Asymmetric Dihydroxylation of Cinnamonitrile to *trans*-3-[(5*S*,6*R*)-5,6-Dihydroxycyclohexa-1,3-dienyl]-acrylonitrile using Chlorobenzene Dioxygenase in *Escherichia coli* (pTEZ30)

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Abstract: Asymmetric *cis*-dihydroxylations of aromatic compounds are catalyzed by bacterial dioxygenases. In order to prevent through conversion, either dihydrodiol dehydrogenase blocked mutant strains or recombinant bacterial cells are used as biocatalysts for synthetic purposes. We characterized the *cis*-dihydroxylation of cinnamonitrile by chlorobenzene dioxygenase (CDO) in recombinant *E. coli* on different reaction scales. The absolute stereochemistry of the product was determined to be *trans*-3-[(5S,6R)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile. The cells showed a maximum specific activity of 3.76 U/g cdw in shake-flask experiments. Stable expression of the dioxygenase genes in *E. coli* JM101 (pTEZ30) resulted in increasing volumetric productivities. The maximum volumetric productivities of 80 and 92 mg product/L/h were achieved on 2-L and 30-L scales, respectively. The specific growth rate correlated with the volumetric productivity during the biotransformations. An average volumetric productivity of 40 mg product/L/h in reactors on 2-L and 30-L scales resulted in 0.96 and 16.4 g of isolated product at the end of the biotransformations. This points out the need for metabolically active cells and controllable expression systems for achieving high volumetric productivities for cofactor dependent biooxidations. We have now applied this concept for the asymmetric dihydroxylation of the non-natural substrate cinnamonitrile using multicomponent CDO in tailored *E. coli* JM101 in long-term reactions.

Keywords: asymmetric synthesis; biocatalysis; biotransformations; chlorobenzene dioxygenase; dihydroxylation; scale-up

Introduction

Oxidation of aromatic compounds to *cis*-dihydrodiols is catalyzed by multicomponent bacterial dioxygenases (Figure 1).[1,2] These reactions are of special interest, since *cis*-dihydrodiols are valuable building blocks for the stereoselective synthesis of molecules containing multiple chiral centers.[3–5]

Thus far, several ring-hydroxylating dioxygenases have been identified[6] including naphthalene,[7–9] toluate,[10] benzene,[11] toluene,[12] benzoate,[13] biphenyl,[14] 2-nitrotoluene,[15] chlorobenzene[16] and tetrachlorobenzene dioxygenases.[17] Since Gibson and co-workers first described the isolation of an optically active diol,[18] more than 300 vicinal arene *cis*-diols have been identified.[4,19] Biocatalysts were *cis*-dihydrodiol dehydrogenase blocked mutants of *Pseudomonas* species, *Sphingomonas yanoikuyae* and *Alcaligenes eutrophus*.[2,20] Alternatively, the biotransformations used recombinant cells containing dioxygenases like toluene dioxygenase,[12,21]

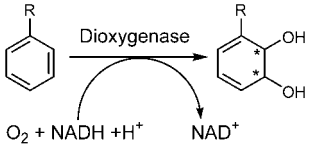

Figure 1. Dioxygenase catalyzed biotransformation of aromatic compounds to the corresponding *cis*-dihydrodiols.

naphthalene dioxygenase,[22–24] biphenyl dioxygenase,[25] chlorobenzene dioxygenase,[16] and phenanthrene dioxygenase[26,27]. Some of the biotransformation processes using mutant strains have been well characterized, developed and scaled up.[28–31] Biotransformations with recombinant cells expressing dioxygenase genes were performed on laboratory and industrial production scale yielding gram to multi-kilogram amounts of *cis*-cyclohexadiene diols.[32]

In general, aromatic nitriles are interesting synthons, because the enzymatic dihydroxylation may be coupled to a selective hydrolysis of the nitrile group or subse-

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quent cycloaddition reactions to increase the pool of chiral synthons. We now examine the efficiency of a bioprocess for the highly selective asymmetric dihydroxylation of non-natural cinnamonitrile to *trans*-3-[(5*S*,6*R*)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile (**1**) by chlorobenzene dioxygenase (CDO) in recombinant *E. coli* JM101 (pTEZ30).[33] We examined the efficiency of the biocatalyst with a focus on the volumetric productivity by comparing biotransformations on different scales. We discuss advantages and limitations of our recombinant expression system based on the alkane oxidation promoter P_{alkB} for arene *cis*-diol production as well as the determination of the absolute stereochemistry of the title compound.

Results

The specific activity of CDO in *E. coli* JM101 (pTEZ30) for cinnamonitrile was determined in 20-mL shake flasks (Figure 2). CDO showed a maximum specific activity of 3.8 Units per gram cell dry weight (U/g cdw) and decreased to 0.3 U/g cdw during the biotransformation resulting in an average specific activity of 2.25 U/g cdw.

The maximum specific activity of CDO in *E. coli* JM101 (pTEZ30) for cinnamonitrile is in the same range as the specific activities of CDO for benzonitrile and naphthalene (3.9 and 2.86 U/g cdw, respectively).[33]

Biotransformation of Cinnamonitrile on a 2-L Scale

Specific activities of whole cell biocatalysts are frequently lower under process conditions in bioreactors than in short term assays, e.g., in shake flasks.[34–38] The biotransformation of cinnamonitrile was therefore performed in a 2-L reactor and process parameters were determined. The biotransformation was started by the addition of 1.6 mM cinnamonitrile, after which the cells grew to 8.2 g cdw/L at a growth rate similar to that before substrate addition. The substrate concentration decreased to 1.1 mM (Figure 3). After addition of another 1 mM substrate, the total substrate concentration increased to 2.1 mM and the cell concentration stayed almost constant afterwards. At time 9 h and 23 h additional 2 mM and 1 mM substrate was added, respectively.

Three distinct phases of product formation could be distinguished: a rapid linear product formation over 3.5 h up to a product concentration of 1.4 mM with an average specific activity of 0.9 U/g cdw, a slower linear increase in product concentration to 2.16 mM with an average specific activity of 0.27 U/g cdw for the next 5 h, and a period towards the end of the biotransformation where the product concentration did not increase any further. The second phase might be longer as there is product formation between 9 and 21 hours. However, an average volumetric productivity of 0.14 U/g cdw in this time range points to a decrease in volumetric productivity towards the third phase. The product formation rate decreased continuously after the cells stopped growing (Figure 5). This suggests a relationship between the metabolic state of the cells and specific activity of the cells. In total, 5.6 mM of substrate was added and the product concentration reached 2.93 mM after 21 h, which corresponds to 0.48 g/L. Total mass balance showed that part of the substrate was stripped during the biotransformation and this resulted in a 52% yield after 25 h.

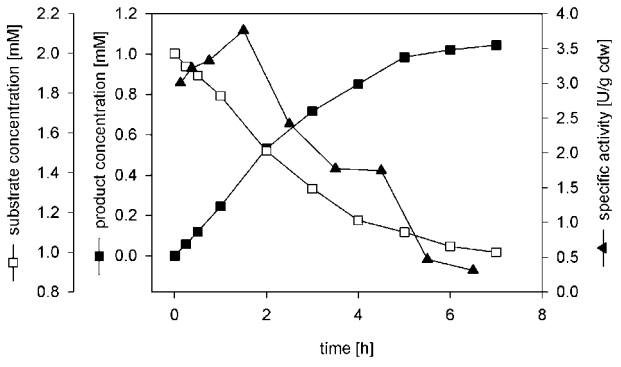

Figure 2. Biotransformation of cinnamonitrile using *E. coli* JM101 (pTEZ30) in shake flasks. Reaction was performed using resting cells (1.29 g cdw/L) in 20-mL shake flasks.

Biotransformation of Cinnamonitrile on a 30 L-Scale

Scale-up of whole cell biocatalytic reactions requires not only an increase in volume but also establishment of appropriate conditions for cell growth, efficient synthesis of the relevant enzyme, and a bioreactor environment that ensures stable bioconversion of substrate over a

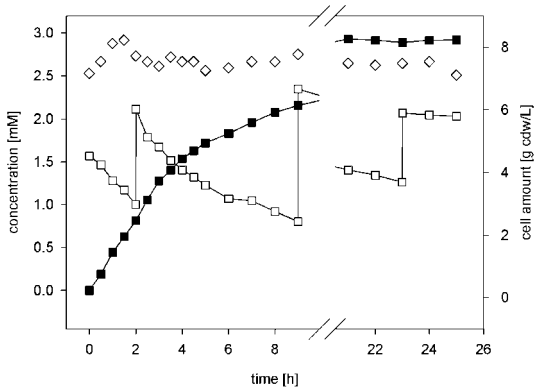

Figure 3. Biotransformation of cinnamonitrile using *E. coli* JM101 (pTEZ30) in a bioreactor on a 2-L scale: cell amount (◇); product concentration (■); substrate concentration (□). After a batch fermentation, glycerol was fed to the reactor and the cells were induced for 3 h with DCPK. Substrate was added sequentially.

long period of time. In order to evaluate the scale-up potential of *cis*-dihydroxylations using recombinant cells we carried out the biotransformation of cinnamonitrile using *E. coli* JM101 (pTEZ30) in a reactor with a 30-L working volume. Four stages of growth could be distinguished (Figure 4): a rapid linear growth with a specific growth rate of 0.74 h^{-1} for 3 h to 15.2 g cdw/L before the biotransformation was started, a stationary period during the first hour of biotransformation at almost 14 g cdw/L, a less rapid but still linear growth with a specific growth rate of 0.47 h^{-1} for the next 5 hours to 25.5 g cdw/L, and a final stationary phase at almost 23 g cdw/L. During the first period, the cell growth did not appear to be affected by induction. Cell growth deceased in the second phase after the addition of the substrate, resumed in the third phase, and leveled off in the last stage, which is probably because of the toxic effect of the product which accumulated in the reactor. Toxic effects of dienediols on cell growth have been observed before.[31,39,40] At toxic substrate and product concentrations, the pores formed in the cytoplasmic membrane may be large enough to cause complete loss of the membrane integrity, resulting in cell death.[41]

Formation of *trans*-3-[(5*S*,6*R*)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile (**1**) started directly after the addition of cinnamonitrile with a rate of 0.29 U/g cdw until 6.5 h reaction time. Then net cell growth stopped, but the rate of product formation decreased only slowly until the end of the transformation period giving a final product concentration of 3.32 mM (0.55 g/L). The initial specific activity increased from 0.23 U/g cdw after induction to a maximum of 0.52 U/g cdw at 2.5 h then decreased again to 0.07 U/g cdw at 12 h. During this time, *E. coli* JM101 (pTEZ30) produced 14.6 g **1**. This corresponded to an average volumetric activity of 4.2 U/L or an average volumetric productivity of 40 mg/L/h of **1**.

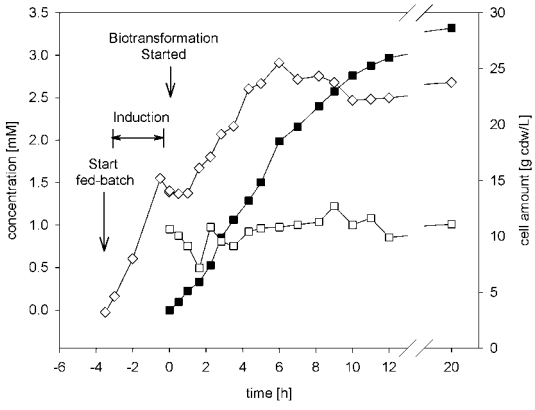

Figure 4. Biotransformation of cinnamonitrile using *E. coli* JM101 (pTEZ30) in a bioreactor with a 30-L working volume: cell amount (◇); product concentration (■); substrate concentration (□). Cells were induced with DCPK and the substrate concentration in the reactor was maintained below 1.2 mM through manual additions of it.

The maximum and the average volumetric productivities, as well as the activity profile of **1** formation are similar on the 2-L and 30-L scales. The decrease of productivity towards the end of the biotransformation was also observed in the shake flask experiment and may have been the consequence of toxicity of the substrate and product. The decrease in specific activity of the biocatalyst correlated with the decrease in volumetric productivity (Figure 5). After cell growth, the dissolved oxygen tension was constant (20–40% of saturation) throughout the biotransformation. This constant oxygen consumption indicates metabolic activity of the biocatalyst even in the stationary phase with concomitant product formation. This relationship points to a coupling of the metabolic state of the cells with the specific activity of CDO as depicted in Figure 5.

HPLC analysis of the products did not point to the formation of by-products during the reaction. *cis*-Dihydrodiols are highly soluble in aqueous solutions but can dehydrate to the corresponding phenols[42] under acidic conditions and high temperatures. This happened during the evaporation step for product recovery: less than 2% of **1** were dehydrated to the phenol. Here, continuous phase extraction with ethyl acetate might be used as an alternative to the evaporation of the aqueous phase.[32] Table 1 lists the process parameters of our cinnamonitrile biotransformation using *E. coli* JM101 (pTEZ30) on different scales. The downstream processing and the purification yields with the corresponding purities are given in Table 2. The *cis*-dihydroxylation of cinnamonitrile using *E. coli* JM101 (pTEZ30) is a scaleable process as cell growth and stability of the recombinant expression system are not significantly affected by the scale-up. The process is limited by the volumetric productivity, which can be improved by keeping the cells in a metabolically active state. High specific activities using growing cells compared to resting cells were pointed out by Walton and Stewart.[43] It

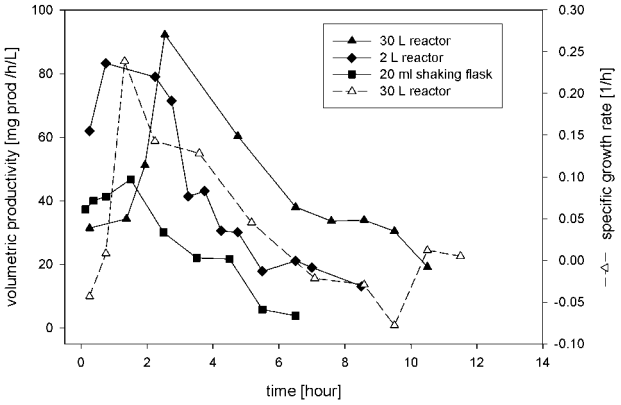

Figure 5. Volumetric productivities of biotransformation of cinnamonitrile using *E. coli* JM101 (pTEZ30) in shake-flasks, and in reactors on 2-L and 30-L scales, and the specific growth rate of the cells in a 30-L reactor.

was shown that growing *E. coli* BL21(DE3) pMM4 have higher activities for the oxidation of cyclohexanone to ε-caprolactone than the resting cells. Higher volumetric productivities were obtained with resting cells, but this is mainly due to high cell densities used during the biotransformations. Thus, induction and start of biotransformation at high density of growing cells may result higher volumetric activities.

Proof of Absolute Stereochemistry

The crude diol **1** (sticky, crystalline solid in appearance) was purified by recrystallization from CH_2Cl_2-EtOAc/hexane to yield beige microscopic needles. The pure material had a melting point of 119–121 °C and α_D +337.9° (EtOH, *c* 1.0).

To determine the absolute stereochemistry of **1** we decided on a convergent match with a compound that could be rapidly synthesized from diol **2** whose absolute configuration[44] as well as enantiomeric purity[45,46] are well known and proven by several total syntheses.[47–53] To this end a crude sample of **1** (as isolated from the fermentation) was subjected to a reduction with potassium azodicarboxylate (PAD) in order to increase the stability of subsequently synthesized intermediates. The PAD reduction provided selectively nitrile **3**, which was purified and converted to acetonide **4** as shown in Figure 6.

This material, purified by flash chromatography, gave an α_D of +208.3° (CH_2Cl_2, *c* 1.0). Diol **2** was obtained from the whole cell fermentation of bromobenzene with *E. coli* JM109 (pDTG 601), a strain developed by David Gibson and expressing the toluene dioxygenase genes.[12,32] This material was also reduced with PAD and the diol protected as acetonide **5**.[54] Heck reaction[55,56] of **5** with freshly distilled acrylonitrile in the presence of $Pd(OAc)_2$, PPh_3, Et_3N in DMF at 100 °C gave, after 20 h, a 20% yield of **4**. The yield of **4** was improved to 76% [taking into account recovered starting material (54%)] by using an excess of acrylonitrile (3.0 equivs.) and a longer reaction time (68 h).

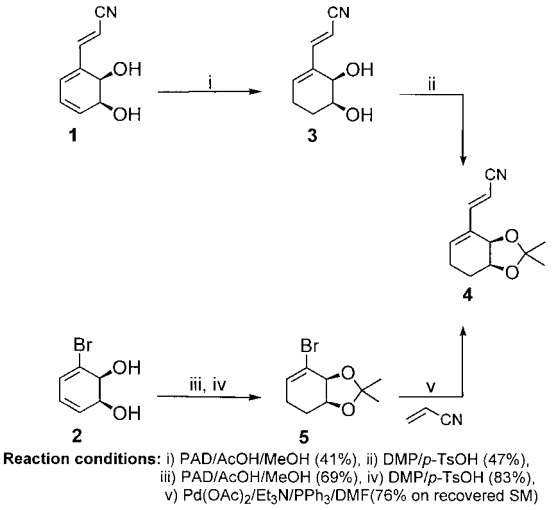

Figure 6. Determination of the absolute stereochemistry of diol **1** by chemical correlation.

The proton NMR as well as IR of **4** obtained from diol **1** matched exactly those of the synthetically derived **4**. Mixed ^{1}H-NMR showed no deviation from the spectra of either sample. The optical rotation of synthetic **4** was almost identical at +207.5° (CH_2Cl_2, *c* 1.0).

Thus the absolute stereochemistry of the hydroxyl groups in diol **1** is β, as expected from the toluene dioxygenase processing of monosubstituted aromatics. To date there has been no deviation observed in the β-configuration of the diols derived from such compounds (excepting the diols of various benzoic acids which also possess different 1,2-regiochemistry).[47]

Discussion

Biocatalytic oxidations use oxygen as a cheap and environmentally friendly oxidant, and often have high regio-, enantio- and stereoselectivities.[57] The oxidation of aromatic compounds to *cis*-dihydrodiols catalyzed by bac-

Table 1. Bioprocess data of the *cis*-dihydroxylation of cinnamonitrile on different scales

Parameter	Unit	Biotransformation of cinnamonitrile in		
		Shake flask	Bioreactor, lab scale	Bioreactor, technical scale
Working volume	L	0.02	2	30
Total amount of product formed	g	0.0034	0.96	16.4
End product concentration	g/L	0.17	0.48	0.55
Bioconversion	%	49	72[a]	74[b]
Maximum growth rate	1/h	–	0.14	0.24
Maximum specific activity	U/g cdw	3.76	0.97	0.52
Average specific activity	U/g cdw	2.25	0.54[a]	0.24[b]
Maximum volumetric productivity	mg prod/L/h	0.94	80	92
Average volumetric productivity	mg prod/L/h	0.49	40[a]	40[b]

[a] Value in 9 h.
[b] Value in 12 h.

Table 2. Downstream processing data.

	Amount of product [g]	Yield [%]	Purity
Reactor	16.4	74[a]	–[b]
Volume reduction	13.7	62	–[b]
Acetonitrile precipitation	11.6	52	–[b]
Purification on silica gel column[c]	9.2		62
	3.7		72
	2.1		76
	2		69
Absolute amount[d]	11.4	52	
Recrystallization		48[e]	100

[a] Bioconversion yield.
[b] Not isolated.
[c] Four different fractions collected.
[d] Total pure product amount.
[e] Yield of the recrystallization step.

terial dioxygenases is especially interesting since the chemical equivalent of such a reaction is unknown. There is only a single report of tetrahydroxylation of benzene under photolytic osmylation conditions.[58] The vast majority of the reported *cis*-dihydroxylations were performed with whole cells as biocatalysts, due to a low stability of the multicomponent system of dioxygenases and requirements of expensive cofactor.[2] As a result of such extensive studies the average volumetric productivity of the (mutated) wild-type cells on their natural substrates could be increased to 0.24–2 g/L/h over biotransformation times between 7–96 hours (Table 3).

Although the mutant cells have high activities towards their natural substrates, they often show lower activities for non-physiological compounds.[20,59] As presented in Table 3 average volumetric productivities of wild-type strains towards their natural substrates like toluene and naphthalene were as high as 400 mg prod/L/h, whereas the productivities of the *P. putida* and *Rhodococcus* sp. B264-1 towards a non-natural substrate indene were 44 and 32 mg prod/L/h, respectively. We ob-

Table 3. Microbial *cis*-dihydroxylation processes.

Strain	Enzyme	Substrate	Scale [L]	Biotrans. time [h]	Max. sp activity [U/gcdw]	cdw [g/L]	Avg. prod. [g/L/h]	Product conc. [g/L]	Ref.
P. putida UV4	TDO	Toluene		96	n.m	1.2[a]	0.24[b]	22.7[b]	[59]
P. putida UV4	TDO	Toluene	1.7	20	26–33	4–6[c]	0.41	8.2	[31]
P. putida UV4	TDO	Toluene	1.7[d]	12.5	158	4–6	2	25	[30]
P putida UV4	TDO	Toluene	1.7	7	132	1–1.5	1.0	6.8	[72]
P. putida	TDO	Indene	70[e]	8	n.m.	n.m.	0.044	0.35	[28]
Rhodococcus MA7249	n.m.	Toluene	1.5	70	n.m.	n.m.	0.25	18	[73]
P. putida (strain NG1)	TDO	Toluene	3.5	24	105	1.5[f, g]	0.8	24.0	[63]
P. fluorescens TTC1	NDO	Naphthalene	1.0	15	40	2.1	0.4	6.5	[20]
P. fluorescens TTC1	NDO	Dibenzofuran	0.8	10	n.m.	n.m.	0.160	1.6	[74]
Rhodococcus sp. B264-1	n.m.	Indene	23[e]	150	n.m	n.m.	0.032	4.8	[28]
E. coli JM109 (pKST11)	TDO	Toluene	1.5	96	n.m.	0.03[a]	0.014[b]	1.32[b]	[59]
E. coli TG2 (p1/1)	TDO	Toluene	5	7.9	35	1.2[g]	0.13	1.02	[75]
E. coli D160-1	TDO	Indene	15	24	n.m.	n.m	0.05	1.2	[76]
E. coli D160-1	TDO	Indene	23[e]	24	n.m	n.m	0.042	1	[28]
E. coli JM109 (pDTG601)	TDO	Diverse	10–15	n.m	n.m	n.m	n.m	0.06–15[h]	[32]

[a] Average cell dry weight.
[b] Average cell dry weight was used for the calculation of the value.
[c] 4–6 g/L paste of cells was resuspended.
[d] Two phase system consisting of 510 mL organic (10% toluene) phase and 1190 mL aqueous phase.
[e] Total volume of the reactor.
[f] Assumption: 50% of the cell dry weight is carbon.
[g] Average cell dry weight during the biotransformation time given.
[h] The minimum and the highest values obtained for the given substrates.

served 40 mg prod/L/h with *E. coli* JM101 and the non-natural substrate cinnamonitrile. Overexpression in a recombinant host does not necessarily result in higher activities than in the wild-type (Table 3).[59] However, recombinant hosts like *Escherichia coli* are well defined and therefore easy to handle. They can be grown to high cell densities on glucose as a cheap and non-toxic carbon source.[60,61] Induction of gene expression can be controlled by easily controllable promoters using non-aromatic inducers,[62] which are not substrates for the dioxygenases and can easily be separated from the dihydrodiols. Scale-up of recombinant cell biocatalysis is significant for synthetic purposes. The key points in scale-up of such systems are: efficient synthesis of the enzyme to high specific activities; high biocatalyst stability, namely high volumetric productivity over time; substrate and product toxicity; and downstream processing, product recovery. The last two points have been improved using (mutated) wild-type cells based on the use of different feeding strategies,[63] regulated substrate addition,[31] two-liquid phase reaction media,[30] and *in situ* product recovery.[64,65] Here we have described, on three different scales, the enzyme synthesis, and the biocatalyst stability for the production of an interesting product **1** with a focus on specific activity, and volumetric productivity over the reaction time, which determine the efficiency of the *cis*-dihydroxylation process (**1**). We determined the absolute stereochemistry of the hydroxyl groups in title compound **1** as β, as expected from the toluene dioxygenase processing of monosubstituted aromatics by matching a derivative with a material prepared by independent synthesis from a compound of known absolute stereochemistry (i.e., diol from bromobenzene). Efficient expression of the dioxygenase genes under the control of the *alk* promoter P_{alkB} in recombinant *E. coli* JM101 (pTEZ30) resulted in maximum volumetric productivities of 80 and 92 mg prod/L/h in a 2-L and a 30-L reactor, respectively. On both scales, the average volumetric productivity was 40 mg product/L/h (over 9 h in 2 L and over 12 h in 30 L). This points to a straightforward scalability of the *cis*-dihydroxylation of cinnamonitrile using recombinant *E. coli* JM101 (pTEZ30). Yet, the decrease in volumetric productivity at the end of the biotransformation indicated that the biocatalyst stability is limited and correlates with the metabolic activity of the cells which therefore should be examined in detail during the biotransformation. The link of growth rate of *E. coli* expressing *alk* genes with its biocatalytic activity has also been described earlier.[60] In our case, the factor limiting metabolic activity of the cells is most probably the presence of the product. Therefore, the *cis*-dihydroxylation processes using recombinant cells might be further improved by the regulated addition of the substrate[31] or the integration of *in situ* product removal[64,65] to minimize the toxic effect of the substrate and the product to the cells. This would allow a further increase of the volumetric productivity resulting in higher yields.

The activity of *E. coli* JM101 (pTEZ30) for cinnamonitrile in a reactor on a 2-L scale was very low (0.97 U/g cdw), compared to the activity of the (mutated) wild-type strains for toluene conversion (26–158 U/g cdw) in small bioreactors. However, the average volumetric productivity of 40 mg prod/L/h in both 2-L and 30-L scales made it possible to obtain 0.96 and 16.4 g amounts of **1** respectively, with a clear perspective of improvement in productivity. We expect that tightly controllable recombinant whole cell biocatalysts will allow optimization of the expression and enzyme activity. This will be very useful for *cis*-dihydroxylations of non-natural substrates like aromatic nitriles. This will also have a high impact on the synthetic applicability of engineered dioxygenases, which are steadily increasing in number.[66–69]

Conclusion

Recombinant cells have generally been used to overexpress dioxygenases for the production of *cis*-diols from various different substrates. Here we characterized the product and described the biotransformation of cinnamonitrile to *trans*-3-[(5*S*,6*R*)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile (**1**) using *E. coli* JM101 (pTEZ30) in different scales. The results show that recombinant hosts with a strong expression system, such as *E. coli* JM101 (pTEZ30), can be used to produce dioxygenases efficiently to perform *cis*-dihydroxylations up to technical scales. The cells should be maintained in a metabolically active state during the biotransformation in order to increase and maintain the volumetric productivity for long-term reactions. Controlled substrate addition and *in situ* product removal techniques can be integrated into the *cis*-dihydroxylation processes using recombinant cells in order to scale-up and improve the performance of these processes.

Experimental Section

Strain, Media, Cultivation and Chemicals

We used *E. coli* JM101 {*supE thi* Δ(*lac-proAB*) F′[*traD36 lacI*^{q}-Δ(*lacZ*)*M15 proAB*$^+$]}[70] as a recombinant host strain and pTEZ30 as an expression vector[33] containing chlorobenzene dioxygenase genes *tcbAa*, *tcbAb*, *tcbAc*, and *tcbAd* under the control of the *alk* regulatory system of *P. oleovorans* GPo1.[71] Luria-Bertani broth (LB) complex (Difco, Detroit, MI.) or M9 minimal medium[70] supplemented with 50 mg/L kanamycin were used as a medium. When necessary the medium was solidified with 1.5% agarose (Difco). Glucose, thiamine, and US* the composition of which is described elsewhere[35] were added to M9 medium at a final concentration of 0.5%, 0.001%, and 0.1%, respectively. All incubation and growth steps were carried out at 30 °C. Liquid media was incubated on a shaker at 220 rpm. Chemicals were obtained from Fluka AG (Buchs, Switzerland).

Biotransformations in Shake-Flasks

Freshly transformed *E. coli* JM101 cells were plated on LB agar plates. After incubation overnight, 5 mL LB medium was inoculated with a single colony and incubated for 10 h. 1 mL of this LB preculture was used to inoculate 100 mL of M9 medium. After incubation overnight, 100 mL of M9 medium were inoculated with the M9 preculture to a final cell concentration of A_{450} of 0.1, induced with 0.05% dicyclopropyl ketone (DCPK) and further incubated for 4 h. Then the cells were centrifuged at 10,000 × g for 10 minutes and the pellet was resuspended in 20 mL 50 mM potassium phosphate buffer (pH 7.4) supplemented with 1% glucose to a final cell concentration of 1.29 g cdw/L and further incubated. Substrate was added to a final concentration of 2 mM. Samples were taken, centrifuged at 20,000 × g for 5 minutes and supernatant was analyzed. Activity of the cells was calculated based on the product formation. One unit (IU) is defined as 1 μmol of product formed in one minute.

Biotransformation of Benzonitrile in a 2-L Reactor

Biotransformation of cinnamonitrile was carried out in a stirred tank reactor with two turbine impellers, four baffles, and a total volume of 3 liters. The reactor contained 1775 mL of M9 medium, 2 mL of trace elements solution US*, thiamine and antibiotics. The pH was adjusted to 7.2 with 25% NH_4 OH and 25% phosphoric acid. Temperature was kept at 30 °C. Silicone oil-based antifoam agent (Sigma) was used to control foaming. After inoculation with 200 mL of the preculture (prepared as M9 preculture for biotransformations in shake flasks) the reactor was aerated at a rate of 2 liters per minute and stirred at 1,500 rpm for *ca.* 10 h (overnight), which resulted in a culture in the stationary phase which contained 3.48 g (dry weight) of cells per liter. The medium was then supplemented with 8 mL of trace elements solution US* and 8 mg of thiamine. Subsequently, the culture was fed at a rate of 6 mL/h with an aqueous solution containing 50% glycerol and 1% $MgSO_4$. Half an hour later, the cells were induced by addition of 0.05% DCPK to the reactor. Three hours after induction, substrate was added to a final concentration of 1.57 mM and then added repeatedly afterwards. Samples were taken at different time points and diluted 10 times with ice-cold acetonitrile and centrifuged at 5000 rpm at 4 °C. Supernatants were filtered through a cellulose membrane filter (Spartan, Schleicher & Schuell GmbH, Germany) and analyzed. Biotransformations were carried out twice and nearly identical results were obtained.

Biotransformation of Benzonitrile in a 30-L Reactor

The 30-L biotransformations were performed in a 42-L stirred tank reactor with regulated temperature, pH, stirrer speed, and internal pressure (New MBR, Zurich, Switzerland). The pH was kept at 7.2 by the addition of 25% (wt/vol) ammonium water or 30% phosphoric acid. Data collection occurred every 30 s with the Caroline II software (PCS, Wetzikon, Switzerland) on an OS/9 operating system. Mixing was achieved with two 6-bladed Rushton turbine impellers. Before sterilization the reactor contained salts equivalent to 30 L of M9 medium dissolved in 20 L of water. After sterilization at 121 °C for 40 min, the reactor contents were supplemented aseptically with glucose solution, US*, magnesium sulfate, thiamine, kanamycin, and antifoaming agent. The volume was adjusted to 27 L with water. The reactor was inoculated with 3 L of M9 preculture (prepared as M9 preculture for biotransformations in shake flasks) and batch cultivation was performed at a stirring speed of 500 rpm and an aeration of 15 L/min for about 10 h. Silicone oil-based antifoam agent (Sigma) was used to control foaming. Fed-batch was started with pumping of the feed consisting of 46% glucose and 1% $MgSO_4$ with a rate of 10 g/L/h into the reactor. Stirring speed was increased to 700 rpm. After 1 h of fed-batch growth, additional kanamycin (1.5 g) US* (30 mL) and thiamine (0.3 g) were added and the expression of CDO genes was induced by the addition of 0.05% DCPK. 3 h later the biotransformation was started by the addition of substrate. Substrate was added repeatedly to keep the concentrations below 1.5 mM. Samples were taken at different time points and diluted 10 times with ice-cold acetonitrile and centrifuged at 5000 rpm at 4 °C. Supernatants were filtered through a cellulose membrane filter (Spartan, Schleicher & Schuell GmbH, Germany) and analyzed. Volumetric productivities and specific growth rates were calculated for the intervals between two sampling points.

Downstream Processing

Reaction medium (30 L) was centrifuged at 5000 rpm and at 4 °C for 15 minutes. The supernatant was removed and evaporated in a rotary evaporator at room temperature to a volume of approximately 1 L. The dissolved salts and proteins were precipitated with the addition of 4 L of acetonitrile. The mixture was centrifuged again under the same conditions and the supernatant was evaporated down to 20 mL. Product was run on a silica gel (60) column with 60% ethyl acetate and 40% hexane as eluent and fractions with different purities were collected. For the absolute stereochemistry determination, a part of the product was further purified by recrystallization from CH_2Cl_2-EtOAc/hexanes to yield beige microscopic needles. Part of the product from the biotransformation on a 2-L scale was purified by recrystallization from EtOAc/hexane and used as a standard for quantifications. Purities were quantified based on the comparison of the UV absorption area of the standard and the samples from the fractions.

Chemical Analysis

To analyze the metabolites, 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) was used with a mobile phase of 69.93% of H_2O-30% acetonitrile-0.07% H_3PO_4. The UV detector was set at a wavelength range of 210–600 nm. NMR spectra were recorded on a Bruker DPX 300 in $CDCl_3$, IR measurements were performed with a Perkin-Elmer "Spectrum One" FT-IR. The optical rotations were measured on a Perkin-Elmer 341 polarimeter.

Determination of Absolute Stereochemistry

***trans*-3-[(5*S*,6*R*)-5,6-Dihydroxycyclohexa-1,3-dienyl]-acrylonitrile (1):** Crude diol (0.250 g), a pasty beige solid, was purified

by recrystallization from CH_2Cl_2-EtOAc/hexanes to give beige microscopic needles; yield: 0.120 g (48%); mp 119–121 °C; $[\alpha]_D^{26}$: +337.9° (*c* 1.0, EtOH); R_f (EtOAc) = 0.57; ^{1}H NMR: δ = 6.99 (d, 1H, *J* = 16.5 Hz), 6.19 (d, 1H, *J* = 3.0 Hz), 5.98 (bs, 2H), 5.60 (d, 1H, *J* = 16.4 Hz), 4.37 (d, 1H, *J* = 6.2 Hz), 4.17 (d, 1H, *J* = 6.2 Hz), 3.37 (s, 2H); ^{13}C NMR: δ = 149.9, 136.3, 134.5, 132.1, 123.2, 118.6, 95.3, 69.7, 64.8. IR: ν = 3313, 3056, 3036, 2974, 2210, 1590, 1557 cm^{-1}.

***trans*-3-[(5*S*,6*R*)-5,6-Dihydroxycyclohex-1-enyl]-acrylonitrile (3):** Potassium azodicarboxylate (0.968 g, 4.99 mmol, 1.8 equivs.) was added to a cooled (−4 °C) stirred solution of *trans*-3-[(5*S*,6*R*)-5,6-dihydroxycyclohexa-1,3-dienyl]-acrylonitrile (**1**; 0.452 g, 2.77 mmol) in methanol (10 mL). Then a solution of glacial acetic acid (630 μL, 0.658 g, 10.97 mmol, 2.2 equivs. to PAD) in methanol (10 mL) was added dropwise over a 2 hour period while maintaining the temperature of the reaction mixture below 0 °C. Upon completion of the addition the reaction mixture was allowed to slowly warm up to room temperature over 5 h at which time the TLC analysis indicated completion. Excess acetic acid was neutralized by the addition of saturated sodium bicarbonate solution (5 mL) and most of methanol was removed under reduced pressure. The residue was diluted with water (5 mL) and aqueous phase was extracted with ethyl acetate (6 × 20 mL). Organic extracts were washed with brine (2.5 mL) and dried over anhydrous sodium sulfate. Drying agent was then removed by filtration and the solvent was removed under reduced pressure. The residue was almost completely dissolved in minimum amount of hot ethyl acetate (*ca.* 50 °C), and a small amount of brown solid, which remained was decanted. The supernatant was then carefully reheated and hexanes were added until the hot solution appeared cloudy. Upon cooling to room temperature, the flask was placed in freezer. The product was isolated as yellowish needles by filtration and dried under vacuum; yield: 0.186 g (41%); mp 84–85 °C; $[\alpha]_D^{27}$: −14.3° (*c* 1.0, CH_2Cl_2); R_f (EtOAc) = 0.47; ^{1}H NMR: δ = 6.96 (d, 1H, *J* = 16.5 Hz), 6.22 (t, 1H, *J* = 4.0 Hz), 5.64 (d, 1H, *J* = 16.5 Hz), 4.29 (d, 1H, *J* = 4.0 Hz), 3.78 (m, 1H), 2.29 (m, 2H), 1.76 (m, 2H); ^{13}C NMR: δ = 151.0, 141.1, 134.7, 118.5, 95.5, 68.9, 65.0, 25.3, 24.6; IR: ν = 3400, 3061, 3017, 2935, 2216, 1631, 1598 cm^{-1}.

***trans*-3-[(3a*R*,7a*S*)-2,2-Dimethyl-3a,6,7,7a–tetrahydrobenzo[1,3]dioxol-4-yl]-acrylonitrile (4):** *trans*-3-[(5*S*,6*R*)-5,6-Dihydroxycyclohex-1-enyl]-acrylonitrile (**3**; 0.300 g, 1.82 mmol) was dissolved in 2,2-dimethoxypropane (DMP) (10 mL, 81.5 mmol, 45 equivs.). To a stirred solution was then added a catalytic amount of *p*-toluenesulfonic acid monohydrate, and the reaction progress was monitored by TLC. Upon completion the reaction was quenched with saturated sodium bicarbonate solution (5 mL) and water (5 mL), excess DMP was removed under reduced pressure. The aqueous phase was extracted with methylene chloride (5 × 20 mL) and the organic extracts were dried over anhydrous sodium sulfate overnight. After removing the drying agent the filtrate was evaporated to dryness with small amount of silica. Thus pre-adsorbed crude product was then purified by column chromatography (silica, hexane:EtOAc, 90:10). A colorless oil was obtained, which crystallized upon standing; yield: 0.176 g (47%); mp 61–62 °C; $[\alpha]_D^{27}$: +208.3° (*c* 1.0, CH_2Cl_2); R_f (hexane:EtOAc, 40:60) = 0.72; ^{1}H NMR: δ = 6.91 (d, 1H, *J* = 16.5 Hz), 6.25 (t, 1H, *J* = 4.0 Hz), 5.59 (d, 1H, *J* = 16.5 Hz), 4.56 (d, 1H, *J* = 5.5 Hz), 4.37 (m, 1H), 2.36 (m, 1H), 2.13 (m, 1H), 1.94 (m, 1H), 1.75 (m, 1H), 1.37 (s, 3H), 1.31 (s, 3H); ^{13}C NMR: δ = 151.0, 141.0, 134.1, 118.9, 109.4, 96.4, 73.1, 70.6, 28.0, 26.6, 24.9, 21.8; IR: ν = 3064, 3017, 2986, 2216, 1632, 1600 cm^{-1}; anal. calcd.: C 70.22%, H 7.37%; found: C 69.97%, H 7.39%.

***trans*-3-[(3a*R*,7a*S*)-2,2-Dimethyl-3a,6,7,7a–tetrahydrobenzo[1,3]dioxol-4-yl]-acrylonitrile (4):** To a solution of (3a*S*, 7a*S*)-7-bromo-2,2-dimethyl-3a,4,5,7a–tetrahydrobenzo[1,3]-dioxole (**5**; 0.680 g, 2.92 mmol) in dry dimethylformamide (5 mL) was added $Pd(OAc)_2$ (0.066 g, 0.292 mmol, 0.1 equiv.), triphenylphosphine (0.153 g, 0.584 mmol, 0.2 equivs.), dry triethylamine (3.25 mL, 23.30 mmol, 8 equivs.) and, finally, freshly distilled acrylonitrile (576 μL, 8.76 mmol, 3.0 equivs.). The reaction mixture was briefly degassed by a stream of argon and heated in a sealed tube to 100 °C for 68 h. Upon cooling the reaction mixture was poured into water (20 mL) and extracted with ether (10 × 20 mL). Organic layers were washed with brine (10 mL) and dried over anhydrous magnesium sulfate. After removal of drying agent, the solvent was evaporated under reduced pressure and the crude mixture was separated by column chromatography to give unreacted starting material (0.364 g, 54%) as colorless oil and the desired product; yield: 0.212 g (76% based on the recovered starting material); mp 62–63.5 °C, $[\alpha]_D^{27}$: +207.5° (*c* 1.0, CH_2Cl_2).

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