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Continuous testing system for Baeyer–Villiger biooxidation using recombinant *Escherichia coli* expressing cyclohexanone monooxygenase encapsulated in polyelectrolyte complex capsules

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ABSTRACT

An original strategy for universal laboratory testing of Baeyer–Villiger monooxygenases based on continuous packed-bed minireactor connected with flow calorimeter and integrated with bubble-free oxygenation is reported. Model enantioselective Baeyer–Villiger biooxidations of rac-bicyclo[3.2.0]hept-2-en-6-one to corresponding lactones (1R,5S)-3-oxabicyclo-[3.3.0]oct-6-en-3-one and (1S,5R)-2-oxabicyclo-[3.3.0]oct-6-en-3-one as important chiral synthons for the synthesis of bioactive compounds were performed in the minireactor equipped with a column packed with encapsulated recombinant cells *Escherichia coli* overexpressing cyclohexanone monooxygenase. The cells were encapsulated in polyelectrolyte complex capsules formed by reaction of oppositely charged polymers utilizing highly reproducible and controlled encapsulation process. Encapsulated cells tested in minireactor exhibited high operational stability with 4 complete substrate conversions to products and 6 conversions above 80% within 14 repeated consecutive biooxidation tests. Moreover, encapsulated cells showed high enzyme stability during 91 days of storage with substrate conversions above 80% up to 60 days of storage. Furthermore, usable thermometric signal of Baeyer–Villiger biooxidation obtained by flow calorimetry using encapsulated cells was utilized for preparatory kinetic study in order to guarantee sub-inhibitory initial substrate concentration for biooxidation tests.

1. Introduction

Baeyer–Villiger biooxidations catalyzed by Baeyer–Villiger monooxygenases (BVMOs, EC 1.14.13.xx) are attractive for industrial biotechnology processes since they enable biotransformations of cyclic ketones to enantiomerically pure lactones as chiral precursors of bioactive compounds [1–4]. Moreover, recently described syntheses of enantiomerically pure β-amino alcohols [5], novel fused ring systems [6], and modified nucleosides [7] exploiting chiral biotransformations mediated by BVMOs demonstrated innovative and novel applications.

Cyclohexanone monooxygenase (CHMO, EC 1.14.13.22) from the bacteria *Acinetobacter calcoaceticus* NCIMB 9871 overproduced in recombinant *Escherichia coli* cells is the most intensively exploited enzyme from the group of BVMOs [8,9]. The broad substrate profile of CHMO was illustrated by the possibility of the enzyme to convert more than 100 synthetic substrates with high enantioselectivity [1]. Furthermore, stabilization of isolated CHMO [10] was achieved by immobilization, which is considered to be a key technique for improvement of industrial biocatalyst's properties [11]. In this regard, immobilization of whole-cells expressing CHMO by encapsulation in semipermeable polyelectrolyte complex (PEC) capsules [12] may provide extra process benefits for whole-cell mediated biooxidations by BVMOs. Encapsulation of recombinant cells of *E. coli* overexpressing a phylogenetically related enzyme to CHMO – cyclopentanone monooxygenase – within the PEC capsules preserved the viability of the cells during encapsulation process and improved the enzyme storage stability [12].

In further extending the encouraging results of our group [12], this study is devoted to examine both storage and operational stability of encapsulated recombinant whole cells expressing CHMO in continuous Baeyer–Villiger biooxidations of rac-bicyclo[3.2.0]hept-2-en-6-one (1) to (1S,5R)-2-oxabicyclo-

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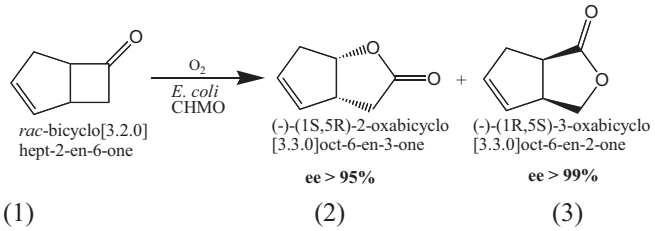

Fig. 1. Enantioselective Baeyer–Villiger biooxidation of rac-bicyclo[3.2.0]hept-2-en-6-one (1) to corresponding lactones (1S,5R)-2-oxabicyclo-[3.3.0]oct-6-en-3-one (2) and (1R,5S)-3-oxabicyclo-[3.3.0]oct-6-en-3-one (3) catalyzed by CHMO encapsulated in PEC capsules used in this work.

[3.3.0]oct-6-en-3-one (2) and (1R,5S)-3-oxabicyclo-[3.3.0]oct-6-en-3-one (3) shown in Fig. 1. These model compounds are important chiral synthons for the synthesis of prostaglandins and nucleosides [10]. In this regard, a continuous packed-bed minireactor with encapsulated cells integrated with a bubble-free oxygenation is considered as a substantial part of the experimental set-up (Fig. 2). Additionally, monitoring of Baeyer–Villiger biooxidations using gas chromatography may be substituted by innovative on-line measurements *via* sensitive flow calorimetry utilizing temperature changes of oxygenations. Therefore, the thermometric signal of Baeyer–Villiger biooxidation measured by flow calorimetry using encapsulated *E. coli* producing CHMO was investigated. The aim of this work is to develop precise and universal laboratory testing system for the family of recombinant BVMOs. Moreover, this work represents the premise for potential production scale-up of continuous packed-bed reaction system for enantioselective Baeyer–Villiger biooxidations catalyzed by immobilized biocatalysts.

2. Materials and methods

2.1. Materials

Bicyclo[3.2.0]hept-2-en-6-one and (1R,5S)-3-oxabicyclo-[3.3.0]oct-6-en-3-one were from Fluka, (1S,5R)-(−)-2-oxabicyclo-[3.3.0]oct-6-en-3-one was from Aldrich. LB_{amp} medium for cell growth contained (in g/l): peptone 10; yeast extract 5; NaCl 10; ampicillin 0.2. TB_{amp} for cell production medium contained in (g/l): tryptone 12; yeast extract 24; glycerol 5; $K_2HPO_4{\cdot}3H_2O$ 16.4; KH_2PO_4 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 and 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 [13].

2.2. Cultivation of cells

Recombinant *E. coli* cells overexpressing CHMO (EC 1.14.13.22) from *A. calcoaceticus* NCIMB 9871 [14] were used as follows: *E. coli* from the frozen (−70 °C) glycerol stocks were cultured on LB_{amp} agar plates 1.5% (w/v) for 12 h at 37 °C. A single colony was inserted into 10 ml LB_{amp} medium and cultivated at 37 °C for 12 h on rotary shaker (150 rpm). Afterwards, 100 ml of TB_{amp} medium was inoculated with 1% (v/v) of the latter inoculum and cultivated for another 8 h at 37 °C followed by activation of cells with 0.25 mM IPTG for 2 h at 25 °C. Biomass was collected by centrifugation for 15 min at 4000 s^{-1} and 25 °C and used for encapsulation.

2.3. Preparation of CHMO–PEC capsules

Encapsulation of *E. coli* with CHMO in PEC capsules (CHMO–PEC capsules) was performed in a custom-made coaxial air-stripping extrusion device fitted with a multiloop reactor [15] as reported previously [13]. The polyanion (PA) 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 centrifuged biomass of *E. coli* cells was suspended in PA solution to obtain concentration of wet cells of 160 mg/ml PA. Drops of PA solution with cells were extruded at an air pressure of 0.65 bar, a flow rate of stripping air in coaxial nozzle of 1.5 l/min and a PA flow rate of about 0.6 ml/min into the stream of polycation (PC) solution that was continuously flowing in the 5-loop reactor. The PC solution consisting of 1.8% (w/v) poly(methylene-*co*-guanidine), 1.0% (w/v) $CaCl_2$ and 0.9% (w/v) NaCl was delivered from the 5 l container into the 5-loop reactor at an air pressure of 0.08 bar and a flow rate of 54 ml/min providing the reaction time for capsule formation of 80 s. The reaction was quenched by collecting CHMO–PEC capsules at the exit of the reactor in 150 ml of 0.9% (w/v) NaCl in 1 min intervals. Capsules were then treated for 5 min with an excess of 50 mM citrate solution 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 [13].

2.4. Flow calorimetry

The flow microcalorimeter (FC) (3300 Thermal Assay Probe, Advanced Biosensor Technology, AB, Lund, Sweden) and the procedure have been described elsewhere [16]. The thermostatic cell contained the column packed with the CHMO–PEC capsules. The column is operated as a packed bed minireactor. The temperature difference between the column input and output is the signal S (in mV) measured by the enzyme thermistor. The column (7 mm i.d. × 20 mm) was packed with wet CHMO–PEC capsules (500 mg of wet weight) prepared according to procedure described above. Phosphate buffer (0.05 M, pH 7.0) was passed through the system at a flow rate 1 ml/min as long as the thermal equilibration (25 °C) and oxygen saturation was reached. Used temperature (25 °C) and phosphate buffer of pH 7.0

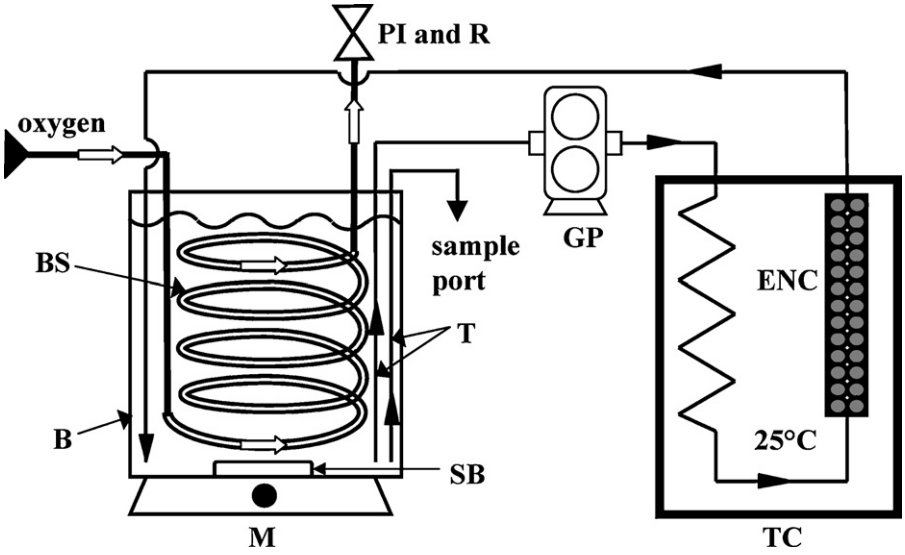

Fig. 2. Scheme of continuous packed-bed minireactor for testing of encapsulated whole-cell CHMO connected with flow calorimetry and integrated with bubble-free oxygenation. B: beaker with substrate solution, closed with parafilm; BS: bubble-free system consisting of silicone membrane insert for bubble-free oxygenation *via* oxygen permeation through the membrane into the liquid (white arrows indicate the direction of oxygen flow); PI: oxygen pressure indicator; R: oxygen pressure regulator; M: magnetic stirrer; SB: stirring magnetic bar; T: silicone tubings for conduction of reaction buffer and withdrawing of samples with the directions indicated by the arrows; GP: gear pump; TC: thermostated cell of the flow calorimeter; and ENC: plastic mini-column packed with CHMO–PEC capsules.

was based on optimization achieved previously [12,14]. Afterwards the buffered solutions of substrate (1) (0.92–9.2 mM) saturated with oxygen were continuously pumped through the FC column until the steady-state heat production was obtained. The change of temperature due to the Baeyer–Villiger biooxidation reaction was calculated from the Labtech software as described previously [16].

2.5. Operational and storage stability

The operational stability of encapsulated *E. coli* with CHMO during repeated BV biooxidation cycles in continuous mode was assessed by monitoring of substrate conversion to products *via* gas chromatography (GC) as described below. A home-made apparatus was constructed and tested for the continuous Baeyer–Villiger biooxidations of substrate (1) to products (2) and (3) (Fig. 1) catalyzed by encapsulated *E. coli* with CHMO as depicted in Fig. 2. 1.7 g of fresh wet CHMO–PEC capsules were placed into the plastic column (97 mm × 6 mm i.d.). The column temperature was equilibrated in a thermostated cell of the flow calorimeter (3300 Thermal Assay Probe, Advanced Biosensor Technology AB, Lund, Sweden). The column was operated as a small packed bed reactor in a continuous mode [16]. After thermal equilibration at 25 °C, 100 ml of the buffer solution pH 7.0 consisting of (g/l): $K_2HPO_4{\cdot}3H_2O$ (16.4), KH_2PO_4 (2.3), glucose (4), ampicillin (0.2) and substrate (1) (1.85 mM, 0.2 g/l) was continuously circulated by the gear pump from the stirred reservoir through the column at a flow rate 0.4 ml/l, saturated with oxygen *via* bubble-free system (Silicone membrane insert UniVessel® 1l, Sartorius BBI Systems GmbH, Melsungen, Germany) with the oxygen pressure 1.2 bar and oxygen flow rate of 0.3 l/min. The concentration of dissolved oxygen was determined using an oxygen meter SevenGo Pro (Mettler Toledo GmbH, Schwerzenbach). The glucose concentration in buffer solution was measured periodically by a spectrophotometric horseradish peroxidase assay described elsewhere [17] and maintained at the concentration of 4 g/l. Samples (150 μl) were withdrawn every 30 min from the sample port, extracted with 150 μl CH_2Cl_2 (with 1 mg/ml of internal standard methylbenzoate) and the organic phase was analyzed by GC as described below. After 12 h, BV biooxidation was terminated, the column was flushed with the fresh buffer solution (without substrate) for 30 min. After addition of substrate (1) (1.85 mM, 0.2 g/l) to the next batch of the buffer solution (100 ml) in the reservoir, the next biotransformation cycle was started. Overall 14 continuous BV biooxidations were performed and operational stability of encapsulated whole-cell CHMO was expressed as the percentage of substrate conversion to products after each repeated BV biooxidation, analyzed by GC. The mixture of produced lactones was isolated from the reaction solution by a procedure described previously [18] and analyzed by GC.

Another plastic column of the flow calorimeter, filled with 1.7 g of fresh wet CHMO–PEC capsules was used for measurement of storage stability. One BV biooxidation cycle for 12 h was performed as reported above and the sample withdrawn at the end of BV biooxidation was analyzed by GC. The column was flushed with the fresh buffer solution (without substrate) for 30 min, stored in the same buffer solution at 4 °C and used again for single BV biooxidation cycles after 14, 26, 35, 48, 60, 70 and 91 days of storage. Storage solution was changed periodically by fresh buffer solution after glucose concentration measurement in order to maintain the glucose concentration of 4 g/l. The storage stability of encapsulated *E. coli* with CHMO was expressed as the percentage of substrate conversion to products after each single BV biooxidation during storage, analyzed by GC.

2.6. Gas chromatography

Concentrations of substrate (1) and products (2, 3) were determined by GC [14] using a HP 5890 Series D gas chromatograph with FID detector R and BGB-5 column (30 m × 0.32 mm i.d., 0.25 μm film). Samples were extracted with CH_2Cl_2 (volume ratio 1:1) containing 1 mg/ml of methyl benzoate as internal standard and the organic layer was analyzed by GC. The conversion degree was determined as the percentage of the substrate (1) conversion to products from the area under the substrate peaks in GC chromatograms.

3. Results and discussion

3.1. Encapsulation of recombinant E. coli cells with CHMO

The CHMO–PEC capsules, formed by encapsulated cells *E. coli* with CHMO within the PEC capsules (Fig. 3a), were uniform in size and membrane thickness. The mean diameter of capsules was 0.80 ± 0.04 mm and the mean membrane thickness was 0.08 ± 0.005 mm. The CHMO–PEC capsules at the end of biotransformation tests were slightly deformed (Fig. 3b) as compared to spherical shape of capsules before the tests (Fig. 3a) due to the compression caused by the flow of reaction solution through the column. Nevertheless, capsules acquired almost spherical shape immediately after removal from the columns due to their elasticity. Thus, the continuous setup shown in Fig. 3b can be considered as a highly appropriate mode for the biooxidation using immobilized BVMOs. Importantly, in this setup the bursting of capsules by the shear forces, which was experienced in the bubbled batch-wise reactor [12], is avoided.

3.2. Temperature response of the Baeyer–Villiger biooxidation by flow calorimetry

Encapsulation of *E. coli* with CHMO in PEC capsules enabled continuous operation of Baeyer–Villiger biooxidations in packed-bed reaction system with bubble-free oxygenation (Fig. 2). This system permitted constant pumping of the substrate solution through the column without formation of oxygen bubbles inside the tubings. Moreover, the use of the encapsulated cells was advantageous since the continuous mode using free cells is not feasible due to a high risk of the cell washout and clogging of the column. Firstly, the concentration-dependent temperature response of the Baeyer–Villiger biooxidation of substrate (1) (Fig. 1) was measured by a flow calorimetry in order to prevent anticipated substrate inhibitory effect [14] to encapsulated cells. Recombinant *E. coli* cells expressing CHMO encapsulated in the PEC capsules exhibited substrate inhibitory behavior as depicted in Fig. 4. A mild substrate inhibition was apparent when the substrate concentration exceeded 4.0 mM. The measurements were performed at constant oxygen saturation concentration of 0.625 mM (25 °C, p_{atm}) [17] at the entrance of the column with encapsulated cells. The consumption of oxygen was assumed to be low due to minimum substrate

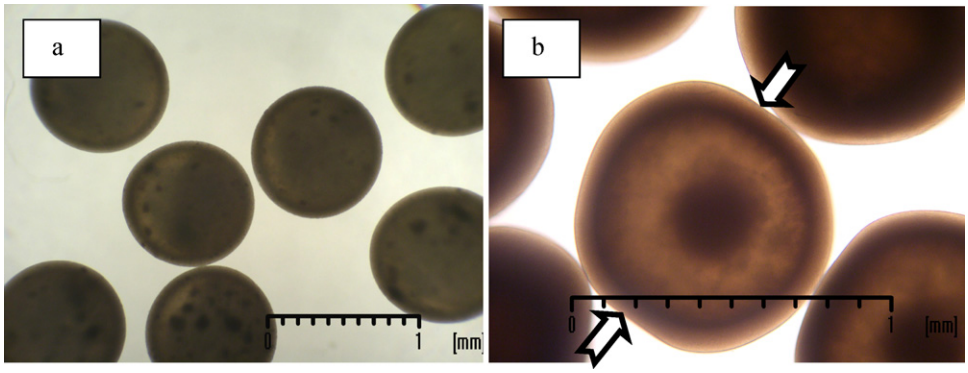

Fig. 3. Photomicrographs of CHMO–PEC capsules. (a) Spherical shape of CHMO–PEC capsules after encapsulation and before placement into the column of the enzyme thermistor (original magnification 40×); (b) typical shape of intact CHMO–PEC capsule withdrawn from the column after the 14th repeated cycle of continuous Baeyer–Villiger biooxidation (original magnification 100×). Arrows indicate deformation zones of the capsule as a result of compression forces within the column during operation. Turbid appearance of the capsules in both pictures was caused by presence of high concentration of dispersed cell biomass. The length of the bars depicted in both pictures equals to 1 mm.

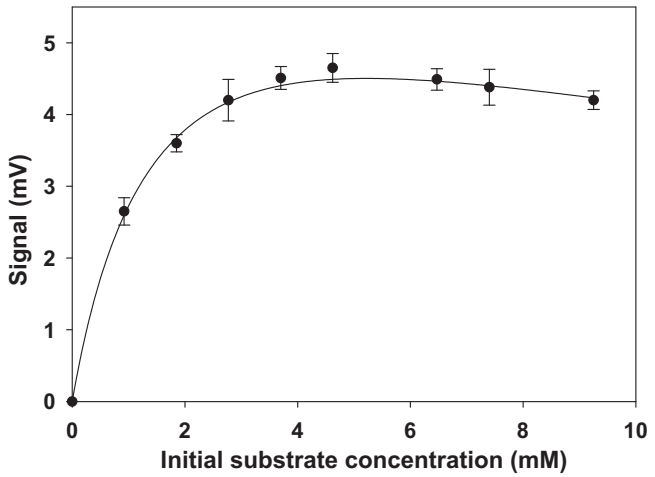

Fig. 4. Effect of initial substrate concentration on temperature response (measured as the voltage) by the flow calorimeter during Baeyer–Villiger biooxidations of bicyclo[3.2.0]hept-2-en-6-one catalyzed by CHMO–PEC capsules. Experimental conditions – temperature: 25 °C; flow rate of mobile phase: 1 ml/min. Initial substrate concentrations were validated by GC. The tests were performed in triplicates.

conversion required for measurement of initial reaction rate utilizing the principle of differential bed [19]. Thus the apparent kinetic parameters were derived from Michaelis–Menten model with substrate inhibition using the equation:

$$S = \frac{v'_m C_s}{K'_m + C_s + (C_s^2/K'_i)}$$

where contribution of constant oxygen concentration was included in the parameter v'_m, which is an apparent maximum detector response proportional to a maximum reaction rate. Derived parameters were as follows: $v'_m = 6.8 \pm 0.4$ mV, $K'_m = 1.4 \pm 0.2$ mM, $K'_i = 20.5 \pm 3.9$ mM, where S is a signal measured by flow calorimetry, C_s is a substrate concentration, K'_m is an apparent Michaelis–Menten constant and K'_i is an apparent substrate inhibition constant. As a result, initial substrate concentrations of 1.85 mM (0.2 g/l) were used in all experiments regarding operational and storage stability of CHMO–PEC capsules. Consequently, negligible effect of the substrate inhibition as the possible reason for decrease in conversions was guaranteed for biooxidation studies in this work. Moreover, in view of our previous results [19,20] and to the best of our knowledge, presented kinetic results using flow calorimetry represent an original study based on the temperature changes due to Baeyer–Villiger biooxidation using encapsulated whole-cell CHMO.

3.3. The operational and storage stability of CHMO–PEC capsules

Fig. 5 depicts the operational stability of CHMO–PEC capsules during 14 repeated BV biooxidations of substrate (1) (Fig. 1). Substrate conversion to products (2) and (3) increased from 77% to complete conversion within the first 4 cycles in a total of 14 biotransformation cycles and remained at complete conversion during the following 3 cycles. Conversion then decreased gradually to 75% during the 14th (and last) cycle. In contrary to these results, sharp conversion drop after third cycle of repeated Baeyer–Villiger biooxidations was reported using immobilized isolated CHMO [10]. Isolation of product was demonstrated after complete biooxidation in the 4th cycle. Combined yield of both lactones and their ratio was in accordance with previous results [18], i.e. 74% and 51:49, respectively. Thus it is clear, that CHMO–PEC capsules are able to preserve the catalytic efficiency of encapsulated cells in viable state during 14 repeated continuous Baeyer–Villiger biooxidations in sub-inhibitory substrate concentrations.

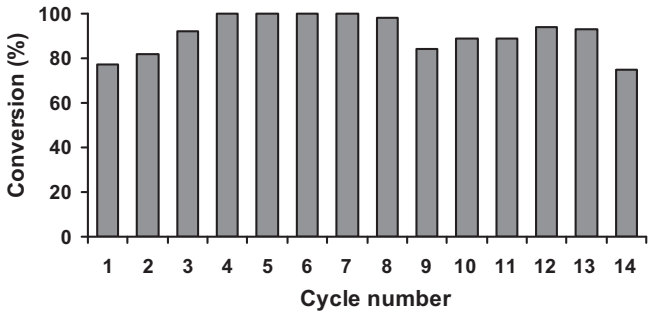

Fig. 5. Operational stability of CHMO–PEC capsules expressed as the evolution of substrate conversion degree to products during repeated Baeyer–Villiger biooxidations of rac-bicyclo[3.2.0]hept-2-en-6-one to (1R,5S)-3-oxabicyclo-[3.3.0]oct-6-en-3-one and (1S,5R)-2-oxabicyclo-[3.3.0]oct-6-en-3-one. Biooxidations were performed in continuous mode at 25 °C.

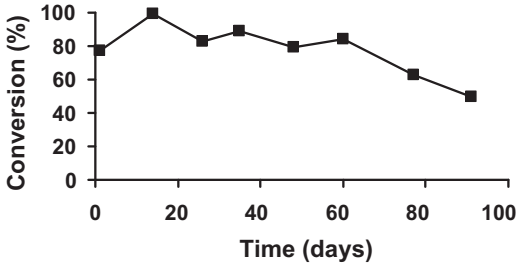

Fig. 6. Storage stability of CHMO–PEC capsules expressed as the evolution in substrate conversion degree to products during single Baeyer–Villiger biooxidations of rac-bicyclo[3.2.0]hept-2-en-6-one to (1R,5S)-3-oxabicyclo-[3.3.0]oct-6-en-3-one and (1S,5R)-2-oxabicyclo-[3.3.0]oct-6-en-3-one performed in selected storage times at 25 °C. The CHMO–PEC capsules were stored in a refrigerator at 4 °C and tested in continuous single biooxidations.

Evolution of storage stability of CHMO–PEC capsules is shown in Fig. 6. The conversions of substrate (1) reached the maximum value 14 days after encapsulation in PEC capsules, i.e. complete conversion. Afterwards, the conversion values above 80% were observed during subsequent 4 biooxidation tests up to 60 days of storage at 4 °C. The final value of 50% was found at 91st day of storage. These results proved superior properties of PEC capsules regarding long-term stabilization of encapsulated whole-cell BVMOs biocatalysts in a view of our previous storage stabilization tests during 4 days using encapsulated *E. coli* with CPMO [12]. Thus, the real results of operational and storage stability tests showed, that the CHMO–PEC capsules packed in the column of the enzyme thermistor may be considered as useful biocatalyst in a form of ready-to-use "encapsulated BVMOs cartridge" at least for versatile continuous Baeyer–Villiger biooxidation studies in laboratory scale.

4. Conclusions

The results of this work demonstrate that the stability of encapsulated cells was indeed improved in a view of previously published results, expressed in long-term stabilization of catalytic efficiency during storage and high operational stability within repeated Baeyer–Villiger biooxidations of bicyclo[3.2.0]hept-2-en-6-one to corresponding lactones. Importantly, continuous mode of the latter Baeyer–Villiger biooxidation performed in model packed-bed minireactor appears to be an appropriate configuration with convenient mechanical conditions for optimal performance of CHMO–PEC capsules. Additionally, an original approach for determination of kinetic properties of Baeyer–Villiger biooxidations by flow calorimetry was introduced. It is evident that investigation of the whole-cell CHMO encapsulated in PEC capsules and used in the

form of continuous packed-bed minireactor is an easy-to-use tool for further development of immobilized BVMOs.

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