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Whole cell immobilization and catalysis in a Centrifugal Partition Chromatograph

J. Krause, R. Krutz, G. Schembecker, J. Merz*

Laboratory of Plant and Process Design, Department of Biochemical and Chemical Engineering, TU Dortmund University, D-44227 Dortmund, Germany

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ABSTRACT

Biphasic reaction systems using enzymes as catalysts were recently operated in Centrifugal Partition Chromatography (CPC) devices. Since complex biotransformations involving co-factors are commonly operated using whole cell catalysts, this study deals with the implementation of these reaction systems in a CPC device. Therefore, cells from *Saccharomyces cerevisiae* in a biphasic system consisting of an aqueous phase (100 mM phosphate buffer, pH = 7.0) including the co-substrate glucose and heptane as organic phase were used to reduce two β-ketoesters of different polarity. It was found that biocompatibility and extractability in this process have opposed effects and need to be compromised. The cells were immobilized for 5 h using the CPC reactor and the substrate was reduced steadily. The mechanism of immobilization was evaluated visually and traced back to the centrifugal forces as well as the inability of the cells to overcome the liquid–liquid interface.

All in all, the CPC reactor for whole cells has conceptual advantages like easy continuous operation, stable catalyst immobilization without the use of a solid phase and *in situ* product removal, which may open possibilities for the small scale synthesis of fine chemicals using whole cells.

1. Introduction

Various biochemical production processes for the production of bulk chemicals to pharmaceuticals have been established using fermentations or specific bioconversions. The high selectivity that can be achieved using whole cells or free enzymes enables the efficient production of bulk chemicals from renewable feed stocks or highly specific molecules like building blocks for the production of various drugs or chiral pharmaceuticals. One major research field is the production of chiral molecules, especially the reduction of β-ketoesters to their corresponding hydroxyesters as building blocks for the production of chiral pharmaceuticals, fine chemicals, flavors or fragrances [1–3]. Various studies showed the potential of *Saccharomyces cerevisiae* as a suitable organism with a broad substrate spectrum and a strong solvent tolerance [4,5]. Since long chain β-ketoesters can be cytotoxic to cells and are often hardly soluble in aqueous media the use of a second phase to enable the biocatalytic reaction and to reduce the toxic effect on the biocatalyst is common practice [6,7]. However, using multiple phases in the reaction system increases the complexity for phase selection and reactor design. On the one hand the biocompatibility of the phases, the forces at the interface and the substrate accessibility have to be addressed to achieve a high activity and stability of the biocatalyst. On the other hand mixing and phase separation have to be taken into account to achieve an efficient product formation and recovery. For the selection of the phase system scouting and screening experiments are necessary to preserve the catalytic activity and adjust partition coefficients for substrate supply and product recovery. In case of reactor design various reactor concepts for biphasic reaction systems exist. Besides the use of special reactor designs like loop or membrane reactors an immobilization of enzymes or cells is mandatory to avoid catalyst loss, maintain catalyst activity and to enable continuous processing. Drawbacks of these known reactor concepts are often mass transport hindrances, inefficient immobilization and the high effort (time or energy) to fully separate the phases after reaction. For industrial applications the use of free enzymes is costly as they need to be produced via fermentation and purified before usage. Therefore, using the cells directly is common practice. Main advantages of using whole cells for catalysis are an easy production, the lack of purification and the use of an integrated co-factor regeneration system by adding cheap co-substrates like glucose, ethanol or glycerol instead of expensive co-factors like NAD(P)H.

* Corresponding author.
E-mail address: juliane.merz@bci.tu-dortmund.de (J. Merz).

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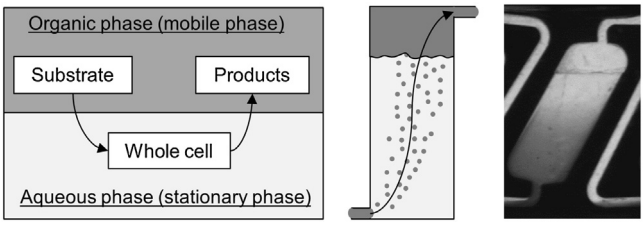

Fig. 1. From left to right: Basic scheme of biphasic biocatalysis in CIR; CIR chamber with immobilized phase und mobile phase flow (Organic mobile phase (darker) and aqueous, catalytically active immobilized cell suspension (light)); Filled FCPC®-chamber without mobile phase flow. At the top of the chamber organic phase, the interfacial area and the cell suspension are shown (The color gradient in the stationary (on the picture: lower) phase is caused by the cell suspension and the centrifugation effect on the cells). Adapted from [8].

In this study the Centrifugal Partition Chromatograph (CPC) is presented as a continuous reactor for multiphase reactions using whole cells as catalysts. The CPC is commonly used as a preparative liquid–liquid chromatograph for the separation of complex mixtures. Multiple chambers are connected in series and arranged around a rotary axis. By rotating this system a centrifugal field is generated that allows the immobilization of one phase of a biphasic system in the chambers. By pumping the mobile phase through this stationary phase a mixture of components can be separated by their partition coefficient. Besides a high capacity of the stationary phase, strong mixing and the ability to use the heavier phase (ascending mode) or the lighter phase (descending mode) as stationary phase are advantageous and make the CPC a powerful device for downstream processing.

Recently the CPC has been used for enzymatic reactions [8–11]. The substrate was supplied by the mobile phase, converted in the stationary phase, where the catalyst is solved and the product partitions back to the mobile phase and can be recovered from the system. It was shown, that the batch CPC-reactor (with recycled mobile phase feed) performed similar or better compared to classical batch stirred tank experiments. This is assumed to be caused by the steady dispersion and coalesces of one phase and therefore an increased mixing. Also the volume distribution to multiple chambers was stated to be favorable for biphasic catalysis [8,9].

In this study the reduction of β–ketoesters using *Saccharomyces cerevisiae* as biocatalysts is demonstrated and the behavior of the cells in the CPC or "Centrifugal Immobilization Reactor" (CIR), shown in Fig. 1, is investigated. A previously proposed methodology for the design of biphasic reaction systems for enzymatic reactions is used to select the reaction system and the reduction of two β-ketoesters with different polarity is investigated as potential reaction CIR reactions.

Main challenge for selecting a biphasic reaction system is the preservation of the cells activity influenced by the pH value, oxygen transfer rate (also referred to as k_La-value), shear forces and/or solvents. However, contrary to classical fermentation certain enzyme cascades for product conversion and co-factor regeneration need to be active only and the complete vitality of the cell is of minor importance [12].

In this study a biphasic reaction system was designed for the reduction of two β–ketoesters of different polarity. Besides influences of the pH value, different buffers and the organic phase, different ways of co-factor regeneration were evaluated. The suitability of biphasic systems for the reduction of substrates of different polarity is discussed regarding the biocompatibility of the organic solvents and the influence of the partitioning on the reactions. Afterwards the reduction of a β-ketoester was transferred to the CIR. A transparent single disc rotor and video recording system was used to evaluate the behavior of the aqueous cell suspension in the CIR chambers. Influences of the volume flow rate and the rotational speed on the cells' activity were evaluated by operating reductions in batch mode in small scale. Results from the batch mode were then transferred to the CIR using a commercial rotor and operated continuously.

2. Materials and methods

2.1. Chemicals

Ethyl acetoacetate (EAA), ethyl 3-hydroxybutyrat (EHB), ethyl 3-oxohexanoate (EOH), ethyl 3-hydroxyhexanoate (EHH), ethyl acetate, butyl acetate, 1-pentanol, toluene, xylene, 1-hexanol, heptane, methyl *tert*-butyl ether, ethanol, 2-propanol, glucose, glycerol, 1,3-bis(tris(hydroxymethyl)methylamino)propane (Bis-Tris propane), 3-[[1,3-dihydroxy-2-(hydroxylmethyl)propan-2-yl]amino]-2-hydroxypropane-1-sulfonic acid (TAPSO), *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 4-morpholinepropanesulfonic acid (MOPS), dipotassium hydrogen phosphate, potassium dihydrogen phosphate were purchased from Sigma-Aldrich Co. LLC, Germany. Substances purities were at least 98% and all organic solvents were of analytical grade.

2.2. Model reaction system

Unmodified and dried wild type *Saccharomyces cerevisiae* (yeast) was purchased from Biovegan®. The yeast was solved in buffers at fixed pH values. No cultivation or purification was performed prior the reaction experiments. Two different industrial relevant β-ketoesters were used and the overall product formation rate was measured to evaluate the performance, whereas enantioselectivity was not investigated in this study. The reaction mechanism is displayed in Fig. 2. Ethyl acetoacetate (EAA) was reduced to ethyl 3-hydroxybutyrat (EHB) as building block for the production of Decarestrictin L, a cholesterol biosynthesis inhibitor metabolite, and ethyl 3-oxohexanoate (EOH) was reduced to ethyl 3-hydroxyhexanoate (EHH), which can be used as building block for the production of Neopeltolid, a potent inhibitor of the in vitro proliferation of the A-549 human lung adenocarcinoma [3,13].

2.3. Analytics

2.3.1. Product quantification in organic solvents

Product concentrations in organic samples were quantified using the gas chromatograph Agilent 7890A (Agilent Technologies, Inc.), helium as carrier gas and a flame ionization detector (FID). An OPTIMA WAXplus® (25 m) column plus a 10 m pre-column was used to separate the sample molecules. The system was calibrated using standard components. In Table 1 the used methods as well as the resulting retention times are displayed.

2.3.2. Biomass quantification

The dry biomass purchased from Biovegan® was used as received. For quantifying the cell concentration the optical density

Table 1
Methods for the quantification of EAA/EHB/EOH and EHH using Gas-Chromatography.

Parameters		EAA/EHB	EOH/EHH
$T_{Injector}$	[°C]	200	200
T_{FID}	[°C]	230	230
T_{Column}	[°C]	100	130
$\dot{V}_{Helium}$	[mL/min]	0.5	0.5
Split ratio	[–]	20:1	20:1
$t_{Retention}$	[min]	8.1/8.9	6.5/7.1