Journal of Biotechnology 191 (2014) 196–204

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Whole-cell double oxidation of *n*-heptane

Christina A. Müller[a,1], Alexander Dennig[a,2], Tim Welters[a], Till Winkler[b], Anna Joelle Ruff[a], Werner Hummel[b], Harald Gröger[c], Ulrich Schwaneberg[a,*]

[a] Institute of Biotechnology, RWTH Aachen, Worringerweg 3, 52074 Aachen, Germany
[b] Institute of Molecular Enzyme Technology at the Heinrich-Heine-University of Düsseldorf, Research Centre Jülich, Stetternicher Forst, 52426 Jülich, Germany
[c] Faculty of Chemistry, Bielefeld University, Universitätsstrasse 25, 33615 Bielefeld, Germany

ARTICLE INFO

Article history:
Received 13 March 2014
Received in revised form 25 May 2014
Accepted 2 June 2014
Available online 9 June 2014

Keywords:
Cascade reaction
Double oxidation
Monooxygenase
Alcohol dehydrogenase
Directed evolution

ABSTRACT

Biocascades allow one-pot synthesis of chemical building blocks omitting purification of reaction intermediates and expenses for downstream processing. Here we show the first whole cell double oxidation of *n*-heptane to produce chiral alcohols and heptanones. The concept of an artificial operon for co-expression of a monooxygenase from *Bacillus megaterium* (P450 BM3) and an alcohol dehydrogenase (RE-ADH) from *Rhodococcus erythropolis* is reported and compared to the widely used two-plasmid or Duet-vector expression systems. Both catalysts are co-expressed on a polycistronic constructs (single mRNA) that reduces recombinant DNA content and metabolic burden for the host cell, therefore increasing growth rate and expression level. Using the artificial operon system, the expression of P450 BM3 reached 81 mg g^{-1} cell dry weight. In addition, *in situ* cofactor regeneration through the P450 BM3/RE-ADH couple was enhanced by coupling to glucose oxidation by *E. coli*. Under optimized reaction conditions the artificial operon system displayed a product formation of 656 mg L^{-1} (5.7 mM) of reaction products (heptanols + heptanones), which is 3-fold higher than the previously reported values for an *in vitro* oxidation cascade. In conjunction with the high product concentrations it was possible to obtain *ee* values of >99% for (*S*)-3-heptanol. Coexpression of a third alcohol dehydrogenase from *Lactobacillus brevis* (Lb-ADH) in the same host yielded complete oxidation of all heptanol isomers. Introduction of a second ADH enabled further to utilize both cofactors in the host cell (NADH and NADPH) which illustrates the simplicity and modular character of the whole cell oxidation concept employing an artificial operon system.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Molecular oxygen can be introduced into non-activated carbon atoms under mild reaction conditions in a selective manner employing biocatalysts (Hollmann et al., 2011). 'Operational stability' of biocatalysts and cofactor regeneration during oxidative biocatalysis are challenging tasks (Hollmann et al., 2011). Limitations in the performance of enzymes under non-natural conditions are often solved by protein engineering (Ruff et al., 2013; Tee and Schwaneberg, 2007). Simple and cost-effective solutions are favored since cofactors expenses often have a high impact on reaction economics.

Biocatalytic processes for the functionalization of alkanes have numerous advantages over traditional synthetic routes (Hollmann et al., 2011; Schrewe et al., 2013). For example, chemo-catalysts often are limited with respect to regio- and stereoselectivity and commonly perform with lower turnover numbers (Bordeaux et al., 2012). Additionally, synthetic efforts can yield expensive catalysts (metal complexes or metal-oxides) that pose challenges in waste disposal and often require harsh reaction conditions such as elevated temperatures (>200 °C) (Crabtree, 2001; Shilov and Shul'pin, 1997).

One-pot double oxidation of alkanes in cell-free systems has recently been published employing P450 BM3 monooxygenase variants and alcohol dehydrogenases (Müller et al., 2013; Staudt et al., 2013) with product titers up to 0.8 g L^{-1} and 0.2 g L^{-1} and TONs of 11641 and 3591 for cyclooctane and *n*-heptane as substrates respectively. P450 BM3 is a self-sufficient industrially

* Corresponding author. Tel.: +49 241 80 24170; fax: +49 241 80 22387.
E-mail address: u.schwaneberg@biotec.rwth-aachen.de (U. Schwaneberg).
[1] Permanent address: Institute of Environmental Biotechnology, Graz University of Technology, Petersgasse 12, 8010 Graz, Austria.
[2] Permanent address: Department of Chemistry, Organic and Bioorganic Chemistry, University of Graz, Heinrichstrasse 28, 8010 Graz, Austria.

http://dx.doi.org/10.1016/j.jbiotec.2014.06.001
0168-1656/© 2014 Elsevier B.V. All rights reserved.

attractive monooxygenase that is capable of hydroxylating a broad range of substrates, ranging from alkanes to steroids (Dennig et al., 2012; Whitehouse et al., 2012). Major limitations for a commercial application are relatively low stabilities and cofactor dependency of monooxygenases (Urlacher and Eiben, 2006) that are often associated with low coupling efficiencies for non-natural substrates (Bernhardt, 2006; Müller et al., 2013; Whitehouse et al., 2012).

Scheme 1 shows the developed whole cell double oxidation system which overcomes the main limitations of the *in vitro* one pot double oxidation by employing resting cells with expressed oxidoreductases as catalysts in a buffered medium, with supplemented glucose for cofactor regeneration, oxygen as oxidant and *n*-heptane as model substrate. Oxidation reactions that require regeneration of a cofactor or multi-enzymatic pathways can be operated efficiently in whole cells (Schrewe et al., 2013). In this way, the cell metabolism ensures intracellular availability and regeneration of reduction equivalents that are then employed as cofactors in the oxidative catalysis (Blank et al., 2010). A parallel resolution and separation of products (e.g. employing a two-liquid-phase system) (Lilly, 1982) was employed to minimize purification efforts and reduce process expenses.

The double oxidation of aliphatic alkanes enables direct synthesis of ketones and/or chiral alcohols, using molecular oxygen as sole oxidant in aqueous solution (see Scheme 1). The oxidation cascade products, ketones and chiral alcohols, are important building blocks for the pharmaceutical, agrochemical and food industries (Breuer et al., 2004). For instance, aliphatic ketones are employed in the production of flavors and fragrances (Weissermel and Arpe, 2003). The developed double oxidation process for *n*-heptane represents to our best knowledge the first one in a whole cell system.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals used were of analytical grade or higher and were purchased, if not stated otherwise from Sigma–Aldrich (Steinheim, Germany), AppliChem (Darmstadt, Germany), TCI Europe (Eschborn, Germany), Merck (Darmstadt, Germany) and Carl Roth (Karlsruhe, Germany). Enzymes and dNTPs were purchased from Fermentas (St. Leon-Rot, Germany) and New England Biolabs (Frankfurt, Germany). All employed HPLC-purified oligonucleotides were obtained from Eurofins MWG Operon (Ebersberg, Germany).

2.2. Strategies for coexpression of P450 BM3 and ADHs

Different coexpression strategies for the heterologous expression of P450 BM3 variants (WT^{NADH}, 19A12, $19A12^{NADH}$, CM1, $CM1^{NADH}$) and ADHs (RE-ADH and Lb-ADH) in *Escherichia coli* (*E. coli*) cells were investigated. An overview of the generated coexpression strains can be found in Supplementary Data (Table S1). For generation of strains containing two plasmids chemically competent *E. coli* BL21(DE3) Gold $lacI^{Q1}$ cells (Blanusa et al., 2010) were transformed with 50 ng of each plasmid (pAlXtreme-1a and pKA1) simultaneously and were spread on agar plates containing both antibiotics (50 μg mL^{-1} kanamycin, 34 μg mL^{-1} chloramphenicol). Construction of the coexpression systems with the commercial vector pACYCDuet-1 from Merck (Darmstadt, Germany) was performed by restriction cloning. The RE-ADH gene was cloned employing restriction sites *NdeI* and *EcoRV* in the second multiple cloning site (MCS2). A restriction site *EcoRV* was introduced at the end of the RE-ADH gene-sequence by PCR (40 ng pKA1 RE-ADH as template, 1 U of Phusion DNA polymerase, 0.2 mM dNTPs, 1x Phusion buffer and 0.4 μM of each primer, final volume of 50 μL). PCR conditions: 98 °C for 2 min, 25 cycles (98 °C for 30 s, 57 °C for 30 s, 68 °C for 30 s), 72 °C for 10 min, employing forward primer (RE-ADH_F_*NdeI*) 5′-GGAGATATACATATGAAGGCAATCCAG-3′ and reverse primer (RE-ADH_R_*EcoRV*) 5′-GCAGCCGATATCTTACAGACCAGGGACCACAACC-3′. The P450 BM3 monooxygenase variant $CM1^{NADH}$ was cloned into MCS1 using restriction sites *EcoRI* and *NcoI*. The resulting plasmid was transformed into chemically competent *E. coli* BL21 (DE3) Gold cells.

Construction of the artificial operon was performed using the PLICing (Phosphorothioate-based ligase-independent gene cloning) method (Blanusa et al., 2010). Four phosphorothioated oligonucleotide primers (Table 1) were designed for amplification of the P450 BM3 monooxygenase gene together with the pALXtreme-1a vector backbone (no. 1–2, larger fragment; vector DNA) and the RE-ADH gene (no. 3–4, smaller fragment; insert DNA).

Using the forward primer for amplification of the RE-ADH gene (Table 1, no. 3) an additional ribosome binding site (RBS) for translation of the ADH in the artificial operon construct was introduced upstream of the ADH gene. The RBS contains the Shine–Dalgarno sequence (SD: UAAGGAGGU) (Ringquist et al., 1992; Shine and Dalgarno, 1975) and an optimal sequence spacing of five nucleotides (Chen et al., 1994) between SD and translational initiation codon (ATG). A schematic representation of the generated artificial operon can be found in Supplementary Data, Fig. S1.

The vector pALXtreme-1a containing the respective P450 BM3 (WT^{NADH}, $19A12^{NADH}$, $CM1^{NADH}$) was used as template DNA for amplification of the monooxygenase together with the vector backbone. Prior to amplification it was linearized by digestion using *EcoRI*. RE-ADH was amplified directly from the circular pKA1 plasmid. A two-stage PCR was employed (Wang and Malcolm, 1999). The PCR mixture (50 μL) contained template DNA (20 ng), 1 U Phusion DNA polymerase, 0.2 mM dNTPs, 1x Phusion buffer and 0.2 μM forward or reverse primer. Following temperature program was used for the first amplification step: initial denaturation at 98 °C for 2 min, followed by 5 cycles of denaturation at 98 °C for 30 s, annealing at 55 °C for 1 min and elongation at 72 °C for 9 min (P450 BM3) or 2 min (RE-ADH). Subsequently, 25 μL of forward and 25 μL of reverse amplified reaction products were mixed together and 0.5 U of Phusion DNA polymerase were added. PCR program for the second amplification step: initial denaturation at 98 °C for 2 min, followed by 25 cycles of denaturation at 98 °C for 30 s, annealing at 55 °C for 1 min and elongation at 72 °C for 6 min (P450 BM3) or 1 min (RE-ADH), and a final elongation step at 72 °C for 5 min.

2.3. Cultivation of E. coli in shake flasks

Cultivation and expression in shake flask of the *E. coli* constructs containing only one P450 BM3 monooxygenase variant (CM1 and P450 BM3 $CM1^{NADH}$) was performed as previously described (Staudt et al., 2012). Coexpression of two or three proteins (P450 BM3, RE-ADH, LB-ADH) in the generated *E. coli* strains (see Supplementary Data, Table S1), was achieved with the same protocol with minor modifications. The corresponding antibiotics, depending on the plasmid(s) present (50 μg mL^{-1} kanamycin for pALXtreme-1a; 34 μg mL^{-1} chloramphenicol for pKA1 or pACYCDuet-1) were supplemented to the culture media. In addition, for expression of RE-ADH, the main culture was supplemented with 1 mM $ZnCl_2$ directly after induction with IPTG. Cell pellets were stored at −20 °C until further use.

Download English Version:

https://daneshyari.com/en/article/23015

Download Persian Version:

https://daneshyari.com/article/23015

Daneshyari.com