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Modular micro reaction engineering for carboligation catalyzed by benzoylformate decarboxylase

Abstract: The downscale of different unit operations for the biocatalytic carboligation of benzaldehyde and acetaldehyde catalyzed by benzoylformate decarboxylase from *Pseudomonas putida* was investigated. The reactor volume was reduced to 115 µl thus enabling a substrate and enzyme saving by a factor of 52 in comparison to standard laboratory techniques. Additionally, the successful downscale of membrane based liquid-liquid contactors was shown, which allows, for example, the screening of solvents for extraction as well as the feed of a substrate. Here, comparable volumes as well as residence times were realized, enabling the integration of all three unit operations.

Keywords: biocatalysis; downscale; enzyme; membrane; screening.

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List of abbreviations

AA	Acetaldehyde
ASE	Advanced silicon etching
BA	Benzaldehyde
BFD	Benzoyl formate decarboxylase
CI	Concentration indication
HPLC	High pressure liquid chromatography
HPP	Hydroxyl phenylpropanone
µ-CaR	Micro capillary reactor
µ-SiR	Micro silicon reactor
PVDF	Polyvinylidene fluoride
PI	Pressure indication
PW	Process window
RTD	Residence time distribution
TC	Temperature control
ThDP	Thiamine diphosphate

List of symbols

D_{ax}	Axial dispersion coefficient
$E(\theta)$	Density curve of dimensionless residence time
θ	Dimensionless residence time
u	Flow velocity
t_n	Normalized time
Re	Reynolds number
$F(\theta)$	Sum curve of dimensionless residence time
$\dot{V}$	Volume flow

1 Introduction

In comparison to chemical processes where heat exchangers as well as temperature and pressure control of the reaction are crucial parts, the key parts of bioprocesses are the biocatalysts, which can be enzymes or whole cells. These biocatalysts exhibit a certain activity and selectivity towards a specific substrate. Both activity and selectivity regarding a certain reaction can be characterized by kinetics as well as thermodynamics of the reaction [1, 2]. Apart from parameters such as temperature or pH, the solvent also plays an important role in enzyme activity and especially enzyme stability. All this needs to be quantified when setting up a bioprocess. Finally, questions regarding the choice of reactor type, catalyst recovery and downstream processing need to be answered [3].

Commonly accepted advantages of bioprocesses are the high selectivity of most biocatalysts as well as the usually mild reaction conditions, both resulting in low amounts of side products and thus high product quality [4]. When setting up a bioprocess first of all the catalyst must be selected and characterized with an understanding of kinetics and appropriate reaction conditions. However, catalysts and very often also the substrates are relatively expensive. Additionally, the biocatalyst has a limited stability. Therefore, in the early stage of

bioprocess development there is a strong interest in a low consumption of consumables as well as in a high reproducibility and reliability of the data obtained from the characterization [5–7]. A crucial point for bioprocesses is also the product recovery, because often relatively low product concentrations are obtained and products must be isolated from a liquid phase. Here an appropriate downstream process or *in situ* recovery strategy has to be chosen [8].

Classical approaches for the screening of enzyme activity are based on the use of multititer plates mostly made from polystyrene or glassy materials [9]. These multi-titer plates represent an open batch system, which has two main drawbacks. Firstly, organic solvents must be circumvented because they might attack the plastic material. Secondly, volatile substrates or products cannot be used, because their evaporation results in huge errors of measurement. Therefore, the first set-up for the evaluation of a biocatalyst or reaction conditions for a bioprocess is a stirred tank reactor with a volume of usually 30–500 ml. Here, mostly standard glass hardware is used [10]. This approach is fairly labor intensive and automation is difficult. Later, a high amount of substrate as well as enzymes is necessary to obtain the desired data in a sufficient density. However, for the process development and scale-up, (kinetic) data obtained at real process conditions are necessary.

This is where micro reaction technology can contribute its promising potentials [11, 12]. Using micro reactors a downscale of the reaction can be realized [13, 14], and also other unit operations, such as membrane separation processes as they are often used in biotechnology, can be characterized on a small scale [15, 16]. Usually the characterization of each unit operation of a process is done independently, where every single unit operation operates with its own conditions and limitations ('process window'). However, micro system technology offers the possibility to characterize combinations of unit operations, whereby the process window of the combined systems is restricted by the overlap of the unit operations. Initially only process windows, which are acceptable for all necessary unit operations, are evaluated [17]. Therefore, this approach can drastically reduce development times by omitting laborious investigations of conditions which are not applicable within the final process at any rate (Figure 1).

A biocatalytic process, consisting of continuous substrate feed, enzymatic reaction and continuous product removal, is presented below. The process was subdivided into its unit operations, which were considered individually.

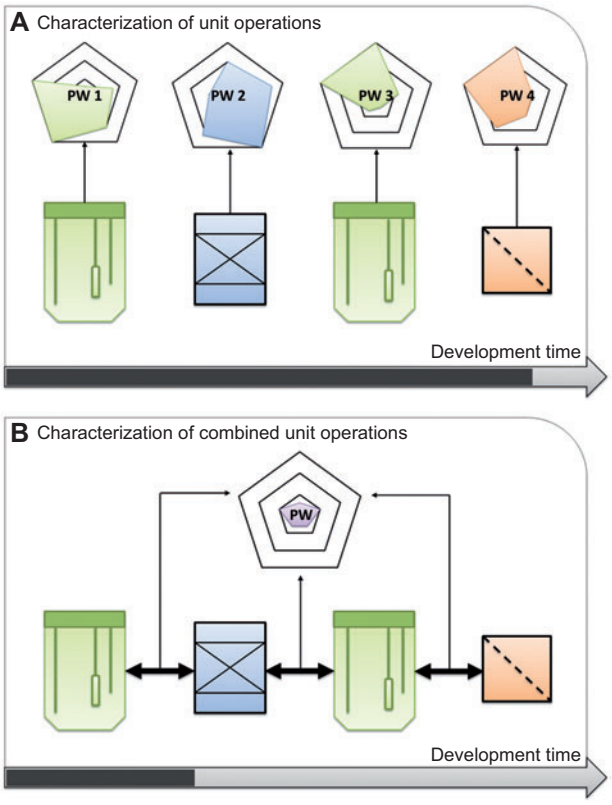

Figure 1 Illustration of a possible sequence of unit operations within a bioprocess comparing the development time to characterize all unit operations within their specific process windows (PWs) with the development time when characterizing the combined unit operations in a common smaller process window.

First two different types of micro reactors for a carboligation catalyzed by benzoylformate decarboxylase [18, 19] are presented and one of these reactors is characterized in view of residence time distribution and flow conditions. In addition, the enzymatically catalyzed reaction was investigated in stirred vessel as well as in two micro reactors. Here, the comparability of stirred vessel and flow reactors should be reached [20].

To utilize a flow system in micro scale, a continuous product removal in a similar scale is required. Therefore, a product removal with membranes operating as contactor devices should be established and described in view of the degree of extraction and process stability.

Additionally, the usage of membranes for substrate feed was evaluated during this study. The relatively low solubility of benzaldehyde in the aqueous buffer led to a new approach for continuous substrate feed in a membrane module. A reproducible and constant benzaldehyde transfer rate across the membrane was the goal.

2 Materials and methods

2.1 Enzymatic assay

All chemicals used in this study were purchased from Sigma-Aldrich (Steinheim, Germany), unless indicated otherwise.

To obtain benzoyl formate decarboxylase (BFD) fermentation, harvesting and purification of the enzyme was done as described in [18, 19]. Enzymatic activity determination was carried out according to [19]: one unit is defined as the amount of enzyme in milligrams transforming 1 mmol benzaldehyde and acetaldehyde to hydroxyl phenylpropanone (HPP) per min in 100 mM phosphate buffer containing 0.5 mM thiamine diphosphate (ThDP) and 2 mM $MgCl_2$, 400 mM acetaldehyde and 40 mM benzaldehyde at pH 7.5 and 30°C. The tenfold acetaldehyde excess was chosen according to the K_m values determined for BFD in previous studies [19].

2.2 Micro reactors and characterization

Two different types of micro reactors were used for this work. The µ-CaR was made of a stainless steel capillary with an inner diameter of 200 µl. Connectors and valves were provided by Swagelok. Analysis of residence time distribution was done as depicted in Figure 2. The dimensionless sum of exit age distribution F(θ) was calculated according to [21].

The µ-SiR was made of 500 µm borosilicate glass (Pyrex®, Plan Optik AG, Elsoff, Germany) and silicon wafers (Si-Mat, Kaufering, Germany) using standard photolithography and dry etching techniques to machine the channel pattern to the silicon. With advanced silicon etching (ASE) an anisotropic etch occurs resulting in rectangular channel geometry. The channels have a width of 300 µm, a height of 500 µm as well as a length of 77 mm. The fluidic connectors were etched to the Pyrex® using hydrofluoric acid. Finally, both wafers were mated irreversible by anodic bonding.

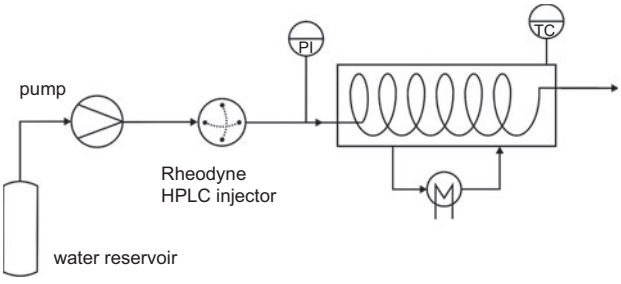

Figure 2 Set-up for the analysis of the residence time distribution.

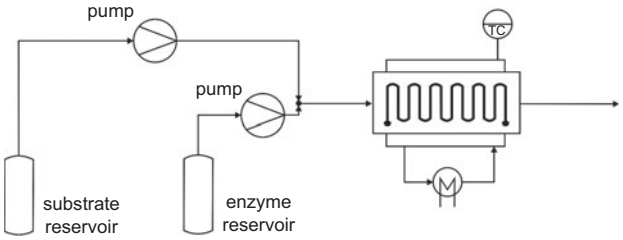

Figure 3 Set-up for the analysis of the enzymatic reaction.

Enzymatic reactions were carried out as depicted in Figure 3 at 30°C. Substrate concentrations were adjusted as described for the enzymatic assay by keeping ratio of flow rates constant for all residence times. The aqueous substrate solution and the aqueous enzyme solution were pumped into the reactor and samples were taken at the outflow. The product concentration was determined using high pressure liquid chromatography (HPLC).

2.3 Membrane module and characterization

The membrane module is made of polytetrafluorethylen (Teflon®). Separated channels with an inner diameter of 2 mm and an inner length of 75 mm are available. This allows a fine adjustment of the accessible membrane area or parallel use of different membranes or different solvents. Figure 4 shows a model of one channel and one ferrule for potting and sealing the hollow fiber on both ends with an epoxide two-part glue Loctite 3340 (Henkel, Düsseldorf,

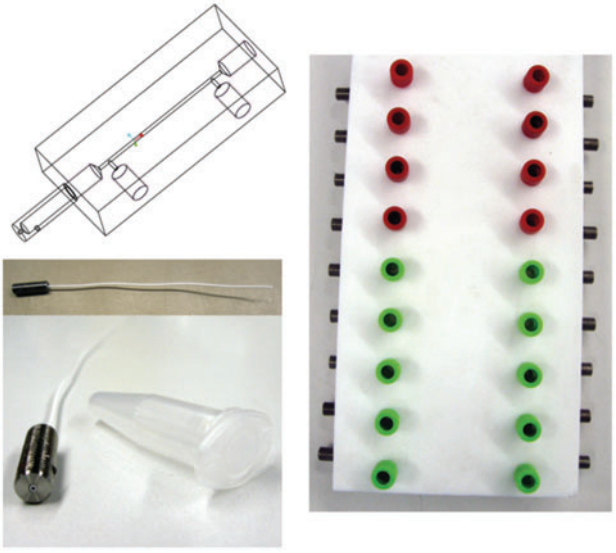

Figure 4 Model of one channel and one ferrule for potting and sealing the hollow fiber on both ends (left). Membrane module with nine single channels (right).

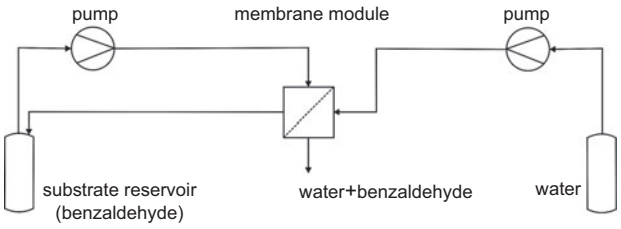

Figure 5 Set-up for the analysis of the substrate feed.

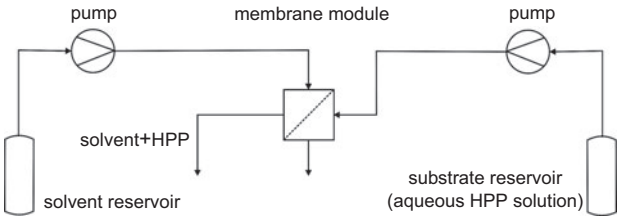

Figure 6 Set-up for the analysis of the product extraction.

Germany). The ferrules have a ¼ inch-28 UNF thread for easy capability of connecting tubes. The module can be operated co- or counter-current. Figure 5 illustrates the set-up for the analysis of the substrate feed. Figure 6 illustrates the set-up for the analysis of the product extraction.

Parameters such as temperature, enzyme concentration, flow rates and the reaction volume for the two micro reactors and the membrane processes are listed in Table 1.

2.4 Analytics

Benzaldehyde (BA) and HPP concentrations in reaction buffer were determined by HPLC-UV analysis at 254 nm using a Merck KGaA (Darmstadt, Germany) Chromolith RP-8 phase at 40°C. Triethanolamine buffer and methanol (1:1) were used as an eluent with an isocratic flow rate of 1 ml/min. The reaction was quenched by adding stop solution, consisting of 90% acetonitrile and 5% phosphoric acid.

For the determination of HPP concentration in ethyl acetate a gas chromatography with flame ionization detector was used. An isocratic flow of 1.8 ml/min hydrogen was applied to a Varian CP-Chirasil-DEX CB phase from Agilent Technologies (Böblingen, Germany) at 110°C.

	Temperature (°C)	Enzyme concentration (mg/ml)/(U/ml)	Flow rates (ml/min)	Volume (ml)
Stirred vessel	30	0.35/1.82	–	30
µ-CaR	30	0.875/4.55	0.05–1	0.79
µ-SiR	30	0.6/1.92	0.05–0.25	0.79
Membrane module for substrate feed	20	–	0.3	0.03
Membrane module for product removal	20	–	0.03–0.1	0.03

Table 1 Reaction conditions for stirred vessel, flow reactors and membrane modules.

3 Results and discussion

Micro reactors are promising tools for screening of catalysts and reaction conditions [22]. Here two different types of micro reactors were evaluated for the investigation of an enzymatic reaction utilizing volatile substrates.

As a model system the carboligation of BA and acetaldehyde (AA) catalyzed by the enzyme BFD from *Pseudomonas putida* was chosen [19, 23]. The product (*S*)-2-hydroxyphenyl propanone (HPP) is formed in an enantiomerically pure form (Figure 7). However, to date, the analysis of this reaction has been carried out in a glass vessel operated as a stirred tank reactor [24]. Here, the evaporation of the highly volatile substrate acetaldehyde (boiling point 20°C) is problematic and requires additional equipment, such as reflux and/or closed pressure apparatus. Thus, using micro reactors can be highly beneficial [25].

The first micro reactor type used was a micro capillary reactor (µ-CaR) consisting of a steel capillary of 1.6 m and an inner diameter of 200 µm. Including connectors and valves the resulting reaction section volume was 790 µl. This reactor was characterized in view of residence time distribution (RTD) using the substrate benzaldehyde as a tracer and by measuring the absorption at 280 nm in the outflow of the reactor. The resulting F(θ) curves are shown in Figure 8.

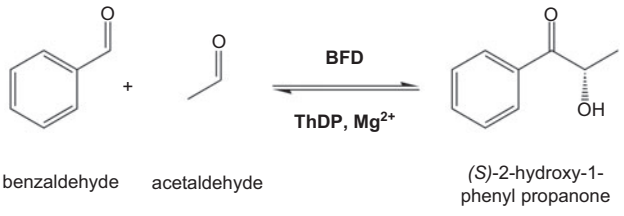

Figure 7 Reaction scheme for the ligation of benzaldehyde (BA) and acetaldehyde (AA) catalyzed by the enzyme benzoyl formate decarboxylase (BFD). Reaction conditions: 40 mM BA, 400 mM AA, 100 mM phosphate buffer [pH 7.5, 0.5 mM thiamine diphosphate (ThDP) and 2 mM $MgCl_2$], T = 30°C.

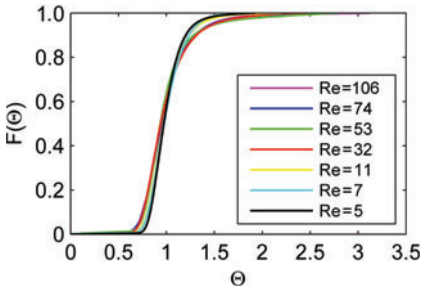

Figure 8 Flow scheme for the analysis of the residence time distribution and the resulting F(θ) curves for the μ-CaR. Conditions: Ocean Optics flow cell, injection of 5 μl 25 mM benzaldehyde as a tracer.

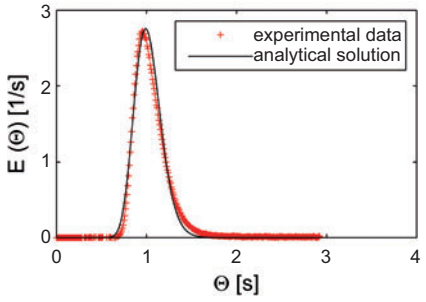

Figure 9 Analytical solution of the boundary condition for open-open systems at given Bodenstein numbers to fit the experimental data for a residence time of 116 s at a Reynolds number of 11.

To compare the μ-CaR to the stirred tank reactor plug performance of the flow reactor was evaluated. According to the first order boundary condition for open-open systems, an analytical solution from the dispersion model was used to determine Bodenstein numbers. Therefore the $E(\theta, D_{ax})$ curves were fitted to experimental data. Figure 9 illustrates the comparison between experimental data and the analytical solution, resulting in a Bodenstein number of 95. Various flow rates were adjusted to validate the flow conditions. The resulting Bodenstein numbers are summarized in Table 2.

The resulting Bodenstein numbers vary between 78 and 110. Thus, laminar plug-flow can be assumed for all evaluated Reynolds numbers [26]. A RTD analysis of the stirred vessel is not required because of premixed substrates and the addition of enzyme dissolved in reaction buffer.

$\dot{V}$ (ml/min)	u (m/s)	Re	τ (s)	Bo
1.00	0.53	106	13	80
0.70	0.37	74	19	75
0.50	0.27	53	24	95
0.30	0.16	32	43	78
0.10	0.05	11	116	95
0.07	0.04	7	144	60
0.05	0.03	5	234	110

Table 2 Flow rates and Reynolds numbers applied for the characterization of the μ-CaR.
For conditions, see Figure 8.

Because the investigated biocatalytic reaction (see Figure 7) has a typical initial reaction rate of 4 mM/min at a substrate concentration of 20 mM BA and 200 mM AA residence times of up to 15 min should be realized, thus flow rates were varied in the range of 0.05 to 1.00 ml/min.

The second reactor (μ-SiR) was made from borosilicate glass and silicon. Rectangular channels were etched into the silicon, capped with glass on both sides. This allows a visual access to the reactor. The reaction section volume was 115 μl and a Y-shaped connector was implemented directly on the microchip.

Figure 10 depicts concentration over time curves for the two investigated micro reactor types in comparison to the formerly used stirred vessel ('batch') (30 ml). To compare the stirred vessel with flow systems the reaction conditions must be comparable. Owing to the reactor set-up and enzyme solubility an equal enzyme concentration in all reactors was not achieved. For that reason, a normalized time t_n, multiplying the applied enzyme activity in units per milliliter (U×ml) reaction volume with the batch and residence time, is used for comparison.

Evidently, the resulting curves are very similar and it can be concluded that the downscale of reaction volume has no impact on the reaction progress. When comparing the results of the continuous flow experiments with the batch experiments one has to keep in mind that each data point necessitates at least one residence time. Therefore, for five data points a reduction of enzyme and substrate consumption in comparison to the batch experiment at 30 ml scale by a factor of 7.6 and 52.2 for the μ-CaR and the μ-SiR, respectively, was achieved. The reactor volume was reduced at these experiments by a factor of 38 and 261, respectively.

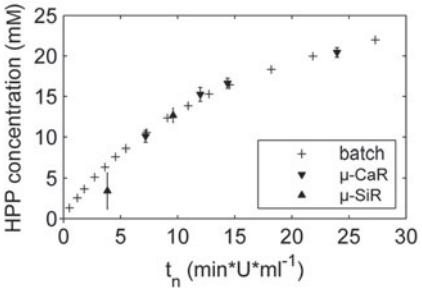

Figure 10 HPP concentration in the course of the reaction. Reaction time (batch) as well as residence times (micro reactors) were normalized in view of the enzyme activity applied. Concentration time plot for the μ-CaR with a volume of 790 μl and for the μ-SiR with a volume of 115 μl. Reaction conditions: 40 mM BA, 400 mM AA, 100 mM phosphate buffer [pH 7.5, 0.5 mM thiamine diphosphate (ThDP) and 2 mM $MgCl_2$], T = 30°C.

A crucial point for many bioprocesses is product recovery, because often relatively low product concentrations are obtained and products must be isolated from a liquid phase. Here, the choice of an appropriate downstream process or *in situ* recovery strategy has to be done. To analyze the substrate supply as well as the product extraction, the respective unit operations were implemented on small scale applying membrane contactors [27, 28].

The biocatalytic reaction of BFD requires the substrate benzaldehyde. However, this substrate has a limited solubility of only 40 mM in aqueous solution. Dissolving the substrate in batch mode is a time-consuming step that requires intensive mixing for around 30 min. Therefore, a feed via a membrane is a good way to circumvent this procedure and enables the direct integration of the substrate supply into the micro system. Benzaldehyde is a hydrophobic compound that easily wets hydrophobic membrane materials such as polyvinylidene fluoride (PVDF), whereas the aqueous/polar reaction phase cannot go into the pores. The membrane acts as an interface between the two liquid phases and benzaldehyde diffuses into the reaction system. These membranes are available as flat membrane filters as well as micro hollow fibers. The huge advantage of micro hollow fibers is their low inner diameter of around 700 µm, which excellently fits to the micro reactors illustrated above [29]. Therefore, this membrane type was chosen for the investigated system. The volume of the used hollow fiber was 30 µl at a length of 7.5 cm. For the biocatalytic reaction the pore size is a also crucial parameter, because a leaching of the enzyme through the membrane reduces the catalytic activity in the reactor. However, two immiscible liquid phases, the hydrophilic enzyme containing phase (aqueous) and the hydrophobic substrate phase, were applied, no leaching of the enzyme in the direction of the organic phase was observed and the micro hollow fiber was therefore operated as a liquid-liquid contactor [30].

As illustrated in Figure 11 saturation of the membrane with the substrate benzaldehyde and thus a constant benzaldehyde transfer rate (156 µmol/min in this case) is achieved within 90 min at a constant volume flow of 0.3 ml/min representing a residence time of 0.1 min [31]. Both the residence time of 0.1 min as well as the transfer rate of 156 µmol/min are completely sufficient to provide enough substrate for the biocatalytic reaction. Carrying out the reaction with an activity of around 4 mM/min in a volume of 115 µl and at a residence of 0.1 min, a maximal consumption of 0.046 µmol is expected whereas a feed of 15.6 µmol is ensured.

The principle of a liquid-liquid contactor in the form of a micro hollow fiber was also applied for product extraction. Here, an organic solvent (ethyl acetate) was used to extract the product from the aqueous phase. The degree of extraction at different residence times up to 1 min was investigated using a 20 mM HPP solution (Figure 12). At a residence time of 1 min a degree of extraction of 50% was obtained. Comparing these results again with results obtained for the downscaled biocatalytic reaction, a product concentration of 2 mM HPP is to be expected in the case of a residence time of 1 min, illustrating the effectiveness of this established procedure.

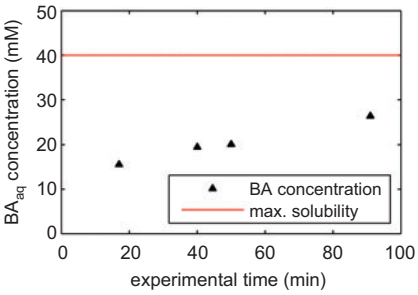

Figure 11 Feed of the substrate benzaldehyde via a hollow fiber membrane. Membrane parameters: hydrophobic PVDF membrane, d_i = 700 µm, pore diameter 0.1 µm, area 165 mm². Experimental conditions: T = 20°C, flow of aqueous phase 300 µl/min, residence time 0.1 min.

4 Conclusions

In this study, the successful downscale of different unit operations for the biocatalytic carboligation of benzaldehyde and acetaldehyde was demonstrated. The reactor volume was reduced to 115 µl, thus enabling a substrate and enzyme saving by a factor of 52 in comparison to standard laboratory techniques. Additionally, the successful downscale of membrane based liquid-liquid contactors was shown, which allow, for example, the screening of solvents for extraction. Comparable volumes as well as

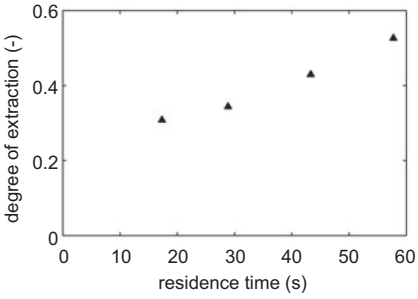

Figure 12 Extraction of the product 2-hydroxy phenylpropanone via a hollow fiber membrane using ethyl acetate for extraction. Mem-brane parameters: hydrophobic PVDF membrane, d_i = 700 µm, pore diameter 0.1 µm, area 165 mm². Experimental conditions: starting concentration of HPP 20 mm, T = 20°C, flow of aqueous phase: 30–100 µl/min.

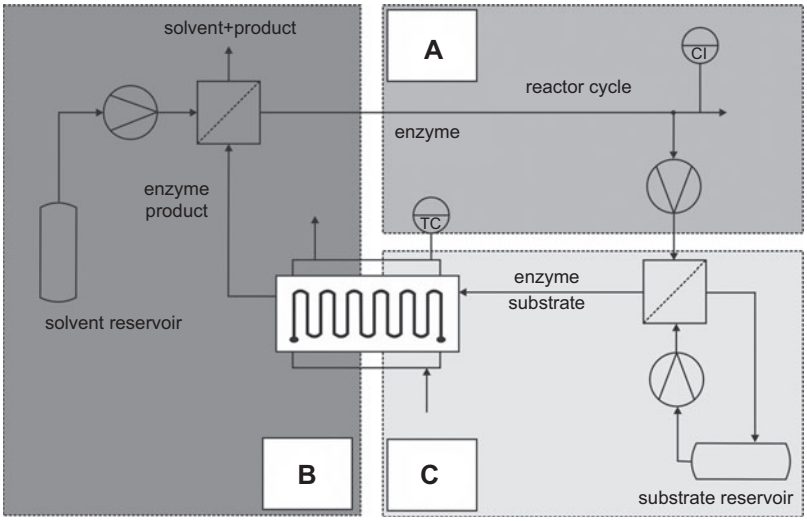

Figure 13 Possible flow scheme for the continuous analysis of the combined unit operations introduced. The reactor cycle with a sampling valve as well as two membrane cycles for substrate feed and product removal is shown.

residence times were realized, enabling the integration of all three unit operations.

Future work will deal with the combination of different unit operations (Figure 13) and the analysis of these combinations compared to the analysis of single unit operations. A more detailed analysis of the flow stream of the individual components within the channel is aimed for both micro reactors [32].

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