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Dedicated to Professor Florin Dan Irimie on the Occasion of His 65th Anniversary

CONTINUOUS-FLOW ENZYMATIC KINETIC RESOLUTION MEDIATED BY A LIPASE NANOBIOCONJUGATE

MĂDĂLINA ELENA MOISĂ, LÁSZLÓ CSABA BENCZE, CSABA PAIZS, MONICA IOANA TOŞA*

ABSTRACT. This study describes the development of a continuous-flow procedure for the enzymatic kinetic resolution of *rac*-1-phenylethan-1-ol with the clickable acylating agent 2,2,2-trifluoroethyl 2-(prop-2-yn-1-yl-oxy)acetate, which allows facile click reaction-based downstream, enabling the large-scale chemo-enzymatic synthesis of both secondary alcohol stereoisomers with high enantiopurity. The influence of flow rate, acylating agent and substrate concentration upon the productivity of the packed-bed reactor was investigated. In addition to this, the performances of continuous-flow and batch reactors were compared.

Keywords: *enzymatic kinetic resolution, secondary alcohol, lipase, continuous-flow process, batch process, productivity*

INTRODUCTION

An important aspect when developing a new process is its applicability on industrial scale, consequently scaling-up becomes pivotal. Currently the use of continuous-flow technology is gaining more and more attention as it generally affords processes with increased efficiency and productivity [1,2,3]. Moreover, facile automatic control, reproducibility, lower costs and increased mass and heat transfer make continuous-flow processes an advantageous alternative to traditional batch processes [4].

Babeş-Bolyai University, Faculty of Chemistry and Chemical Engineering, Biocatalysis and Biotransformations Research Center, 11 Arany Janos str., RO-400028, Cluj-Napoca, Romania
**Corresponding author: mtosa@chem.ubbcluj.ro*

Flow technology represents an attractive tool for developing efficient biocatalytic processes and numerous comparative studies prove its superior performance over batch processes [5-8].

Generally, for enzyme-catalysed reactions the preferred type of reactor is the packed-bed reactor, which consists in a column filled with the enzyme (usually immobilized). With the aid of a pump, allowing control of residence time, the reaction media (substrate and reagent dissolved in a solvent) is passed through the enzyme-packed column. The reactor is frequently connected to a thermostat permitting temperature control. Beside the simple construction, the packed-bed reactor has the advantages of extended biocatalyst lifetime through the lack of stirring, which might damage the biocatalyst particles and also through simplified reaction workup since catalyst removal at the end of the process is not required [9-11].

Enzymatic kinetic resolution (EKR) is one of the most employed biocatalytic methods to access enantiomerically pure compounds, mainly due to its simplicity and versatility. The major disadvantage of this method is represented by the maximal theoretical yield of 50%, requiring separation of the two enantiomers at the end of the process which raises practical and economical issues at industrial scale.

Encouraged by our previous results [12], in order to apply the newly developed click reaction-aided EKR products separation strategy for large-scale production of both enantiomerically enriched stereoisomers of secondary alcohols (see **Figure 1**), the EKR step was investigated in continuous-flow mode using a packed-bed reactor.

Accordingly, the efficient clickable acylating agent 2,2,2-trifluoroethyl 2-(prop-2-yn-1-yl-oxy)acetate was applied in the continuous-flow enzymatic transesterification of racemic 1-phenylethan-1-ol (frequently employed as model aromatic chiral alcohol) mediated by the highly stable and thermotolerant lipase B from *Candida antarctica* immobilized on single-walled carbon nanotubes (CaL-B-SWCNT), with reported increased operational stability [13].

RESULTS AND DISCUSSION

Preliminary experiments with CaL-B-SWCNT in continuous-flow acylations of various (hetero)aromatic ethanols revealed 60 °C as the optimal temperature, therefore this biocatalyst was employed in the continuous-flow acylation of *rac*-1-phenylethan-1-ol *rac*-**1** with 2,2,2-trifluoroethyl 2-(prop-2-yn-1-yl-oxy)acetate **2** in *n*-hexane at 60 °C in a packed-bed reactor (30 × 4.6 mm, **Figure 1**).

The productivity (the specific reaction rate, r) was employed as a measure of system performance and was calculated with **Equations 1** and **2** for continuous-flow (r_{flow}) and batch reactions (r_{batch}) [14].

$$r_{batch} = \frac{n_P}{t \times m_e}\left[\frac{\mu mol}{min \times g}\right]$$ **Equation (1)**

$$r_{cont} = \frac{[P] \times f}{m_e}\left[\frac{\mu mol}{min \times g}\right]$$ **Equation (2)**

where n_p: the amount of formed product [µmol]
t: the reaction time [min]
m_e: the mass of the applied biocatalyst [g]
[P]: the product concentration [µmol mL^{-1}]
f: the flow rate [mL min^{-1}]

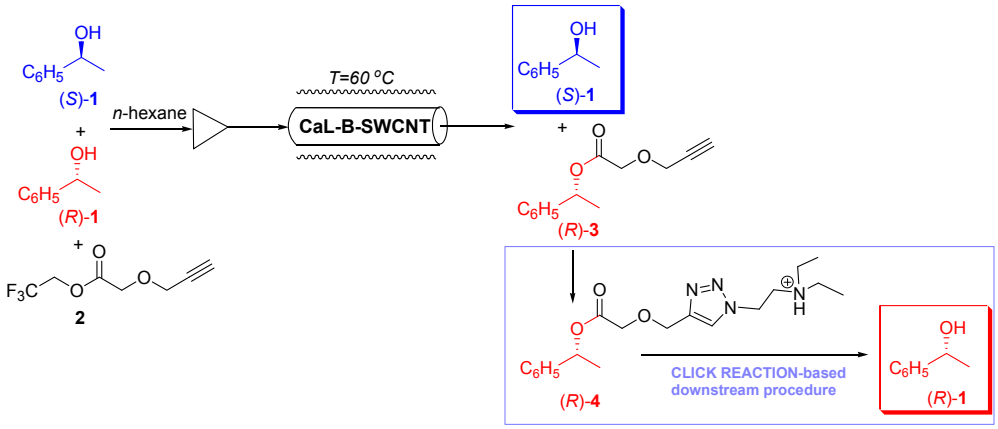

Figure 1. Transesterification of *rac*-1-phenylethan-1-ol *rac*-**1** with 2,2,2-trifluoroethyl 2-(prop-2-yn-1-yl-oxy)acetate **2** in *n*-hexane at 60 °C in a continuous-flow packed-bed reactor, followed by click reaction-based downstream procedure

In order to effectively apply the click chemistry-type downstream, the presence of the reactive enantiomer of the substrate in the effluent is not desired, therefore the flow rate and the acyl donor and substrate concentrations must be well adjusted to ensure complete transformation.

The continuous-flow EKR was initially studied using a 10 mM substrate concentration and 0.75 equiv. of acyl donor **2** at 60 °C, varying the flow rate in the range of 0.1 – 0.5 mL min^{-1}. The maximum conversion attained within this experiment was 42% at 0.1 mL min^{-1} (**Figure 2A**). Consequently, with the aim to reach the maximum conversion of 50%, higher amounts of the acylating agent **2** were tested (2, 3 and 4 equiv.).

The obtained results (**Figure 2A**) confirm the beneficial impact of the increased molar *ratio* of acyl donor on the conversion in the continuous-flow EKR. Accordingly maximum conversion was reached at 0.1 mL min^{-1} flow rate with 3 and 4 equiv. of ester **2**, allowing a productivity of 4.2 μmol min^{-1} g^{-1}. Moreover, when using 4 equiv. of the ester the 50% conversion was also reached at 0.2 mL min^{-1}, affording a two times higher productivity (**Figure 2B**). Since no considerable variations in conversion were seen from 0.1 to 0.5 mL min^{-1} using 4 equiv. of **2** (48-50% *c*), this amount of acylating agent was selected as optimal for the next experiments. Noteworthy, the continuous-flow transesterification proceeded with high enantioselectivity (*E*»200), affording product (*R*)-**3** with maximum enantiomeric excess (ee_P>99%) in all experiments.

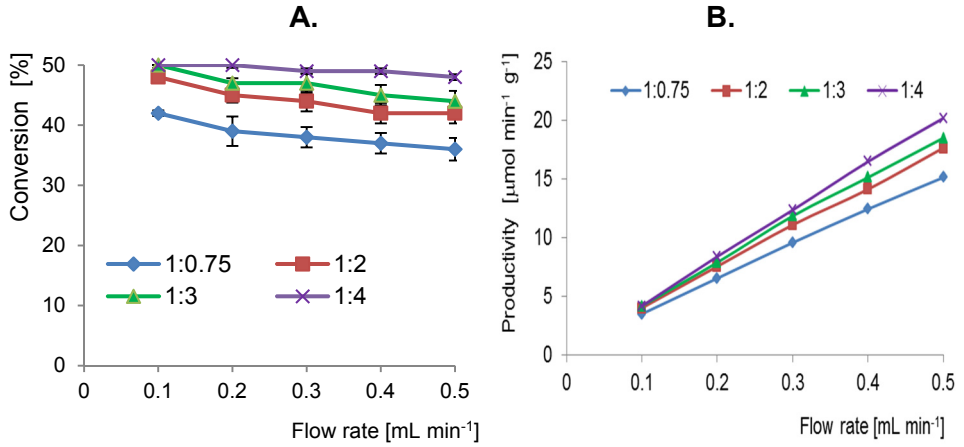

Figure 2. Influence of substrate: acylating agent *ratio* on the conversion (**A**) and productivity (**B**) of CaL-B-SWCNT-mediated continuous-flow transesterification of *rac*-**1** (10 mM) with trifluoroethyl 2-(prop-2-yn-1-yl-oxy)acetate **2** in *n*-hexane at 60 °C. Error bars represent standard deviations from average. The productivity was calculated using the average value of conversion.

Further, in order to increase the productivity, different substrate concentrations (10 – 100 mM) were tested at 0.1 and 0.5 mL min^{-1} flow rate using 4 equiv. of acyl donor (**Figure 3**). As expected, the productivity increased with the substrate concentration, the maximum value being obtained at 100 mM *rac*-**1** and 0.5 mL min^{-1} flow rate (88.2 μmol min^{-1} g^{-1}, **Figure 3B**). Although higher productivities were obtained at the higher flow rate, the significant decrease in conversion at 0.5 mL min^{-1} (**Figure 3A**) must be considered when deciding which flow rate is more advantageous. Analyzing the theoretical maximum productivity calculated for the maximum conversion (represented with dotted

lines in **Figure 3**), it can be noticed that at 0.1 mL min^{-1} flow rate the actual productivity values are close to the maximum theoretical ones, while at 0.5 mL min^{-1} flow rate the difference in actual and theoretical values drastically increases with substrate concentration.

It is worth mentioning that even at the highest studied substrate concentration (100 mM) the system afforded a high conversion of *rac*-**1** (45% at 0.1 mL min^{-1}). In addition to this, the enantioselectivity of the process was not altered throughout the investigated substrate concentration domain (*E*»200).

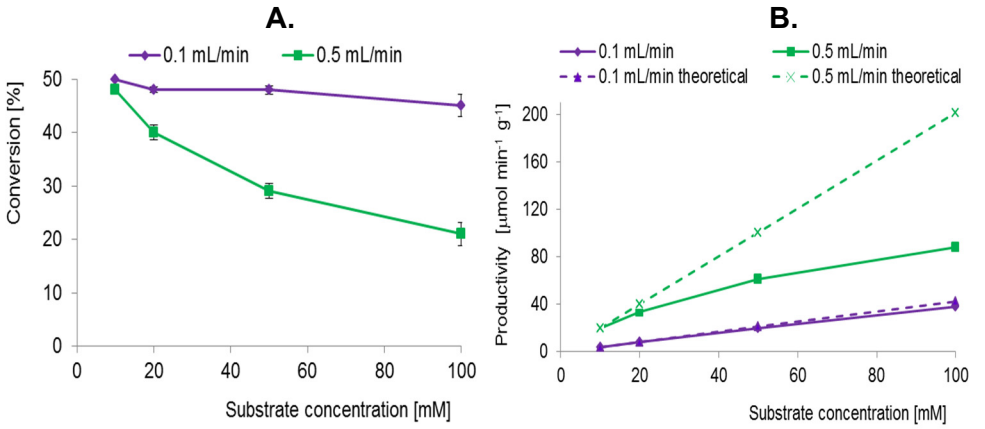

Figure 3. Influence of substrate concentration on the conversion (**A**) and productivity (**B**) of CaL-B-SWCNT-mediated continuous-flow transesterification of *rac*-**1** with trifluoroethyl 2-(prop-2-yn-1-yl-oxy)acetate **2** (4 equiv.) in *n*-hexane at 60 °C. Error bars represent standard deviations from average. The productivity was calculated using the average value of conversion. The theoretical productivity was calculated for the maximum conversion (obtained at 10 mM *rac*-**1**) without considering the decrease in conversion with increased substrate concentration.

Furthermore, the productivities obtained in continuous-flow and batch systems were compared under identical experimental conditions (substrate and acyl donor concentrations, solvent and temperature) at the same degree of conversions [14]. The continuous-flow reactor proved remarkable productivity as compared to the batch system (r_{batch} = 1.0 µmol min^{-1} g^{-1}, r_{flow} = 17.6 µmol min^{-1} g^{-1}, at 42% conversion, 60 °C, 10 mM substrate concentration, 2 equiv. of acyl donor **2** and 0.5 mL min^{-1} flow rate), demonstrating the superior performance of the continuous-flow system over the batch process.

CONCLUSIONS

An efficient continuous-flow procedure for performing lipase-mediated kinetic resolution of *rac*-1-phenylethan-1-ol with 2,2,2-trifluoroethyl 2-(prop-2-yn-1-yl-oxy)acetate was developed using a packed-bed reactor. By combining this continuous-flow EKR with the click reaction-based downstream, the large-scale production of both enantiomers of secondary alcohols can be effectively achieved.

EXPERIMENTAL SECTION

Materials and methods

Chiral HPLC (Agilent 1200 Series instrument) using LUX-3 Phenomenex column (4.6 × 250 mm) and a mixture of *n*-hexane:2-propanol 9:1 v/v at 1 mL min^{-1} flow rate as mobile phase was employed for the quantitative analysis of the enzymatic kinetic resolution [(*S*)-**1**: 6.5 min; (*R*)-**1**: 7.1 min; (*S*)-**3**: 15.4 min; (*R*)-**3**: 18.0 min; 25 °C].

The enantiomeric excesses of the substrate ee_S and product ee_P were determined from peak areas of HPLC chromatograms. The enantiomeric *ratio* *E* was calculated with the equation $E = \ln[(1-c)(1-ee_S)]/\ln[(1-c)(1+ee_S)]$, using the calculated conversion: $c = ee_S/(ee_S+ee_P)$ [15].

CaL-B-SWCNT was obtained by covalently immobilizing lipase B from *Candida antarctica* on carboxy-functionalized single-walled carbon nanotubes ($SWCNT_{COOH}$) as previously described [13].

HPLC grade *n*-hexane and 2-propanol were purchased from VWR.

Batch enzymatic reactions were performed in a Heidolph Vibramax 110 shaker equipped with an incubator module (Heidolph, Germany) while continuous-flow experiments were carried out in a stainless steel column (30 × 4.6 mm) attached to the pump and thermostat modules of an Agilent 1200 Series HPLC instrument.

Enzymatic procedures

All experiments were performed in triplicate.

1. Continuous-flow lipase-mediated transesterification of *rac*-1-phenylethan-1-ol *rac*-1 with 2,2,2-trifluoroethyl 2-(prop-2-yn-1-yl-oxy)acetate 2 in a packed-bed reactor

Continuous-flow reactions were performed in a thermostated stainless steel column (30 × 4.6 mm) filled with the immobilized lipase (119 mg of CaL-B-SWCNT) attached to an Agilent 1200 Series HPLC pump. Prior to performing

the reaction, the lipase-filled column was washed with *n*-hexane at 0.5 mL min^{-1} for 30 min. The solution of the substrate *rac*-**1** (10, 20, 50 or 100 mM) and 2,2,2-trifluoroethyl 2-(prop-2-yn-1-yl-oxy)acetate **2** (0.75, 2, 3 or 4 equiv.) in *n*-hexane was pumped through the reactor thermostated at 60 °C at different flow rates (0.1 – 0.5 mL min^{-1}). Samples of 50 µL were collected periodically, diluted with *n*-hexane (500 µL) and analysed by HPLC. The steady-state condition was reached within 30-90 min, depending on the flow rate. Between experiments the enzyme-filled reactor was washed with *n*-hexane at 0.5 mL min^{-1} for 30 min and stored overnight at 4 °C.

2. Batch mode lipase-mediated transesterification of *rac*-1-phenylethan-1-ol *rac*-1 with 2,2,2-trifluoroethyl 2-(prop-2-yn-1-yl-oxy)acetate 2

Into the solution of *rac*-**1** (10 µmol) in *n*-hexane (1 mL), 2,2,2-trifluoroethyl 2-(prop-2-yn-1-yl-oxy)acetate **2** (2 equiv.) and the immobilized lipase CaL-B-SWCNT (2.4 mg) were added and shaken at 700 rpm at 60 °C until 42% conversion was reached (28 hours). Samples taken as previously described were subjected to HPLC analysis.

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