Encapsulation of recombinant cells *E. coli* catalysing Baeyer-Villiger oxidations

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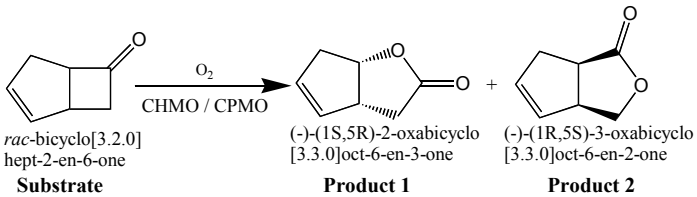
INTRODUCTION

Enzyme mediated biooxidations are interesting for industrial biotechnology processes (so called white biotechnology) due to high enantioselectivity and high redox potential of oxidative enzymes, which may catalyse reactions of chemically resistant substrates (Burton S.G. 2003). Baeyer-Villiger (BV) biooxidations catalysed by Baeyer-Villiger monooxygenases (BVMOs, E.C. 1.14.13.xx) are highly active field of biooxidations (Mihovilovič M.D. 2006). Conversion of cyclic ketones to enantiomerically pure lactones via BV biooxidations allows access to chiral precursors of bioactive compounds (Mihovilovič M.D. 2006). Cyclohexanone monooxygenase from *Acinetobacter* sp. NCIMB 9871 (CHMO, EC 1.14.13.22) and cyclopentanone monooxygenase from *Comamonas* sp. NCIMB 9872 (CPMO, EC 1.14.13.16) have been proposed as the prototypes of the two main groups of cycloketone-converting BVMOs: CHMO- and CPMO-type clusters (Rial D.V. 2008). Lately, the BVMOs production cost was too high despite enzyme production improvement via overexpression of BVMOs in *E. coli* (Alphand V. 2003). Immobilization is known as an effective technology to achieve catalyst stabilization and reuse and thus decrease of catalyst production cost (Alphand V. 2003). Significant improvements were achieved recently by encapsulation of whole-cell and enzyme biocatalyst in polyelectrolyte complex capsules made of sodium alginate (SA), cellulose sulfate (CS), poly(methylene-*co*-guanidine) (PMCG), $CaCl_2$ and NaCl (SA-CS/PMCG capsules) (Bučko M. 2005, Vikartovská A. 2007). The aim of this work was encapsulation of recombinant cells *E. coli* overexpressing either CHMO or CPMO within SA-CS/PMCG capsules. Preliminary investigations regarding storage stability of encapsulated CHMO and productivity optimization of encapsulated CPMO have been performed. Enantioselective BV biooxidation of *rac*-bicyclo[3.2.0]hept-2-on-6-ene to its regioisomeric lactones (-)-(1S,5R)-2-oxabicyclo-[3.3.0]oct-6-en-3-one (product 1) and (-)-(1R,5S)-3-oxabicyclo-[3.3.0]oct-6-en-3-one (product 2) was used as the model reaction for investigation of encapsulated CHMO and CPMO (Mihovilovič M.D. 2006) (Scheme 1).



Scheme 1. Model Baeyer-Villiger biooxidation used for investigation of encapsulated whole-cell CHMO and CPMO (Mihovilovič M.D. 2006).

MATERIAL AND METHODS

Materials. Recombinant cells *Escherichia coli* with overexpressed CHMO and CPMO were kindly donated by prof. M.D. Mihovilovič (IAS, VUT Vienna, Austria). Substrate and standard of product 2 were from Fluka, standard of product 1 was from Aldrich. LB_{amp} medium for cell growth contained peptone (10 $g \cdot l^{-1}$), yeast extract (5 $g \cdot l^{-1}$), NaCl (10 $g \cdot l^{-1}$) and 200 $mg \cdot l^{-1}$ of ampicillin. Agar (15 $g \cdot l^{-1}$) was added into LB_{amp} medium and used for plates. TB_{amp} medium contained tryptone (12 $g \cdot l^{-1}$), yeast extract (24 $g \cdot l^{-1}$), glycerol (5 $g \cdot l^{-1}$), $K_2HPO_4 \cdot 3H_2O$ (16.4 $g \cdot l^{-1}$) and KH_2PO_4 (2.3 $g \cdot l^{-1}$). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was from Takara Bio Inc. (Otsu, Japan). High viscosity sodium alginate (SA) was from ISP Alginates (Girvan, Ayrshire, UK), cellulose sulfate (CS), sodium salt, was from Acros Organics (New Jersey, NJ, USA), poly(methylene-*co*-guanidine hydrochloride) (PMCG) (Scientific Polymer Products Inc., Ontario, NY, USA) supplied as 35% (w/v) aqueous solution was lyophilized prior use. SA, CS and PMCG were characterized as reported previously (Bučko et al. 2005). Compressed oxygen had a purity of 99.5%. All other chemicals were of analytical grade.

Cultivation conditions. E. coli expressing either CHMO or CPMO were incubated at 37°C on LB_{amp} plates for 12-15 hours. A single colony was selected and 10 ml of LB_{amp} media as a pre-culture was inoculated and cultivated (150 rpm, 12-15 hours, 37°C). 200 ml batches of the growth TB_{amp} media were inoculated with 1% (v/v) of the pre-culture and cultivated (150 rpm, 37°C). The biomass was centrifuged at 4000 rpm for 15 min and encapsulated. Cultivation time of *E. coli* with CHMO for storage stability tests was 8 hours. *E. coli* with CPMO were withdrawn for encapsulation after 2, 5, 8, 12 and 16 hours of cultivation for productivity optimization.

Encapsulation. A coaxial air-stripping extrussion device fitted with multiloop reactor (Anilkumar A.V. 2001) was used for encapsulation of *E. coli* with either CHMO or CPMO. Cells were suspended in polyanion solution (PA) prepared from SA and CS in 0.9% (w/v) NaCl at pH 7.0 to achieve the following concentrations: 0.9% (w/v) SA, 0.9% (w/v) SA and 16% (w/w) of wet CHMO cells or 10% (w/w) of wet CPMO cells. The latter suspensions were air-stripped through a concentric nozzle of the encapsulator into the stream of polycation solution (PC) in reactor, consisting of 1.8% (w/v) PMCG, 1.0% (w/v) $CaCl_2$ and 0.9% (w/v) NaCl. PC flow rate of 68 ml/min provided reaction time of 60 s. The reaction was quenched by collecting the capsules at the exit of reactor in 150 ml batches of 0.9% (w/v) NaCl solution in the time intervals of 1 min. SA-CS/PMCG capsules with cells were incubated in sodium citrate (50 mM) for 5 min and washed with 0.9% (w/v) NaCl solution. Size analysis of capsules was performed as reported previously (Bučko M. 2005).

Storage stability of encapsulated whole-cell CHMO. 1.7 g of SA-CS/PMCG capsules with immobilized CHMO were activated in solution (50 ml) of 0.25 mM IPTG in reaction buffer (RB) consisting of 16.4 $g \cdot l^{-1}$ $K_2HPO_4 \cdot 3H_2O$, 2.3 $g \cdot l^{-1}$ KH_2PO_4, 200 $mg \cdot l^{-1}$ ampicillin and 4 $g \cdot l^{-1}$ glucose for 1 h at 25°C (stirred by oxygen bubbling). Capsules were placed inside the column (7 mm i.d. x 120 mm) and mounted into the enzyme thermistor (Vikartovská A. 2007), used as a model continuous column reactor for investigation of storage stability. Stirred beaker was filled with 100 ml of RB and 0.2 $g \cdot l^{-1}$ of substrate (10% v/v solution in ethanol) was added. RB with substrate was passed through the column at a flow rate of 0.3 $l \cdot min^{-1}$ and 25°C and recirculated to a stirred beaker. Recirculating solution was saturated with oxygen. Withdrawn samples (150 µl) were extracted with CH_2Cl_2 and analysed by gas chromatography. Storage stability was determined as the percantage of substrate conversion to products after 12 hours of BV biooxidation using fresh capsules stored in RB at 4°C, withdrawn in time intervals of 1, 14, 26 and 48 days.

Productivity optimization of encapsulated whole-cell CPMO. 2 g of capsules with CPMO were activated in mixed solution (18 ml) of 0.25 mM IPTG in RB (placed in erlenmayer flasks) for 1 hour at 25°C. Substrate was added to achieve concentration of 0.5 $g \cdot l^{-1}$. BV biooxidations were performed in batch-vise mode for 6 hours at 150 rpm and 25°C. Samples were analysed by GC as mentioned above.Productivity of encapsulated *E. coli* was determined by enzyme activity (A) and space-time yield of the product (STY). Cells with different incubation times (mentioned above) were encapsulated. One unit of A was [$U \cdot g^{-1}_{dry\ weight}$] which corresponds to production of 1 µmol of the product – mixture of product 1 and 2 in 1 min under specific conditions. One unit of STY of the product was [$g \cdot l^{-1} \cdot day^{-1}$], determined from the product concentrations in time of substrate total conversion.

RESULTS AND DISCUSSION

Figure 1 depicts recombinant cells *E. coli* expressing CPMO encapsulated in SA-CS/PMCG capsules. Average size of capsules determined by digital image analysis was 0.8 ± 0.03 mm and average membrane thickness was 0.13 ± 0.01 mm. Consequently, high uniformity of capsules was achieved with the standard deviation in size of 4 % and membrane thickness of 8 %. The storage stability of encapsulated cells with CHMO, evaluated after 12 hours of continuous BV biooxidations as the percentage of substrate conversion to products, measured within 48 days after encapsulation, is shown on Figure 2. The substrate conversion percentage reaches the maximum value of 100% 14 days after encapsulation. Encapsulation of *E. coli* with CPMO in SA-CS/PMCG capsules provides a favourable microenvironment for cells, since 79 % of substrate conversion was achieved 48 days after encapsulation and storage of the biocatalyst.

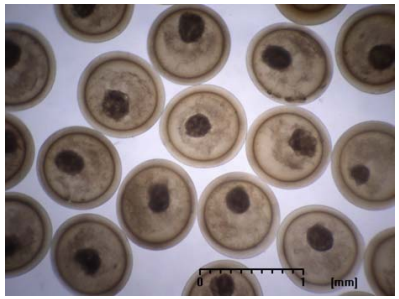


Figure 1. Photomicrography of *E. coli* with CPMO encapsulated in SA-CS/PMCG capsules

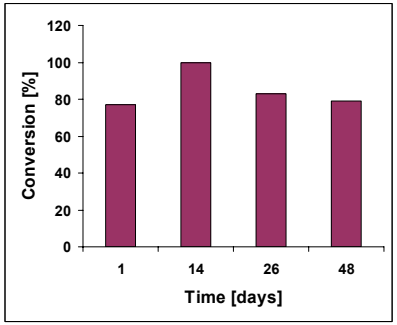


Figure 2. Storage stability of *E. coli* with CHMO encapsulated in SA-CS/PMCG capsules

Figure 3 shows the productivity optimization of encapsulated whole-cell CPMO, evaluated as the enzyme activity (A) and space-time yield (STY) of the product using cells with different cultivation times. The highest values of A of 2.5 $U \cdot g^{-1}_{dry\ weight}$ and STY of 3.4 $g \cdot l^{-1} \cdot day^{-1}$ were obtained using encapsulated cells with cultivation time of 12 h. Figure 4 depicts the course of BV biooxidation using the latter encapsulated cells.

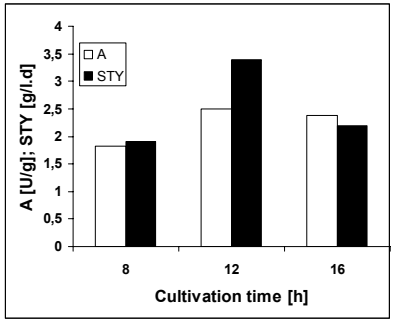


Figure 3. Enzyme activity of encapsulated *E. coli* with CPMO (A) and space-time yield of the product (STY) using encapsulated cells with different cultivation times

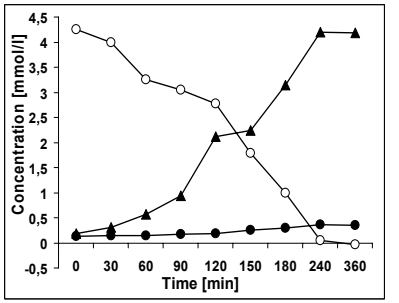


Figure 4. Course of BV biooxidation using encapsulated *E. coli* with CPMO (with cultivation time of 12 h). (○) – substrate, (▲) – product 1, (●) – product 2

CONCLUSIONS

Encapsulation in SA-CS/PMCG capsules resulted in stabilisation of whole-cell CHMO within storage and productivity optimization of whole-cell CPMO. Further experiments are needed to fully investigate the effects of SA-CS/PMCG capsules on whole-cell recombinant BVMOs. It is clear, however, that SA-CS/PMCG capsules provide a useful tool to assist in modeling the performance of BVMOs as the prerequisite for development of „biotechnological BVMO process".

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