Controlled encapsulation of biocatalysts for chiral immobilized biotechnology

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Introduction

The unrivalled selectivity of biocatalysts for production of chiral pharmaceuticals is the key to success of industrial biotransformations (Straathof 2006). Presented concept of chiral immobilized biotechnology is based on stereospecific production of model enantiomers – L-(+)-tartaric acid and D-gluconic acid utilizing encapsulated biocatalysts. *Nocardia tartaricans* cells with *cis*-epoxysuccinate (CES) hydrolase activity and enzyme glucose oxidase (GOD), respectively, were encapsulated 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) (Lacík 1998) and used as biocatalysts for production of L-(+)-tartaric acid from disodium CES and D-gluconic acid from D-glucose, respectively (Bučko 2005 and 2006, Vikartovská 2007). In addition, enantioselective synthesis of (1S,6S)-3,9-dioxabicyclo[4.2.1]non-7-en-4-one via Baeyer-Villiger (BV) biooxidation of 8-oxabicyclo[3.2.0]oct-6-en-3-one using encapsulated cells is proposed (http://www.gemokat.chem.sk). The latter reaction is catalysed by recombinant cells *Escherichia coli* with overexpressed enzyme Cyclopentanone Monooxygenase (CPMO) from *Comamonas sp.* NCIMB 9872 (EC 1.14.13.16) and the product is potential precursor in synthesis of natural compounds (Mihovilovič 2005). In this work, viable recombinant cells *E. coli* with CPMO, encapsulated in SA-CS/PMCG capsules, were used to assess biocompatibility of the whole encapsulation procedure. Tight control of the whole encapsulation process, i.e. uniform polyanion droplet (SA, CS and biocatalyst) production by an air-stripping nozzle followed by defined and continuous reaction with gelling ions (Ca^{2+}) and polycation (PMCG) in multiloop reactor (Anilkumar 2001) enabled precise adjustability of capsule properties such as their size, membrane thickness, molecular weight cut-off (MWCO) and mechanical resistance (Lacík 1998). The latter properties are prerequisites for successful immobilization of model biocatalysts and improvement of their biocatalytic efficiency as compared with entrapment in calcium pectate gel (CPG) beads. The main objective of the present work was to demonstrate the pros and cons of controlled encapsulation protocol for model biotransformations producing chiral compounds, observed during long-term examination of SA-CS/PMCG capsules for immobilization of the model whole-cell and enzyme biocatalysts. The following parameters were tested to show the main differencies in biocatalytic performance of biocatalysts encapsulated in SA-CS/PMCG capsules as compared to biocatalysts in free form and entrapped in CPG beads: a) enzyme activity during repeated biotransformations, i.e. operational stability of *N. tartaricans* cells and GOD, b) maximum enzyme activity during storage of *N. tartaricans* cells and space-time yield of produced L-(+)-tartaric acid, d) loss of GOD during encapsulation in SA-CS/PMCG capsules, e) biocompatibility of the whole encapsulation protocol, tested by viability measurement of encapsulated recombinant cells *E. coli* with CPMO by imaging of propidium iodide penetration in necrotic cells and flavine autofluorescence in cells using confocal laser scanning microscopy (CLSM).

Material and methods

Materials. Nocardia tartaricans ATCC 31191 cells were grown, centrifuged, lyophilized and stored as reported elsewhere (Bučko 2005). Recombinant cells *Escherichia coli* with overexpressed CPMO were kindly donated by prof. M.D. Mihovilovič (IAS, VUT Vienna, Austria). GOD from *Aspergillus niger* was from Biozyme Laboratories Ltd. (Gwent, UK). High viscosity SA was from ISP Alginates (Girvan, Ayrshire, UK); CS (sodium salt) was from Acros Organics (New Jersey, NJ, USA); PMCG hydrochloride (Scientific Polymer Products Inc.; Ontario, NY, USA) was lyophilized prior use; potassium pectate was prepared from commercial apple pectin (Pectin-Fabrik; Smiřice, Czech Rep.; http://www.chem.sk/products). Disodium CES was prepared as reported previously (Vikartovská 2004). All other chemicals were of analytical grade.

Encapsulation. A coaxial air-stripping extrussion device fitted with multiloop reactor (Anilkumar 2001) was used for encapsulation of *N. tartaricans* cells (Bučko 2005 and 2006), GOD (Vikartovská 2007) and *E. coli* with CPMO (http://www.gemokat.chem.sk). Generally, the latter biocomponents were suspended or dissolved in polyanion solution (PA) prepared from 0.8-0.9% (w/v) SA and 0.8-0.9% (w/v) CS in 0.9% (w/v) NaCl at pH 7.0 and air-stripped through concentric nozzle into the stream of polycation solution (PC) consisting of 1.8% (w/v) PMCG, 1.0% (w/v) $CaCl_2$ and 0.9% (w/v) NaCl at the flow rates from 45 to 50 ml/min providing reaction times from 70 to 100 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. Additionally, the second polyanion layer was prepared for capsules with immobilized GOD by subsequent 10 min treatment of capsules with an excess of 0.1% (w/v) CS solution.

Entrapment of *N. tartaricans* cells in hardened CPG beads was performed using Encapsulator Var E (Nisco Engineering, Zürich, Switzerland) as reported previously (Vikartovská 2004). GOD was entrapped in CPG beads using above described air-stripping device without multiloop reactor (Vikartovská 2007).

Enzyme activity of CES hydrolase in *N. tartaricans* cells was assessed via monitoring of L-(+)-tartrate production using HPLC technique (Bučko 2005). Enzyme activity of GOD was determined spectrophotometrically and flow calorimetry (FC) was used for determination of operational stability of GOD-SA-CS/PMCG capsules as reported previously (Vikartovská 2007). Enzyme loss during encapsulation of GOD was assessed by mass spectrometry (ESI-MS) (Vikartovská 2007).

Viability of cells was assessed by simultaneous imaging and counting of propidium iodide stained necrotic cells and flavine autofluorescence for visualisation of all cells using confocal laser scanning microscope Zeiss LSM 510 Meta on Axiovert 200M inverted microscope (Zeiss).

Results and Discussion

Figure 1 depicts the most important results regarding encapsulation of *N. tartaricans* cells for production of L-(+)-tartaric acid. Cells encapsulated in SA-CS/PMCG capsules retained 95 % of initial CES hydrolase activity (on average) after 10 repeated biotransformation cycles as compared to 38 % of retained activity in free cells (Bučko 2006). The maximum CES hydrolase activity during storage was 208 U/mg for encapsulated cells, which is about 2-fold as compared to the value obtained with CPG beads, which equals to 92 U/mg (Bučko 2005). The space-time yield of the product, 1550 g/l·d, was roughly 2-fold using encapsulated cells as compared to the value of 850 g/l·d for cells entrapped in CPG beads.

Quantification of the amount of GOD in each step of enzyme immobilization in SA-CS/PMCG capsules is summarized in Figure 2. The largest portion of GOD (56 %) was lost either to polycation solution or washing NaCl solution during the first step of the encapsulation, i.e. during the initial capsule and membrane formation (in the course of 70 s). During further encapsulation stages including washing in NaCl solution, treatment with citrate solution and coating with CS, the loss of the enzyme was less than 1 %. Thus the overall encapsulation efficiency was 43 % and no enzyme was lost during subsequent storage and biotransformation experiments (Vikartovská 2007).

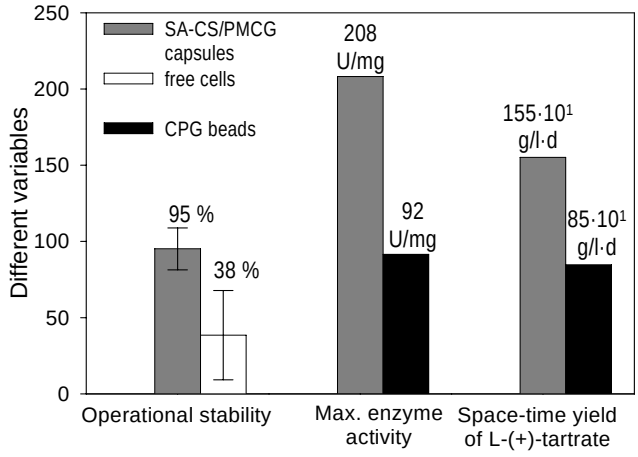

Figure 1. Summarization of the most important results regarding encapsulation of *N. tartaricans* cells

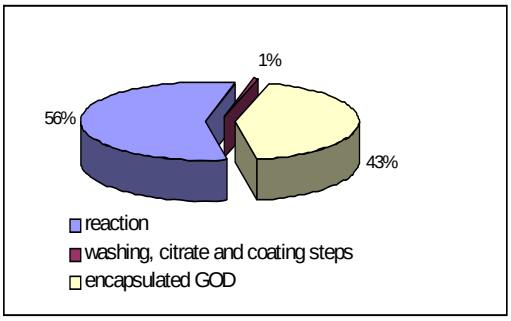

Figure 2. Division of GOD in encapsulation process. "Encapsulated GOD" – enzyme immobilized in the capsules. "Reaction" – enzyme loss in reactor. "Washing, citrate and coating steps" – enzyme loss in remainder post-encapsulation stages.

The latter statement was also proved by the observation that GOD-SA-CS/PMCG capsules retained 92 % of initial enzyme activity after 45 repeated biooxidations during assessment of operational stability (Figure 3). Enzyme activity of GOD entrapped in CPG beads disappeared after 12 biooxidation cycles due to uncontrolled porosity of CPG beads (Vikartovská 2007).

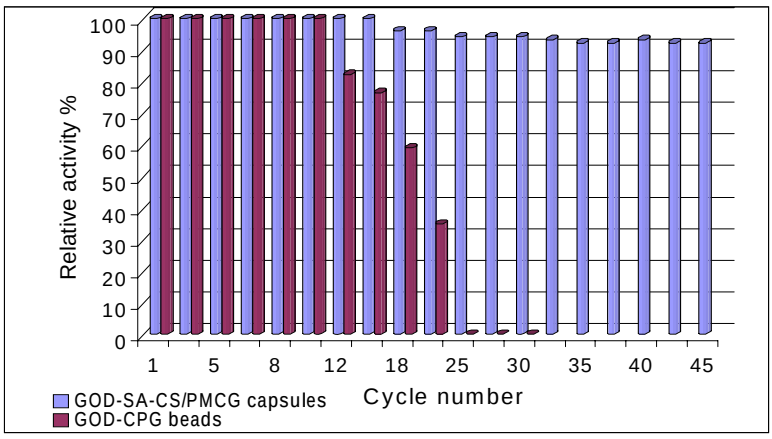

Figure 3. Operational stability of GOD-SA-CS/PMCG capsules and GOD-CPG beads by flow calorimetry.

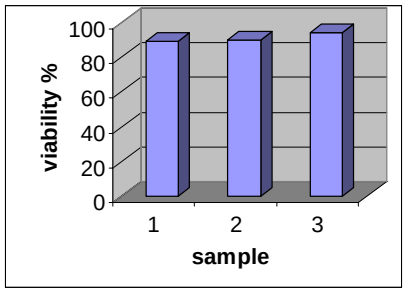

Figure 4. Viability of *E. coli* with CPMO by CLSM in different encapsulation stages: 1 – free cells after cultivation and centrifugation, 2 – cells after mechanical release from SA-CS/PMCG capsules, 3 – cells from the sample 2 after 1 day incubation at 5 °C

More than 90 % of viability of *E. coli* with CPMO was retained after encapsulation procedure as depicted on Figure 4. Developed method enables assessment of encapsulated cell viability which is important for future optimization of cultivation and encapsulation conditions with the aim to achieve maximum catalytic efficency of cells during BV biooxidations (www.gemokat.chem.sk).

Conclusions

Controlled encapsulation in SA-CS/PMCG capsules resulted in significant stabilisation of tested biocatalysts. Improvement of the product space-time yield, elimination of enzyme loss from capsules after encapsulation and cell viability preservation after encapsulation procedure are another possitive features of used encapsulation protocol. Elimination of enzyme loss during encapsulation, minimization of presumptive mass transfer resistance of capsules, capsule production up-scale as well as further increase of their mechanical resistance are challenging tasks for the future. It can be concluded, that SA-CS/PMCG capsules provide a useful tool to assist in understanding and modeling the performance of biocatalyst for chiral immobilized biotechnology.

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