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Optimization of the organic solvent-stable asymmetric hydrogen transfer system of *Rhodococcus ruber* DSM 44541: an activity-growth study

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Abstract

The organic solvent-stable redox-system of *Rhodococcus ruber* DSM 44541, which allows the efficient oxidation/reduction of *sec*-alcohols/ketones at the expense of acetone/2-propanol, respectively, as cosubstrate was optimized with respect to a maximum of alcohol dehydrogenase activity during cell growth. Comparison of the fermentation of *R. ruber* DSM 44541 in shake flasks cultures (1 l flask with 250 ml medium) and in a bioreactor (15 l with 10 l working volume) revealed that the desired organic solvent-stable alcohol dehydrogenase activity reached its maximum during the log phase for the bioreactor. In contrast, in shake flasks the maximum of activity was reached during the stationary phase.

Keywords: *Rhodococcus ruber*; *sec*-Alcohol dehydrogenase; Fermentation; Activity-growth

1. Introduction

The use of isolated *sec*-alcohol dehydrogenases for the enantioselective oxidation of *sec*-alcohols and the asymmetric reduction of ketones using acetone or 2-propanol for cofactor [NAD(P)$^+$] recycling, respectively, is state of the art. However, this method is still unsuitable for large-scale applications, because the *sec*-alcohol dehydrogenases known to date are not stable towards elevated concentrations of substrate/product and cosubstrate (acetone/2-propanol) [1,2],[1] which are required to shift the (reversible) reaction towards completion. Thus, the use of a cheap sacrificial ketone/alcohol for the cofactor recycling bears its limitations [3,4] and more complex alternative methods are used. In addition, the use of fermenting cells requires also low substrate concentrations and thus sets a low ceiling for the productivity of these processes [5–7]. Recently, we discovered that whole resting cells of *Rhodococcus ruber* DSM 44541 possess an alcohol dehydrogenase system which is exceptionally stable towards 2-propanol (or acetone), which allows the preparative reduction of ketones and

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[1] In general, the substrate concentration was below 0.15 mol/l and cosubstrate concentration was below 3% (v/v) (15% (v/v) cosubstrate 2-propanol, 0.12 mol/l substrate).

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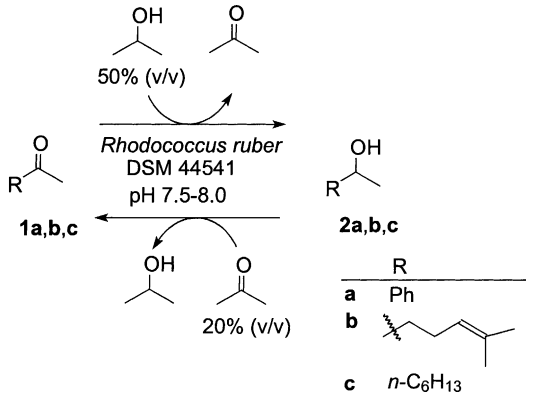

Scheme 1. Oxidation of *sec*-alcohols/reduction of ketones employing whole cells of *R. ruber* DSM 44541.

the oxidation of *sec*-alcohols, respectively (Scheme 1) [8]. In the reduction mode, 2-propanol can be employed at a concentration of up to 50% (v/v) as cosubstrate and at high substrate concentrations of up to 1.8 mol/l. Similarly, highly efficient oxidation of *sec*-alcohols was achieved in the presence of the cosubstrate acetone at a concentration up to 25% (v/v).

In order to provide a reproducible protocol for the optimized large-scale production of this biocatalyst by fermentation, we investigated the course of alcohol dehydrogenase activity during cell growth in 1 l shake flasks (250 ml medium) and in a 15 l bioreactor (10 l medium) and compared the alcohol dehydrogenase activity of the cells during growth. It has been reported that the expression of some dehydrogenase activities of the yeast *Kluyveromyces lactis* [9] varied markedly during growth depending on the presence of glucose. In contrast, the activity of an alcohol dehydrogenase of *Sulfolobus solfataricus* was not affected by cell density [10]. In order to obtain a maximum in activity, the determination of the point of cell harvest is crucial for preparative-scale biotransformations.

2. Materials and methods

2.1. Medium

The following components of the medium were sterilized in separate groups: Group I: Glucose (10 g/l, Fluka 49150). Group II: Yeast extract (10 g/l, Oxoid L21), bacteriological peptone (10 g/l, Oxoid L37). Group III (salts): NaCl (2 g/l, Roth 9265.1), $MgSO_4 \cdot 7H_2O$ (0.15 g/l, Fluka 63140), NaH_2PO_4 (1.3 g/l, Fluka 71496), K_2HPO_4 (4.4 g/l, Merck 5101).

2.2. Strain maintenance

R. ruber DSM 44541 was maintained on agar plates using the above described medium (18% agar). Sub-culturing was performed every 12 weeks and plates were stored at 4 °C.

2.3. Shake flask cultures

Cells were grown at 30 °C on a rotary shaker at 120 rpm in four 1 l baffled Erlenmeyer flasks using 250 ml of above described medium each.

2.4. Determination of dissolved oxygen in shake flask cultures

The shaking of the flasks was stopped and the dissolved oxygen was measured using a $p(O_2)$-electrode (Braun, Biostat E). To avoid infection, a number of shake flask experiments was started simultaneously. Each shake flask was used for a single measurement only.

2.5. Bioreactor cultivation 10 l scale

Large-scale growth was performed in a Biostat E bioreactor (Braun, Melsungen, Germany) with a stainless steel vessel ES-10 of 10 l working volume. Peptone and yeast extract were autoclaved in the reactor vessel (121 °C, 60 min), other components were sterilized separately in groups as described above and added after cooling to room temperature. The oxygen electrode was calibrated by saturation of the medium with air and the $p(O_2)$ was regulated at 80% using valve-control with an aeration pressure of 1.5 bar (13 l/min, normalized) and a constant stirrer rotation (disc agitator) of 220 rpm. The pH of 7.0 could be controlled within ±0.1 by automatic addition of acid/base (HCl 2N, NaOH 2N). Antifoam 289 (Sigma No. A-5551, 0.5 l) in water (0.5 l) was used. Cells were grown on 10 l of medium at 30 °C starting with 1 l inocolum precultured in shake flasks as described above. The cell growth was monitored by analyzing samples (50 ml) for (i) cell dry weight (CDW), (ii)

optical density, (iii) glucose concentration and (iv) alcohol dehydrogenase activity. The end of the exponential growth phase was reached when the biomass (CDW and OD) was constant after the exponential growth. The cells were harvested after 32 h.

2.6. Determination of optical density OD_{546}

OD_{546} was measured on a Shimadzu UV-Vis scanning spectrophotometer UV-2101 PC.

2.7. Determination of cell dry weight

The CDW was determined on a thermobalance (Sartorius MA 30) heating a filtered (pore size 0.45) sample (20 ml) until constant weight at 130 °C.

2.8. Determination of glucose concentration

Glucose was monitored using a commercial glucose hexokinase test kit (Sigma, GAHK-20).

2.9. Assay for alcohol dehydrogenase activity

Every 2 h, 10 samples (each 1.8 ml) were centrifuged (13.000 rpm, 2 min), the medium was decanted and the centrifuged cells were shock-frozen to analyze all samples at the end of the fermentation. In a typical procedure, Tris–HCl buffer (0.9 ml, 10 mm, pH 7.5) was added to the frozen cells, the mixture was left to warm to room temperature before 1-phenyl ethanol **2b** (1.0 μl, 8.3 μmol) and acetone (100 μmol) as hydrogen acceptor was added. The mixture was shaken at 30 °C and 130 rpm in 1.5 ml Eppendorf vials for 15 min, before the reaction was stopped by addition of ethyl acetate (0.5 ml). After centrifugation, the organic phase was separated, dried (Na_2SO_4) and analyzed by GC (Varian 3800, FID). Temperature program for the determination of *rac*-1-phenyl ethanol **2a**/acetophenone **1a** on a chiral column (Chrompack Chirasil Dex, 25 m × 0.32 mm × 0.25 μm; H_2): start temperature 100 °C, hold 3 min, 12 °C/min, until 160 °C, hold 2 min. R_T [(*R*)-1-phenyl ethanol]: 4.7 min, R_T [(*S*)-1-phenyl ethanol]: 4.9 min, R_T (acetophenone): 2.1 min. *rac*-Sulcatol **2b**/sulcatone **1b** was analyzed on an achiral column (HP-1301, 30 m × 0.25 mm × 0.25 μm; N_2): start temperature 80 °C, hold 2 min, 10 °C/min, until 130 °C, hold 2 min. R_T (*rac*-sulcatol): 4.7 min, R_T (sulcatone): 4.5 min. Peak intensities were corrected using a calibration curve.

2.10. Preparation of lyophilized cells

Cells from the 10 l fermentation were harvested by centrifugation (300 × g, 20 min) and resuspended in Tris–buffer (pH 7.5, 50 mM). Lyophilisation gave ~87 g of dry cells from a total volume of 10 l of culture. The cells could be stored at +4 °C for several months without significant loss of activity.

2.11. Preparative-scale reactions

For reduction experiments, lyophilized cells (1.0 g) were rehydrated in phosphate buffer (4.0 ml, 50 mM, pH 7.5) for 30 min at 30 °C. Cosubstrate 2-propanol (5.0 ml) and substrate sulcatone (1.0 g, 7.9 mmol) were added and the mixture was shaken at 30 °C for 6 h. The reaction was stopped by extraction with ethyl acetate (3 ml × 20 ml) and centrifugation. The products were purified by conventional silica gel chromatography.

For oxidation experiments, lyophilized cells (0.6 g) were rehydrated in phosphate buffer (6.0 ml, 50 mM, pH 8.0) for 30 min at 30 °C. Cosubstrate acetone (1.0 ml + 2.0 ml after 6 h) and substrate *rac*-2-octanol (1.0 g, 7.7 mmol) were added and the mixture was shaken at 30 °C for 16 h. The work up was performed as described above.

3. Results and discussion

3.1. Shake flasks experiments

The fermentation of *R. ruber* DSM 44541 in baffled shake flasks (1 l) with 250 ml of a complex medium lasted 60 h (Fig. 1) until no further increase of the OD or CDW was observed. Since the pH in the shake flask could not be kept constant for technical reasons and insufficient buffer capacity, an increase of the pH at the final stage of the growth was observed (Fig. 1). Measuring the oxygen saturation in the shake flasks revealed a constant saturation during growth, thus no oxygen limitation occurred [11]. From the shake flask fermentation 7.0 g lyophilized cells per litre medium were obtained with a final optical density $OD_{max} = 46.6$ and a dry cell weight $CDW_{max} = 8.2$ g/l.

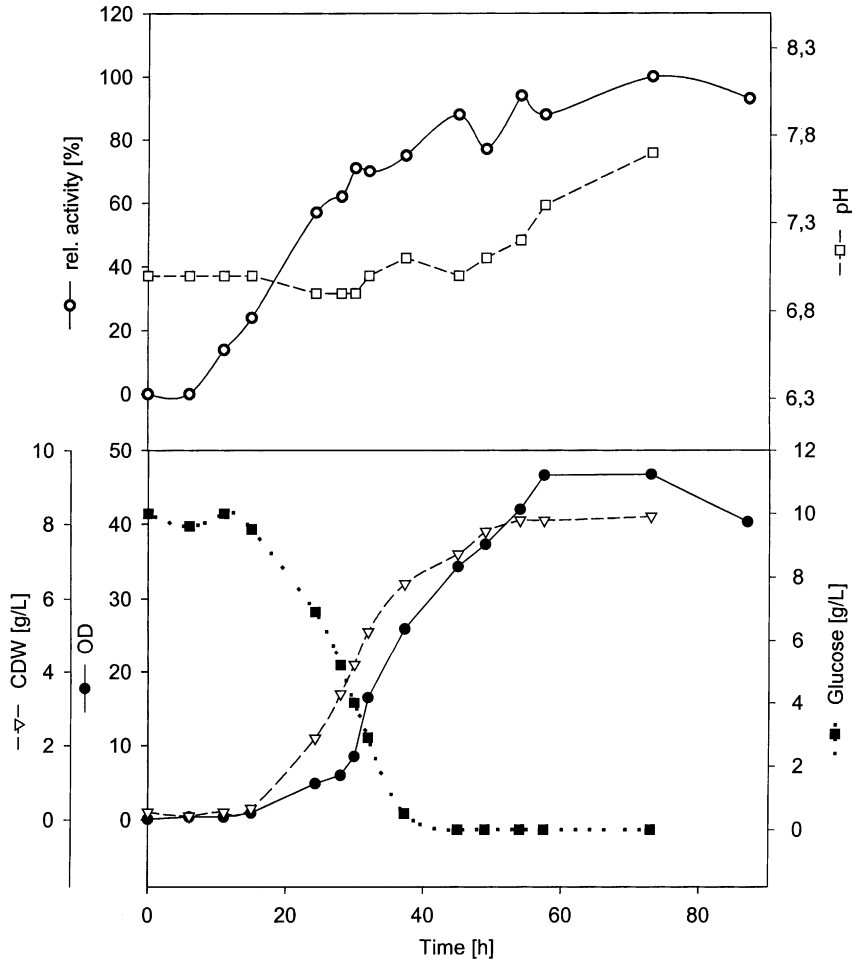

Fig. 1. Fermentation of *R. ruber* DSM 44541 in shake flasks (250 ml medium): (●) optical density; (▽) cell dry weight (CDW); (■) glucose; (□) pH; (○) relative activity for oxidation of *rac*-sulcatol *rac*-**2b**.

3.2. Bioreactor experiments

The up-scaling of the shake flask experiment to a 10 l scale in a bioreactor using the above mentioned medium required optimization. Using only air for the fermentation as oxygen source showed that oxygen was limiting, however, it was not possible to maintain a higher oxygen level either by increasing the air pressure or by increasing the rotation speed of the stirrer ($OD_{max} = 39.7$). In a previous experiment it was shown that a rotation speed greater 240 rpm caused destruction of the cells due to mechanical shear. Furthermore, the cells cultivated in the shake flasks produced much less mucous substances[2] and (as judged by the color) less carotenoids than in this bioreactor experiment. This result is especially significant in view of enzyme purification, which is simpler for cells containing less mucous substances. Performing a fermentation by using not only air as oxygen source but adding additional oxygen to effect a final oxygen concentration of 51% resulted in the

[2] After cell disruption the cell lysate was filtered through sterile syringe filters (cellulose acetate membrane, acrylic, 0.45, Iwaki, Japan), which were rapidly blocked by the lysate from the first two 10 l fermentation but not by the lysate from cells obtained from shake flask cultivation.

production of cells possessing a low content of mucous substances. However, the maximal optical density obtained ($OD_{max} = 32.2$) was lower than before, glucose was not consumed at all and the cells showed almost no *sec*-alcohol dehydrogenase activity (see below). In a simplified hypothesis we assumed that enough oxygen is needed to keep the amount of mucous substances low, but oxygen must be kept under a certain concentration to ensure that glucose is consumed to obtain cells with high *sec*-alcohol dehydrogenase activity. In all previous bioreactor experiments the pH was kept constant at 7.0. However, since in the shake flask experiment the non-regulated pH had no adverse effect, the next fermentation was performed without automatic pH controlling. To inhibit glucose consumption right from the beginning of the growth the oxygen concentration in the 'air' was set to 51% for the first 6.5 h before to total oxygen concentration was set to 31%. The fermentation of *R. ruber* DSM 44541 in a bioreactor displayed a lag phase of ~14 h (Fig. 1) when a 24 h preculture was used. In the period between the 10th to the 14th hour after the start of the fermentation it was necessary to add 2.5 ml of antifoam every hour. The fermentation was finished after 32 h when no further increase of the OD or CDW was observed. The exponential growth rates μ could be determined to be $\mu = 0.351\,h^{-1}$ (18–20 h). In comparison to the shake flask experiment ($OD_{max} = 46.6$, $CDW_{max} = 8.2$ g/l) and to the previous bioreactor experiments the highest OD ($OD_{max} = 50.7$) and highest CDW was obtained ($CDW_{max} = 8.9$ g/l). In total 8.7 g lyophilized cells per litre medium were obtained.

3.3. Alcohol dehydrogenase activity

The cells obtained from shake flask- and bioreactor-fermentation showed comparable solvent stability and could be employed as biocatalyst for hydrogen transfer reactions using acetone (up to 25% (v/v)) and 2-propanol (up to 50% (v/v)) as hydrogen acceptor and donor, respectively. However, the maximum of activity markedly differed between shake flask- and bioreactor-experiments. The activity, expressed as μmol product formed per gram dry cell weight per hour, reached its maximum in the bioreactor during the log phase, when glucose was entirely consumed [750 μmol product/(h g CDW) for the kinetic resolution of *rac*-sulcatol **2b**]. In contrast, the activity in 1 l shake flasks increased with increasing CDW, thus the maximum was reached at the stationary phase [1033 μmol product/(h g CDW) for the kinetic resolution of *rac*-sulcatol **2b**]. Furthermore, when using another substrate, *rac*-1-phenylethanol **2a**, the same activity maxima were obtained, indicating that both substrates were transformed by the same enzyme(s) system(s). The activity was measured at an increased concentration of acetone to avoid the interference of other alcohol dehydrogenases in the cells, which are not stable towards increased concentration of acetone and thus are rapidly deactivated.[3] From Figs. 1 and 2 it can be concluded that glucose—known to cause catabolite repression—had no inhibiting effect on the production of the organic solvent-stable alcohol dehydrogenase, since enzyme activity increased while glucose was still present in significant concentrations.

3.4. Application

Due to the enhanced dehydrogenase activity of cells obtained from shake flasks in higher yields, these cells were employed for large-scale kinetic resolution of *rac*-2-octanol *rac*-**2c** (111 g/l, 0.84 mol/l) employing 0.6 g cells and acetone (22% (v/v)) to furnish 2-octanone **1c** (37% isolated yield) and (*R*)-2-octanol (*R*)-**2c** in 48% isolated yield and 96% e.e., which corresponds to an enantioselectivity of $E > 100$. In this case, the space–time yield reached an impressive 18 mmol/(h l) or 0.8 g product per gram cells. The reduction of sulcatone **1b** (0.75 mol/l, 95.2 g/l) employing lyophilized cells (1.0 g) and 2-propanol (48% (v/v)) as hydrogen donor was stopped after 6 h at 79% conversion to give exclusively (*S*)-sulcatol (*S*)-**2b** in 76% isolated yield and >99% e.e., which corresponds to a space–time yield of 98 mmol/l h or 0.77 g product per gram cells.

In conclusion it was shown that the fermentation of *R. ruber* DSM 44541 possessing a highly efficient biocatalytic hydrogen transfer system can be performed in 250 ml medium in 1 l shake flasks and in 10 l medium in a fermentor. Highest cell yield was obtained in the fermentor, however, higher activity

[3] Protein purification revealed (at least) the presence of six alcohol dehydrogenases in total, but only one is stable at elevated acetone concentration and can efficiently use acetone as hydrogen acceptor (unpublished results).

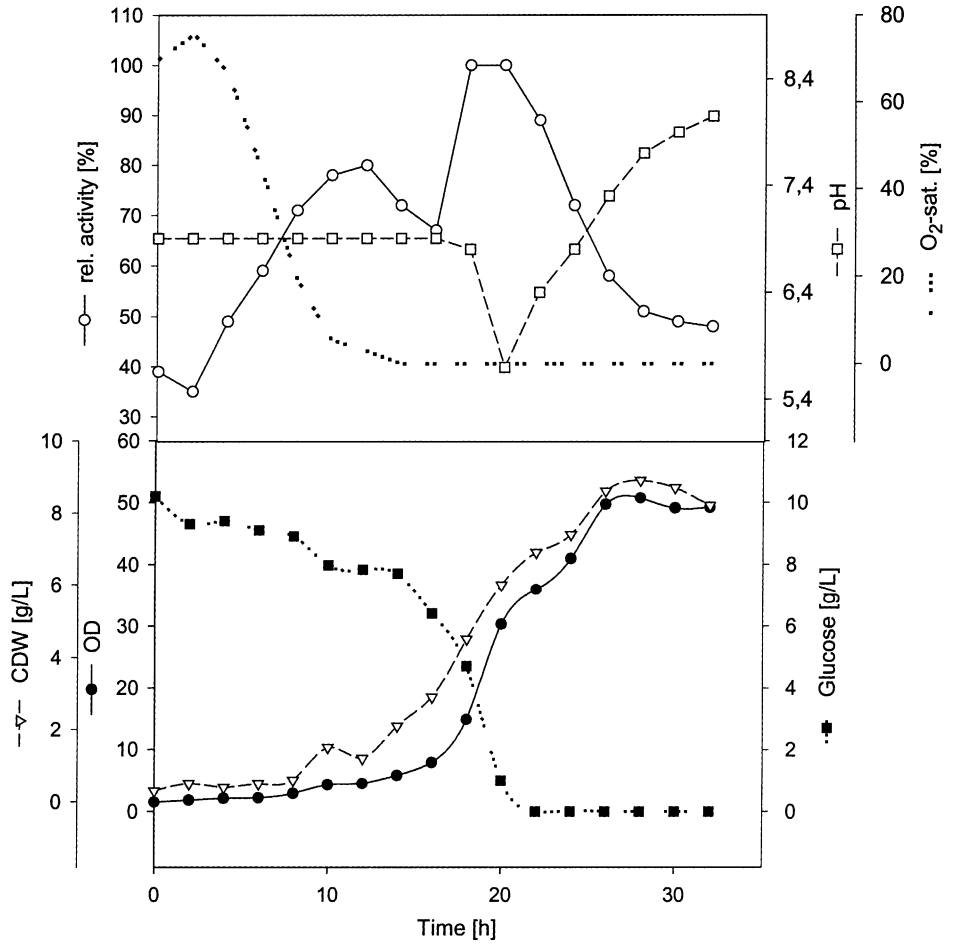

Fig. 2. Fermentation of *R. ruber* DSM 44541 in a 15 l bioreactor (10 l medium): (●) optical density; (▽) cell dry weight (CDW); (■) glucose; (○) relative activity for oxidation of *rac*-sulcatol *rac*-**2b**; (···) O_2 saturation; (□) pH.

was obtained for the shake flask experiment at the end of the fermentation.

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References

[1] M. Wolberg, W. Hummel, C. Wandrey, M. Muller, Angew. Chem. Int. Ed. Eng. 39 (2000) 4306.
[2] C. Heiss, R.S. Phillips, J. Chem. Soc. Perkin Trans. 1 (2000) 2821.
[3] R. Devaux-Basseguy, A. Bergel, M. Comtat, Enzyme Microb. Technol. 20 (1997) 248.
[4] W. Hummel, Adv. Biochem. Eng. Biotechnol. 58 (1997) 145.
[5] H.I. Pérez, H. Luna, N. Manjarrez, A. Solís, Tetrahedron: Asym. 12 (2001) 1709.
[6] G. Fantin, M. Fogagnolo, A. Medici, P. Pedrini, S. Fontana, Tetrahedron: Asym. 11 (2000) 2367.
[7] H.I. Pérez, H. Luna, N. Manjarrez, A. Solís, M.A. Nuñez, Biotechnol. Lett. 21 (1999) 855.
[8] W. Stampfer, B. Kosjek, C. Moitzi, W. Kroutil, K. Faber, Angew. Chem. Int. Ed. Engl. 41 (2002) 1014.
[9] M. Saliola, C. Falcone, Mol. Gen. Genet. 249 (1995) 665.
[10] C. Schiraldi, M. Acone, M. Giuliano, M. Carteni, M. de Rosa, Extremophiles 5 (2001) 193.
[11] M.-F. Pepin, J. Archambault, C. Chavarie, F. Cormier, Biotech. Bioeng. 47 (1995) 131.