and analyzed with HPLC for benzonitrile or BNDD.

Determination of Adsorption Isotherms

Adsorbent (XAD4, XAD7, charcoal, and Lewatit 1065, Sepabeads) was added (0.10–1.00 g) to a 10 mL aqueous solution of benzonitrile (30 mg) or BNDD (30 mg), the mixture was sealed and stirred at 22 °C. After 90 min, the liquid phase was analyzed for benzonitrile or BNDD using HPLC as described above. Their concentrations in the solid phase were derived from the mass balance.

Determination of Effects of Adsorbents and Benzonitrile on Biomass Accumulation

The *E. coli* JM101 (pTEZ30) cells were grown in 19 mL M9 medium to a cell concentration of 0.35 g cdw/L and then 0.5 g adsorbent was added. The suspension was incubated on a shaker at 30 °C and after 9 h the amount of cells was determined. These experiments were repeated with the addition of benzonitrile together with the adsorbents.

Biotransformation of Benzonitrile with Integrated Product Recovery

Biotransformation of benzonitrile in a 2-L reactor was performed as described. 7.5 h after biotransformation had started, the bioreactor medium was passed over an external loop, packed with 35 g of activated charcoal by means of a peristaltic pump. Details about the dimensions, set up, and preparation of the external loop are described elsewhere.[61] A second adsorption cycle was performed after 17 h. Thereafter the biotransformation continued for another 7 hours.

Acknowledgements

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