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Preparation of an epoxide-hydrolyzing biocatalyst: *Rhodococcus ruber* DSM 44540—an activity-growth study

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Abstract

The constitutive epoxide hydrolase activity of *Rhodococcus ruber* DSM 44540 strongly depends on the status of the cells and appears to be regulated by a catabolic switch: activity peaked when glucose was exhausted and peptone/yeast extract consumption started. The activity-maximum for the kinetic resolution of a 2,2- and the enantioconvergent asymmetric biohydrolysis of a 2,3-disubstituted oxirane coincided. In order to obtain a maximum yield, cells should be harvested after ca. 17 h.

Keywords: *Rhodococcus ruber*; Epoxide hydrolase; Fermentation; Activity-growth

1. Introduction

Bacterial epoxide hydrolases have been shown to be highly versatile biocatalysts for the preparation of nonracemic *vic*-diols [1,2]. In particular, sterically more demanding 2,2-disubstituted epoxides were hydrolyzed with excellent selectivities (*E*-values up to >200) via kinetic resolution. In addition, we recently reported the asymmetric biohydrolysis of (±)-*cis*-2,3-dialkyl oxiranes, which led to the formation of the corresponding internal *vic*-diols in high enantiomeric purities [3]. In case of haloalkyl-substituted derivatives, enzyme triggered cascade reactions were observed [4,5]. The remarkable feature of the latter biotransformations is the fact that—governed by the relative *cis*-configuration of the substrate—they did not follow a kinetic resolution pathway, but proceeded in an enantioconvergent fashion to furnish a single enantiomeric *vic*-diol as the sole product in 100% theoretical yield [6]. In view of their considerably improved economic balance, such 'deracemization' processes have recently gained considerable attraction [7].

Rhodococcus ruber DSM 44540 shows exceptionally high selectivities for a broad variety of oxiranes bearing lipophilic functional groups. One of the best substrates is (±)-2-methylglycidyl benzyl ether, which is a versatile building block for the asymmetric synthesis of several bioactive compounds [8]. Recently, optimization and up-scaling of this biotransformation with respect to reaction conditions (pH, temperature, buffer type, addition of detergents and substrate concentration) was reported [9]. In order to provide a reproducible protocol for the preparative scale production of this biocatalyst by fermentation, we investigated the course of epoxide hydrolase activity during cell growth. In general, microbial epoxide hydrolase activity is associated with secondary metabolism and

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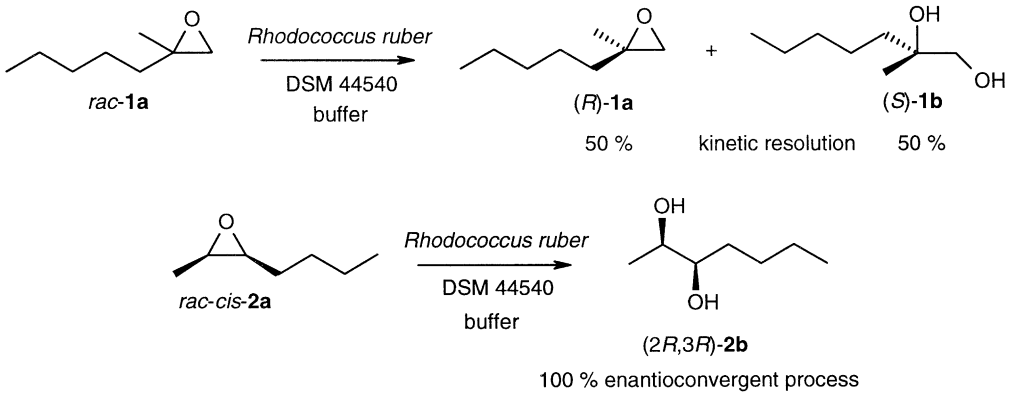


Scheme 1. Kinetic resolution and enantioconvergent biohydrolysis of oxirane *rac*-**1a** and *rac-cis*-**2a**, respectively.

thus is expected to strongly depend on the status of the cells during growth. It has been noted that the expression of epoxide hydrolase activity varied markedly during growth for various fungi [10–13] and a *Bacillus* sp. [14]. In order to obtain a maximum in activity, the determination of the point of cell harvest is crucial for preparative scale biotransformations. In view of the reproducibility of asymmetric biohydrolysis of epoxides, the enantioselectivity (expressed as the enantiomeric ratio, *E*) was determined during various stages of growth. As representative substrates for the study, (±)-2-methyl-1,2-epoxyheptane (*rac*-**1a**) and *rac-cis*-2-heptene oxide (*rac-cis*-**2a**) were selected as examples for kinetic resolution and an enantioconvergent process, respectively (Scheme 1).

2. Experimental

2.1. Synthesis of substrates

(±)-2-Methyl-1,2-epoxyheptane (*rac*-**1a**) was prepared as previously described [15]. *rac-cis*-2-Heptene-oxide (*rac-cis*-**2a**) was synthesized by epoxidation of commercially available *cis*-2-heptene (Aldrich) using *m*-chloroperbenzoic acid [3]. Reference material for diols *rac*-**1b** and *rac-threo*-**2b** were synthesized via acid-catalyzed hydrolysis of the corresponding epoxides *rac*-**1a** and *rac-cis*-**2a** as previously described [3,15].

2.2. Preparation of lyophilized cells of Rhodococcus ruber DSM 44540

2.2.1. Medium

The following components of the medium were sterilized in separate groups: Group I (phosphate buffer): NaH_2PO_4 (1.3 g/l), K_2HPO_4 (4.4 g/l). Group II (mineral salt medium): NaCl (2 g/l), $MgSO_4 \cdot 7H_2O$ (0.15 g/l). Group III (carbon source I): glucose (10 g/l). Group IV (energy source II): yeast extract (10 g/l), peptone (10 g/l).

2.2.2. Strain maintenance

R. ruber DSM 44540 was maintained on agar plates using the above described medium with the addition of agar (20 g/l). Sub-culturing was performed every 6 weeks. Agar plates were left in the incubator for 48 h at 30 °C, long-term storage was at 4 °C.

2.2.3. Shake-flask cultures

Cells for inoculum for the fermentation were precultured for 43 h at 30 °C on a rotary shaker at 120 rpm in four baffled Erlenmeyer flasks using 250 ml of above described medium each.

2.2.4. Bioreactor cultivation

Fermentation was performed in a Biostat E bioreactor (Braun, Melsungen, Germany) with a stainless steel vessel ES-10 of 10 l working volume. Yeast extract and peptone were autoclaved in situ in the reactor vessel (120 °C, 60 min), all other components were

sterilized separately and added after cooling to room temperature. Cells were grown on 10 l of medium at 30 °C, the oxygen electrode was calibrated by saturating the system with air and the $p(O_2)$ was kept at 70% using valve control with an aeration pressure of 1.2 bar (14 l per min, normalized) and a stirring speed of 220 rpm. The pH of 7.3 was controlled within ±0.1 by automatic addition of acid/base (HCl, NaOH, 2 M). Antifoam 289 (Sigma, A-5551, 5 ml) in water (0.5 l) was used. For the inoculum, 1 l of a shake-flask preculture using the same medium as described (30 °C for 43 h, shaking speed 120 rpm) was used. During certain periods of the fermentation, extended foaming controlled by external addition of antifoam agent through a septum. Cell growth monitored by analyzing samples (50 ml each) for (i) cell dry weight (CDW), (ii) optical density (OD, absorbance at 546 nm), (iii) glucose concentration, and (iv) epoxide hydrolyse activity. Cells were harvested after 36 h by centrifugation ($3000 \times g$), washed with Tris–HCl buffer (0.05 mM, pH 7.5) and lyophilized. Lyophilization gave ∼95 g of dry cells from a total volume of 10 l of culture. The cells can be stored at 4 °C for several months without significant loss of activity.

2.2.5. Determination of optical density

A culture sample (100 μl) was placed into a plastic cuvette (1 ml) and diluted with NaCl solution (0.9%, 900 μl). The optical density was measured using a Shimadzu UV–VIS scanning spectrophotometer (UV-2101PC) at 546 nm against NaCl solution (0.9%) as blank. The extinction (ε) should be <1, if necessary, the dilution factor was increased. The OD_{546}-value was obtained via the following formula: $OD_{546} = \varepsilon \times$ dilution factor.

2.2.6. Determination of cell dry weight

The CDW was determined using an automatic thermobalance (Sartorius MA 30 moisture analyzer) at constant weight at 130 °C.

2.2.7. Determination of glucose concentration

Glucose was monitored using a commercially available glucose hexokinase assay kit (Sigma, GAHK-20), based on the hexokinase UV method. A sample of clear filtrate from the CDW determination was diluted with deionized water (1:20, 1:10 or 1:5) and 50 μl of this solution were added to 1 ml of the glucose-UV-test solution and mixed well. After 15 min, the extinction at 340 nm was measured against a sample of the glucose-UV-test as blank, the value should be <1. Glucose concentration was calculated as follows: glucose [g/l] = $\varepsilon \times$ dilution factor × 0.61.

2.3. Assay for epoxide hydrolyse activity

Culture samples (1.8 ml each, in total 8, withdrawn in 2 h intervals) were placed into plastic vials (2 ml) and cells were centrifuged (13,000 rpm, 2 min). Wet cells were stored at −10 °C until use for the activity assay.

For each substrate, *rac*-**1a** and *rac-cis*-**2a**, three parallel values for enhanced reproducibility were measured and results were averaged. Cells were resuspended in Tris–HCl buffer (1 ml, 0.05 M, pH 7.5) for 15 min, substrate (10 μl), *rac*-**1a** or *rac-cis*-**2a**, was then added in one portion and the mixture was agitated on an orbit shaker at 30 °C and 120 rpm. After 24 h (for *rac*-**1a**) and 69 h (for *rac-cis*-**2a**), after centrifugation ($3000 \times g$, 10 min), products were extracted with ethyl acetate (from the supernatant) and with acetone (from the pellet), the combined organic phases were dried (Na_2SO_4) and the resulting samples were analyzed on achiral GC. Conversion was measured on a Varian 3800 gas chromatograph equipped with FID, using a HP1701 capillary column (30 m, 0.25 mm, 0.25 μm film, N_2). The relative activity (expressed as percentage of maximum) was calculated from the conversion of the biotransformation per amount of cell dry weight.

2.4. Determination of ee and absolute configuration

Absolute configurations of both epoxides and diols were determined as previously described [3,15]. Enantiomeric excesses were analyzed on a Varian 3800 gas chromatograph equipped with FID, using a CP-Chirasil-DEX CB column (column A, 25 m, 0.32 mm, 0.25 μm film) with H_2 as carrier gas. *rac*-**1a**: [10 psi, 55 °C (iso), t_{R1} = 9.2 min (*R*), t_{R2} = 9.6 min (*S*)]; *rac*-**1b**: [10 psi, 110 °C (iso), t_{R1} = 12.4 min (*S*), t_{R2} = 12.8 min (*R*)]; *rac-cis*-**2a**: [5 psi, 65 °C (iso), t_{R1} = 7.3 min (2*S*,3*R*), t_{R2} = 7.7 min (2*R*,3*S*)]; *rac-threo*-**2b**: [14 psi, 100 °C (iso), t_{R1} = 7.7 min (2*S*,3*S*), t_{R2} = 8.4 min (2*R*,3*R*)].

2.5. Calculation of enantioselectivity for rac-**1a**

Enantioselectivities are expressed as the enantiomeric ratio (*E*). In order to obtain most accurate values, the method based on enantiomeric excess of epoxide (ee_S) and enantiomeric excess of diol (ee_P) was used [16].

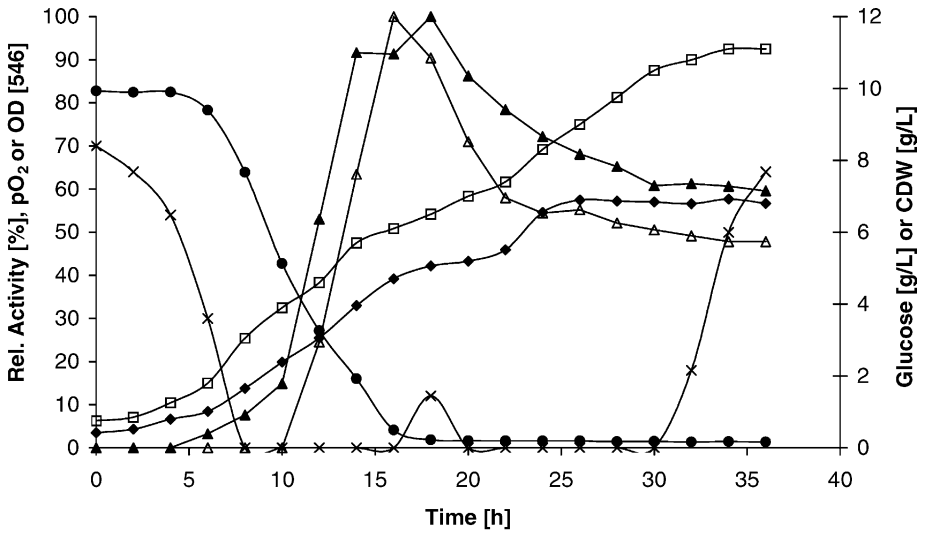
3. Results and discussion

3.1. Fermentation and epoxide hydrolase activity

The results obtained from the bioreactor cultivation are depicted in Fig. 1. After 16 h glucose was completely consumed as the first carbon source. As a consequence, the cell growth slowed down beyond this point for a period of about 6 h (16–22 h) indicated by the optical density and the cell dry weight. During this interphase production of enzymes which utilize the second energy source (yeast extract and peptone) is enhanced. After 26 h, the stationary phase was reached as indicated by the optical density. High oxygen demand started with the beginning of the exponential growth phase (OD). After 30 h from start, the $p(O_2)$ suddenly increased to a value of 70% again, going in hand with constant cell dry weight, which indicated the cease of cell growth after all energy sources have been fully consumed.

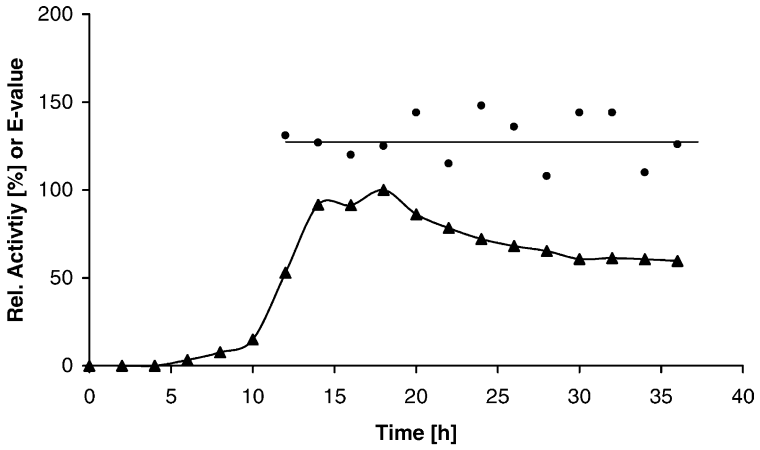
In Fig. 1, the results of the activity assay for epoxides *rac*-**1a** and *rac-cis*-**2a** as example for a kinetic resolution and an enantioconvergent process, respectively, are shown. The course of the activity for both substrates is largely parallel and peaks closely overlap at 17 h, which indicates, that the same epoxide hydrolase is responsible for the transformation of both substrates (for the isolation and characterization of an epoxide hydrolase from *Rhodococcus ruber* DSM 44540 (formerly *Nocardia* EH1) see [17]). Epoxide hydrolase activity was detected during late log-phase growth, exhibiting a maximum of 140 μmol/(h g) (CDW) for *rac*-**1a** and 56 μmol/(h g) (CDW) for *rac-cis*-**2a** at the point at which glucose was fully consumed. The activity remained after glucose was completely exhausted and peptone and yeast extract consumption dominated; it later decreased to ca. 60% over 20 h as the cell density increased. The activity-maximum coincided with the end of glucose consumption and appears to be associated a catabolic switch during the late log-phase.

For both substrates, enzyme activity was low in the presence of glucose and expression of epoxide



Δ Relative activity for *rac-cis*-**2a**, π relative activity for *rac*-**1a**, x pO_2, ◆ optical density, ● glucose, □ cell dry weight

Fig. 1. Growth and epoxide hydrolase activity profile of *Rhodococcus ruber* DSM 44540.



▲ Relative activity for *rac*-**1a**, ● *E*-value

Fig. 2. Epoxide hydrolase activity vs. enantioselectivity during growth.

hydrolase activity was initiated when glucose was almost exhausted. When the cells were grown on glucose as the sole C-source, low epoxide hydrolase activity was observed [18]. This inhibiting effect of glucose—known as catabolite repression—on the synthesis of a variety of unrelated enzymes and for the epoxide hydrolase in particular is well known.

For various fungi, such as *Beauveria bassiana* and *Aspergillus niger* [10,11], and two dematiaceous hyphomycetes [12], it was observed that the epoxide hydrolase activity reached the maximum during the late stationary phase. In contrast, for the fungus *Beauveria densa* the highest activity was detected prior to the stationary phase, going in hand with exponential growth [13], and almost identical results were reported for the bacterium *Bacillus megaterium* ECU 1001 [14], where a second activity-maximum towards the end of the stationary phase indicated the existence of a second epoxide hydrolase.

3.2. Dependence of selectivity on growth

In order to provide a reproducible protocol for the preparative scale preparation of an epoxide-hydrolyzing biocatalyst, the dependence of the enantioselectivity versus cell growth was investigated. (±)-2-Methyl-1,2-epoxyheptane (*rac*-**1a**) was used as a representative substrate for kinetic resolution. In order to obtain most reliable results, *E*-values were calculated from the enantiomeric excess of substrate and product (ee_P, ee_S), respectively, via the method of Rakels et al. [16]. For the 2,3-disubstituted oxirane (*rac-cis*-**2a**), *E*-values are not applicable due to the enantioconvergence of the biohydrolysis. Fig. 2 shows the results for epoxide *rac*-**1a**: beyond ca. 50% of relative activity until the late stage of the fermentation, the selectivity remained on a constant level of *E* 110–135. The observed fluctuations within this range are due to the technical limitations of analysis.

4. Conclusions

In conclusion, we have shown for *R. ruber* DSM 44540 that (i) the enzyme(s) appear to be constitutive and do not require any external inducer, (ii) the expression of epoxide hydrolase activity is regulated by catabolite repression, (iii) the maximum of epoxide hydrolase activity peaks during the late log-phase growth, and (iv) the activity-maximum is independent on a kinetic resolution or an enantioconvergent pathway, which supports the earlier observation that only single protein is responsible for the epoxide hydrolase activity of whole cells.

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References

[1] A. Steinreiber, K. Faber, Curr. Opin. Biotechnol. 12 (2001) 552.
[2] A. Archelas, R. Furstoss, Curr. Opin. Chem. Biol. 5 (2001) 112.
[3] W. Kroutil, M. Mischitz, K. Faber, J. Chem. Soc. Perkin Trans. 1 (1997) 3629.
[4] S.F. Mayer, A. Steinreiber, R.V.A. Orru, K. Faber, Tetrahedron: Asymmetry 12 (2001) 41.
[5] S.F. Mayer, W. Kroutil, K. Faber, Chem. Soc. Rev. 30 (2001) 332.
[6] S.F. Mayer, A. Steinreiber, R.V.A. Orru, K. Faber, Eur. J. Org. Chem. (2001) 4537.
[7] K. Faber, Chem. Eur. J. 7 (2001) 5005.
[8] A. Steinreiber, I. Osprian, S.F. Mayer, R.V.A. Orru, K. Faber, Eur. J. Org. Chem. (2000) 3703.
[9] H. Hellström, A. Steinreiber, S.F. Mayer, K. Faber, Biotechnol. Lett. 23 (2001) 169.
[10] P. Moussou, A. Archelas, R. Furstoss, J.C. Baratti, Enzyme Microb. Technol. 26 (2000) 414.
[11] N. Nellaiah, C. Morisseau, A. Archelas, R. Furstoss, J.C. Baratti, Biotechnol. Bioeng. 49 (1996) 70.
[12] G. Grogan, S.M. Roberts, A.J. Willetts, FEMS Microbiol. Lett. 141 (1996) 239.
[13] G. Grogan, C. Rippé, A. Willetts, J. Mol. Catal. B 3 (1997) 253.
[14] Y.F. Tang, J.H. Xu, Q. Ye, B. Schulze, J. Mol. Catal. B 13 (2001) 61.
[15] M. Mischitz, W. Kroutil, U. Wandel, K. Faber, Tetrahedron: Asymmetry 6 (1995) 1261.
[16] J.L.L. Rakels, A.J.J. Straathof, J.J. Heijnen, Enzyme Microbiol. Technol. 15 (1993) 1051.
[17] W. Kroutil, Y. Genzel, M. Pietzsch, C. Syldatk, K. Faber, J. Biotechnol. 61 (1998) 143.
[18] Y. Genzel, M.Sc. Thesis, University of Stuttgart, 1997.