INNOVATIVE DOWN-STREAM PROCESSING IN WHOLE-CELL BIOCATALYSIS USING SUPERCRITICAL CARBON DIOXIDE

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I Introduction

The biotechnological production of certain bulk and fine chemical has come into scientific as well as industrial interest during the last decades. This refers mainly to the fact that certain biocatalytic processes using microorganisms or enzymes offer an interesting possibility to produce those products with a high selectivity and efficiency. The biocatalytic functionalization of hydrocarbons is of special interest as it allows a higher productivity and enantiomeric excess as compared to conventional chemical processes. To achieve high product concentrations and to avoid inhibitory / toxic effects of apolar products and substrates, a two-liquid phase system can be applied, in which a second organic phase serves as a substrate reservoir and as a product sink (Figure 1) [1-3]. In this work, the stereospecific epoxidation of styrene to (*S*)-styrene oxide using recombinant *Escherichia coli* was considered as the model reaction [4-6] (Figure 1).

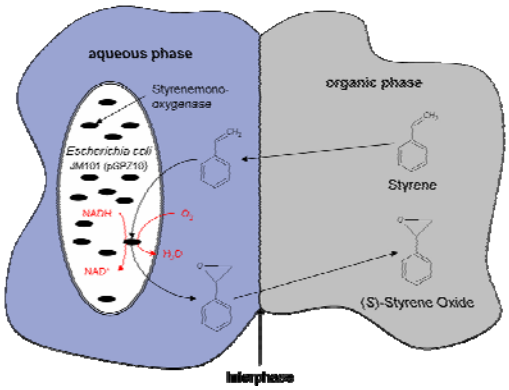

Figure 1: Biocatalytic styrene epoxidation in a two-liquid phase system.

Besides biocatalyst and reaction engineering, an efficient downstream processing is a key factor for the industrial application of such biphasic biocatalytic processes. The industrial implementation of whole-cell biocatalysis in organic/aqueous emulsion systems however faces major challenges with respect to efficient and cost-effective downstream processing. Complications mostly arise from the stabilization of the emulsions by biological macromolecules, which make phase separation difficult. This phenomenon is typical for the biocatalytic production of apolar fine chemicals using high-cell-density two-liquid phase cultures. On a laboratory scale, this problem is often solved by excessive centrifugation, which may lead to acceptable results in terms of phase separation, but often is not applicable

on an industrial scale (see Figure 2). Other approaches include filtration or membrane separation techniques but none of them lead to an effective downstream processing [7].

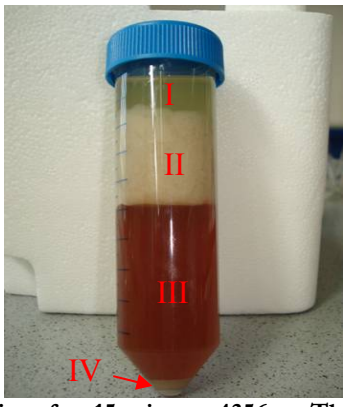

Figure 2: Emulsion after centrifugation for 15 min at 4356 g. The organic phase (I), the emulsified interphase (II), the aqueous phase (III) and the cell pellet (IV) are indicated.

It is known from literature that the addition of carbon dioxide to technical oil-in-water emulsions has an influence on the phase separation behavior [8-10]. In this work, the application of supercritical carbon dioxide was for the first time investigated with respect to its ability to break stable emulsions originating from whole-cell bioconversions and to act as extraction medium in the downstream processing of such biotransformations.

II Materials and Methods

After the two-liquid-phase biotransformation of styrene to (*S*)-styrene oxide, the reaction mixture was cooled down to 4°C. For all further experiments, the emulsion was centrifuged in a RC-5B Refrigerated Superspeed Centrifuge (Thermo, Waltham, Massachusetts) for 15 min at 4°C to remove the bulk of whole *E. coli* cells. The complete supernatant was then transferred into a sterile bottle and stored at 4°C.

High-Pressure Variable-Volume View Cell

For optical investigations on the phase separation behavior of emulsions containing compressed carbon dioxide, a High-Pressure Variable-Volume View cell (HPVVV, New Ways of Analytics, Lörrach, Germany) was used.
The equilibrium cell of the HPVVV has a variable volume of 30 - 60 ml, which can be varied by moving a sapphire piston. The pressure is adjusted with a manual hydraulic-press M(O) 189 (Maximator, Zorge, Germany). A sapphire window opposite to the piston allows the observation of the whole inner volume of the cell. The HPVVV stands pressures up to 700 bars and temperatures up to 180°C. The system can be homogenized by a magnetically coupled stirrer. For dosing and sampling purposes, four 1/8″ ports are available. By adjusting the pressure in the system, it is possible to follow the pressure-dependent phase-separation behavior without changing the composition of the system. The temperature can be measured directly inside the equilibrium cell as well as at the heating jacket to ensure constant temperatures.

Dosing of compressed carbon dioxide

Compressed carbon dioxide was added into the HPVVV by a 260D syringe pump (ISCO, Lincoln, New England) (p_{max}=560 bar). The mass of added carbon dioxide was calculated from the volume of carbon dioxide introduced into the view cell at constant pressure and temperature using the IUPAC certified equation of state as proposed by Span and Wagner [11].

Direct sampling out of the supercritical phase

To allow a direct sampling at high pressure and at temperatures of up to 100°C, the HPVVV was equipped with a sampling valve from VICI Valco® (Schenkon, Switzerland), which was modified for application under supercritical conditions. This valve allows sampling via an internal loop followed by an expansion into an external loop. The external sample loop can be heated by an auxiliary heating system to avoid condensation of organic compounds at decreased pressures. The sample is then directly injected into the carrier gas stream of the GC (see below for analytics).
Prior to sampling, the tubing was flushed with fresh sample over a pressure restrictor. Pressure decrease in the HPVVV remained within an acceptable range at pressures up to 250 bars.

Phase separation and extraction behavior

After transferring a defined amount of the emulsion into the HPVVV, the system was closed, homogenized, and tempered. Afterwards, carbon dioxide was added in a controlled manner as described above. The system was then kept at a defined temperature for at least 10 min. For phase separation experiments, the pressure in the system was raised by moving the back sapphire piston. The behavior was visualized by means of a cold light source positioned behind the HPVVV.
To evaluate extraction into carbon dioxide, the system was pressurized to a defined pressure. After equilibration, a sample was taken from the gas phase and analyzed directly by gas chromatography. Each measurement was repeated at least 3 times before a new pressure was applied.

III Results

Phase-separation behavior

The phase-separation behavior of different mass fractions of emulsion was analyzed under compressed carbon dioxide in a pressure range between 70 to 650 bars. Experiments were carried out using a reaction mixture, from which the recombinant *E. coli* cells used for stereospecific styrene epoxidation had been removed by centrifugation. For high mass fractions of emulsion, phase separation did not change as compared to the original emulsion, of which the phases did not separate for months. At mass fractions of emulsion lower than 0.25 and pressures of at least 120 bars, phase separation kinetics changed dramatically and phase separation was achieved within a few minutes as shown in Figure 3.

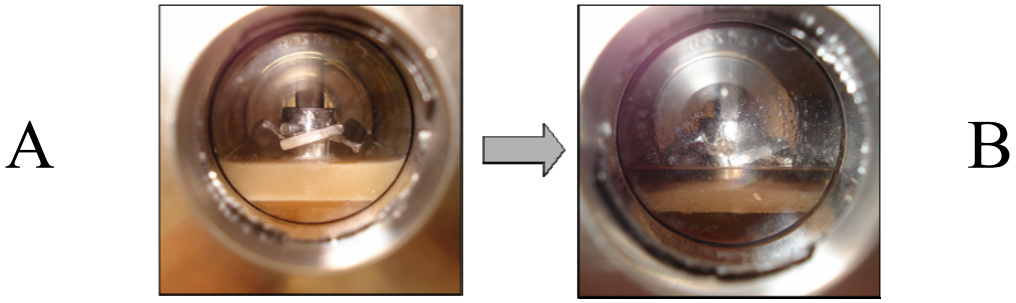

Figure 3: Comparison of the phase separation behavior of the emulsion before and after treatment with compressed carbon dioxide. A: State after filling the HPVVV. B: Behavior after the treatment with compressed carbon dioxide at elevated pressure and temperature.

In comparison to the original emulsion (Figure 3A), the treated emulsion (Figure 3B) showed an improved phase-separation behavior. A sharp phase boundary became visible. Astonishingly, cell fragments, which presumably stabilized the emulsion before $scCO_2$-treatment, settled gravimetrically within the organic phase. Thus, the use of $scCO_2$ as an

emulsion breaker enabled not only the separation of the two phases in a much simpler way than by continuous centrifugation, but also allowed the separation of cell fragments present in the emulsion. Even after depressurization, the emulsion treated with compressed carbon dioxide still showed an improved phase-separation behavior.

Extraction behavior

In order to investigate the potential for direct supercritical extraction out of the emulsion, samples from the CO_2-rich phase were analyzed. To avoid plugging problems with the original emulsion, a model system of the organic phase consisting of standard chemicals was used. The model system consisted of 55.6 mM 2-phenylethanol, 19 mM octane, and 605 mM (*S*)-styrene oxide dissolved in bis-2(ethylhexyl)phthalate (BEHP). In analogy to the experiments concerning the phase-separation behavior, the extraction behavior was investigated for different mass fractions of the model emulsion at pressures ranging from 80 - 250 bars. As shown in Figure 4, (*S*)-styrene oxide could selectively been extracted out of the organic phase.

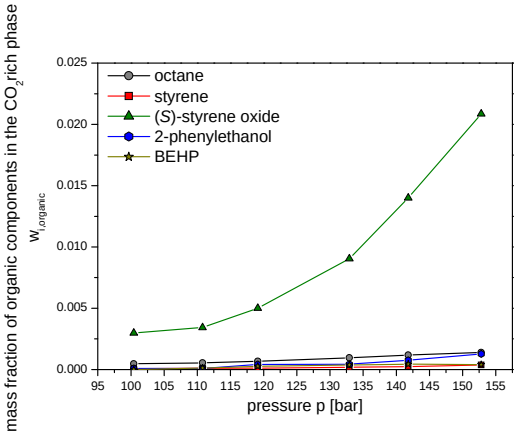

Figure 4: Solubilities of the organic phase components in $scCO_2$. The mass fractions of the different compounds in CO_2 are plotted against the pressure.

This offers the possibility of a supercritical product extraction directly out of the emulsion without any pretreatment.

IV Conclusion

In our work, we demonstrated for the first time that stable emulsions caused by biosurfactants can efficiently be broken by addition of carbon dioxide at elevated temperature and pressure. This considerably facilitates downstream operations of biphasic bioprocesses. Our results can now be used to design a phase separation step prior to further product purification. Furthermore, we showed that $scCO_2$ can selectively extract the product out of the organic phase allowing product isolation prior, after, or simultaneously with phase separation.
As the separation of highly stabilized emulsions marks a general problem in two-liquid phase biotransformation processes, the use of $scCO_2$ might represent a platform technology for product isolation from bioprocesses based on whole cells.

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