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FERMENTATION, CELL CULTURE AND BIOENGINEERING

The dynamic influence of cells on the formation of stable emulsions in organic–aqueous biotransformations

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Abstract Emulsion stability plays a crucial role for mass transfer and downstream processing in organic–aqueous bioprocesses based on whole microbial cells. In this study, emulsion stability dynamics and the factors determining them during two-liquid phase biotransformation were investigated for stereoselective styrene epoxidation catalyzed by recombinant *Escherichia coli*. Upon organic phase addition, emulsion stability rapidly increased correlating with a loss of solubilized protein from the aqueous cultivation broth and the emergence of a hydrophobic cell fraction associated with the organic–aqueous interface. A novel phase inversion-based method was developed to isolate and analyze cellular material from the interface. In cell-free experiments, a similar loss of aqueous protein did not correlate with high emulsion stability, indicating that the observed particle-based emulsions arise from a convergence of factors related to cell density, protein adsorption, and bioreactor conditions. During styrene epoxidation, emulsion destabilization occurred correlating with product-induced cell toxification. For biphasic whole-cell biotransformations, this study indicates that control of aqueous protein concentrations and selective toxification of cells enables emulsion destabilization and emphasizes that biological factors and related dynamics must be considered in the design and modeling of respective upstream and especially downstream processes.

Keywords Two-liquid phase biotransformation · Whole-cell biocatalysis · Pickering emulsion · Emulsion stability · *E. coli*

Introduction

The use of biocatalysts for the production of bulk and fine chemicals has enjoyed increased interest in recent years, as green alternatives to traditional chemical transformations have been sought [46, 48]. Whole cells have the distinct advantage of being able to regenerate and stabilize the required co-factors and enzymes, avoid enzyme isolation and purification, and increase the economic and ecological efficiency of highly regio- and stereoselective reactions [41]. The demands of oxygenases are particularly intricate as oxidative stress, instabilities of multicomponent enzyme systems, membrane association, and cofactor regeneration must be dealt with [17, 39]. Whole cells can compensate for these issues, but can entail drawbacks. In cases where the product or substrate is toxic to the cells, it can be difficult to maintain cell viability and avoid enzyme inhibition while at the same time achieving high product titers. In situ product removal offers solutions, e.g., the application of a second liquid organic phase [45, 47]. By using a suitable solvent (biocompatible, favorable partitioning, etc.), cellular exposure to toxic components can be reduced enabling higher productivities. The partitioning behavior of reactants may also be exploited to enable control of multistep reaction kinetics [8, 42]. However, the tendency

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of these biphasic systems to form stable emulsions represents a major hurdle for their implementation on an industrial scale. Emulsion formation during biotransformation is often advantageous, particularly when dealing with mass transfer limited systems, but stable emulsions are notoriously difficult to handle downstream [40, 47]. Recently, we have reported methods for phase separation and product extraction based on supercritical carbon dioxide, which would be amenable to implementation on industrial scale [6, 7].

The characteristics of emulsions derived from biphasic (aqueous/organic) whole-cell biotransformations have been described [10, 29, 40]. Four main categories of surface-active compounds were identified: inorganic electrolytes (nutrients and salts), detergents such as antifoam, macromolecules (membranes, proteins, etc.), and particles (i.e., cells). The contributions of these components to overall emulsion stability vary according to the system. For example, the tendency of some microbial species to produce/release surfactants during cultivation leads to a greater contribution of the detergent fraction [26]. Other systems, especially those utilizing microorganisms with hydrophobic surface properties, have been shown to produce stable, primarily cell-based emulsions [10, 18]. In our previous work on the two-liquid phase biotransformation of styrene to (*S*)-styrene oxide, bacterial cells (i.e., recombinant *E. coli* JM101) have been identified as the major emulsion-stabilizing factor [6].

In this work, we examine the formation of stable emulsions in this biphasic biocatalytic whole-cell system with the aim of identifying and understanding the factors influencing both biocatalyst and emulsion stability. This will allow coupling of productive biocatalysis with the ability to control the emulsion in downstream processing. The stereoselective styrene epoxidation chosen as the model biotransformation is based on *E. coli* JM101 (pSPZ10) as whole-cell biocatalyst containing the styrene monooxygenase StyAB from *Pseudomonas taiwanensis* VLB120. This enzyme's biochemical characteristics, use in isolated form, and application in whole cells have been well described [31, 32, 34, 35]. The optimized process utilizes bis(2-ethylhexyl) phthalate (BEHP) as a secondary organic phase and cells growing in a fed-batch mode [21, 33, 36]. In this system, we have examined the kinetics of emulsion stability during the biotransformation and have identified previously unknown fluctuations in emulsion stability. Fast initial emulsion stabilization is linked to both hydrophilic *E. coli* cells and hydrophobic biomass fractions present at the interface. Decreases in emulsion stability during biotransformation correlate with cell toxification by respective products. Thus, as a major difference to chemical processes involving emulsions, this study emphasizes that, beyond physico-chemical properties of individual substances, biological factors and related dynamics have to be considered in bioprocess design and modeling, especially regarding DSP.

Materials and methods

Bacterial strains and plasmids

Escherichia coli JM101 (*supE thi-1* Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]), a derivative of *E. coli* K-12, was used as a recombinant host strain [28]. The expression plasmid pSPZ10 is based on the pBR322 vector and contains the styrene monooxygenase genes *styAB* from *Pseudomonas taiwanensis* VLB120 under the control of the *alk* regulatory system from *Pseudomonas putida* GPo1 [35]. Alternatively, the plasmid pA-EGFP_B [16] encoding a GFP-tagged styrene monooxygenase was used.

Two-liquid phase biotransformation using *E. coli* JM101 (pSPZ10)

Unless otherwise stated, batch and fed-batch cultivation (in 1 L aqueous Riesenberg medium) and two-liquid phase biotransformation (after addition of 1 L organic phase composed of 910 mL BEHP, 80 mL styrene, and 10 mL octane serving as the inducer of *styAB* expression) in 3 L stirred-tank bioreactors (KLF 2000, Bioengineering, Wald, Switzerland) as well as analytics were performed as previously reported [22]. Samples for emulsion stability determination (2 × 14 mL) were taken every hour and stored in Falcon tubes overnight at 4 °C.

Two-liquid phase cultivation of *E. coli* JM101 (pSPZ10) using different organic phases

Biphasic cultivations of *E. coli* JM101 (pSPZ10) with different organic phases were performed as reported above except for the following variations: 1) In purely aqueous cultures, cells were grown to approximately 18 g_{CDW} L^{-1} using an incrementally increased glucose feed rate (feed composition: 730 g L^{-1} glucose, 19.6 g L^{-1} $MgSO_4 \cdot 7H_2O$), which was then kept constant at 0.6 g min^{-1} until the biomass had increased to approximately 40 g_{CDW} L^{-1}. At this point, the feed was reduced to 0.2 g min^{-1} and kept constant for the remainder of the experiment. Samples for external emulsification (2 × 7 mL) were taken every hour and stored in falcon tubes at 4 °C prior to emulsification. 2) Biphasic cultures grown only in the presence of BEHP were, prior to organic phase addition, performed as described under 1). At a cell concentration of approximately 18–19 g_{CDW} L^{-1}, an equal volume (1 L) of BEHP was introduced and the glucose feed was maintained at a rate of 0.6 g min^{-1} until

the cell concentration had increased to approximately 40 g_{CDW} L^{-1}. Then, the feed was reduced to 0.2 g min^{-1} and the procedure was the same as described for 1). 3) Biphasic cultures grown in the presence of inducer (octane) or substrate (styrene) differed from 2) in that the organic phase added consisted of 10 mL octane or 80 mL styrene in 990 or 920 mL BEHP, respectively. 4) Biphasic cultures grown in the presence of increasing concentrations of products (styrene oxide or 2-phenylethanol) differed from 2) in that styrene oxide or 2-phenylethanol were added to the stirring biphasic cultivation at rates of 100 or 8 mmol h^{-1} (11.4 or 1.0 mL h^{-1}), respectively, for 6 h via a peristaltic pump.

Cultivation and two-liquid phase biotransformation using resting cells

Precultivation, batch, and fed-batch cultivation (in 1.5 L aqueous Riesenberg medium) as well as analytics were performed as previously reported [17, 22]. When the concentration of induced *E. coli* JM101 (pSPZ10) had reached ~22 g_{CDW} L^{-1} during aqueous fed-batch cultivation, cells were harvested by centrifugation. The harvested cells were resuspended to a concentration of 20 g_{CDW} L^{-1} in nitrogen-free M9-medium containing 1 % (w/v) glucose, US* trace element solution (1 mL), and thiamine (0.001 % w/v). The cell suspension (1 L) was transferred back into a bioreactor and equilibrated for 10 min at 30 °C, 1,500 rpm, and 1.5 L min^{-1} aeration. Then, a second organic phase (1 L) consisting of bis(2-ethylhexyl)phthalate (BEHP) containing 1 % (v/v) octane and 8 % (v/v) styrene was added and the stirring rate increased to 2,800 rpm. A glucose feeding rate of 0.13 g min^{-1} was applied and the pH was maintained at 7.2 by addition of 30 % (v/v) phosphoric acid and 10 N sodium hydroxide solutions. Samples for external emulsification (14 mL) were taken every hour and treated as described above. Concentrations of styrene, styrene oxide, and 2-phenylethanol were measured by a TRACE GC Ultra (ThermoFisher Scientific Inc., Waltham, MA, USA) equipped with a Factor-Four VF-5 ms column (Varian, Inc., Palo Alto, CA, USA). The oven temperature was raised from 50 to 140 °C at a heating rate of 15 °C min^{-1}, followed by heating at a rate of 100 °C min^{-1} to 300 °C, which was maintained for 3.5 min. Detection was achieved by a flame ionization detector (FID). Cell concentrations were determined spectrometrically at a wavelength of 450 nm (OD450) on a Libra S11 spectrophotometer (Biochrom Ltd., Cambridge, UK), whereby one OD450 unit corresponded to a cell dry weight (CDW) of 0.166 g L^{-1}.

Preparation of cell lysate to evaluate its effect on emulsion stability in a biphasic cultivation

Cells were grown in a 2 L batch bioreactor setup on 2 % (w/v) glucose yielding 16.03 g_{CDW}, harvested by centrifugation at 4000*g* (30 min, 4 °C), resuspended in 120 ml ice cold 50 mM potassium phosphate buffer (pH 7.2), and lysed by three passages through a French press at 20000 psi. The resulting lysate was centrifuged (4000*g*, 30 min, 4 °C) to remove non-lysed cells. After overnight storage at 4 °C, the supernatant was injected into a biphasic culture.

Biphasic setup to compare protein extraction in the presence and absence of cells and to visualize cells via fluorescence microscopy

Escherichia coli JM101 (pA-EGFP_B) was cultivated in batch and fed-batch mode as described above on a 1.5 L scale. When a cell concentration of 19 g_{CDW} L^{-1} was reached, 0.5 L culture broth was removed from the reactor and centrifuged (4000*g*, 30 min, 4 °C), while a regular biphasic cultivation was started with the remaining 1 L culture via addition of 1 L BEHP as described above. Cell-free supernatant (150 ml of the centrifuged broth) was transferred into a RALF bioreactor (Bioengineering) and an equal volume of BEHP was added. Protein concentrations in the aqueous phase were monitored for 8 h in both the biphasic cultivation and the cell-free setup. In the biphasic cultivation system, cells were visualized in the emulsion fraction by fluorescence microscopy using an Observer D1 microscope equipped with a Plan-Apochromat 40 × 0.95 Korr M27 objective and a HXP 120 V illumination system (Carl Zeiss, Jena, Germany). GFP fluorescence was excited at 470 nm and fluorescence emission was detected at 525 nm after passing a 38 HE filter set using an AxioCam MRm3 (Carl Zeiss).

Determination of emulsion stability

The emulsion stability was determined as previously described [6] and is reported as either the emulsion fraction (EF) of the total two-liquid phase sample after standard centrifugation in 15 mL Falcon tubes (60 min at 4000*g* and 4 °C in an Eppendorf Centrifuge 5804R with rotor A-4-44, Hamburg, Germany) or as a relative stability, given in % of the highest EF observed in the respective experiment. For samples taken from purely aqueous cultures, the emulsion stability after external emulsification was determined as follows. Samples (7 mL) of aqueous cultivation broth were brought to room temperature and then emulsified with pure BEHP (7 mL) using an "Ultra-Turrax tube drive" (IKA, Staufen, Germany) with appropriate mixing unit (ST-20) at a rotational speed of 3300 min^{-1} for 60 s. The emulsified samples were subjected to standard centrifugation followed by EF and relative stability determination as described above.

Three-phase contact angle measurements and CDW determination

Samples (2 × 50 mL in Falcon tubes) were taken at each time point and centrifuged (60 min at 4500*g* and 4 °C). The aqueous and organic supernatants were separated from the resulting cell pellet, which was then washed using 20 mL 50 mM potassium phosphate buffer (pH 7.2), centrifuged again, and finally resuspended in 40 mL of the same buffer. The suspension was filtered in 5 mL aliquots through a Whatman ME 25 filter (Sigma Aldrich, Steinheim, Germany) to form a smooth layer of cells, which was again washed with 10 mL potassium phosphate buffer (pH 7.2) and then allowed to dry for 10 min with air passing through the filter under aspirator vacuum. The filter was placed in a small glass Petri dish and covered with BEHP. The advancing three-phase contact angle was measured using a Keyence VHX-500F Digital microscope (Keyence, Osaka, Japan) and water (5 µL water, dyed with methyl blue). All values are given as an average of five independent measurements. The biomass (in g_{CDW}) was measured by filtration of cells as above and drying of the cells in a vacuum oven (12 h, 50 °C) until a constant mass was achieved.

The mass and contact angle of the interfacial material were likewise determined after its isolation using a phase inversion protocol. For the latter, samples (2 × 50 mL in Falcon tubes) were centrifuged as described above. The aqueous/organic mixture was separated from the cell pellet, transferred to a new Falcon tube, and centrifuged again under the same conditions. Aqueous broth (20 mL) was removed from the samples with a syringe and replaced with BEHP (20 mL). Thorough mixing of the resulting emulsions (IKA Ultra-Turrax tube drive, 3300 rpm, 2 min) induced phase inversion (O/W to W/O) and emulsion destabilization and, after centrifugation, gave rise to new cell pellets. These pellets were separated from the aqueous and organic phases, washed using 20 mL 50 mM potassium phosphate buffer (pH 7.2) and then combined before being centrifuged and resuspended to a final volume of 20 mL in the same buffer. Two cell samples were prepared by filtering 10 mL each of the suspension through a 0.1 µm pore size hydrophilic membrane filter as described above and were used for CDW and contact angle determinations of the interface cell fraction.

Protein analyses

The protein concentration in cell-free aqueous samples originating from biphasic cultivations was estimated using a Bio-Rad DC protein assay kit (Bio-Rad, München, Germany) and the protein content analyzed via SDS-PAGE [23]. Gel staining was performed using Coomassie brilliant blue. Alternatively, the protein concentration was measured by Bradford assay.

Results

Emulsion stability dynamics during biotransformation

To investigate the parameters influencing the formation of stable emulsions during biotransformation reactions, biphasic styrene epoxidation was performed using *E. coli.* JM101 (pSPZ10) under standard conditions [21]. The emulsion stability, in terms of emulsion fraction (EF) after standardized centrifugation [6], was measured in samples taken every hour (Fig. 1). Time 0 h represents the time point at which the organic phase, containing styrene and octane (the inducer of recombinant *styAB* expression) in BEHP, was introduced into the fed-batch culture to an organic–aqueous phase ratio of 1:1. The emulsion stability values given for samples taken before the organic phase addition are based on external emulsification of samples with BEHP at the same phase ratio using a standardized setup [6]. Before organic phase addition, the emulsion stability slowly increased correlating with the cell concentration. Within the first hour after organic phase addition, a rapid increase in emulsion stability was observed, reaching a maximal EF of 0.36 (100 %) with a thick gel-like appearance. In the following course of the biotransformation, the emulsion stability continuously decreased reaching an emulsion stability of 30 % after 5 h. The cells continued to grow at a decreasing rate over this time period, reaching a final concentration of 40 $g_{CDW}\ L^{-1}$ (CDW: cell dry weight), when cells entered an apparent stationary phase. This growth behavior has been shown to relate to cell toxification by the major product (*S*)-styrene oxide, as well as the side product, 2-phenylethanol (Fig. 1a) [35, 36]. This large variation in emulsion stability under constant stirring and aeration conditions was unexpected. Whereas the stability increase correlated with induction, biotransformation start, and emulsification in the bioreactor, the stability decrease correlated with decreasing substrate and increasing product concentrations. Thus, the investigation of the emulsion stability during two-liquid phase cultivation in the absence of inducer, substrate, and products appeared to be the next logical step.

Influence of organic phase composition

Possible causes for the observed emulsion stability dynamics can be divided into effects related to mere cultivation conditions (i.e., BEHP addition, reactor operation) and effects related to the biotransformation reaction (i.e., induction, substrate consumption, formation of toxic products). When only BEHP was added during a standard fed-batch cultivation of *E. coli* JM101 (pSPZ10) (Fig. 2a), cells were forced to enter a stationary phase by reducing the glucose feed rate 5 h after addition. Immediately after organic

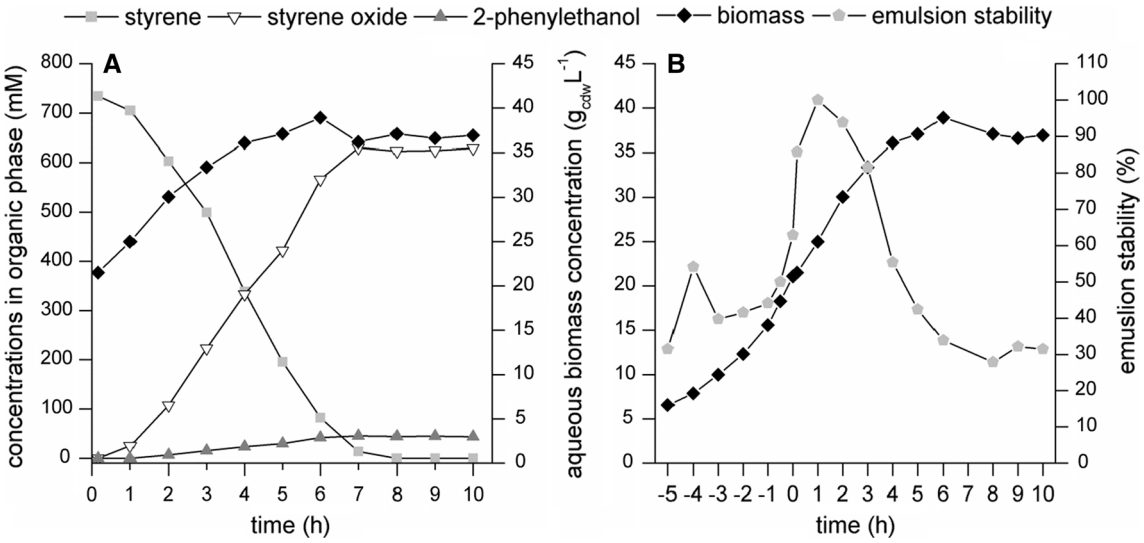

Fig. 1 **a** Biphasic biotransformation of styrene to (*S*)-styrene oxide with *E. coli* JM101 (pSPZ10) growing in fed-batch mode. **b** Course of emulsion stability given as percentage of the maximum emulsion fraction (length of emulsion/total length of sample after standardized centrifugation) observed in the experiment (0.35)

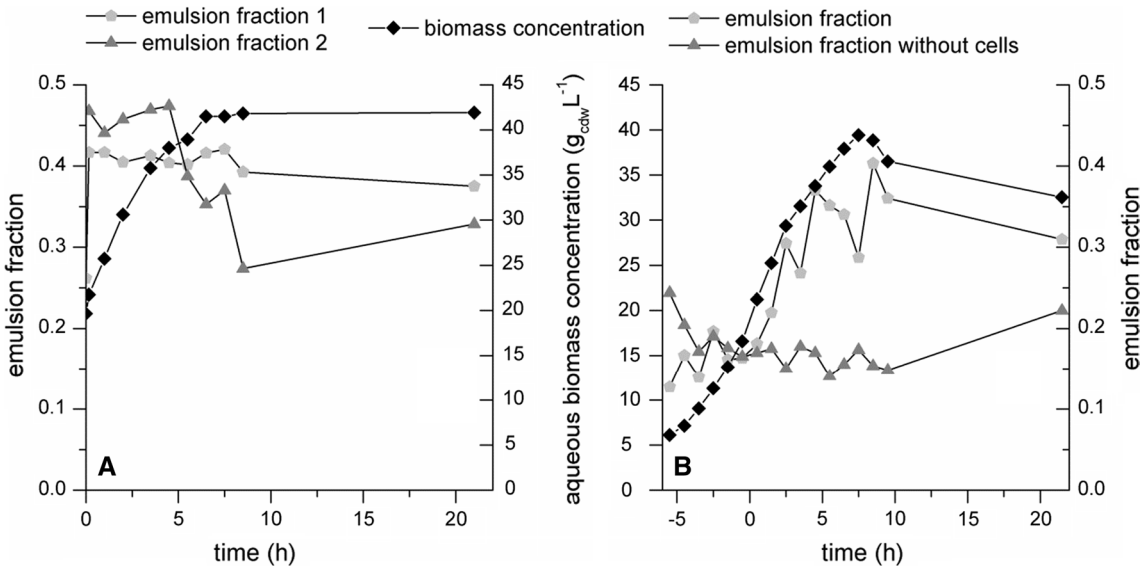

Fig. 2 **a** Emulsion stability given as emulsion fraction (EF) during biphasic fed-batch cultivation of *E. coli* JM101 (pSPZ10) with BEHP (EF = length of emulsion/total length of sample after standardized centrifugation; see "Materials and methods"). Emulsion fraction 1 is derived from samples taken directly from the bioreactor while emulsion fraction 2 represents the same samples further diluted with BEHP (4 ml BEHP added to 10 ml sample). **b** Emulsion stability of samples taken from an aqueous fed-batch cultivation of *E. coli* JM101 (pSPZ10) and emulsified externally with an equal volume of BEHP before or after removal of cells. Cells were forced to enter a stationary phase by reducing the glucose feed rate from 0.6 to 0.2 g feed L_{aq}^{-1} min^{-1} at the time point 5 h

phase addition, the emulsion stability (given as emulsion fraction 1) increased to an EF $\geq$0.4 where it remained. This was above the detection limit of the assay, as no visible organic phase was present at the top of all centrifuged samples. Thus, no real stability trend could be obtained. A relative stability trend was generated by determining the emulsion stability after addition of fresh BEHP (4 ml) to the emulsion samples (10 ml) and re-emulsification (giving emulsion fraction 2). This trend indicates some emulsion destabilization which coincided with the reduction of the glucose feed rate and the start of the stationary growth phase. The effective change in aqueous/organic phase ratio

between emulsion fractions 1 and 2 makes it impossible to directly compare them in terms of absolute stability. Yet, this strategy enabled the determination of the previously hidden stability trend. Results were confirmed by repeating the experiments. Summing up, the emulsion stability in the absence of biotransformation was much greater than that observed during biotransformation, especially in the stationary phase.

Another series of emulsions were generated by fed-batch cultivation of *E. coli* JM101 (pSPZ10) without organic phase and external emulsification of samples with an equal volume of pure BEHP (Fig. 2b). In this case, the emulsion stability correlated with the biomass concentration. The initial and rapid increase in emulsion stability, as observed upon organic phase addition to the bioreactor (Fig. 2a), did not occur. This indicated that emulsion formation was influenced by the operational conditions in the bioreactor (aeration, cell growth). Cell-free supernatants of centrifuged aqueous samples were also subjected to the same external emulsification with equal volumes of BEHP. The relatively flat trend and small emulsion fractions highlight the comparatively low contribution of solubilized surfactants present in the cell-free aqueous phase (i.e., fragments, membranes, proteins, and metabolites of lysed cells) to emulsion stabilization in the two-liquid phase cultivation system.

To investigate the biotransformation-related effects on emulsion stability, the composition of the organic phase was varied in a series of experiments: The sole addition of the inducer octane, the substrate styrene, the product styrene oxide, or the by-product 2-phenylethanol to the BEHP phase was tested (Fig. 3). Octane addition resulted in the induction of *styAB* expression in the absence of biotransformation, whereas styrene addition to uninduced cells resulted in a slowly decreasing styrene concentration due to evaporation and no product accumulation. The effect of increasing concentrations of toxic oxidation products on emulsion stability was investigated by addition of styrene oxide or 2-phenylethanol to uninduced cells at rates corresponding to the respective formation rates during biotransformation. In all cases, cells were cultivated to biomass concentrations of approximately 40 g_{CDW} L^{-1}. Growth ceased either due to glucose limitation upon feed rate reduction (octane and styrene experiments) or toxification by increasing concentrations of oxidation products (styrene oxide and 2-phenylethanol). The presence of the inducing agent octane or the substrate styrene in the organic phase appeared to have little or no impact on the stability trend. In both cases, highly stable emulsions were observed. During styrene oxide addition at a rate of 100 mmol L_{org}^{-1} h^{-1} over 6 h, the emulsion stability decreased by 35 %, eventually reaching a final value of 55 % (of the original maximum) after 9 h. A similar trend of decreasing emulsion stability

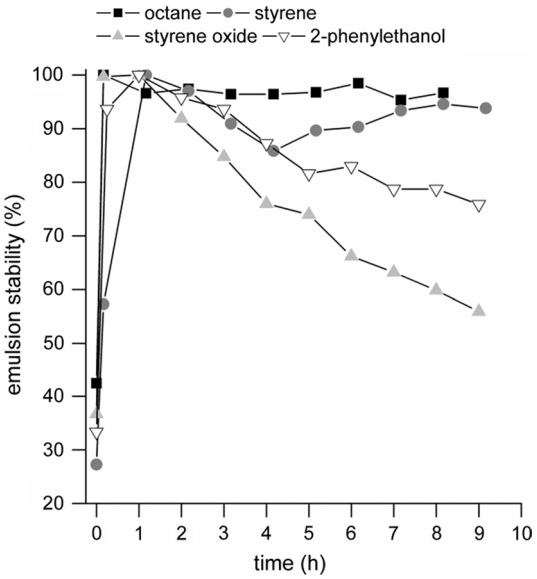

Fig. 3 Emulsion stability trends during biphasic cultivations of *E. coli* JM101 (pSPZ10) with organic phases containing either the inducer octane, styrene, styrene oxide, or 2-phenylethanol. At time 0 h, the organic phase was added to the bioreactor. Styrene (8 % v/v) and octane (1 % v/v) were present in the organic phase added, whereas styrene oxide (11.4 mL h^{-1} for 6 h) and 2-phenylethanol (1.0 mL h^{-1} for 6 h) were fed continuously to mimic product accumulation. Emulsion stability is given as percentage of the highest emulsion fraction (length of emulsion/total length of sample after standard centrifugation) observed in the experiment (octane: 0.37, styrene: 0.43, styrene oxide: 0.34, 2-phenylethanol: 0.43)

was observed during 2-phenylethanol addition (8 mmol L_{org}^{-1} h^{-1} over 6 h), where an emulsion stability decrease of 25 % was observed. In both cases, the addition of toxic organic components resulted in acetic acid accumulation and the eventual cessation of glucose uptake/catabolism (data not shown). The observed decrease in emulsion stability correlated with the increasing microbial toxicity of the organic phase. This effect was less pronounced than that observed during biotransformation, where these two products accumulate simultaneously leading to additive toxic effects (Fig. 1). Differences associated with external addition versus intracellular production of the compounds may further promote toxification [36] and possibly emulsion destabilization during biotransformation. These results indicate a link between toxification of the cells and emulsion destabilization. It has to be noted that the emulsions obtained at the end of biotransformations were themselves originally considered to be highly stable, severely complicating phase separation [6].

Finally, since all previous experiments showed maximum emulsion stability in the growth phase, it was interesting to investigate emulsion stabilization and destabilization during biotransformations performed with resting cells [17]. To do this, a biotransformation with a resting

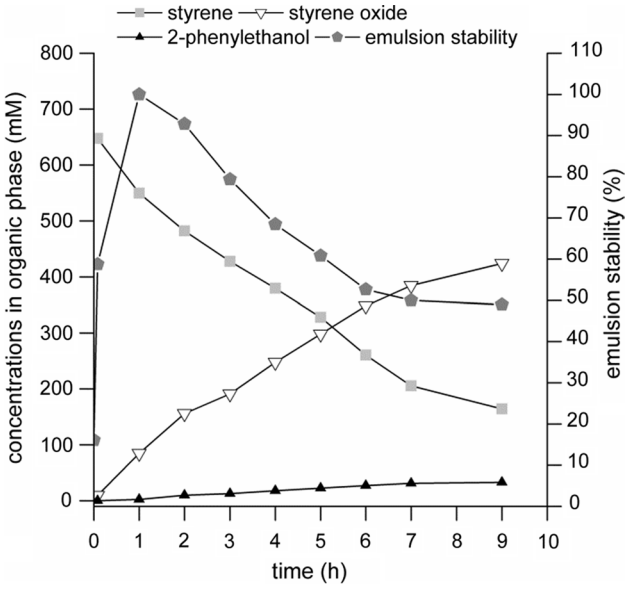

Fig. 4 Biphasic biotransformation and emulsion stability with resting cells of *E. coli* JM101 (pSPZ10) (20 g_{CDW} L^{-1}). The biphasic system was composed of nitrogen-free M9 medium containing 1 % (w/v) glucose (which additionally was fed; see experimental section) and BEHP containing 1 % (v/v) octane and 8 % (v/v) styrene at an aqueous:organic phase ratio of 1:1 (v/v). Emulsion stability is given as percentage of the emulsion fraction (EF = length of emulsion/total length of sample after standard centrifugation) maximally observed in the experiment (EF = 0.18)

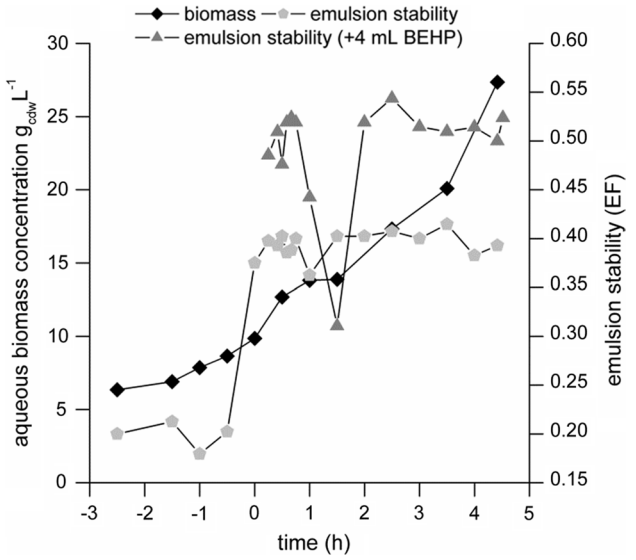

Fig. 5 Effect of crude cell extract addition on emulsion stability during biphasic cultivation of *E. coli* JM101 (pSPZ10). Cells were grown in 1 L Riesenberg minimal medium in fed-batch mode and provided with 1 L BEHP as second organic phase at 0 h. Emulsion stability is given as emulsion fraction (EF = length of emulsion/total length of sample after standard centrifugation). Thirty minutes after organic phase addition, crude cell extract equivalent to 5 g_{CDW} was injected into the reactor

cell concentration of 20 g_{CDW} L^{-1} was performed leading to styrene oxide and 2-phenylethanol accumulation over 9 h to final organic phase concentrations of 420 and 33 mM, respectively (Fig. 4). The emulsion stability trend was similar to that observed with growing cells. In both cases, the maximum emulsion stability was reached approximately 1 h after organic phase addition. The time required to reach 50 % stability was longer for resting (5 h) as compared to growing cells (3 h), correlating with the faster product accumulation in the latter case. Growing cells reached a greater overall emulsion stability (emulsion fraction of 0.36 for growing vs 0.18 for resting cells), while the minimum emulsion stabilities reached at the end of the biotransformations were similar (0.10 for growing vs 0.09 for resting cells). Being less compact, dense, and gel-like, the appearance of the emulsion obtained with resting cells differed significantly from that obtained with growing cells. These results indicate that the application of growing cells, as opposed to non-growing cells, leads to a higher emulsion stability. This was also observed for non-toxified cells, when linear growth and stationary phases are compared (Fig. 2a), and provides further evidence for a correlation of emulsion destabilization with the accumulation of toxic products in a manner which is independent of cell growth. Thus, the evaluation of the effect of cell lysis (an inherent effect of toxification) on emulsion stability was envisioned.

Artificial in situ toxification during biphasic cultivation (e.g., by acidification or enzymatic lysis) would change the physical conditions and consequently influence the emulsion characteristics in a toxification-independent way. Therefore, an indirect approach was chosen. Toxification-induced cell lysis was simulated by adding lysed cells (crude cell extract) to a standard biphasic fed-batch culture (Fig. 5). After establishing the biphasic system by addition of an equal volume (1 L) of BEHP, emulsion stability immediately increased giving emulsion fractions of 0.41 and 0.53 (with and without dilution by 4 ml BEHP, respectively) which exceeded the limits of the assay, as previously observed (Fig. 2a). Thirty minutes after organic phase addition, crude cell extract equivalent to 5 g_{CDW} of lysed cells (in 20 ml) was added and, 15 min later, emulsion stability indeed started to decrease reaching a minimum (0.31 with dilution by 4 ml BEHP) after 45 min. This was followed by an increase of the stability to the initial maximum after another 30 min. The decrease of emulsion stability after the addition of cell extract supports the hypothesis that cell lysis is a major factor causing emulsion destabilization during biotransformation, where it is caused by toxification. In contrast to the biotransformation, cells were kept in a growing and viable state in this spiking experiment. These growing cells appeared to override the emulsion destabilizing

effect of crude cell extract, restoring the initial emulsion stability. Besides the increasing cell concentration, the degradation of the cell extract by living cells may be a possible cause. However, these results indicate that toxicity-induced cell lysis leads to emulsion destabilization confirming the correlation of toxification and emulsion destabilization.

Correlation of cell surface properties with emulsion stability

The identification of the cells as the major trigger for the formation of stable particle-based (Pickering) emulsions [6] and the reliance of this type of emulsion on the surface properties of the involved particles [4] led us to investigate the hydrophobic/hydrophilic character of cells present in the emulsion. After centrifugation of samples collected from biphasic systems, cells were found in both the pellet and the emulsion fraction. The cells in the emulsion fraction could be recovered via phase inversion as described in "Materials and methods". Both cells from the interface and the cell pellet were isolated and quantified. Cell surface hydrophobicities were determined via three-phase contact angle measurements. This approach was followed for biphasic cultivations of *E. coli* JM101 (pSPZ10) with and without biotransformation (Fig. 6 a, b and c, d, respectively).

Upon emulsification outside of bioreactors using equal volumes of BEHP and aqueous culture harvested from the bioreactor (at time points ≤0 h), cells isolated from the emulsion fractions showed contact angles of 50°–60°, indicating that these cells possess a slightly hydrophilic character. Cells harvested from the pellet displayed a lower contact angle of 22°–26° and can be considered hydrophilic. After organic phase addition and 2 h of biphasic cultivation, both with and without biotransformation, the contact angle of cells from the emulsion fraction had increased to 140° indicating that cells became hydrophobic (Fig. 6a, b). In the following 2 h, the contact angle of cells from the emulsion fraction decreased slightly to around 120° and then remained constant throughout the rest of the cultivations, whereas cells from the pellet fraction maintained a relatively constant contact angle (22°–26°). Remarkably, cells from cultivations with and without biotransformation showed virtually identical surface hydrophobicity behaviors, although the emulsion stability courses differed. The stability of various Pickering type emulsions has been demonstrated to rely not only on the surface character, but also on the quantity of the particles [5]. Accordingly, the biomass in terms of CDW was determined in the total systems as well as in the pellet and emulsion fractions (Fig. 6b, d). After organic phase addition, the biomass in the emulsion fraction increased from less than 1 to 3–4 g_{CDW} per liter aqueous phase (L_{aq}) corresponding to a slight increase from ~6 to ~10 % with respect to total biomass. Again, cultivations with and without biotransformation showed the same behavior.

In conclusion, the increase in surface hydrophobicity and number of cells in the emulsion fractions correlates with the strong increase in emulsion stability upon organic phase addition to the bioreactor. For the decrease in emulsion stability during biotransformation, such a correlation was not observed. This indicates that the appearance of hydrophobic cells plays a role in emulsion stabilization and that the emulsion destabilization during biotransformation is a superimposed toxification-related effect which is not mediated by the number and hydrophobicity of interface-associated cells.

The fate of proteins solubilized in the aqueous cultivation broth upon emulsification

Proteins are among those macromolecular components present in organic–aqueous biotransformations that have been linked to emulsion stabilization [6]. To investigate the fate of this macromolecular component during emulsification, the proteins solubilized in the aqueous phase were quantified during biphasic cultivations with and without biotransformation (Fig. 7). During styrene epoxidation with *E. coli* JM101 (pSPZ10) in the biphasic model system, the protein concentration in the aqueous phase was observed to drop sharply from 1.5 to 1.1 g L^{-1} upon organic phase addition. This coincided with the sharp rise in emulsion stability (Fig. 7a) and the increase in hydrophobicity and amount of cells in the emulsion fraction (Fig. 6a, b). The drop in aqueous protein concentration was followed by a steady increase along with cell growth. When cell growth slowed because of toxification by styrene oxide and 2-phenylethanol, the aqueous protein concentration continued to increase, possibly due to toxicity-induced cell lysis. As observed before, emulsion stability remained constant for approximately 3 h after the initial rapid increase and then decreased steadily. During biphasic cultivation without biotransformation, a similar initial drop in aqueous protein concentration was observed, again coinciding with rapid emulsion stabilization (Fig. 7b) and the increase in hydrophobicity and amount of cells in the emulsion fraction (Fig. 6c, d). Whereas the emulsion stability remained high, the aqueous protein content increased more modestly than its biotransformation counterpart. This can be explained by the lack of toxification-induced cell lysis.

The apparent loss of protein from the aqueous phase just after addition of the organic phase in both cultivation regimes (with and without biotransformation) prompted us to investigate if this effect was inherent to the emulsification of the two liquid phases applied or depended on conditions intrinsic to biphasic, stirred-tank cultivation

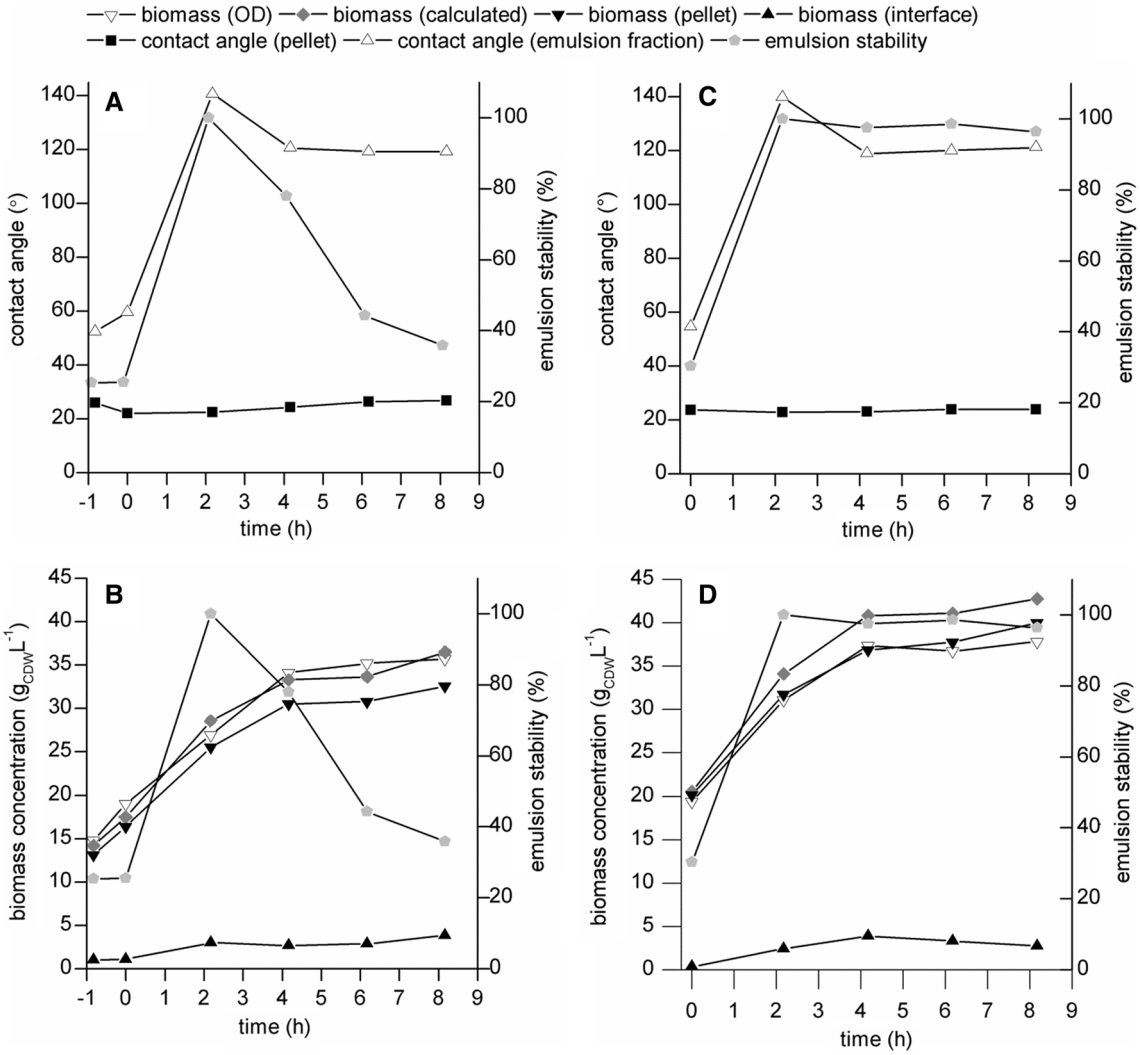

Fig. 6 Cell hydrophobicities of different cell fractions (pellet; emulsion fraction) represented as three-phase contact angles and emulsion stability during biphasic fed-batch cultivation of *E. coli* JM101 (pSPZ10) with (**a**) and without (**c**) biotransformation. Emulsion stability is given as percentage of the maximum emulsion fraction (length of emulsion/total length of sample after standard centrifugation) observed in the experiments (0.43 and 0.41, respectively). **b**, **d** show the respective biomass concentrations determined via cell dry weight measurements for pellets (biomass pellet) and emulsion fractions (biomass interface) with the sum giving the total biomass concentration (biomass calculated). Pellet-derived biomass concentrations additionally were determined via the standard optical density measurement at 450 nm (biomass OD)

(including microbial growth and aeration). *E. coli* JM101 (pSPZ10) was grown solely in aqueous medium with growth being limited after 8 h of standard fed-batch cultivation by reducing the glucose feed rate. The solubilized protein content was determined before and after emulsification of individual samples in an external Turrax mixing drive system. The protein concentration increased steadily in the growth phase and, at a lower rate, also in the stationary phase (Fig. 7c). External emulsification appeared to have little effect on the aqueous protein concentration. This is in sharp contrast to the characteristic decrease upon organic phase (BEHP) addition to the operating bioreactor. This correlated with a slower increase in the stability of Turrax drive-derived emulsions, which reached a maximum after around 5 h (100 %, EF = 0.45) and, 4 h later, started to decrease to about 60 % of the maximum. This slow stability increase as well as the decrease during the stationary phase is in contrast to the emulsion behavior in the bioreactor. The emulsion appearance lacked the thick, gel-like character observed in biphasic cultivations. Thus, it can be concluded that reactor operation, including aeration and cell growth, promoted emulsion stabilization correlating with a characteristic decrease in aqueous protein concentration upon organic phase addition.

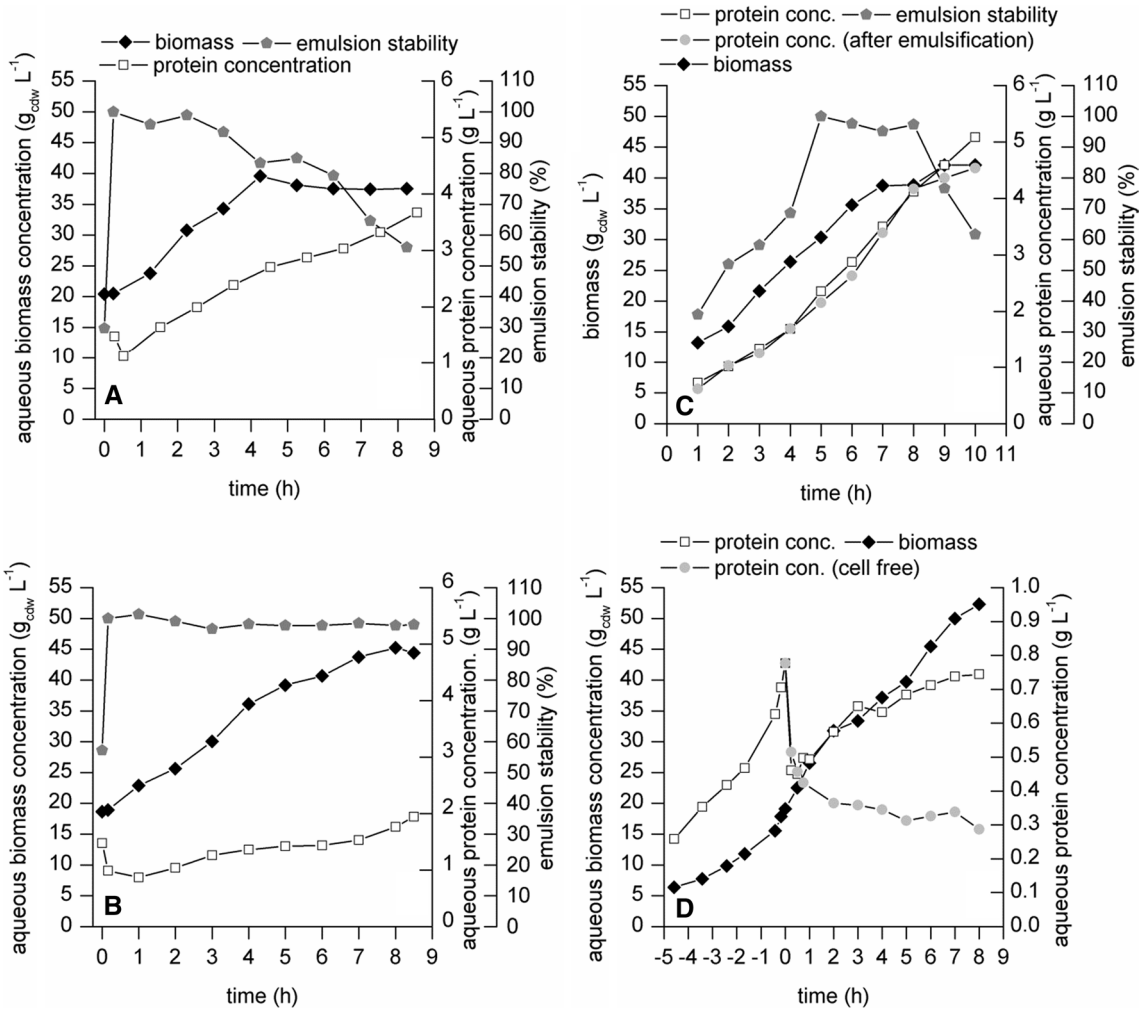

Fig. 7 Correlation of extracellular aqueous protein concentration with cell concentration and emulsion stability during biphasic fed-batch cultivations. **a**, **b** Cultivations of *E. coli* JM101 (pSPZ10) with and without biotransformation, respectively. **c** Aqueous fed-batch cultivation of *E. coli* JM101 (pSPZ10) and determination of extracellular protein concentrations before and after external emulsification of samples with BEHP. **d** Course of aqueous protein concentrations during a biphasic cultivation of induced *E. coli* JM101 (pA-EGFP_B) without biotransfomation and in a corresponding cell-free biphasic system. Proteins were quantified according to Bradford [25] instead of Lowry (see text for details). These two systems were established by removing a culture volume of 0.5 L from an 1.5 L fed-batch culture and adding 1 L of BEHP to the culture at $t = 0$ h (standard two-liquid phase culture). The removed culture broth was centrifuged and the cell-free supernatant was incubated in a second bioreactor supplied with an equal amount of BEHP at $t = 0$ h. Emulsion stabilities are given as percentage of the maximum emulsion fractions (length of emulsion/total length of sample after standard centrifugation) observed in respective experiments ($A = 0.44$, $B = 0.42$, $C = 0.45$)

These prominent differences in emulsion stability and aqueous protein concentration trends indicate that, in the operating bioreactor, protein adsorption to the cells and/or the interface between the two liquid phases contributed to the observed increase in emulsion stability. Further, the fact that the emulsion stability increase and the decrease in aqueous protein concentration coincided with an increase in hydrophobicity and amount of cells in the emulsion fraction (Fig. 6) suggest that the appearance of hydrophobic cells is caused by protein adsorption to the cells. Does the mechanism of observed aqueous protein loss (in the bioreactor setup) proceed through adhesion of solubilized protein to cells (making the cells hydrophobic), or are proteins extracted to the interface independently of the presence of cells? To answer this question, the effect of organic phase addition on the aqueous protein concentration in cell-free culture broth was investigated and correlated with the behavior of a cell-containing emulsion. When a cell concentration of 17.9 g_{CDW} L_{aq}^{-1} was reached in a standard batch and fed-batch cultivation setup, the culture was split in two. One part (1 L) was used to perform a biphasic cultivation with standard settings, while the other (0.5 L) was centrifuged to remove cells. Cell-free supernatant was then incubated with an equal volume of BEHP. As observed

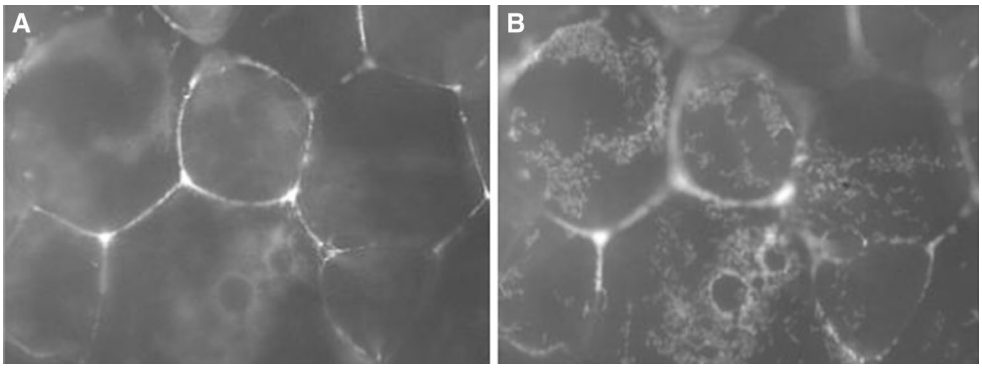

Fig. 8 Fluorescence microscope image of the stable emulsion fraction obtained during biphasic cultivation of induced *E. coli* JM101 (pA-EGFP_B). Cells were induced by 0.5 mM IPTG 2 h after organic phase addition and a sample of emulsion was taken 8 h after organic phase addition and centrifuged for 1 h under standardized conditions. The biphasic cultivation experiment corresponds to the one shown in Fig. 7d. See legend of Fig. 7 for further details. Microscopic pictures focused on the center (**a**) and on the surface (**b**) of an organic droplet layer are shown. No cover glass was applied for the microscopic analysis

before, the aqueous protein concentration increased during fed-batch cultivation (correlating with the increasing cell concentration), decreased steeply upon BEHP addition, and then increased again in parallel to cell growth (Fig.7 d). Upon BEHP addition to the cell-free system, the same steep decrease in the aqueous protein concentration was observed before stabilizing at roughly half the initial concentration. This indicated that the loss of proteins from the aqueous phase of a bioreactor-based emulsion does not depend on the presence of cells. The cell-free emulsion reached a lower stability (EF = 0.16) than the cell-containing emulsion. This indicated that hydrophobic cells, whose appearance is connected with protein extraction from the aqueous phase of an operating two-liquid phase bioreactor, constitute the main, although not the only, emulsifying agent in the cultivation-derived emulsion.

To exclude a bias associated with the application of the Lowry method for an emulsion-derived aqueous cultivation broth, an alternative protein quantification method (according to Bradford [25]) was used in the experiment shown in Fig. 7d. Using this method, the same relative behavior of the aqueous protein concentration was observed upon organic phase addition to a growing culture. As reported before [25], the estimation of the protein concentration according to Bradford gave lower absolute protein concentrations as compared to the estimation according to Lowry. Furthermore, an alternative expression vector containing a *styA-eGFP* gene fusion was applied in this experiment to visualize cells in the emulsion fraction via green fluorescence. Microscopic analysis of the emulsion fraction showed that cells were distinctly absent in the solvent droplet interior (Fig. 8a focusing on the center of an organic droplet layer), whereas they were readily observed at the surface of the solvent droplets (Fig. 8b focusing on the surface of an organic droplet layer). This confirmed the surface-active character of the hydrophobic cells in the emulsion fraction, promoting emulsion stabilization.

SDS-PAGE analyses of the aqueous supernatant of samples from biphasic fed-batch cultures of *E. coli* JM101 (pSPZ10) confirmed the immediate decrease in aqueous protein concentration upon BEHP addition and the observed increase parallel to cell growth (Fig.9). Additionally, it revealed a significant change in the extracellular protein pattern. Most prominently, the intensity of one band located between 40 and 50 kDa and of two others between 85 and 100 kDa decreased with the addition of the organic phase. During growth in the two-liquid phase system, the intensity of these bands recovered but to a much lower level compared to the protein background (Fig. 9a). The protein pattern in the cell-free setup changed in a similar way upon BEHP addition (Fig. 9b). This indicates that these proteins are either not being produced to the same degree following organic phase addition or that they are being disproportionately removed from the aqueous phase, presumably by some agglomeration or adsorption mechanism.

Discussion

Investigations on liquid–liquid biphasic microbial biocatalysis have often focused on specific activity and volumetric productivity [36, 47], oxygen transfer [30], control of biotransformation selectivity through partitioning [8, 12, 42], or economical and/or environmental efficiency [21, 22, 27]. While this has led to the development of a number of highly productive and useful reaction and process systems, far less work has been done to address a major drawback of these systems, namely the formation of stable emulsions and the respective detrimental impact on downstream processing. In this study, the emulsion stability behavior

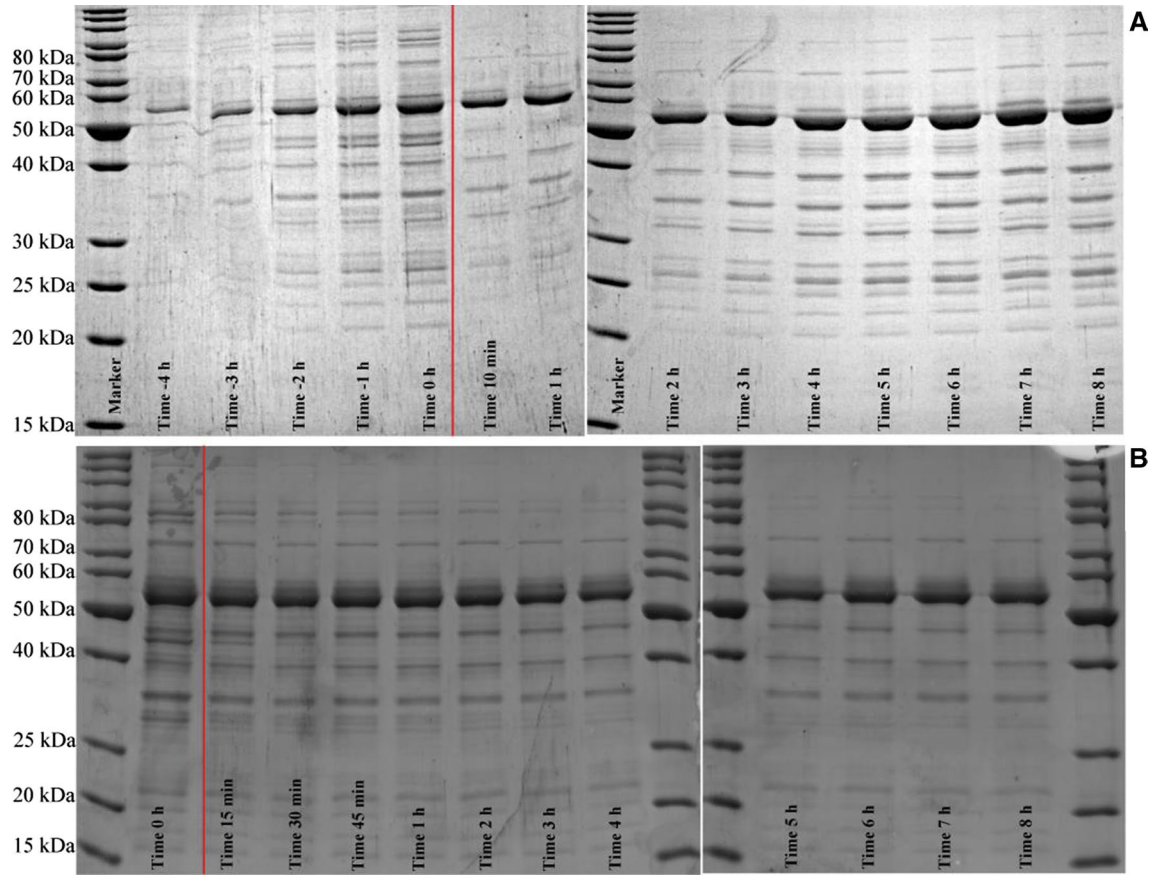

Fig. 9 SDS-PAGE analysis of aqueous samples before and after BEHP addition (separated by *red line*) to a fed-batch culture of *E. coli* JM101 (pSPZ10) (**a**) and to a cell-free biphasic system (**b**). Samples correspond to data points in Fig. 7d. See legend of Fig. 7 for details

in a model biphasic biotransformation was systematically investigated revealing factors which influence emulsion stability during biotransformation.

Emulsion destabilization, but not its initial stabilization, correlates with changes in cell physiology

Previous investigations have focused on emulsion stability and separation at the end of the biotransformation process [6, 7]. While the cells themselves have been described as the primary stabilizing factor, we were interested in understanding the formation of these stable emulsions during biotransformation. The rapidly increasing and then decreasing emulsion stability trend during biotransformation (Fig. 1b) was surprising, since it did not directly correlate with the cell concentration, a correlation which can be expected for cell-based Pickering emulsions. Further, it was expected that toxicity-induced cell lysis would stabilize and not destabilize the emulsion because of the release of surface-active compounds and cell fragments.

In all experiments conducted in 3 L stirred-tank bioreactors, stable emulsions formed shortly after organic phase addition (within 10 min). However, viability and growth behavior in terms of growth rates and yields were not affected, as previously observed for this model biotransformation [22, 36]. Thus, the fast emulsion stabilization, which was absent upon external emulsification via Ultra-Turrax tube drive, appeared not to result from changes in cell physiology. This is corroborated by the similar course of emulsion stability in two-liquid phase biotransformations based on growing and resting cells (Figs. 1 and 4). This comparison indicated that the growth stage has an influence on the absolute emulsion stability reached (resting cells at 20 g_{CDW} L^{-1} caused lower emulsion stabilities than growing cells at the same concentration), but is not responsible for the fast emulsion stabilization after organic phase addition. The fast emulsion stabilization is further discussed below. As the biotransformation progresses, cell physiology is affected by recombinant gene expression and product toxicity [9, 20, 21, 36]. The toxification of the cells by the oxidation products styrene oxide and 2-phenylethanol is evident from the decrease of the biomass growth rate beginning 2 h after the start of biotransformation (Fig. 1a). This decrease correlates with the beginning

of the decrease in emulsion stability (Fig. 1b). The effects of various hydrocarbons on cells have been reviewed [44]. Among them are hydrocarbon–protein interactions [11], changes in membrane structure [43], and altering of membrane function [44]. In the case of the styrene epoxidation system, cell permeabilization and lysis have been reported as the major effect [36]. Two-liquid phase cultivations without biotransformation confirmed this correlation between the observed decrease in emulsion stability and cell toxification (Figs. 2a, 3, and 5), which was further supported by the transient emulsion stability decrease observed upon introduction of cell lysate during a biphasic cultivation (Fig. 5). The mechanism by which cell toxification contributes to emulsion destabilization in these Pickering emulsions may be linked to changes in cell surface character and/or in the extracellular environment, i.e., released cellular components or cell debris. The amount and surface hydrophobicity of cells recovered from emulsion and aqueous fractions did not change upon toxification (Fig. 6) and can be excluded as causes for the decrease in emulsion stability. This together with the effect of cell extract addition (Fig. 5) indicates that surface-active cellular components and cell debris are responsible for this stability decrease, possibly via a displacement of cells from the organic–aqueous interface and a weakening of the Pickering type of emulsion stabilization.

Changes in cell hydrophobicity lead to initial emulsion stabilization

The stability of Pickering emulsions has been shown to depend on the concentration of the stabilizing particle [24, 38]. Such a behavior has also been reported for biphasic biotransformations [29]. In this work, a strictly positive correlation between cell concentration and emulsion stability was not observed during biphasic cultivation and biotransformation (Figs. 1 and 2a). This is corroborated by the similar course of emulsion stability in two-liquid phase biotransformations based on growing and resting cells (Figs. 1 and 4). In contrast, the emulsion formed via external emulsification of samples from purely aqueous cultures did show increasing stability with increasing cell concentrations (Fig. 2b). This could be the result of differences in the mixing or medium environments (stirrer geometry, aeration, glucose availability).

Besides particle concentrations, the surface properties of particles can have a significant effect on emulsion stability in Pickering emulsions [3, 4]. In the investigated model bioemulsion, two groups of particles displaying distinct surface properties were observed. In both samples taken from biphasic cultivation and those collected from external emulsification of aqueous cultures, the majority of the biomass settled as a cell pellet upon centrifugation. In both cases, these cells displayed a relatively low hydrophobicity (~24° contact angle) throughout biotransformation/cultivation. The total amount of these hydrophilic cells increased with the overall cell concentration. The large quantity of hydrophilic particles and the existence of an O/W emulsion are consistent with the Bancroft rule and findings of Binks and Lumsdon [5]. The continuous (aqueous) phase is the phase in which the majority of the particle surfactants, in this case hydrophilic cells, reside. Despite this correlation, the highest emulsion stabilities during two-liquid phase cultivation were uncoupled from the concentration of hydrophilic cells. The addition of the organic phase brought the appearance of a second group of hydrophobic cells, albeit in lower concentration (1–4 $g_{CDW}\ L^{-1}$) compared to the hydrophilic cell fraction. The emergence of this small quantity of highly hydrophobic cells, nonetheless, correlated with the formation of highly stable emulsions and did not occur upon external emulsification. To our knowledge, this is the first report on a monoseptic biphasic cultivation containing cells developing heterogeneous surface properties. While an enhancement of emulsion stability due to the presence of particle mixtures is unknown for biphasic whole-cell systems, this has been studied extensively in abiotic particle systems [1, 2, 5, 15]. Binks and others have demonstrated that the addition of hydrophobic particles to O/W emulsions created using hydrophilic silica particles leads to increased creaming with little change to coalescence. In the present work, centrifugation of the reactor-derived emulsions containing hydrophobic cells provided essentially compete creaming, with the higher emulsion stability indicating a lower degree of coalescence as compared to emulsification outside the reactor. The decrease in coalescence appears to be directly linked to the advent of the hydrophobic cell fraction. Considering the behavior of well-defined Pickering emulsion systems, one would expect a decrease in mean droplet size and aqueous conductivity upon the appearance of the hydrophobic cell fraction. Future experiments will be aimed at the elucidation of these interrelations.

The emergence of the hydrophobic cell fraction appears to be linked to the extraction of solubilized proteins from the aqueous phase

Two events have thus far been correlated with the formation of stable emulsions in the biphasic biotransformation model system: the appearance of hydrophobic cells in the emulsion fraction and a sharp decrease in the aqueous protein concentration. Organic solvents have been extensively explored as antisolvents for the precipitation of proteins, but these procedures almost universally deal with solvents which are miscible with water [19]. The organic phase in this model system (BEHP) possesses a log *P* of 7.45 [14].

Given the sparse solubility of BEHP in water, it is reasonable to infer that it is not behaving as an antisolvent in this case. This was confirmed by the absence of an aqueous protein concentration decrease when culture broths derived from purely aqueous cultivations were mixed vigorously with BEHP (Fig. 6). Protein denaturation and agglomeration are known to occur both in aqueous/organic liquid–liquid and gas–liquid systems with the resulting denatured proteins tending to remain at the interface [13, 37]. In the presented model two-liquid phase cultivation/biotransformation system, the appearance of the interface-associated hydrophobic cell fraction consistently correlated with the loss of solubilized protein from the aqueous phase, which itself did not depend on the presence of cells but on operative bioreactor conditions. One plausible explanation for the appearance of hydrophobic cells is that, in the highly dispersed gas–liquid–liquid three-phase system established in the reactor, the proteins in question become amphiphilic via the interaction with gas–liquid interfaces and adsorb to hydrophilic cells making them hydrophobic. This in turn leads to emulsion stabilization. The fact that only a small portion of cells becomes hydrophobic may be explained by extraction/saturation effects, i.e., the limited availability of such proteins, or by population heterogeneity in terms of cell surface affinity for such proteins. However, further investigations are required to clarify the mechanistic connection between the emulsion stabilization, the decrease of aqueous protein concentrations, and the appearance of an interface-associated hydrophobic cell fraction.

Conclusions

For the first time, emulsion characteristics during organic–aqueous two-liquid phase cultivations and biotransformations have been investigated revealing unexpected dynamics in emulsion stability and previously unknown factors affecting emulsion formation and stability. The rapid stabilization of a primarily cell-based Pickering emulsion observed upon organic phase addition was found to depend on operative bioreactor conditions (including aeration) and to correlate with a sudden loss of solubilized protein from the aqueous phase. This further coincided with the emergence of a hydrophobic cell fraction at the phase boundary. While it was previously thought that cells were the dominant factor in emulsion stabilization, this indicates that aqueous-soluble proteins present in the culture broth before organic phase addition also play a crucial role. Given the observed correlations, it is suggested that this may be occurring via protein adsorption to cells and phase boundaries. In experiments involving biocatalytic styrene epoxidation (the model whole-cell biotransformation), the initial formation of a highly stable emulsion was followed by a decrease in emulsion stability. This was in stark contrast to the high emulsion stability which persisted throughout biphasic cultivations performed in the absence of biotransformation. The decrease in emulsion stability correlated with biocatalyst toxification and cell lysis. This connection suggests that selective toxification of whole-cell biphasic cultures can be exploited as a novel strategy for the initial downstream processing of the described emulsions. This would enable future applications of biphasic biocatalytic techniques by significantly reducing the impact of their primary shortcoming, the persistence of the formed emulsions in the downstream.

Obviously, emulsion characteristics in bioprocesses are inherently linked to biological factors, such as microbial cells and dissolved proteins, along with related dynamics, such as changes in cell physiology and protein adsorption to cells and phase boundaries. These systems cannot be described based on physico-chemical properties of individual substances as it is typical for chemical processes. Thus, biological factors and related dynamics which play a decisive role in the design of upstream and downstream processes must be considered. The influence of the biocatalyst (whole cells), beyond simply its catalyst function, must also be considered in future process models if the full potential of biphasic whole-cell techniques is to be realized.

Overall, this work forms the basis for future work on general reaction engineering and downstream processing methods enabling the development of integrated whole-cell two-liquid phase processes. Current work in our laboratories, for example, is focused, besides the toxification strategy, on the treatment with supercritical carbon dioxide [6, 7] and catastrophic phase inversion to facilitate phase separation.

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