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Encapsulation of recombinant *E. coli* expressing cyclopentanone monooxygenase in polyelectrolyte complex capsules for Baeyer–Villiger biooxidation of 8-oxabicyclo[3.2.1]oct-6-en-3-one

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Abstract Recombinant *Escherichia coli* cells, over-expressing cyclopentanone monooxygenase activity, were immobilized in polyelectrolyte complex capsules, made of sodium alginate, cellulose sulfate, poly(methylene-co-guanidine), CaCl_2 and NaCl. More than 90% of the cell viability was preserved during the encapsulation process. Moreover, the initial enzyme activity was fully maintained within encapsulated cells

while it halved in free cells. Both encapsulated and free cells reached the end point of the Baeyer–Villiger biooxidation of 8-oxabicyclo[3.2.1]oct-6-en-3-one to 4,9-dioxabicyclo[4.2.1]non-7-en-3-one at the same time (48 h). Similarly, the enantiomeric excess above 94% was identical for encapsulated and free cells.

Keywords Baeyer–Villiger biooxidation · Cyclopentanone monooxygenase · Encapsulation · Immobilization · Poly(methylene-co-guanidine)

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Introduction

Enzyme-mediated Baeyer–Villiger biooxidations of synthetic ketones catalysed by Baeyer–Villiger monooxygenases (BVMOs, EC 1.14.13.xx) offers “green chemistry” approach for the production of chiral lactones as versatile intermediates for synthesis of bio-active compounds (Mihovilovic et al. 2006a). BVMOs belong to the flavin monooxygenases family and are NADPH-dependent. Two different approaches have been exploited for cofactor regeneration: (i) isolated BVMOs with regeneration of NADPH utilizing an auxiliary dehydrogenase, (ii) utilization of whole-cells expressing BVMOs (Mihovilovic 2006). The first approach was recently developed into a fusion-biocatalyst concept by covalently connecting both catalytic domains (Pazmino et al. 2008). The second concept of overexpression of BVMOs in non-pathogenic strain such as *Escherichia coli* provides “easy-to-use”

catalytic system for organic syntheses (Mihovilovic et al. 2006b). Cyclopentanone monooxygenase from *Comamonas* sp. NCIMB 9872 (CPMO, EC 1.14.13.16), overexpressed in *E. coli* was chosen as a representative candidate from the family of BVMOs, since it displayed a remarkable substrate profile and stereoselectivity (Rudroff et al. 2006). The latter enzyme catalyses preparatively useful Baeyer–Villiger biooxidation of synthetic 8-oxabicyclo[3.2.1]oct-6-en-3-one to 4,9-dioxabicyclo[4.2.1]non-7-en-3-one with excellent enantioselectivity (Mihovilovic et al. 2005).

It is assumed that immobilization of whole-cell CPMO may preserve high regio- and enantioselectivity of Baeyer–Villiger biooxidation, which is a prerequisite for the pharma industry (Patel 2008). Therefore it is desirable to employ a controlled encapsulation of whole-cell CPMO within polyelectrolyte complex (PEC) capsules, which proved to be suitable for encapsulation of *Nocardia tartaricans* cells (Bučko et al. 2005) and enzyme glucose oxidase (Vikartovská et al. 2007). Encapsulation may provide an universal immobilization technique for the family of whole-cell and isolated BVMOs and their synthetic substrates since it allows (i) efficient immobilization process with controlled encapsulation parameters for nearly monodisperse capsules, (ii) biocompatible microenvironment for viable cells, (iii) available space for cell growth and movement, (iv) adjustability of mechanical resistance and permeability of membranes, (v) tolerance of water-miscible and water-immiscible solvents, (vi) increase of O₂ solubility using O₂ carriers (Bučko et al. 2009), (vii) appropriate storage and operational stability, (viii) adaptability and ability to increase an encapsulation throughput. The aim of this work was to perform a first approach for an encapsulation of recombinant cells *E. coli* with CPMO within PEC capsules based on sodium alginate, cellulose sulfate and poly(methylene-co-guanidine). Viability and storage stability of encapsulated cells, the course and enantioselectivity of above mentioned Baeyer–Villiger oxidation were investigated.

Methods

Materials

Substrate and product of Baeyer–Villiger biooxidation, 8-oxabicyclo[3.2.1]oct-6-en-3-one and

4,9-dioxabicyclo[4.2.1]non-7-en-3-one, respectively, were prepared as previously described (Kim and Hoffmann 2000; Mihovilovic et al. 2006b). Bicyclo[3.2.0]hept-2-en-6-one and (1*R*,5*S*)-3-Oxabicyclo-[3.3.0]oct-6-en-3-one were from Fluka (1*S*,5*R*)-(-)-2-Oxabicyclo-[3.3.0]oct-6-en-3-one was from Aldrich. LB_{amp} medium for cell growth contained in (g l⁻¹): peptone 10; yeast extract 5; NaCl 10; ampicillin 0.2. TB_{amp} medium contained in (g l⁻¹): tryptone 12; yeast extract 24; glycerol 5; K₂HPO₄·3H₂O 16.4; KH₂PO₄ 2.3; ampicillin 0.2. IPTG was from Takara Bio Inc. (Otsu, Japan). High viscosity sodium alginate from ISP Alginates (Girvan, Ayrshire, UK) and cellulose sulfate, sodium salt, from Acros Organics (New Jersey, NJ, USA) were used directly, poly(methylene-co-guanidine hydrochloride) from Scientific Polymer Products Inc. (Ontario, NY, USA), supplied as 35% (w/v) aqueous solution, was lyophilized prior to use to be characterized (altogether with sodium alginate and cellulose sulfate) as reported previously (Bučko et al. 2005).

Gas chromatography

Substrates and products were determined by GC (Rudroff et al. 2006) using a HP 5890 Series D gas chromatograph with detector R, BGB-5 column (30 m × 0.32 mm ID, 0.25 μm film) and HP-INNO-Wax column (30 m × 0.32 mm ID, 0.25 μm film). Enantiomeric excess was determined using a chiral BGB-175 column (30 m × 0.25 mm ID, 0.25 μm film). Samples were extracted with CH₂Cl₂ (1:1 v/v) containing 1 mg methyl benzoate ml⁻¹ as internal standard and organic layer was analysed by GC.

Cultivation of cells

Recombinant *E. coli* over-expressing CPMO (EC 1.14.13.16) from *Comamonas* NCIMB 9872 (Rudroff 2006) was used as follows: *E. coli* was cultured on LB_{amp} agar plates for 12–15 h at 37°C. A single colony was inserted into 10 ml LB_{amp} medium and cultivated at 37°C for 12–15 h on rotary shaker (120 rpm). After, 100 ml TB_{amp} medium was inoculated with 1% (v/v) of the latter inoculum and cultivated for another 8 h. Biomass was collected by centrifugation for 15 min at 4,000 s⁻¹ and 23°C and used for encapsulation. Equivalent amounts of wet cells (normalized via dry cell wt of 20%) were

used in biotransformations using encapsulated and free cells.

CPMO-PEC capsules preparation

Encapsulation of *E. coli* with CPMO was performed in a custom-made coaxial air-stripping extrusion device fitted with a multiloop reactor as reported previously (Bučko et al. 2005; Vikartovská et al. 2007). The polyanion solution was prepared from 0.9% (w/v) sodium alginate and 0.9% (w/v) cellulose sulfate in 0.9% (w/v) NaCl at pH 7.0. A freshly centrifuged paste of *E. coli* cells (20% of dry cell wt) was suspended in polyanion solution to obtain concentration of wet cells of 100 mg ml⁻¹ polyanion. Drops of polyanion solution with cells were extruded at an air pressure of 0.4 bar, a flow rate of stripping air in coaxial nozzle of 1.5 l min⁻¹ and a polyanion flow rate of about 0.5 ml min⁻¹ into the stream of polycation solution that was continuously flowing in the 5-loop reactor. A polycation solution consisting of 1.8% (w/v) poly(methylene-co-guanidine), 1% (w/v) CaCl₂ and 0.9% (w/v) NaCl was delivered from the 5 l container into the 5-loop reactor at an air pressure of 0.1 bar and at 50 ml min⁻¹ providing the reaction time for capsule formation of 70 s. The reaction was quenched by collecting CPMO-PEC capsules at the exit of the reactor in 150 ml 0.9% (w/v) NaCl at 1 min intervals. Capsules were then treated for 5 min with an excess of 50 mM citrate in 0.9% (w/v) NaCl, followed by washing in 0.9% (w/v) NaCl. Imaging and determination of average size and membrane thickness was performed as reported previously (Bučko et al. 2005).

Cell viability measurement by confocal laser scanning microscopy (CLSM)

Transmission and fluorescence contrast modes of the confocal laser scanning microscope LSM 510 META coupled to inverted microscope stand Axiovert 200 M (both Zeiss, Germany) were employed for imaging of the cells. A 40× C-Apochromat/1.2 water objective and pinhole aperture of 1 Airy unit corresponding to the 2 μm thick optical slice in the object plane was used. The cell viability was quantified by the intake of propidium iodide (PI) into necrotic cells, which was measured simultaneously with the intrinsic cell flavin fluorescence (AF) and yielded the identification of the label-free bacteria. The cells were withdrawn

after each encapsulation step including cultivation, centrifugation, mixing with polyanion solution, homogenization of CPMO-PEC capsules using a mini-homogenizer into a solution of 0.9% (w/v) NaCl and incubation of the latter suspension in 0.9% (w/v) NaCl at 5°C for 24 h. The cells were incubated for 10 min with PI at 1 μM. Fluorescence of both PI and flavin was excited by 488 nm line of Argon:ion laser. A two-channel detector configuration with BP 500–550 nm bandpass emission filter for AF and LP 560 nm long-pass emission filter for PI fluorescence detection were used for signal separation. The cell viability was evaluated by CLSM as a ratio $(N_{\text{all}} - N_{\text{PI}})/N_{\text{all}}$ where N_{all} is the total number of bacterial cells identified by AF fluorescence, and N_{PI} is a number of necrotic cells identified by PI fluorescence.

Storage stability

The storage stability of free and encapsulated cells was expressed in percentage of initial enzyme activities of both preparatives during storage at 4°C. CPMO activities were investigated using biooxidation of bicyclo[3.2.0]hept-2-en-6-one. Free and encapsulated cells were stored in aqueous solution consisting of (g l⁻¹): K₂HPO₄·3H₂O 16.4; KH₂PO₄ 2.3; glucose 4; ampicillin 0.2. Two grams of stored encapsulated cells or corresponding amount of free wet cells (200 mg wet cells with 20% of dry cell wt) were added to the above mentioned solution to obtain 20 ml of reaction mixture. Cells were activated prior to the biooxidation with 0.25 mM IPTG at 120 rpm in orbital shaker for 2 h at 25°C. The Baeyer–Villiger biooxidation was started by addition of the substrate, bicyclo[3.2.0]hept-2-en-6-one (10% v/v in ethanol), to obtain the concentration of 0.5 g l⁻¹. Samples (150 μl) were analysed by GC as outlined above. Enzyme activities were determined from the concentration of the product—mixture of produced lactones (1*R*,5*S*)-3-Oxabicyclo-[3.3.0]oct-6-en-3-one and (1*S*,5*R*)-(-)-2-Oxabicyclo-[3.3.0]oct-6-en-3-one (Doig et al. 2003). One unit of the CPMO activity corresponds to formation of 1 μmol product per 1 min.

Baeyer–Villiger biooxidation of 8-oxabicyclo[3.2.1]oct-6-en-3-one using CPMO-PEC capsules

Baeyer–Villiger biooxidation of 8-oxabicyclo[3.2.1]oct-6-en-3-one to 4,9-dioxabicyclo[4.2.1]non-7-en-3-one,

catalysed either by encapsulated or free cells *E. coli* with CPMO was performed in batch-wise mode in gas-washing bottle. The bottle was equipped with fritted glass tip for oxygenation and mixing to keep capsules in fluidized bed-like conditions and thermostatted in water bath at 25°C. Pure O₂ was used for oxygenation to ensure O₂ saturation during biooxidation. Five grams CPMO-PEC capsules or corresponding amount of free wet cells (500 mg wet cells at 20% dry cell wt) were added to the solution consisting of (g l⁻¹): K₂HPO₄·3H₂O 16.4; KH₂PO₄ 2.3; glucose 4; ampicillin 0.2 to obtain 50 ml of reaction mixture. Cells were activated prior biooxidation with 0.25 mM IPTG and O₂ bubbling for 2 hs at 25°C. Biooxidation was started by addition of the substrate 8-oxabicyclo[3.2.1]oct-6-en-3-one (10% v/v in ethanol) to give of 0.7 g l⁻¹ together with addition of β -cyclodextrin (10 mol.%). Samples (150 μ l) were analysed by GC as outlined above. Glucose concentrations were determined periodically by a spectrophotometric horseradish peroxidase assay described elsewhere (Vikartovská et al. 2007) and glucose was added to maintain 4 g l⁻¹. The concentration of dissolved O₂ in aqueous reaction mixtures was determined using an oxygen meter.

Results and discussion

Encapsulation of *E. coli* with CPMO in PEC capsules

The CPMO-PEC capsules (shown in Supplementary Fig. 1) were comparably uniform in size and membrane thickness to those reported in our previous work (Bučko et al. 2005). The mean diameter of capsules was estimated to be 0.8 ± 0.03 mm and the mean membrane thickness was to 0.13 ± 0.01 mm. The values of standard deviations were 4% for capsules diameter and 8% for the membrane thickness. The high uniformity of prepared capsules is very important for correct comparison of catalytic efficiency by providing equal mechanical, chemical and diffusional properties of the capsule batches.

Viability of encapsulated cells by confocal laser scanning microscopy

A successful encapsulation of *E. coli* with CPMO in PEC capsules (CPMO-PEC capsules) and the precise measurement of the cell viability in each encapsulation stage represent crucial steps in maintaining and monitoring the cell viability within PEC capsules. The viability of *E. coli* cells (CPMO) during all encapsulation steps, assessed by confocal laser scanning microscopy (CLSM) was $93.8 \pm 3.6\%$. In this regard, PEC capsules may be considered as a highly biocompatible immobilization matrix able to preserve the viability of *E. coli* with CPMO in the entire encapsulation procedure. Thus the CLSM method utilizing simultaneous measurement of PI intake into necrotic cells and the intrinsic cell AF fluorescence is a powerful technique for a quantitative assessment of the cell viability during the encapsulation process.

Effect of cell encapsulation on biocatalytic efficiency of *E. coli* with CPMO

The storage stability of cells encapsulated in PEC capsules, compared with the free cells is shown in Fig. 1. While the initial CPMO activity (determined using Baeyer–Villiger biooxidation of bicyclo[3.2.0]hept-2-en-6-one) decreased to 54% after 96 h for free cells, it was completely maintained for the encapsulated cells. It should be noted that the initial activity for encapsulated cells is about 5-times lower (0.12 U g^{-1}) than that for the free cells (0.58 U g^{-1}) as is typically seen for the encapsulated biocatalysts such as enzyme glucose oxidase encapsulated in the PEC capsules (Vikartovská et al. 2007). The increased stability of encapsulated cells may be explained by the confinement of the cells behind a capsular membrane with controlled pore size (Bučko et al. 2005; Vikartovská et al. 2007), which may lead to the stabilization of the plasmid copy number (Kumar and Schügerl 1990), resulting in preserving the CPMO activity during storage. Moreover, due to the high cell density and the mass transfer resistance of the capsules, the cells may be exposed to lower concentrations of toxic substrate bicyclo[3.2.0]hept-2-en-6-one within the PEC capsules than it is for the free cells.

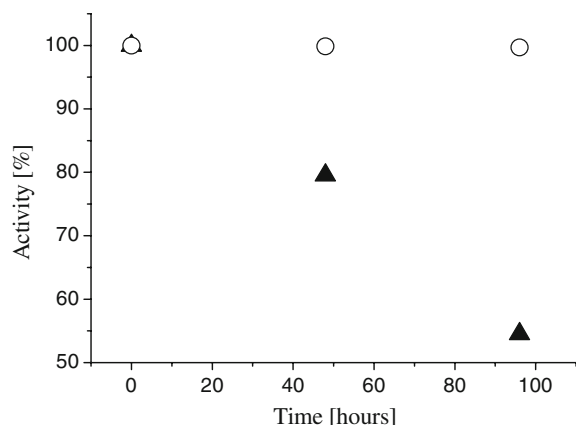


Fig. 1 Storage stability of free (filled triangle) and encapsulated (open circle) *E. coli* cells with CPMO after storage at 4°C using model Baeyer–Villiger biooxidation of bicyclo[3.2.0]hept-2-en-6-one. The value of 100% represents 0.12 and 0.58 U g^{-1} for encapsulated and free cells, respectively. 1 U represents $1 \mu\text{mol min}^{-1}$ of substrate utilization

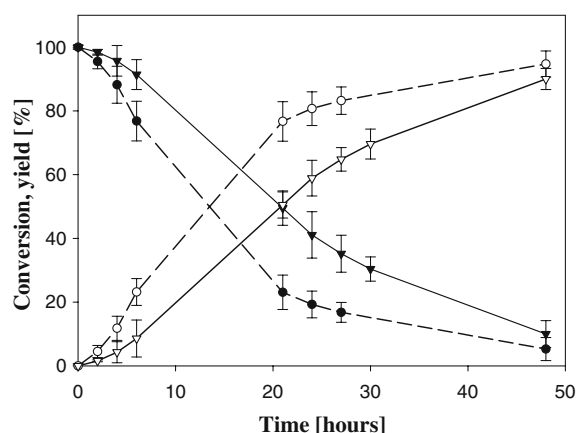


Fig. 2 Baeyer–Villiger biooxidation of 8-oxabicyclo[3.2.1]oct-6-en-3-one to 4,9-dioxabicyclo[4.2.1]non-7-en-3-one using free *E. coli* cells with CPMO and the same cells encapsulated in PEC capsules. Oxygen concentration was 0.625 mM. Indicated conversions using free (filled circle) and encapsulated (inverted filled triangle) cells for the substrate and yields using free (open circle) and encapsulated (inverted open triangle) cells for the product were determined from concentrations measured by GC

The time evolution of 8-oxabicyclo[3.2.1]oct-6-en-3-one conversion to 4,9-dioxabicyclo[4.2.1]non-7-en-3-one using encapsulated and free cells, respectively, is depicted in Fig. 2. The substrate conversion rate during the complete Baeyer–Villiger biooxidation was to a certain degree higher using free cells than the encapsulated cells as shown in Table 1. This behavior can be explained by the mass transfer resistance experienced

Table 1 Comparison of substrate conversion rates at 5 and 48 h, respectively, using free and encapsulated cells *E. coli* with CPMO during Baeyer–Villiger biooxidation of 8-oxabicyclo[3.2.1]oct-6-en-3-one to 4,9-dioxabicyclo[4.2.1]non-7-en-3-one

Form of CPMO biocatalyst	Substrate conversion at 5 h (%)	Substrate conversion at 48 h (%)
Free cells	19.5	94.7
Encapsulated cells	10.3	91

by the cells in CPMO-PEC capsules, which is not present in the case of free cells. Interestingly, the time required to reach the end point of the biooxidation was 48 h for both free and encapsulated cells with the same initial substrate concentration and the same total amount of cells per the reactor volume. Therefore, the CPMO-PEC capsules may be considered as operationally effective immobilization system for *E. coli* with CPMO. On the other side, the intensive bubbling resulted in the turbidity of reaction mixture during biooxidation with encapsulated cells. This indicates on bursting of a negligible amount of CPMO-PEC capsules due to shear forces and the release of encapsulated cells.

Enantiomeric excess percentage (ee) of the produced (+) 4,9-dioxabicyclo[4.2.1]non-7-en-3-one was $94.9 \pm 0.3\%$ using free cells and $94.7 \pm 0.5\%$ using encapsulated cells. Obtained data are in good accordance with previously achieved ee value of 95% using free cells (Mihovilovic et al. 2005). Thus, the encapsulation of *E. coli* with CPMO in PEC capsules did not negatively influence the high CPMO enantioselectivity in Baeyer–Villiger biooxidation of 8-oxabicyclo[3.2.1]oct-6-en-3-one.

Conclusions

PEC capsules provided a biocompatible microenvironment for recombinant *E. coli* cells over-expressing CPMO. A novel approach on the basis of confocal laser scanning microscopy for determination of cell viability during encapsulation of *E. coli* with CPMO in PEC capsules was developed. The performance of encapsulated cells was improved compared to the free cells expressed in a constant CPMO activity during the storage test experiment. This work clearly demonstrates that CPMO-PEC capsules provide an

universal form of immobilized viable cell biocatalyst for the effective catalysis of Baeyer–Villiger biooxidations.

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