Continuous Lipase B-Catalyzed Isoamyl Acetate Synthesis in a Two-Liquid Phase System Using Corning® AFR™ Module Coupled with a Membrane Separator Enabling Biocatalyst Recycle

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The performance of the Corning AFR™ Low Flow (LF) fluidic module for *Candida antarctica* lipase B-catalyzed isoamyl acetate synthesis in an *n*-heptane–buffer two-liquid phase system was evaluated. Obtained flow regime of dispersed *n*-heptane droplets in a continuous buffer phase, which enables *in situ* extraction of the produced isoamyl acetate to the *n*-heptane phase, provides a very large interfacial area for the esterification catalyzed by an amphiphilic lipase B, which positions itself on the *n*-heptane–buffer interface. Productivities obtained were the highest reported so far for this reaction and indicate that Corning Advanced-Flow Reactor™ (AFR™) modules are also very efficient for carrying out biotransformations in two-phase systems. Additionally, for the separation of the *n*-heptane from the aqueous phase, a membrane separator consisting of a hydrophobic PTFE membrane was integrated, which enabled the reuse of biocatalyst in several consecutive biotransformations.

Keywords: microreactor, lipase, esterification, separation, droplet flow

1. Introduction

Scientific and technological advances in tailoring biocatalysts by protein engineering and design of new biosynthetic pathways have led to increased use of biocatalysis as an environmentally friendly alternative to traditional metallo- and organocatalysis. Biocatalysis is becoming a key component of sustainable processing in chemical synthesis, from commodity chemicals to advanced pharmaceutical intermediates, as well as in food, flavor, and fragrance production [1, 2]. The size of the flavor and fragrance industry was estimated at about 24.9 billion US$ in 2014, a total market growth of about 13% from 2010 [3]. One of its important products is isoamyl acetate which, due to its strong banana flavor, is the number one ester required in food industries [4]. Isoamyl acetate is widely used as a flavoring compound in a variety of foodstuff, such as honey, butterscotch, artificial coffee, and beverages. It is also one of the major flavor components of fermented alcoholic beverages, such as sake, beer, and wines [5]. Annual demand for isoamyl acetate in USA alone amounts to about 74,000 kg [6]. This compound is also used in the cosmetics [7] and pharmaceutical industries [8], as well as in solvent-based formulations for automotive, industrial, and wood paints and thinners [9]. Isoamyl acetate production by lipase-catalyzed esterification has been proved to be economically advantageous compared to extraction from natural sources or chemical synthesis [10]. Lipase-catalyzed isoamyl acetate synthesis has been performed in nonaqueous solvents [10–12], in solvent-free [9], or in two-phase systems [13–18]; in the latter, the interfacial catalysis as a consequence of an amphiphilic character of these enzymes is exploited. Benefits of using two-phase systems for enzyme-catalyzed reactions include increase of product and substrate solubility, shift of reaction equilibrium in the direction of esterification, and increase of selectivity towards a desired product [19].

Multiphase microreaction systems are emerging tools for the development of enzyme-catalyzed transformations involving two or more partly immiscible fluids in continuous flow [20]. The possibility to control liquid–liquid flow patterns in microchannels enables the enhancement of specific interfacial area, leading to improved mass transfer among the phases. Furthermore, the use of microfluidic devices prevents the formation of stable emulsions, usually present in batch processes with two-liquid phase systems and, hence, significantly reduces downstream costs and efforts [21]. Due to very efficient mass and heat transfer, leading to better process control and intensification, microreactors were successfully transferred to industrial production scale, mostly in chemical synthesis [22–25]. Recently, their potential for implementation in biocatalytic process development and production has been highlighted. Continuously operated biocatalytic reactions in microreactors, especially reactions at phase boundaries, have been shown to be superior to batch reactors [20, 21, 26–28].

In order to achieve large scale productions within microscale systems, an external numbering-up approach is typically suggested [24, 29, 30]. However, the connection of multiple microdevices with conventional nonstandardized tubing presents neither a compact engineering solution nor an economical one in terms of fabrication and material costs. Therefore, internal numbering-up, based on the parallel connection of the functional elements grouped within common housing, has been chosen as an alternative concept [29–31]. One of the commercial examples of continuous flow reactors, which is comprised of a chain of identical cells with variable cross sections and internal obstacles known as Heat Exchange and Advanced Reactor Technology (HEART), was developed by Corning. The key component of the Corning AFR™ system is a specialty glass or ceramic fluidic module having hydraulic diameters in the range of 0.3 up to a few mm. These devices are able to provide efficient mixing for homogeneous systems and also to create and maintain fine dispersions for multiphase applications [32].

The goal of this study was to experimentally evaluate the performance of Corning LF fluidic module for *Candida antarctica* lipase B (CaLB)-catalyzed isoamyl acetate synthesis in a two-liquid phase system, in order to establish the process at a larger scale. Productivities obtained were compared with the bench reactor with intense mixing, as well as with microfluidic devices with parallel or segmented flow of two phases. Further integration of the LF module with a membrane-based microseparator aimed to reuse the dissolved enzyme in several consecutive biotransformations.

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2. Results and Discussion

2.1. Fluid Flow in an LF Fluidic Module. Corning LF fluidic module with an internal volume of 0.5 mL was preliminarily tested for delivering a stable, uniform droplet flow regime between the aqueous phase and *n*-heptane. Despite the recommendation to operate this module at flow rates in the range of 2 to 10 mL/min, total flow rates between 0.2 and 0.8 mL/min were employed in this study in order to enable residence times needed for the relatively slow process of lipase-catalyzed esterification [11, 16].

Addition of CaLB, which acts as a surfactant, lowers the interfacial tension between *n*-heptane and aqueous phase. The measured surface tension of the system without lipase (38 mN/m) was 3 times higher compared to the value of 12.6 mN/m obtained after the addition of 10% (*v*/*v*) CaLB in the aqueous phase.

A comparison of a flow pattern obtained in the absence and in the presence of the enzyme is shown in Figure 1. The flow of *n*-heptane and an aqueous phase containing enzyme (Figure 1b), which has lower interfacial tension, yielded smaller and more uniform droplets, with an average size of 330±23 μm, than in the absence of CaLB (Figure 1a), where the average size was 795±184 μm. Furthermore, the comparison of the average sizes of the droplets in Corning LF module and at the outlet of the PTFE tubes (334±38 μm) showed no difference in size. This confirmed that the stable emulsion flow, shown in Figure 1b, was created due to its unique geometry composed of HEART-shaped chain of identical cells, where, additionally, Corning LF module also enabled efficient thermal regulation. From the process control point of view, as well as from the production scale increase, this is beneficial as compared to the tubing system. For further scale-up of the developed process, Corning AFR™ systems should be considered as already shown in the literature for the nonenzymatic reactions [32–35]. The obtained emulsion of *n*-heptane droplets in an aqueous phase provided very large interfacial area for the esterification of isoamyl alcohol and acetic anhydride catalyzed by CaLB, which positions itself on the *n*-heptane–buffer interface. Furthermore, the chosen two-phase system enables *in situ* separation of products of primary reaction, namely, isoamyl acetate and acetic acid, in *n*-heptane and aqueous phase, respectively. Acetic acid from the aqueous phase and isoamyl alcohol from the *n*-heptane phase further react at the interface, and isoamyl acetate formed is positioned in the *n*-heptane phase [17].

Employing various microfluidic devices has already shown to be beneficial for CaLB-catalyzed isoamyl acetate production in a two-liquid phase system [13, 16, 17]. Žnidaršič-Plazl and Plazl [17] applied parallel flow achieved by adjusting the flow rates of both phases to form a liquid–liquid boundary in the middle of the microchannel; this enabled the separation of phases at the Y-shaped exit of the microreactor. Pohar et al. [13] used a Ψ-shaped microreactor with three inlets which led to the creation of a unique flow pattern of the *n*-heptane and the hydrophilic ionic liquid 1-butyl-3-methylpyridinium dicyanamide ([bmpyr][dca]). The flow consisted of *n*-heptane long droplets accompanied by very small ones, providing large interfacial area for the reaction and simultaneous product extraction.

The largest estimated specific interfacial area of the droplets obtained in this study at the highest flow rates used (0.4 mL/min of each phase) and at CaLB concentration of 20% (*v*/*v*) in an aqueous phase was of 9×10^3/m. As shown in Table 1, this is approximately 4 times lower than reported for *n*-heptane–ionic liquid two-phase system at 10% (*v*/*v*) of CaLB in [bmpyr][dca] [16] and 2-times larger than in a microreactor with parallel flow [17]. The presented values are similar to the slug flow of a toluene–water two-phase system in a capillary microreactor (i.d. 250 μm) at equal flow rates, while, by increasing the ratio of aqueous phase and having higher flow rates, the surface to volume ratio could be increased [36]. The specific interfacial area obtained in a small batch reactor at mixing speed of 1000 rpm was only 1.6 lower compared to that in the Corning LF module (Table 1). All areas obtained in microfluidic systems are two orders of magnitude higher than in conventional stirring large batch reactors with liquid–liquid systems [37, 38]. The design of the Corning LF fluidic module maintains the mixing along the reaction path, leading to the intensification of the mass transfer. Moreover, increasing the total flow rates of the multiphase system had a positive effect on mass transfer, as finer dispersions were created [39].

In order to increase the residence times in the Corning LF fluidic module, which were between 0.6 and 5 min at flow rates used in this study, a 13-cm long polytetrafluoroethylene (PTFE) tube with 1.59 mm i.d. was added at the module's outlet. Microdroplets of *n*-heptane in an aqueous solution were preserved, and no coalescence of microdroplets inside the PTFE tube was observed, as evident from Figure 2.

2.2. Biotransformation in an LF Fluidic Module. As evident from Figure 3, Corning LF fluidic module system enabled efficient lipase-catalyzed isoamyl acetate synthesis, while, without an enzyme, the reaction yielded up to 1.6% isoamyl acetate within the miniaturized reactor system. The integrated thermal regulation provided an easy way to adjust the reaction temperature, which had a profound influence on the process. The increase of temperature up to 60 °C had a positive effect on the reaction rate and thereby productivity of the reactor system.

The esterification of isoamyl alcohol and acetic anhydride is a two-step reaction; the first reaction is very fast and exothermic and produces isoamyl acetate and acetic acid, which can then further react with surplus isoamyl alcohol, forming isoamyl acetate and water [10]. The second reaction is much slower; therefore, the fast increase in the isoamyl acetate yield in Figure 3 is due to the first reaction [10].

As expected, lower flow rates and thereby higher residence times within the reactor system have led to higher isoamyl acetate yields, while productivities increased by increasing the flow rate as presented in Figure 3b.

The highest yield of 59% was obtained at 60 °C within 8.6 min which corresponded to 0.52 M of isoamyl acetate in

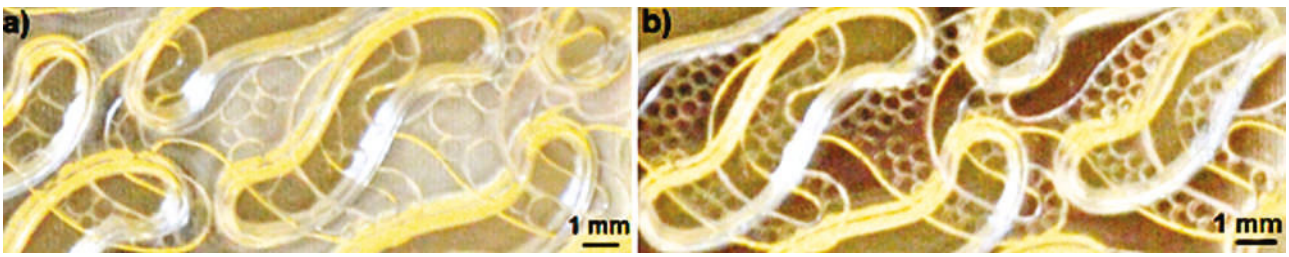

Figure 1. *n*-Heptane droplets in an aqueous phase obtained inside Corning LF flow module at 25 °C and at 0.1 mL/min of each phase a) without CaLB and b) with 5 % (*v*/*v*) of CaLB in reactor volume

Table 1. Comparison of fluid flow characteristics and isoamyl acetate production efficiency in different reactors

Reactor and operation mode	Reactor volume (mL)	Solvent system	Flow type	Maximal total flow rate (μL/min)	Specific interfacial area (/m)	*T* (°C)	Volumetric ($g/m^3 \cdot s$) and biocatalyst [$g/(g_{cat} \cdot h)$] productivity	Productivity (g/h)	Reference
Test tube – batch	4	*n*-Heptane–buffer	Emulsion	–	$5.6 \cdot 10^3$	60	16.8; 37.8	0.24	This work
Y-Y-shaped microreactor – continuous	0.0035	*n*-Hexane–buffer	Parallel flow	400	$4.5 \cdot 10^3$	25	171; 492	$2.2 \cdot 10^{-3}$	[15]
X-shaped microreactor+0.5 mm i.d. tube – continuous	0.01+0.735	*n*-Heptane–[bmpyr][dca]	Droplet flow	60	$3.6 \cdot 10^4$	22	66.1; 225	0.174	[14]
LF Corning+1.59 mm i.d. tube – continuous	0.5+0.6	*n*-Heptane–buffer	Droplet flow	800	$9 \cdot 10^3$	60	400; 548	1.584	This work

[bmpyr][dca]: 1-butyl-3-methylpyridinium dicyanamide

Figure 2. Microdroplets of *n*-heptane in an aqueous solution at the outlet from the PTFE tube connected with Corning LF module. Total flow rate, 0.8 mL/min; 25 °C; 5 *v*/*v*% of added CaLB solution

the *n*-heptane phase. For comparison, in [bmpyr][dca]–*n*-heptane two-phase system with 10% (*v*/*v*) of CaLB in the ionic liquid phase and at the same reactant concentration, a similar yield of 58% was obtained within a X-shaped microreactor at a retention time of 12.3 min [16], while, in a study using the Ψ-shaped microchannel with the same substrates and [bmpyr][dca]–*n*-heptane solvent system, the highest yield of 75% was obtained at the retention time of 33 min [13]. In the water–*n*-hexane system with acetic acid as the acyl donor, performed in a microreactor with Y-shaped inlet and outlet with parallel flow, only 35% yield could be achieved within a single pass through the microreactor at 45 °C [17].

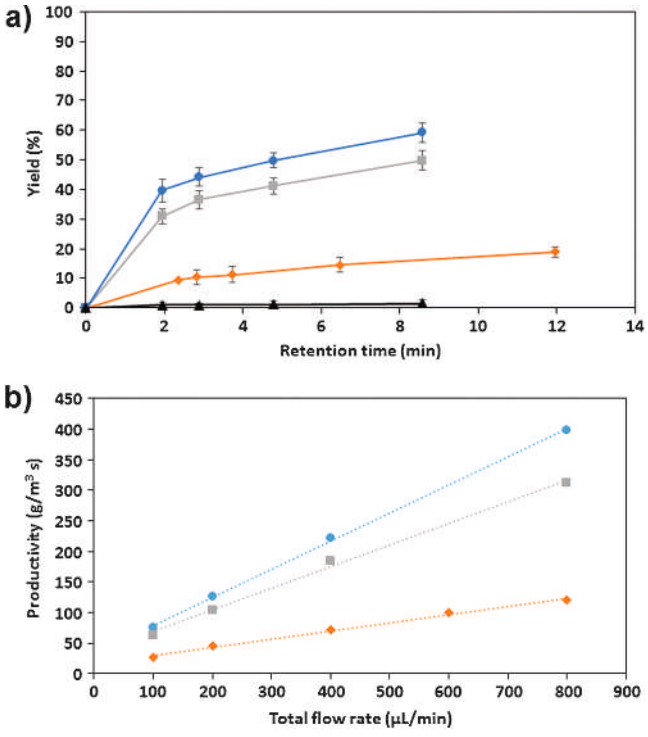

Figure 3. a) Isoamyl acetate yield and b) volumetric productivities obtained at different temperatures and at various total flow rates with the volumetric ratio of both phases 1:1. (●) 60 °C, (■) 50 °C, (♦) 22 °C, (▲) 60 °C, no enzyme

These results are in agreement with the work of Žnidaršič-Plazl and Plazl (2009) on esterification with CaLB in water–*n*-hexane two-phase system, where the increase in temperature from 30 °C to 45 °C showed positive effect on the production of isoamyl acetate [17]. Similarly, in the work of Güvenç et al. (2002), who studied esterification in solvent-free systems with Lipozyme RM IM, enzyme was active at temperatures up to 50 °C and initial rates showed an increasing trend at increased temperature [40]. On the other hand, some studies on esterifications in organic solvents or solvent-free systems have shown that high temperatures may disrupt enzyme tertiary structure resulting in the loss of catalytic activity [41]. For example, Poojari and Clarson (2013) reported on maximal activity of free CaLB for esterification in toluene at 40 °C, while, at 60 °C, it decreased for 40% [42]. Azudin et al. (2013) performed the production of isoamyl acetate in solvent-free medium with CaLB where the increase of temperature from 30 °C to 40 °C and 50 °C showed slight decrease in the initial velocity and lower final yield [9]. This implies that, for the free CaLB, the addition of a nonorganic phase in a two-phase system could improve enzyme stability at higher temperatures.

All processes performed within the miniaturized devices were superior to the batch processes established in conventional equipment, typically in a batch mode. Also, in this study, the volumetric and biocatalyst productivities obtained in the small batch system working at the same temperatures as well as substrate and enzyme concentrations, at a mixing speed where an emulsion was formed, were 24 and 15 times lower than in the Corning LF flow module, respectively (Table 1). Similarly, in the [bmpyr][dca]–*n*-heptane solvent system, the microreactor gave 3 times higher volumetric productivities as compared to the batch vigorously mixed small reactor [13]. Furthermore, optimized batch esterification of isoamyl alcohol and acetic anhydride with immobilized CaLB in *n*-hexane at 40 °C yielded 96% of isoamyl acetate in 2 h, which gave a volumetric productivity of 27.3 g/(m^3 s) and biocatalytic productivity of 89.9 g/(g_{cat} h) assuming 10% of CaLB in immobilized preparation [11], which is evidently several fold worse than in this study with two-liquid flow system processed in the Corning LF flow module.

The chosen solvent system consisting of an aqueous phase and *n*-heptane phase enabled *in situ* product extraction to the *n*-heptane phase, while the side products stayed in the aqueous phase. Studies on the partitioning of the reagents and products of the studied system between phosphate buffer with pH 8 and *n*-heptane revealed that 94% of isoamyl alcohol and 99% of isoamyl acetate are extracted to the *n*-heptane, while only 0.2 and 16% of acetic acid and acetic anhydride were extracted to the *n*-heptane phase, respectively. Corresponding partitioning coefficients (K_p) for isoamyl alcohol, isoamyl acetate, acetic acid, and acetic anhydride in this solvent system were 12, 67.3, 0.002, and 0.19, respectively. This was much better as compared to the [bmpyr][dca]–*n*-heptane system, where only 80% of isoamyl acetate was transferred to the *n*-heptane phase, while 20% of the total produced isoamyl acetate was left in the ionic liquid phase [16]. Additionally, the biocatalyst productivity is 2.4 times higher in this study than in our previous work with the ionic liquid [16], which is also much more expensive than the buffer solution.

2.3. Recycling of the Enzyme. However, the separation of phases at the exit of the microreactor section was necessary to isolate the product and to reuse the enzyme, which is desirable from the economical point of view. To achieve this task, a separator with hydrophobic PTFE membrane was developed. A commercial membrane microseparator which could separate liquid–liquid two-phase system at flow rates up to 0.1 mL/min [16] was not useful for Corning LF fluidic module operated at total flow rates up to 0.8 mL/min. Therefore, a two-plate plug and play membrane separator was developed in order to enable the possibility of recycling of the enzyme (Figure 4).

Continuous runs were performed for minimum of 1 h and the collected aqueous phase containing the enzyme was reused in a next cycle. As evident from Figure 5, more than 90% of the initial yield was preserved in the first 4 consecutive runs, while, after the 5th recycle, it dropped to 77%. The recycled aqueous phase contained the enzyme together with the formed acetic acid, which caused a pH drop from the initial 8 to 4 after the first cycle and a further drop to 3.8 at the end of the 5th recycle. Since separate experiments on CaLB activity in buffers with various pH values indicated a drop of 13% when changing the buffer's pH from 8 to 6 (Figure 6), this is a possible reason for the decline in yield with each additional cycle.

As a membrane, a hydrophobic PTFE material was chosen, so that the enzyme would not go through the pores and only *n*-heptane is wetting the membrane and the aqueous phase is just passing along the membrane surface and the outlet flow of the aqueous phase is equal to the inlet flow by controlled pumping from the membrane separator. All this mentioned is decreasing the effect of the fouling. However, the differences in protein concentration at the outlet of the membrane separator, which effect could be decreasing of the preserved yield, could also be related to the dilution of the proteins in aqueous phase due to water formation during the reaction.

Physical loss of the enzyme on the membrane (fouling), since the same membrane was used for all the cycles, will be further investigated with longer operational times.

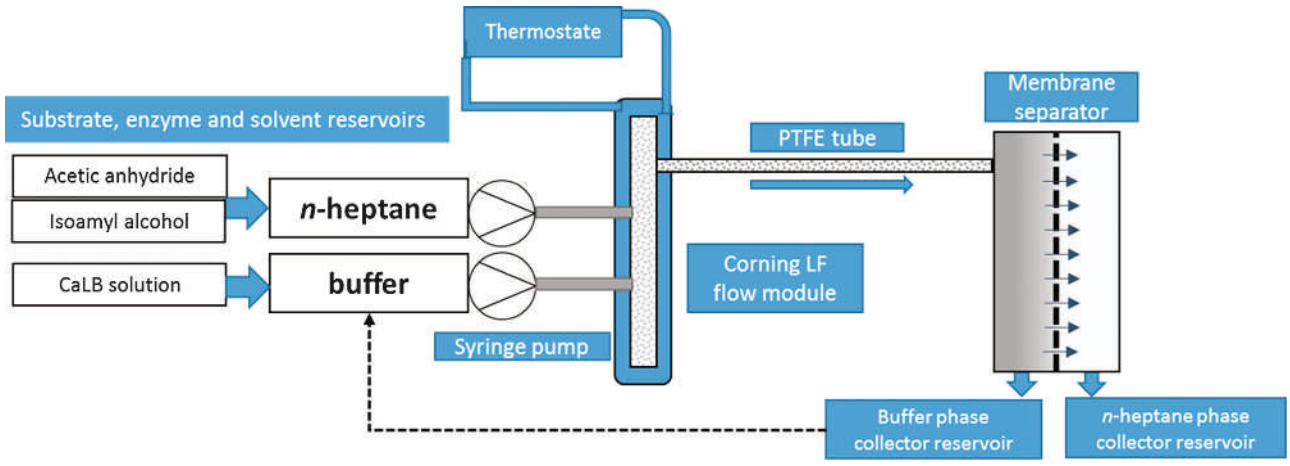

Figure 4. Scheme of the integrated Corning LF fluidic module with a membrane separator

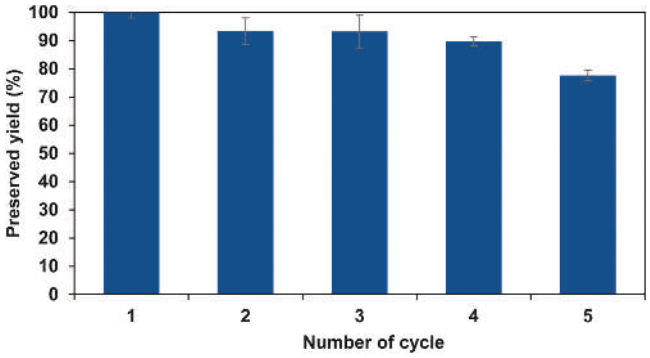

Figure 5. Preserved esterification yield at a total flow rate of 0.8 mL/min after recycling the aqueous phase with dissolved CaLB

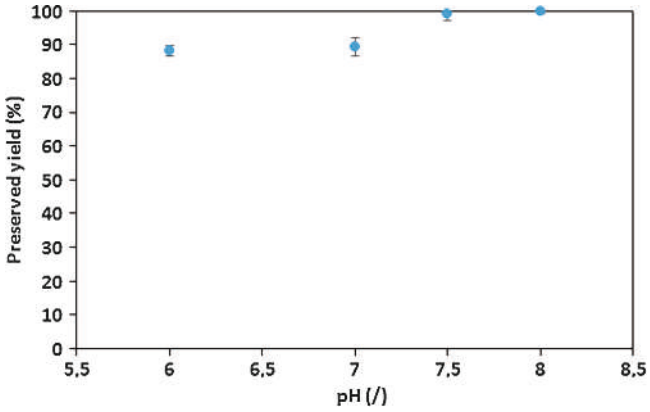

Figure 6. Preserved esterification yield at a total flow rate of 0.8 mL/min at different pH values of the aqueous phase with dissolved CaLB

3. Conclusions

Results obtained in this study confirmed the high efficiency of Corning Advanced-Flow™ also for carrying out biotransformations in two-phase systems. A comparison with bench scale mixing reactor operated in a batch mode, as well as with smaller microfluidic systems, revealed not only that the productivities obtained in the Corning LF fluidic module were, according to the available literature, the highest reported so far for this reaction, but also that the enzyme was more efficiently used. An integrated PTFE membrane enabled continuous phase flow-through separation of product in the organic phase and enzyme in the aqueous phase, which enabled the reuse of the biocatalyst for several continuous runs without significant loss of activity. Such combination results in higher ecological acceptability and cost-effective solution due to recycling of the aqueous phase with the dissolved enzyme. This study opens the possibility for the scale-up and the establishment of a continuous process on a larger scale. Further optimization of process parameters such as the ratio of both phases and the enzyme concentration is under investigation.

4. Experimental

4.1. Materials. Aqueous solution of lipase B from *C. antarctica* (CaLB) with a declared lipase activity of minimum 5000 LU/g of liquid was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Isoamyl alcohol, isoamyl acetate, acetic anhydride, and *n*-heptane were all purchased from Sigma-Aldrich Chemie GmbH and were of analytical grade.

4.2. Esterification in Small Batch Reactor. Batch experiments were carried out at 60 °C in test tubes with integrated thermoregulation, which were mixed at 1500 rpm on a vortex mixer. Esterification was performed in 2 mL of *n*-heptane with dissolved reactants (1.5 M isoamyl alcohol and 0.5 M acetic anhydride) and sodium phosphate buffer pH 8. The reaction was started by the addition of 20% (*v*/*v*) CaLB solution in a sodium phosphate buffer to give final volume of 4 mL. At different reaction times, 200 μL of sample (emulsion) volume was taken from the test tube. Phases were afterwards separated with a centrifuge at 13,000 rpm for 3 min. After separation, the 50 μL of the upper *n*-heptane phase was transferred to the GC vial and analyzed by a gas chromatograph as specified in Section 4.7.

4.3. Esterification in LF Flow System Integrated with Membrane Separator. The whole experimental setup is schematically shown in Figure 6. It comprises four parts: fluid delivery section (syringe pumps, PTFE connecting tubes), reaction system (Corning LF fluidic module connected with PTFE tube of 13 cm length and 1.59 mm i.d.), thermoregulation system, and phase separation system (two-plate PTFE membrane separator). A Corning LF fluidic module with the internal volume of 0.5 mL composed of chains of identical cells — HEART (Corning SAS, Avon, France) was used for the experiments; the module was connected to high-performance syringe pumps (Harvard Apparatus, Holliston, USA), which ensured highly controllable flow rates.

Based on the aqueous–organic solvent used in this study and determined values of the interfacial tension, a membrane with the pore size of 0.5 μm was chosen. The membrane had a surface area of 19 cm^2 and was cut out in two silicone layers to prevent leakage and gave the separator a total volume of 3 mL; the membrane was assembled together with two PMMA plates and tightened up with 6 screws. The picture of the two-plate PTFE membrane (Merck Millipore, Billerica, USA) separator used in the study concerning the reuse of the aqueous phase containing the enzyme is shown in Figure 5a. In order to attain the desired flow rates, the pressure was increased at the outlet of the aqueous side of the separator by connecting an injection pump, which pulls the liquid at the flow rate equal to that of aqueous phase.

The *n*-heptane phase containing the reactants isoamyl alcohol (1.5 M) and acetic anhydride (0.5 M), and 0.1 M potassium phosphate buffer with pH 8 containing 20% (*v*/*v*) of CaLB as an aqueous phase were separately fed to the Corning LF fluidic module. The flow rates used in these experiments were from 50 to 400 μL/min for each phase. Additionally, experiments with different inlet aqueous phase pH values (6–8) were performed at the same conditions as described before. After achieving steady state for selected flow rates, at least three samples were collected and measured on GC as described in Section 4.7. Average values were calculated, and standard deviations have been determined.

4.4. Esterification with Enzyme Recycle. The CaLB solution was added to the aqueous phase to achieve final concentration of 5% (*v*/*v*) of total volume (corresponding to 10% in the aqueous phase) only at the beginning of the first cycle. Each cycle was operated for at least 1 h with equal flow rates of *n*-heptane and buffer phase of 0.4 mL/min. The phases were separated in the membrane separator, and the aqueous phase was collected and reused in 4 consecutive recycles. Fresh organic phase (*n*-heptane containing isoamyl alcohol and acetic anhydride) was supplied, with the same flow rate as in the first cycle. At least five samples were taken from the *n*-heptane phase, as well as from the *n*-heptane aqueous phase extract, and substrate and product concentration were measured on GC as stated in Section 4.7. Mean values were calculated, and standard deviations have been determined.

4.5. Determination of the Interfacial Tension. Measurements of the interfacial tensions between two immiscible liquids were performed with School tensiometer K6 (KRÜSS GmbH, Hamburg, Germany). The ring was first submerged into the lower specific heavier phase (buffer solution or buffer solution

with 10% (*v*/*v*) of added CaLB), and the lighter phase (*n*-heptane) was then pipetted above it with the same volume. Before measurements, the device was levelled to zero-position. The interfacial tension value was reached when the lever arm could no longer be brought into zero-position by slowly lowering the measuring table (over-elongation of the film). All measurements were performed in triplicates.

4.6. Fluid Flow Analysis. The pictures of the flow inside the Corning LF fluidic module as well as in samples from the small batch reactor were taken with digital camera Canon D5100 and later processed with the freeware version of ImageJ 1.47b to determine the average diameter of the formed *n*-heptane droplets. This diameter and the corresponding *n*-heptane fluid flow rate were further used for the calculation of the specific interfacial area of *n*-heptane droplets per total volume of fluid within the reactor [16].

4.7. Analysis of Reactants and Products. Isoamyl acetate, acetic anhydride, and isoamyl alcohol concentrations in buffer phase after extraction in *n*-heptane, as well as in *n*-heptane phase, were determined by a gas chromatograph HP 6890 (Hewlett-Packard, Palo Alto, CA, USA) equipped with a hydrogen flame ionization detector and an HP-INNOWAX column (30 m×0.25 mm i.d. × 0.25 μm). Detailed description of the method has been previously published [14]. Retention times for isoamyl acetate, isoamyl alcohol, and acetic anhydride were 1.49, 1.7, and 2.57 min, respectively.

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