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Enzymatic hydrolysis in an aqueous organic two-phase system using centrifugal partition chromatography☆

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ABSTRACT

Multi-phase reaction systems, mostly aqueous organic systems, are used in enzyme catalysis to convert hydrophobic substrates which are almost insoluble in aqueous media. In this study, a Centrifugal Partition Chromatograph is used as a compact device for enzymatic multi-phase reaction that combines efficient substrate supply to the aqueous phase and separation of both phases in one apparatus. A process design procedure to systematically select the aqueous and organic phase to achieve stable and efficient reaction rates and operation conditions in Centrifugal Partition Chromatography for efficient mixing and separation of the phases is presented. The procedure is applied to the hydrolysis of 4-nitrophenyl palmitate with a lipase derived from *Candida rugosa*. It was found that the hydrolysis rate of 4-nitrophenyl palmitate was two times higher in Centrifugal Partition Chromatography than in comparable stirred tank reactor experiments.

1. Introduction

Since most of the biocatalysts, referred as enzymes, are active in aqueous solutions only, the substrates to be converted are limited to those soluble in aqueous media. For the enzymatic conversion of hydrophobic substrates, approaches like the use of non-aqueous or multi-phase systems as reaction milieu are needed. In case of multi-phase systems, mostly aqueous–organic systems, the hydrophobic substrate is dissolved in the organic phase and mixed with the aqueous phase containing the biocatalyst. Besides the conversion of hydrophobic substrates the main advantage of using multi-phase reaction systems is the ability of "in situ" product removal from the catalytically active aqueous phase to the organic phase. As a consequence, the reaction equilibrium can be shifted toward the product side and product inhibition of the enzyme can be eliminated [1]. However, multi-phase reaction systems lead to different design aspects compared to monophasic aqueous systems [2]. Efficient mixing and separation of the phases need to be realized and mass transport hindrances for the substrate to reach the enzyme and the product to partition in the organic phase need to be taken into account [3–5]. Another challenge of aqueous–organic reaction systems is the preservation of the enzymatic activity. Beside good mixing behavior to increase the interfacial area for mass transport, the effect of the interfacial forces on the activity of the enzyme needs to be considered [6]. Additionally, organic solvents can harm enzymes and significantly decrease their catalytic activity, so solvent screening becomes necessary before setting up the reaction.

Different approaches to enhance the use of multi-phase systems for enzymatic reactions are available like genetic engineering to stabilize the enzymes toward organic solvents and the process conditions applied. Also different reactor concepts to increase mixing and optimize separation of the phases are available [6,7]. These reactor concepts range from classical mixer-settler devices to membrane contactors, and all aim to increase interfacial area to minimize interfacial mass transfer hindrances and simultaneously contacting the aqueous catalytically active phase with fresh organic substrate [7–9].

The CPC can be an efficient mixer that creates a large interfacial area for an efficient distribution of components between the phases and allows settling of the phases in one device. Commonly, CPC is used for liquid–liquid chromatography [10]. In CPC one phase of any two-phase system is immobilized and used as stationary phase. The immobilization is achieved in a chamber system that is arranged around a rotary axis. By rotating the system a centrifugal field is generated which keeps the stationary phase in the chamber cascade. The second phase is pumped through the stationary one and therefore referred to as mobile phase. Depending on the density of the phases used, two main operation modes are defined. In case the lighter phase is used as mobile phase CPC is operated in ascending mode. Separation with the heavier phase as mobile

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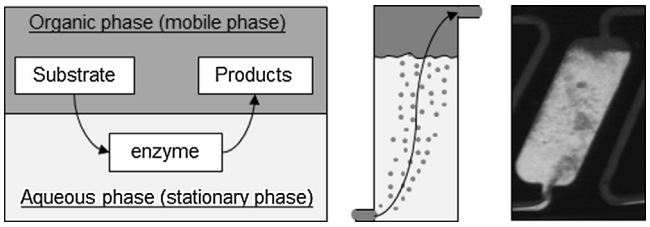

Fig. 1. From left to right: basic scheme of enzymatic multi-phase biocatalysis; CPC chamber in ascending mode (organic mobile phase (darker) and aqueous, catalytically active phase stationary phase (light)); real hydrodynamic in a single CPC chamber.

phase is called descending mode [10,11]. Commonly, the CPC technique is used for the purification of natural extracts including the use of aqueous two-phase systems for the purification of proteins [12–15]. In this study the Centrifugal Partition Chromatography (CPC) is investigated as a new reactor concept for aqueous–organic enzyme catalysis. The stationary liquid phase can be used to immobilize the catalytic active enzyme in solution without handling solid materials or functionalizing surfaces as proposed for other multi-phase reactor concepts [16,17]. The mobile organic phase is used to supply substrate and remove product simultaneously. The concept is presented in Fig. 1.

Utilizing CPC and CCC for reactions is not new. Some initial studies were published in the late 90s. Berthod [18] published a study about a chemical reactions conducted in CCC in 1998. He found that this brought various advantages toward a comparable plug-flow reactor. Studies about enzymatic reactions in CPC were published from 1998 to 1999 by van Hollander, who described the reaction and separation of chiral amino acids in an aqueous two-phase system by pulsing substrate into the CPC. This led to a process that combined reaction and separation of products and non-converted substrates based on chromatographic mechanisms. He also provided a mathematical model to predict concentration profiles in the CPC [19,20]. However, the CPC concept presented is batch mode due to pulsing the substrate solution and, therefore, capacity is limited. Additionally, an aqueous two-phase system was used, not allowing the conversion of hydrophobic substrates as proposed in the multi-phase reaction approach discussed before. Hence, a detailed description of the CPC for aqueous organic enzyme reactions with continuous product removal is not discussed yet.

In this study, a procedure for the selection of an appropriate aqueous organic two-phase system for the conversion of hydrophobic substrates in CPC is developed, following the approach presented by Schembecker and Tlatlik [21] who proposed to determine optimal operating windows for the reaction, separation and the apparatus separately and operating the integrated process in the overlapping zone of these different windows. The procedure is applied to the hydrolysis of 4-nitrophenyl palmitate with a lipase derived from *Candida rugosa*. In their natural inhabitant lipases catalyze the hydrolysis directly at interfaces and are very stable in contact with organic substances. They are the most used enzymes in organic synthesis [6,22,23]. Beside the process design procedure the CPC as apparatus for multi-phase reactions is evaluated. Key issues to be addressed are the long-term stability of the chosen lipase from *Candida rugosa* and a comparison of the CPC technique to a classical stirred tank reactor system.

2. Materials and methods

2.1. Chemicals

4-Nitrophenol (pNP) (spectrophotometric grade), 4-nitrophenyl palmitate (pNPP) (≥98%), 4-nitrophenyl butyrate (pNPB) (≥98%), palmitic acid (PA) (≥99%), 4-morpholinepropanesulfonic acid (MOPS) (≥99.5%), 2-propanol (≥99.5%), n-heptane (≥99%), cyclohexanone (≥99%), methyl tert-butyl ether (≥99.8%), 1-octanol (≥99%), diisopropyl ether (≥98.5%), methyl isobutyl ketone (≥98.5%), trichloroethylene (≥99.5%), ethyl acetate (≥99.8%) and the lipase from *Candida rugosa* (L1754) were purchased from Sigma–Aldrich Co. LLC, Germany.

2.2. Model system

A wild type lipase from *Candida rugosa* is used as model enzyme. For demonstration purposes the hydrolysis of 4-nitrophenyl palmitate (pNPP) to 4-nitrophenol (pNP) and palmitic acid (PA) is investigated. The substrate pNPP is almost insoluble in aqueous media and suitable for the investigation of a multi-phase reaction system and the product pNP is easily detectable due to its strong yellow color in aqueous solutions.

2.3. Analytical procedures

2.3.1. Enzymatic activity assay

For fast determination of lipase activity a test based on 4-nitrophenyl butyrate (pNPB) was adapted from Zhong [24]. The test was modified using 50 mM 4-morpholinepropanesulfonic acid (MOPS) buffered lipase stock (0.15 g/L) and substrate solution (0.75 mM) to ensure no significant pH drop due to production of PA in the measured time frame. Both solutions are mixed in 50:50 volume ratios and absorbance is measured at 410 nm to determine pNP production kinetics. Temperature was kept constant at 25 °C. The enzymatic activity U is defined as 1 μmol pNP produced in 60 s/mg lipase at 25 °C using the substrate pNPB. All reported activities were averaged from triplicates.

2.3.2. pNP quantification in organic solvents

To measure the pNP concentration in organic solvents (mobile phase) a sample (1 ml) is taken from the organic phase and extracted for 10 min with 1 ml of buffered aqueous solution (50 mM MOPS; pH 7.2). Then the absorbance is measured at 410 nm in 500 μl of the aqueous phase.

For calibration, spectrophotometric grade pNP was dissolved in the organic solvent at defined concentrations and then extracted for 10 min into the aqueous phase. The product concentration as function of the absorbance at 410 nm was then derived by measuring the absorbance of the extracted aqueous phase.

2.4. Experimental procedures

2.4.1. Effect of pH-value on enzyme activity

The pH-value for optimum activity of lipase *Candida rugosa* is determined using pNPP as substrate in 50 mM MOPS buffer at different pH-values. In addition, 2-propanol is used to dissolve the product pNPP in small amounts in the aqueous phase. This method is adapted from Ref. [25] and does not influence the lipase activity. The substrate solution, containing 2-propanol, pNPP and 50 mM MOPS buffer, is adjusted to different pH values and mixed with a 50 mM MOPS buffer with the same pH but containing enzyme (0.15 g/L). The activity is measured using the spectrophotometric method as mentioned before but by using the reaction substrate pNPP. The pH value is varied between pH 6.8 and pH 7.6 (pK_s value of MOPS = 7.2). The temperature is kept constant at 25 °C for all experiments. All experiments were performed in triplicates.

2.4.2. Effect of organic solvent on enzyme activity

To screen the enzymatic activity in a two-phase system using different organic phases the lipase *Candida rugosa* is dissolved in