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RESEARCH ARTICLE

Continuous phase separation of stable emulsions from biphasic whole-cell biocatalysis by catastrophic phase inversion

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Abstract

The main bottleneck for the industrial implementation of highly promising multi-phase whole-cell biocatalytic processes is the formation of stable Pickering-type emulsions, hindering efficient downstream processing. Especially for the crucial step of phase separation, state-of-the-art processes require time-consuming and costly process steps (excessive centrifugation/use of de-emulsifiers). In contrast, using the phenomenon of catastrophic phase inversion (CPI), efficient phase separation can be achieved by addition of an excess dispersed phase within minutes. To show applicability of CPI as an innovative process step, a fully automated lab-scale prototype was designed and constructed within this work. A simple mixer-settler set-up enabled a continuous phase separation using CPI termed applied catastrophic phase inversion (ACPI). Test runs were conducted using emulsions from biphasic whole-cell biocatalysis (*Escherichia coli* JM101 and *Pseudomonas putida* KT2440 cells). Solvents used included n-heptane, ethyl oleate or 1-octanol as organic phase. These investigations revealed ideal process settings for a stable ACPI process (e.g., flow/stirring rates and volumetric phase ratios between organic and water phase). The knowledge of the CPI point is most crucial, as only the inverted state of emulsion is successfully destabilized.

KEYWORDS
biphasic whole-cell biocatalysis, catastrophic phase inversion, downstream processing, emulsion phase separation, pickering-type emulsions

1 | INTRODUCTION

1.1 | Pickering-type emulsions as limitation in biphasic whole-cell biocatalysis

Within the transformation from a fossil fuel-based to a bio-based economy, biphasic whole-cell biocatalysis offers highly potent process routes for the industrial production of (green) bulk and fine chemicals, as well as pharmaceutical intermediates/products.[1,2]

The presence of cells (acting as particles) results in the formation of long-term stable Pickering-type emulsions. Various examples of

Abbreviations: ACPI, applied catastrophic phase inversion; DW, cell dry weight; CPI, catastrophic phase inversion; *E. coli*, JM101 *Escherichia coli* JM101; LB, Luria-Bertani medium.; *P. putida*, KT2440, *Pseudomonas putida* KT2440

Nomenclature: h [m], Height; l [m], Length; o [-], Oil/organic phase; S [%], Stability; V [L], Volume.; V_O:V_W [-], Volumetric phase ratio between organic (oil) and aqueous (water) phase; v_w [-], Water volume fraction; $v_{w,crit.}$ [-], Critical water volume fraction, which is needed for phase inversion of an emulsion; w [-], water/aqueous phase

Greek symbols: γ_{ow} [mN m^{-1}], Interfacial tension between organic (oil) and aqueous (water)phase; η [Pa s], Viscosity; Θ_{ow} [°], Three-phase contact angle; $\varkappa$ [mS cm^{-1}], Electrical conductivity; ρ [g cm^{-3}], Density.

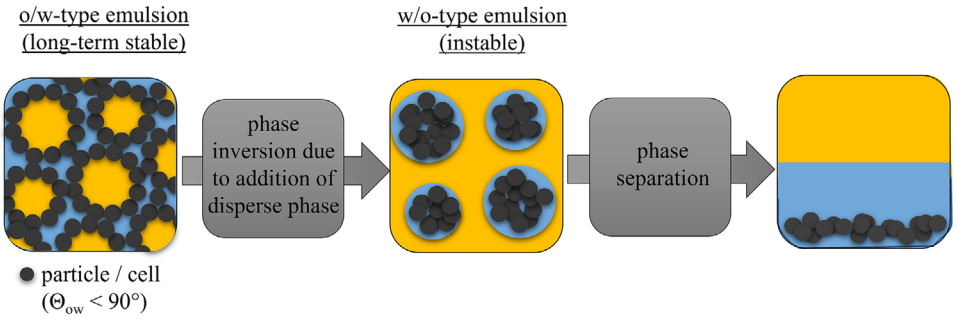

FIGURE 1 Scheme of CPI where an initially o/w-type emulsion stabilized by hydrophilic particles/cells (left) is inverted to w/o-type due to the addition of dispersed (organic) phase (middle), resulting in the loss of stabilizing effect in inverted emulsion as hydrophilic particles/cells remain in the now dispersed (water) phase. Without energy input, phases separate by gravity (right).

such emulsions have already been described in literature, including the biocatalytic epoxidation of styrene by *Escherichia coli* JM101 in a two-liquid (aqueous/organic) system with a volumetric phase ratio between aqueous and organic phase V_o:V_w = 1:1 by Kuhn et al.[3] Besides volumetric phase ratios of 1:1, also other phase ratios have been applied, for example, for the biocatalytic quinaldine hydroxylation by *Pseudomonas putida* in a two-liquid (aqueous/organic) systems with a phase ratio of V_o:V_w = 5:1.[4] Emulsion characteristics depend on the volumetric phase ratio between aqueous and organic phase (V_o:V_w) and the wetting behavior of the cells to form o/w- (more hydrophilic cells) and w/o-type (more hydrophobic) emulsions.[5] In some cases also double or multiple emulsions can occur.[6,7]

As much as emulsification is desired during the upstream processing (yielding in high surface areas and thus high mass transport rates),[1,3,8,9] downstream processing concepts often fail in processing the resulting (highly stable) Pickering-type emulsions.[10–12]

Common concepts for the initial phase separation step of these emulsions, such as centrifugation, use of de-emulsifiers, filtration or membrane separations either fail, or require high effort in both costs and time.[13] In contrast to these common state-of-the-art phase separation concepts, phase separation can be achieved efficiently using the phenomenon of catastrophic phase inversion (CPI), first introduced 1988 by Salager.[14] Phase separation is herein achieved by continuous addition of an excess amount of dispersed phase until the emulsion inverts, thereby switching from oil-in-water (o/w) to water-in-oil (w/o) (or vice versa) (Figure 1).

1.2 | Emulsion phase separation using CPI

Depending on the wettability/wetting behavior of the particles/cells (expressed by the three-phase contact angle Θ_{ow}), the emulsion type that can be stabilized by these particles/cells is fixed. O/w-type emulsions are stabilized by hydrophilic particles/cells (Θ_{ow} < 90°) and w/o-type (Θ_{ow} > 90°) emulsions are stabilized by hydrophobic particles/cells.[6,7]

The process of CPI is schematically shown in Figure 1 for an initially o/w-type emulsion stabilized by hydrophilic particles/cells. In this case, the addition of dispersed (that is organic) phase beyond a certain threshold results in a loss of stabilizing ability of the particles/cells, with the hydrophilic particles/cells residing in the now dispersed (water) phase. Without any further energy input, phase separation is easily achieved by, for example, gravimetric settling.[15]

During the CPI process the apparent drop size within the emulsion is expected to change, as median drop diameters in o/w-type emulsions tend to be much higher than in w/o-type emulsions.[16] Moreover, multiple emulsions (e.g., o/w/o) are formed during the CPI process due to the addition of dispersed phase resulting in an increase of average dispersed phase drop size when approaching the CPI-point.[17] According to the work of Hohl et al.,[18] a lower water content in w/o-Pickering-type emulsions did induce smaller drop sizes. Further effects on emulsion drop sizes are expected for the particle/cell concentration and hydrophobicity. Due to a higher surface coverage, higher particle concentrations result in smaller drops. Increasing particle/cell hydrophobicity led to increasing drop sizes.[18]

Moreover, multiple emulsions (e.g., o/w/o) are formed during the CPI process due to the addition of dispersed phase resulting in an increase of average dispersed phase drop size when approaching the CPI- point.[17]

Various batch experiments[5] already revealed the applicability of CPI as a tool for phase separation of various bioprocess-derived Pickering-type emulsions. Further, previous investigations within our group[5] also revealed, that the mechanisms of emulsions stabilization and destabilizing can be attributed/described by three key parameters, namely the particle size R, the three-phase contact angle Θ_{ow}, and the interfacial tension γ_{ow}. Furthermore, we developed a guideline to calculate/estimate the point at which CPI occurs (critical water volume fraction $v_{w,crit.}$) using these parameters.[5] This allows to define an appropriate solvent system and ACPI operating conditions for a given biphasic whole-cell biocatalytic process.

1.3 | The concept of applied catastrophic phase inversion (ACPI)

Based on the batch experiments two drawbacks to the CPI concept (Figure 1) remain: (1) The product is diluted, as it is preferentially located in the dispersed (mostly organic) phase (circumventing cell

toxification). (2) Removing the excess organic phase reverses phase inversion and thus hinders gravimetric phase-separation. To circumvent these drawbacks, Glonke et al.[13] introduced the concept of ACPI, being the continuous sibling of the batch CPI concept using a continuous mixer-settler set up.

Within this work, we designed and constructed a fully automated lab-scale prototype for continuous phase separation adhering to the ACPI principle. We demonstrate the applicability of the concept for various long-term stable bioprocess-derived Pickering-type emulsions, investigating the influence of both, different organic solvents (n-heptane, ethyl oleate and 1-octanol) as well as biocatalysts (*E. coli* JM101 and *P. putida* KT2440). The critical volumetric phase ratio of organic to water phase (V_o:V_w) which has to be applied to achieve phase inversion, was calculated based on the guideline developed in our previous work.[5] The process windows defined in this regard allowed for robust operating conditions for ACPI. To allow for a detailed investigation of the robustness of the process, we investigated the influence of process parameters (e.g., flow rates) on stability and success of the (continuous) phase separation. Furthermore, we investigated the robustness towards perturbations (e.g., fluctuation in water/organic phase ratio of the feed emulsion).

2 | MATERIALS AND METHODS

2.1 | Strains, media, and process conditions

The Pickering-type emulsions investigated in this work were obtained by biphasic cultivation of *E. coli* JM101 as well as *P. putida* KT2440 in a KLF 2000 reactor (Bioengineering, Wald, Switzerland).

Key parameters for the biphasic cultivation are described in a previous work.[5] The biphasic system consisted of Riesenberg-medium[19] or Luria-Bertani-medium (LB)[20] as an aqueous (cultivation) phase and either n-heptane (Merck, Darmstadt, Germany 100%), ethyl oleate (Alfa Aesar, Kandel, Germany 70%), or 1-octanol (Alfa Aesar, 99%) as organic phase (V_o:V_w = 1:1). No biotransformation was performed within this work, thus after fed-batch cultivation, organic phase was added for about 1 hour to the aqueous cell broth. It was found in previous works (results not shown),[21] that the biotransformation itself only had a negligible influence on emulsion stability and highest values of overall emulsion stability are reached within the first hour of organic phase addition.[22]

2.2 | Characterization of binary aqueous/organic system

For a first estimation of phase system properties (e.g., density and viscosity) of binary aqueous/organic systems, 0.05 M phosphate buffer ($NaH_2PO-K_2HPO_4$) at pH 7 was used as a sufficient simplification of an aqueous phased used during fermentation. 15 ml Falcon tubes were filled with aqueous (0.05 M phosphate buffer at pH 7 as model buffer) and organic phase (either n-heptane, ethyl oleate or 1-octanol) in equal parts. After mixing for 15 min, samples were equilibrated for 24 h at 25°C. Thereby, two phases (aqueous-rich and organic-rich) settled within all samples (complete phase separation).

Density measurements of aqueous and organic phases were performed using a DMA 4100 M by Anton Paar GmbH (Graz, Austria). Viscosity of both phases was measured using a temperature-controlled falling ball viscosimeter (LOVIS 2000 ME) by Anton Paar GmbH (Graz, Austria). All density and viscosity measurements were performed three times, ensuring reproducibility.

2.3 | Monitoring of CPI via electrical conductivity

Within the ACPI prototype, phase behavior of the emulsion is monitored using conductivity sensors (CombiLyz AFI5, Baumer). As described in literature,[23,24] o/w-emulsions show significant values of electrical conductivity κ, whereas in case of w/o-emulsions the value is near zero. Therefore, phase inversion of an o/w-type emulsion to w/o-type caused by addition of organic phase, directly results in a sudden decrease of κ.

2.4 | Process concept of ACPI prototype

Based on a patent filled at TU Dortmund University (publication number EP 2 870 988 A1) and according to the work of Glonke et al.[13] a fully automated lab-scale prototype for continuous phase separation of long-term stable bioprocess-derived Pickering-type emulsions was constructed. Figure 2 shows the process flow diagram of the prototype including the measurement and control equipment.

The two tanks (tank 1 and tank 2 in Figure 2, each 4 L) are used for the constant feed of emulsion (tank 1) and for the (manual) addition of originally disperse (in this work organic) phase to the mixer (tank 2). Within an industrial process, tank 1 would serve as buffer vessel directly after biphasic whole-cell biocatalysis in a bioreactor, ensuring a continuous feed to the ACPI process even if biocatalysis is carried out in (fed)batch mode. To ensure a constant homogenization of the emulsion, tank 1 and the mixing vessel (mixer) are equipped with stirrers (EUROSTAR 60 control, IKA, Germany) and ViscoJET agitators. Using a conductivity sensor (CombiLyz AFI5, Baumer) in tank 1 and the mixer, the apparent emulsion type can be determined through measuring conductivity, as only the o/w-emulsion type show significant values for electrical conductivity κ (see materials and methods section).[23,24]

The feed emulsion is continuously pumped from tank 1 into the mixer (1 L) using tumble piston head pumps (rotarus flow 100 with rotarus TKF QP-Q2 CKC, Hirschmann, Germany). In the mixer, phase inversion is ensured due to the permanent addition of an excess of originally disperse phase, exceeding the critical volumetric phase ratio. To monitor sustained (steady-state) phase inversion, the mixer is equipped with two capacitive sensors (CFDK 25G1125/LN3, Baumer) for level control and a sensor for measuring the electrical conductivity κ (CombiLyz AFI5, Baumer). In the settler (V = 0.5 L, d = 50 mm, h = 260 mm), phase separation of the inverted emulsion is achieved by gravimetric

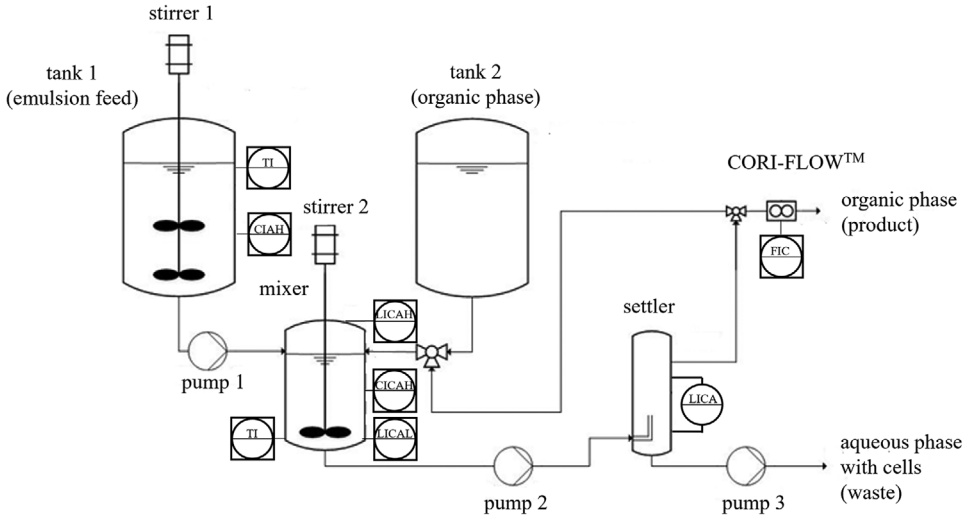

FIGURE 2 Process flow diagram of ACPI prototype including process equipment as well as sensors for process monitoring.

settling. The organic phase (lower density) rises in the settler, whereas the water phase (higher density) and cells settle at the bottom. The location of the phase boundary (upper and lower limit) in the settler is monitored by capacitive sensors (CFDK 25G1125/LN3, Baumer company) on the outer wall of the vessel. For bioprocess-derived Pickering-type emulsions, where the dispersed organic phase serves as substrate reservoir and product sink, the organic phase (containing the product) is further processed. The aqueous cell suspension is discarded/pumped into a waste tank. A mass flow meter/controller (mini CORI-FLOW, Bronkhorst) and automated valve divides the organic phase stream taken from the top of the settler into a product and a recycle stream. The amount of organic phase which is removed from the process, is equal to the amount of organic phase that is introduced into the process by the feed emulsion. The recycle stream is returned to the mixer, minimizing organic phase consumption, avoiding product dilution, and simultaneously ensuring that the threshold value for phase inversion (V_o:V_w) is constantly exceeded.

Figure 3 shows the fully constructed lab-scale prototype, constructed as described above.

2.5 | Determination of emulsion stability and effectiveness of separation process

Three sampling points within the ACPI process are used to determine the emulsion stability and the effectiveness of phase separation. As shown in Figure 4, each sample is centrifuged (Eppendorf 5804R, rotor A-4-44, Hamburg, Germany) for 60 min at 4000 g and 25°C.

2.5.1 | Emulsion stability

The stability (S in [%]) of the emulsion (which is the portion of the still emulsified interphase fraction (v) of the respective sample (i) present within the mixer (sample point 1), was calculated as the ratio of the interphase length (l_I in [mm]) to the total length of all phases (l_{total} in [mm]). The relative stability (S in [%]) was calculated as the ratio of this interphase fraction to the interphase fraction of the reference sample ($v_{i,0}$) prior to any treatment (initial stability).

Afterwards, organic and aqueous phase were removed in two separate steps allowing to determine the volumetric phase ratio V_o:V_w by measuring the respective phase volumes (Figure 4).

2.5.2 | Effectiveness of phase separation

To monitor the composition of aqueous waste and organic product stream (sample point 2 and 3), after centrifugation step, each of the present phases were separated and weighted as shown Figure 4.

3 | RESULTS AND DISCUSSION

3.1 | Influence of emulsion characteristics on phase behavior and phase separation process

Various bioprocess-derived Pickering-type emulsions stabilized by *E. coli* JM101 and *P. putida* KT2440 cells, containing either n-heptane, ethyl oleate or 1-octanol as organic phase were processed within the ACPI prototype.

To investigate the influence of emulsion characteristics on the separation behavior, the aqueous/organic base systems (no cells present) containing three different organic phases were characterized in terms of phase density ρ and viscosity η as well as interfacial tension γ_{ow}. The latter namely between aqueous (0.05 M phosphate buffer) and organic (either n-heptane, ethyl oleate or 1-octanol) phase (Figure 5). Further, the mutual solubility (here expressed as the water volume fraction v_w, present in the water-rich and organic-rich phases) was determined

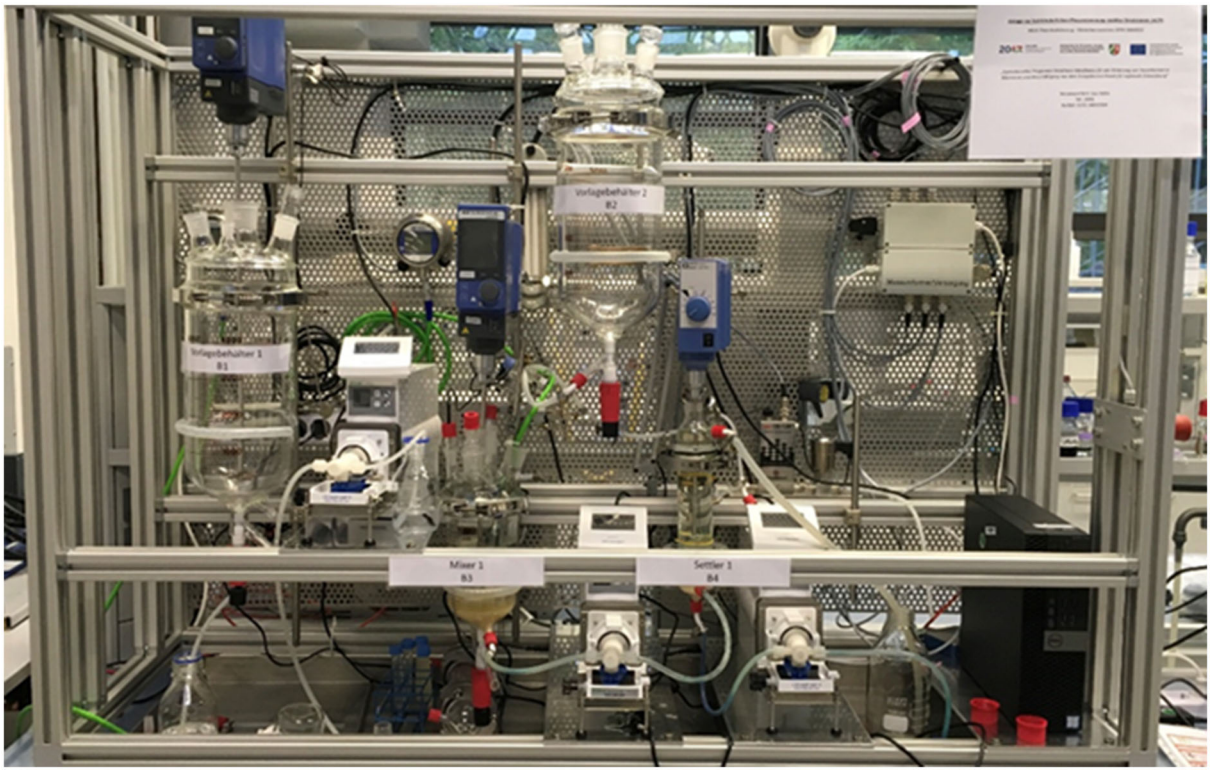

FIGURE 3 Picture of the lab-scale ACPI prototype as constructed described within this work.

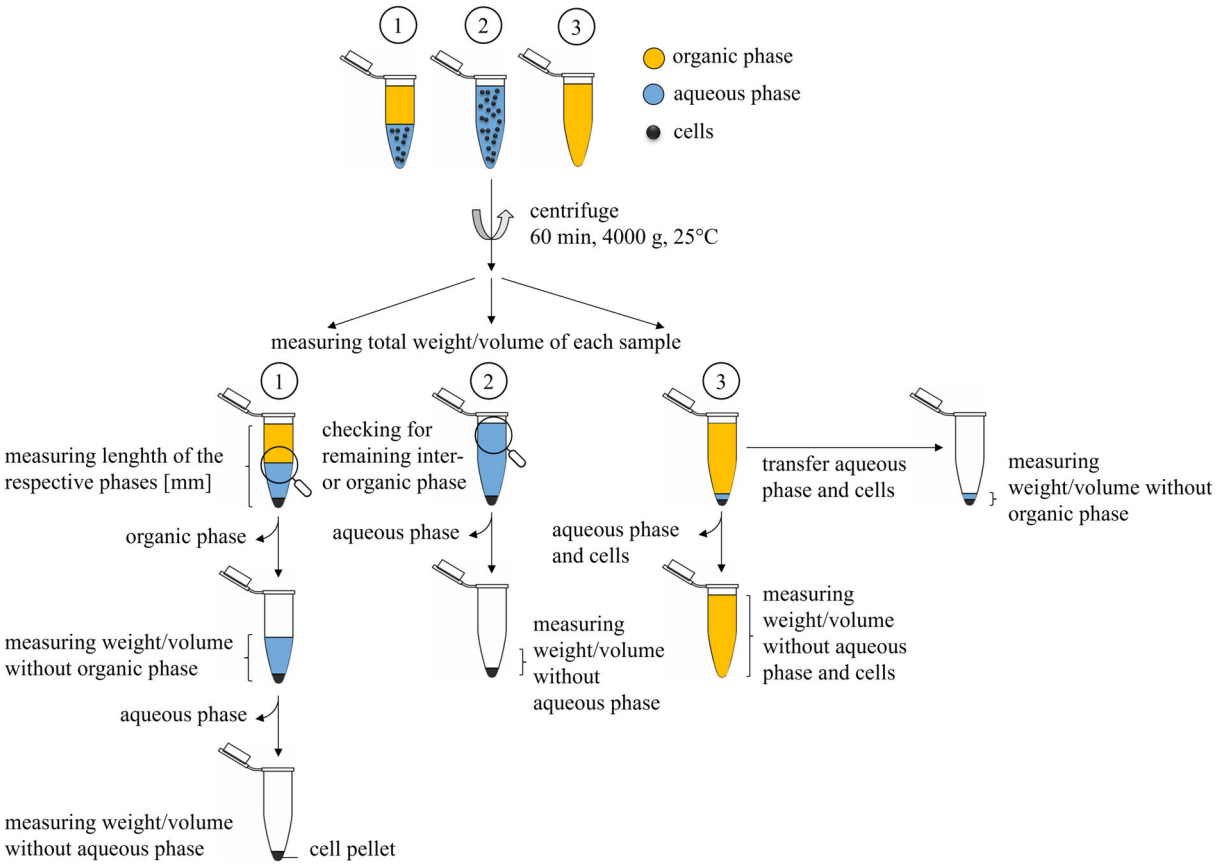

FIGURE 4 Scheme for collection and analysis of samples ensuring monitoring of emulsion stability and stream composition during the continuous ACPI process.

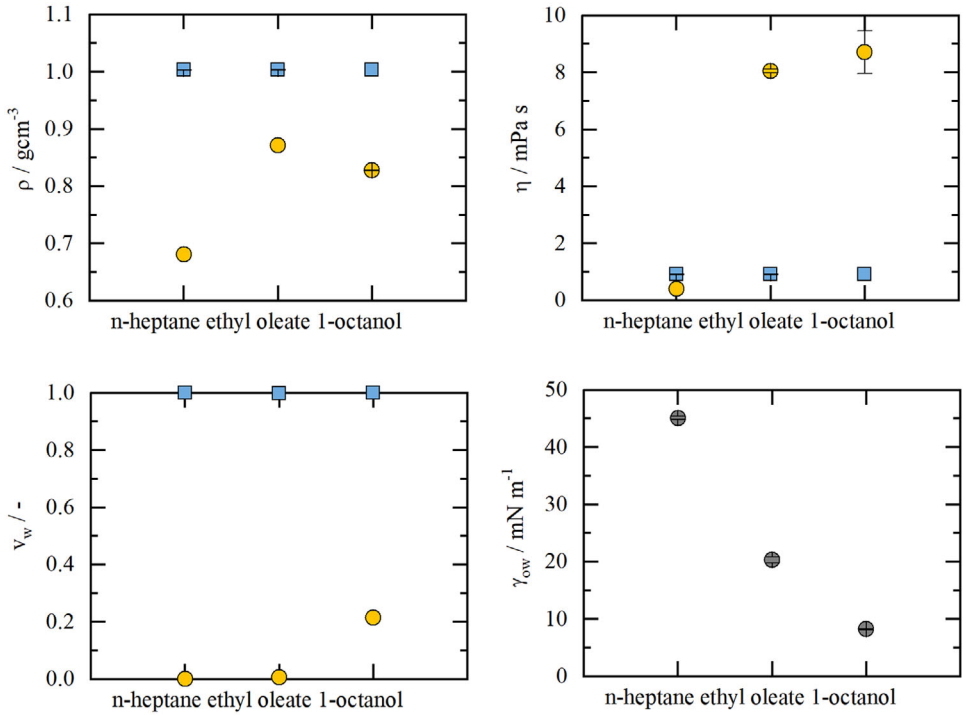

FIGURE 5 (Top) Density ρ and viscosity η for the water-rich (blue squares) and the organic-rich (yellow circles) phases of the equilibrated binary 0.05 M phosphate buffer/organic systems at 25°C and 1 bar. (Bottom, left) Mutual solubility of various organic compounds and water (left) at 25°C and 1 bar. Data for water volume fraction v_w in water-rich (blue squares) and organic-rich (yellow circles) phases were taken from literature: n-heptane,[25] ethyl oleate[26], 1-octanol.[27] (Bottom, right) Interfacial tension γ_{ow} between 0.05 M phosphate buffer and respective organic phase (grey circles) measured at 25°C and 1 bar using pendant drop tensiometry.[5]

(Figure 5) for all binary base systems considered within this work. The impact of the low buffer concentration was found to be negligible and thus only pure water was considered for this data.

For all binary systems, the solubility of the organic solvent (n-heptane, ethyl oleate and 1-octanol) in water is almost zero. The results show, that aqueous phase density ρ and viscosity η in all systems considered are not (notably) affected by the organic solvent, as all values are close to the values measured for pure 0.05 M phosphate buffer at 25°C (1.0044 g cm^{-3} or 0.9145 mPa s). The highest density difference (between aqueous and organic phase) in total can be observed for the 0.05 M phosphate buffer/n-heptane system with a value of 0.32 g cm^{-3}, whereas the system containing 0.05 M phosphate buffer/ethyl oleate shows the lowest density difference with a value of 0.13 g cm^{-3}. As a higher density difference between aqueous and organic phase favors gravimetric phase separation in the settler, the use of n-heptane is preferred in this regard.

The viscosity of organic phases increases with increasing polarity of the solvent (order: n-heptane < ethyl oleate < 1-octanol). The viscosity of the organic phase is three times lower than the viscosity of respective aqueous phase when n-heptane (0.41 mPa s) is used as organic solvent, and around nine times higher if ethyl oleate (8.06 mPa s) or 1-octanol (8.71 mPa s) are used. Depending on the viscosities of the organic phase, the stirring speed needs to be adjusted to ensure constant energy input and homogeneity of the emulsion (feed emulsion and inverted emulsion in mixer). It is known from different studies, that the phase inversion behavior and success is highly affected by viscosity and interfacial tension.[24,28,29] Norato et al.[24] indicated that the ambivalence region, which separates the region of o/w- from the region of w/o-emulsion type, widens, if: (1) The viscosity of disperse increases and/or (2) the interfacial tension between the phases decreases. For the emulsions investigated with this work, widest ambivalence region and therefore the highest volumetric phase ratio needed for CPI is thus to be expected for emulsion containing 1-octanol as organic phase, whereas emulsions containing n-heptane are expected to result in the narrowest ambivalence region and therefore the lowest volumetric phase ratio required. It must be recognized that these estimations are based only on the properties of the aqueous/organic base system, with the cells having a decisive effect on the phase inversion process.

3.2 | Process settings for ACPI prototype

3.2.1 | Determining initial operating flow rates

Characterizing the phase inversion process in the presence of cells is crucial to define operating conditions and a process window for the ACPI prototype. The critical volumetric phase ratios V_o:V_w, required for phase inversion of the bioprocess derived Pickering-type emulsions processed within this work (either stabilized by *E. coli* JM101 or *P.*

TABLE 1 Overview of critical volumetric phase ratios V_o:V_w needed for phase inversion of emulsions originating from biphasic biocatalysis, which were determined experimentally in a previous work.[5]

Biocatalyst	Organic phase	V_o:V_w .
Escherichia coli JM101	n-heptane	10:1
	ethyl oleate	6:1
	1-octanol	2:1
Pseudomonas putida KT2440	ethyl oleate	4:1

TABLE 2 Superficial velocities of the organic phase within the settler ($v_{organic}$) depending on the settler infeed (flow rate of pump 2, P2) and the emulsion processed within the prototype

P2/mL min^{-1}	60	80	100	120
	$v_{organic}$ m s^{-1}	$v_{organic}$ m s^{-1}	$v_{organic}$ m s^{-1}	$v_{organic}$ m s^{-1}
I	$4.7 \cdot 10^{-4}$	$6.2 \cdot 10^{-4}$	$7.8 \cdot 10^{-4}$	$9.3 \cdot 10^{-4}$
II	$4.5 \cdot 10^{-4}$	$5.9 \cdot 10^{-4}$	$7.4 \cdot 10^{-4}$	$8.9 \cdot 10^{-4}$
III	$3.8 \cdot 10^{-4}$	$5.1 \cdot 10^{-4}$	$6.4 \cdot 10^{-4}$	$7.6 \cdot 10^{-4}$
IV	$4.2 \cdot 10^{-4}$	$5.7 \cdot 10^{-4}$	$7.1 \cdot 10^{-4}$	$8.5 \cdot 10^{-4}$

putida KT2440 cells) to occur, were determined experimentally in a previous work[5] and are listed in Table 1.

Based on these values, volumetric phase ratios used within the ACPI process were chosen to be 10% to 20% higher than the critical volumetric phase ratio. Hereby reversibility of phase inversion in the mixer due to an insufficient volumetric phase ratio or in case of small process fluctuations (e.g., by varying emulsion feed) is avoided. For the emulsions stabilized by *E. coli* JM101 cells this results in initial volumetric phase ratios of V_o:V_w = 11:1 (n-heptane), V_o:V_w = 7:1 (ethyl oleate) and V_o:V_w = 3:1 (1-octanol), whereas for the emulsion stabilized by *P. putida* KT2440 cells the initial value is set to V_o:V_w = 5:1 (ethyl oleate). These values show the decisive effect of the cells on the phase inversion behavior of the emulsions. As described above, according to Norato et al.,[24] for the binary emulsions (without cells) it was expected that the highest V_o:V_w needed for CPI is expected for the emulsion containing 1-octanol as organic phase, whereas emulsions containing n-heptane are expected to result in lowest V_o:V_w. In presence of cells opposite trend for the dependence of the present organic phase and the resulting critical volumetric phase ratio, needed for CPI, can be observed. Emulsions stabilized by *E. coli* JM101 cells require the highest V_o:V_w in presence of n-heptane as organic phase, whereas V_o:V_w is lowest for 1-octanol as organic phase.

With the aim to process 1–2 L of emulsion per hour, between 16.67 ml min^{-1} and 33.33 ml min^{-1} of feed emulsion has to be processed. Assuming a constant feed with equal amounts of aqueous and organic phase, half of this stream (8.34 ml min^{-1} to 16.67 ml min^{-1}) leaves the process as product, and the other half as waste stream. The volumetric flow to the settler (pump 2) then only depends on the excess of organic phase and therefore must be set individually for each emulsion, according to the volumetric phase ratios determined previously (e.g., 25 ml min^{-1} for *E. coli* JM101/1-octanol/Riesenberg and 217 ml min^{-1} for *E. coli* JM101/n-heptane/Riesenberg).

3.2.2 | Effect of flow rate on separation efficiency in the settler

The separation efficiency in the settler was investigated for flow rates between 60 and 120 ml min^{-1}. As shown in Figure 6 (left) for pump rates (pump 2) of 60 ml min^{-1} the product stream was not contaminated with aqueous phase and/or cells. For higher flow rates contaminations of the product stream increase, reaching 25.34 $g_{w,cells}$ L_o^{-1} at a flow rate of 120 ml min^{-1}). The respective superficial velocities of the organic phase within the settler are given in Table 2. Within microscopic investigations (results are not shown within this work), for the emulsions stabilized by *E. coli* JM101 cells the average drop size increased from about 50 – 80 μm (V_o:V_w = 1:1) to drop sizes above

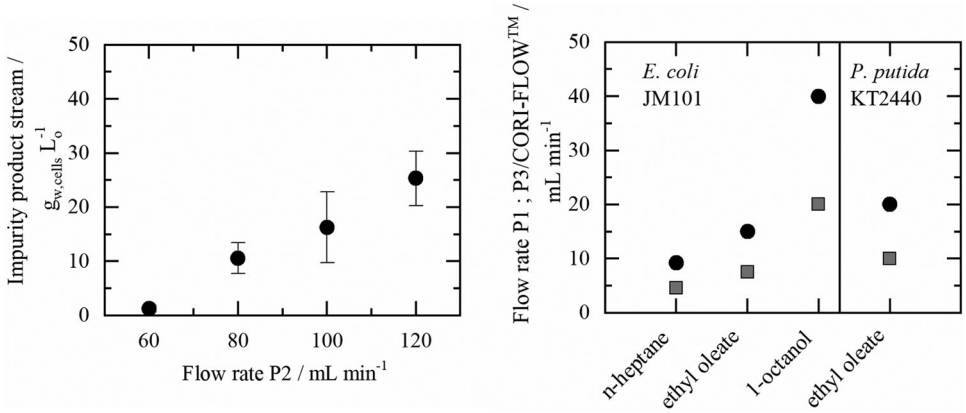

FIGURE 6 (Left) Impurity of organic product stream by aqueous phase and cells depending on pump rate of settler feed (pump 2, P2) exemplary for the emulsion system *Escherichia coli* JM101/ethyl oleate/Riesenberg. (Right) Flow rates of emulsion feed (pump 1, P1, black circles), waste (pump 3, P3), and product (CORI-FLOW) stream (dark grey squares) for emulsions stabilized by *E. coli* JM101 or *P. putida* KT2440 cells at a constant settler feed of 60 ml min^{-1}.

200 – 300 μm by increasing the amount of organic phase. Median drop diameters in the inverted w/o-type emulsion are expected to be smaller.[16] Comparing drop settling velocities of water phase within the settler with the superficial velocities of organic phase (Table 2), critical values for the drop sizes of water phase after CPI can be calculated. The results are critical drop sizes of 30 μm (n-heptane), 100 μm (1-octanol) and 220 μm (ethyl oleate). As droplet coalescence occurred instantaneous after complete phase inversion, we were unable to measure droplet size/droplet size distribution after CPI (and thus calculate real drop settling velocities).

Nevertheless, detailed analysis on the composition of the product stream at the top of the settler (sample point 3) show, that the impurities (Figure 6, right) can be attributed mainly to the presence of cells. A filter at the top of the settler can be implemented to reduce the impurity of the product stream. However, with increasing settler feed-rates (pump 2, P2)/superficial velocities of organic phase within the settler increase and so does the risk of filter clogging.

Thus, to ensure a high purity of organic product stream leaving the settler for further processing, the maximum flow rate of pump 2 was set to 60 ml min^{-1}. This resulted in emulsion feed rates between 9 and 40 ml min^{-1} (Figure 6, right) and throughputs between 0.5 and 2.4 L h^{-1}, depending on the volumetric phase ratio V_o:V_w required for CPI.

Prior to steady state of continuous operation, a start-up routine (duration around 20 min), is performed, ensuring an inverted emulsion state in the mixer and phase separation in the settler. Therefore, depending on the volumetric phase ratio set for the ACPI process, the mixer is filled with stable Pickering-type emulsion and pure organic phase to a filling level of 500 ml. Afterwards, intensive mixing for about 2 min is applied to achieve the initial inverted state, which is monitored measuring electrical conductivity κ. When the κ value is near 0 mS cm^{-1}, inverted emulsion is pumped to the settler, where phase separation can be observed immediately. After complete separation, which is achieved within minutes, the process can be switched into its continuous (steady-state) mode.

3.3 | Automation of ACPI prototype

3.3.1 | Measurement and control concept

Monitoring and control of the fully automated prototype was ensured using LABVIEW. Each vessel within the process is therefore equipped with various sensors as described above. A possible process perturbation can result from fluctuating in the volumetric phase ratio of the emulsion feed (caused by inhomogeneous mixing or unequal amounts of phases in the upstream biocatalysis). To monitor the state of the feed emulsion, electrical conductivity κ is measured, and value is compared to the reference of κ at V_o:V_w = 1:1 (Table 3). Contrary, monitoring of the inverted emulsion state in the mixer, solely on basis of κ does not ensure reliable results as: (1) Increasing amount of aqueous phase, which rapidly endangers the inverted state, is detected late by only using conductivity measurement. (2) κ is additionally influenced by the salt concentration within the emulsion.

TABLE 3 Average electrical conductivity κ of bioprocess-derived emulsion with V_o:V_w = 1:1 (CombiLyz AFI5, Baumer) at ambient temperature

System	κ (V_o:V_w = 1:1)
E. coli JM101/n-heptane/Riesenberg	6.30 ± 0.17 mS cm^{-1}
E. coli JM101/ethyl oleate/Riesenberg	5.66 ± 0.18 mS cm^{-1}
E. coli JM101/1-octanol/Riesenberg	5.68 ± 0.17 mS cm^{-1}
P. putida KT2440/ethyl oleate/LB	6.24 ± 0.19 mS cm^{-1}

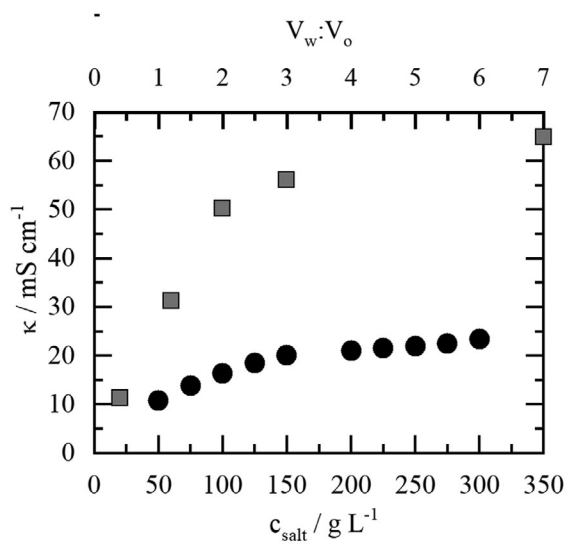

FIGURE 7 Electrical conductivity κ of an ethyl oleate/Riesenberg emulsion stabilized by *E.coli* JM101 cells influenced either by an increase of the reciprocal phase ratio V_w:V_o at a constant salt concentration of 20 g L^{-1} (black circles), or an increase of the NaCl concentration c_{NaCl} at constant phase ratio V_w:V_o = 1:1 (dark grey squares).

As shown in Figure 7, increasing the salt concentration (here NaCl, c_{NaCl}) from 20 g L^{-1} to 150 g L^{-1} at constant volumetric phase ratio V_o:V_w = 1:1 has a higher effect on κ, than increasing the amount of aqueous phase in the system. The later indicated by an increase of the reciprocal phase ratio V_w:V_o at a constant c_{NaCl} of 20 g L^{-1}.

Due to this fact, and to ensure that the inverted state maintained in the settler, monitoring of the inverted emulsion state within the mixer using conductivity sensors alone is not sufficient. Thus, we relied on the conductivity measurements within the mixer and additionally the apparent volumetric phase ratio in the mixer taken from measurements in the settler. The latter can be monitored by the interface level in the settler (height of the interface $h_{interface}$) using two capacitive sensors, which are located slightly above and under the interface, individually aligned for each emulsion using the following equation.

$$h_{interface}\ [mm] = \frac{h_{settler}\ [mm]}{\left(\frac{V_o}{V_w} + 1\right)}$$

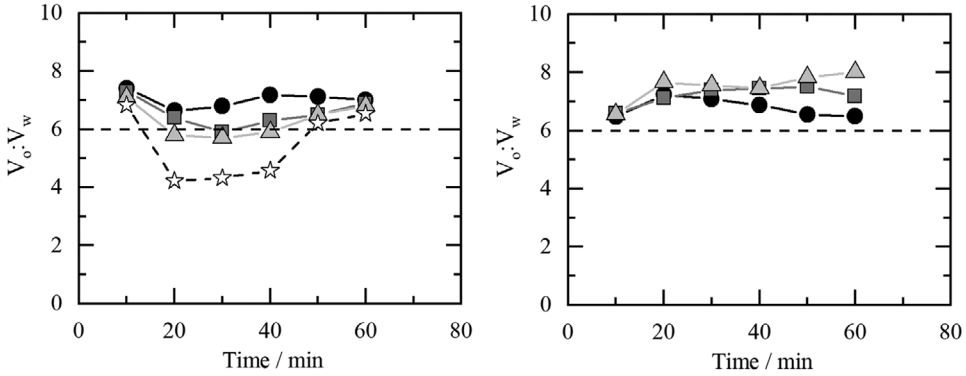

FIGURE 8 Volumetric phase ratio V_o:V_w in the mixer for varying pump rates of pump 3. (Left) Increasing flow rate by 50% (black circles), 60% (dark grey squares), 70% (light grey triangles), or 80% (white stars to regulate emulsion feed with V_o:V_w = 0.5:1. (Right) Decreasing pump rate by 50% (black circles), 40% (dark grey squares), 30% (light grey triangles) to regulate emulsion feed with V_o:V_w = 1.5:1. Dotted lines illustrate the critical volumetric phase ratio (V_o:V_w = 6:1[5]) for CPI of the emulsion ethyl oleate/Riesenberg stabilized by *E. coli* JM101.

3.3.2 | Perturbation of the steady state

The most crucial type of perturbation to be considered are fluctuations in the aqueous/organic phase ratio in the feed. If countermeasures are not initialized in due time, the phase inversion and thus the phase separation will fail as soon as the amount of dispersed phase falls short of the critical phase ratio. Thus, two scenarios were considered:

In case of an increase of aqueous phase in the system, the level of the interface rises (resulting in a signal of upper capacitive sensor), resulting in an automatically controlled increase in flow rate of pump 3 that controls the flow leaving the settler sump/bottom, and closing of CORI-FLOW to avoid reversibility of phase inversion. As illustrated in Figure 8 (left), the increase in the flow rate of dispersed phase is performed gradually, to ensure a constant volumetric phase ratio V_o:V_w above the critical value (indicated by the dotted lines in Figure 8). If the increase in flow rate is too high, aqueous phase is rapidly pumped out of the settler, erroneously indicating the system to be in target state again. Therefore, the maximum increase is set to 50% (black circles in Figure 8, left) of the initial value.

In contrary case of an increasing amount of organic phase, which is detected due to a loss of signal of the lower capacitive sensor, a decrease of the flow rate at settler sump (pump 3) is initiated. A 40% (dark grey squares in Figure 8, right) decrease of the initial value again ensures a gradual return to the steady state and prevents the loss of organic phase in the waste stream (Figure 8, right).

For overall level control of the mixer, pump 1 or CORI-FLOW can be stopped temporarily, ensuring a constant level (950 mL) also in case of varying flow rates.

3.4 | Steady state processing

The measurement and control concept was applied to process various bioprocess-derived Pickering-type emulsions (stabilized by *E. coli* JM101 or *P. putida* KT2440 and either n-heptane, ethyl oleate or 1-octanol as organic phase). As mentioned above, volumetric phase ratios varied between V_o:V_w = 2:1 to V_o:V_w = 11:1 depending on the respective emulsion. Following the start-up routine, process times for the processing of 1 L feed emulsion amounted to 45 min for the *E. coli* JM101/1-octanol/Riesenberg emulsion, 125 min for the *E. coli* JM101/ethyl oleate/Riesenberg emulsion, 208 min for the *E. coli* JM101/n-heptane/Riesenberg emulsion, and 91 min for the *P. putida* KT2440/ethyl oleate/LB emulsion. Figure 9 shows the course of electrical conductivity $\varkappa$ measured within the mixer (left) over time, as well as the results on the actual volumetric phase ratio V_o:V_w determined (right) using sample point 2, located between mixer and settler. The overall amount of destabilized emulsion (right) for emulsions stabilized by *E. coli* JM101 (top) or *P. putida* KT2440 (bottom) and either n-heptane, ethyl oleate or 1-octanol as organic phase.

The results show, that prior to the addition of organic phase, the electrical conductivity $\varkappa$ of all emulsions is about 6 mS cm^{-1}. Directly after the addition of organic phase (exceeding the critical volumetric ratio), phase inversion takes place, resulting in a sudden decrease in electrical conductivity to values near 0 mS cm^{-1}. Values remain constant over time indicating that steady state is reached within minutes. Only for the system *E. coli* JM101/1-octanol/Riesenberg values around 1.8 mS cm^{-1} are detected. The critical phase ratio V_o:V_w at which phase inversion occurs[5] are included in Figure 9 as solid lines and serve as a reference. For the processed emulsions stabilized by *E. coli* JM101 cells, these values vary between V_o:V_w = 2:1 (1-octanol, blue solid line), V_o:V_w = 6:1 (ethyl oleate, red solid line) and V_o:V_w = 10:1 (n-heptane, green solid line), whereas it is V_o:V_w = 4:1 (ethyl oleate, red solid line) for the emulsion stabilized by *P. putida* KT2440 cells. As mentioned before, volumetric phase ratios used within this work (indicated by dotted lines) are intentionally 10%–20% higher than the respective critical values [5] (indicated by solid lines) to avoid process instabilities. A decrease of the apparent phase ratio in the mixer, under the respective threshold will directly lead to phase inversion hindering a successful phase separation process. The results of the volumetric phase ratio measurements during the ACPI runs show, that V_o:V_w can be held

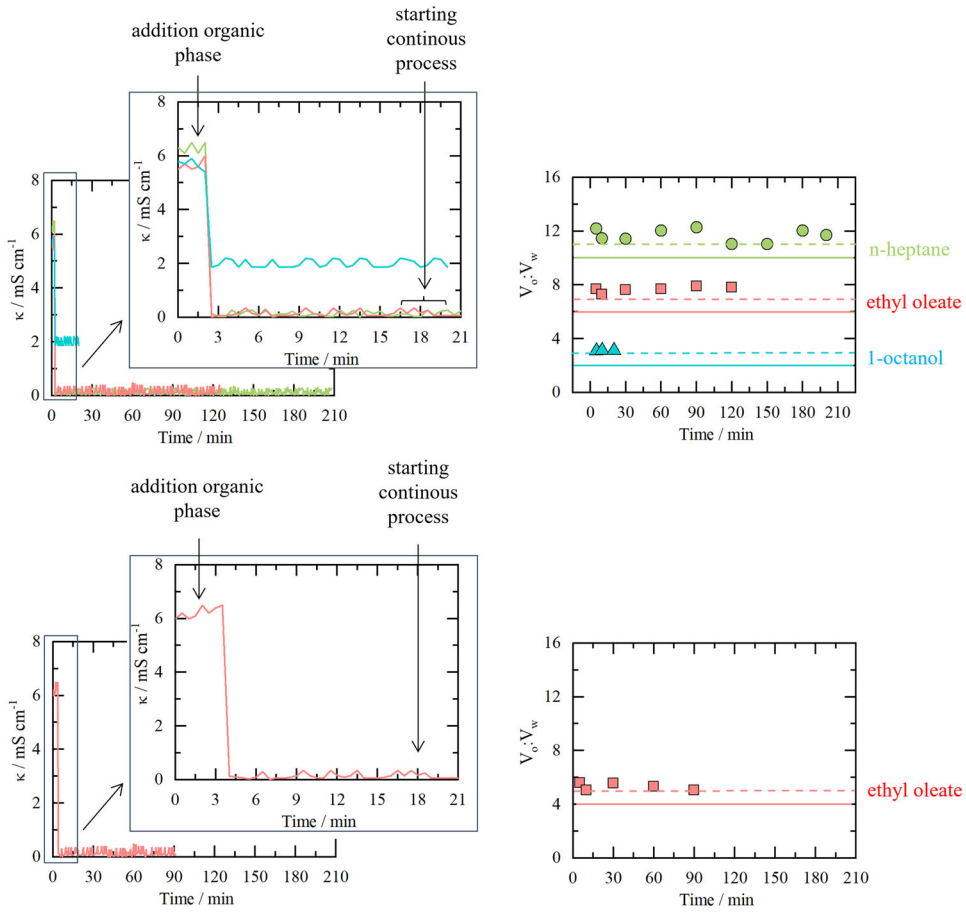

FIGURE 9 Processing of bioprocess-derived Pickering-type emulsions stabilized by *E.coli* JM101 (top) or *P.putida* KT2440 (bottom) in the ACPI prototype. Feed emulsions contained equal parts of Riesenberg- or LB-medium as aqueous phase and either n-heptane (green circles and lines), ethyl oleate (red squares and lines), or 1-octanol (blue triangles and lines) as organic phase. Process is monitored by measuring electrical conductivity κ (left) and determination of the volumetric phase ratio V_o:V_w (right) in the mixer. Solid lines illustrate the critical phase ratios (V_o:V_w) determined previously [5]. Dotted lines illustrate the (actual/safe) values of V_o:V_w set for APCI as described previously (section 3.2).

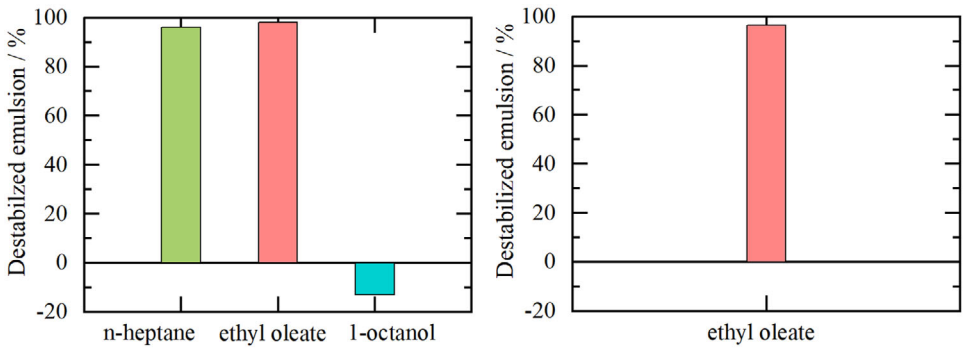

FIGURE 10 Overall amount of destabilized emulsion with either n-heptane (green bars), ethyl oleate (red bars) or 1-octanol (blue bars) as organic phase, which were initially stabilized by *E.coli* JM101 (left) or *P.putida* KT2440 cells (right).

constant (above the intended threshold) during the entire duration of the process, enabling continuous phase separation of the Pickering-type emulsions.

The overall amount of destabilized emulsion for emulsions stabilized by *E. coli* JM101 (left) or *P. putida* KT2440 (right) and either n-heptane, ethyl oleate or 1-octanol as organic phase is illustrated in Figure 10. For the systems *E. coli* JM101/n-heptane/Riesenberg, *E. coli* JM101/ethyl oleate/Riesenberg and *P. putida* KT2440/ethyl/LB oleate 96% to 98% of the long-term stable Pickering-type emulsion can be destabilized/treated successfully within the ACPI prototype.

For 1-octanol as organic phase, the separation process fails. After the startup procedure the settler was apparently filled with the inverted emulsion (low electrical conductivity κ, V_o:V_w above critical value). However, no phase separation could be observed/took place within a time interval of 96 h. Consequently, no switch to continuous mode was performed. The failure in processing the emulsion *E. coli* JM101/1-octanol/Riesenberg can be explained considering the cell wettability, measured in previous work.[5] Within the emulsion, typically two (stabilizing) cell fractions exist, exhibiting different wetting characteristics.[5,30] For the emulsion *E. coli* JM101/1-octanol/Riesenberg the first cell fraction stabilize o/w-emulsion type but, as three-phase contact angle is lower 50.7° no phase inversion to w/o-type is possible, whereas the second cell fraction of these emulsion preferentially stabilize w/o-type emulsions. Results of the conductivity measurements (Table 3) show, that a significant values of κ (5.68 ± 0.17 mS cm^{-1}) was detected, indicating o/w-emulsion type. Assuming the initial emulsion to be o/w-type, phase inversion by addition of organic phase result in w/o-type to be present the mixer, confirmed by significant decrease of κ (to about 2 mS cm^{-1}) (Figure 9). As due to their wetting characteristics (Θ_{ow} = 94°[5]) second fraction cells can also stabilize w/o-emulsion type, no phase separation is achieved. Same results were obtained using volumetric phase ratios V_o:V_w < 1:1 for the ACPI process. As in this case, first fraction cells of the emulsion *E. coli* JM101/1-octanol/Riesenberg stabilize the inverted o/w-emulsion type, no phase separation can be observed within 96 h.

4 | CONCLUSION

Based on the basic idea, described in the work of Glonke et al.,[13] a fully automated prototype was designed and constructed, enabling efficient phase separation of long-term stable Pickering-type emulsions, based on the phenomenon of CPI in a mixer-settler set-up. ACPI ensures a wide applicability to different bioprocess-derived emulsions, requiring no other additives or co-solvents than originally used for biocatalysis.

With the newly developed concept for process monitoring and control, ACPI enables phase separation of emulsions independent of fluctuations in feed emulsions, for example, product concentrations, salt concentration or pH value caused by the biocatalytic process. Even, fluctuations and perturbations to the volumetric phase ratio V_o:V_w, of the feed, can be regulated successfully by simultaneous monitoring of electrical conductivity within the vessels and the aqueous/organic interface level in the settler. In continuous mode, recycling of organic phase within the prototype ensures that product dilution is prevented, lowering the operational costs whilst simultaneously ensuring that a threshold value of volumetric phase ratio is constantly exceeded.

With the exception of the emulsion composed of *E. coli* JM101/1-octanol/Riesenberg, a phase separation efficiency of over 96% of the initial emulsion (1 L) could be achieved with processing times between 0.5 and 2.5 h for all bioprocess-derived Pickering-type emulsions considered within this work.

Therefore, ACPI is an innovative and universal tool overcoming the limitations of the drawbacks in classical downstream processing concepts used in state-of-the-art processing of bioprocess-derived Pickering-type emulsions.

AUTHOR CONTRIBUTIONS

Lisa Janssen: Conceptualization; Data curation; Investigation; Visualization; Writing – original draft. Gabriele Sadowski: Funding acquisition; Project administration. Christoph Brandenbusch: Funding acquisition; Project administration; Supervision; Validation; Writing – review & editing.

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CONFLICT OF INTERESTS STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

PERMISSION STATEMENT

The investigations conducted within this work did not contain any animal or human studies.

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