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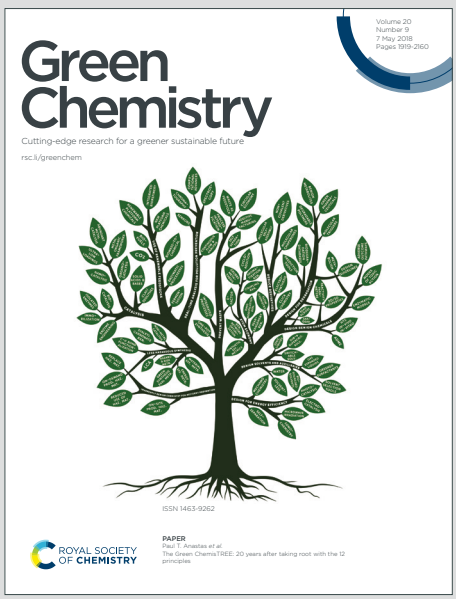

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Selection of a recyclable *in situ* liquid-liquid extraction solvent for foam-free synthesis of rhamnolipids in a two-phase fermentation

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Excessive foaming causes instabilities in fermentation processes, particularly when producing biosurfactants, which can be overcome by intensifying the fermentation via *insitu* product recovery. A reductive, multi-step approach for selecting organic solvents for an *in situ* liquid-liquid extraction of rhamnolipids produced by recombinant *Pseudomonas putida* KT2440 was developed. 1) A database consisting of physicochemical parameters for 183 solvents was composed, allowing a pre-selection by setting respective thresholds. 2) The number of solvents was reduced by evaluating their extraction efficiencies regarding rhamnolipids in cell-free cultivation broth and their impact on the growth of *P. putida* KT2440. 3) The most promising solvent was characterized regarding phase separation, pH-dependency of the extraction, and applicability of back-extraction for product recovery and solvent regeneration. The overall performance was assessed in two-phase (fed)batch fermentations in lab-scale stirred-tank reactors. The solvent selection approach revealed ethyl decanoate to be a highly suitable and sustainable solvent for the *in situ* liquid-liquid extraction of rhamnolipids. During the final two-phase fed-batch fermentation, 30 g/L of produced rhamnolipids accumulated in the organic phase. Integrating extraction and increasing the partition coefficient by moderately lowering the pH prevented foaming during fermentation, thus resolving the initial process instability. Rapid phase separation and back-extractability allowed product recovery and solvent recycling. The here presented reductive, multi-step solvent selection approach was successfully applied to establish a two-phase fermentation producing rhamnolipids by engineered *P. putida* KT2440, resolving the foaming challenge. The approach can serve as a blueprint for selecting solvents for *in situ* liquid-liquid extractions in bioprocesses.

Introduction

The envisaged bioeconomy requires replacements for petrochemically derived chemicals. Promising alternatives for surfactants are rhamnolipids (RLs) produced mainly by bacteria from the *Pseudomonas* genus.[1] The class of rhamnolipids encompasses a high diversity of molecular structures as congeners vary in number of rhamnose moieties (zero to two) as well as chain length and saturation of the alkanoic acid residues[2] (representative structure in Figure 1). For simplicity, they are hereafter collectively referred to as RLs or rhamnolipids. These biosurfactants have potential applications, e.g., in detergents, food, remediation of oil-polluted sites, medicine/pharmacology, plant protection, and agriculture. Specific congeners of RLs might have additional, innovative applications, which have to be fully explored and exploited.[3] Because *P. aeruginosa,* the most prominent producer of RLs,[4,5] is an opportunistic human pathogen, we previously established RL production using recombinant, non-pathogenic *P. putida* KT2440.[6,7]

A main challenge in the production of surfactants in bioreactors is excessive foaming even at small product concentrations caused by the aeration required to continuously provide oxygen for cellular respiration. Microorganisms accumulate in the foam resulting in a substantial loss of the biocatalyst. Although anti-foaming agent has been applied to prevent foaming at high concentrations of biosurfactants[8] it is not only complicating downstream processing[9,10] but is also costly as large amounts are required. Therefore, innovative process alternatives as the integration of *in situ* product removal (ISPR) like foam fractionation[11–14] are advantageous for the biotechnological production of surfactants but pose challenges when transferred to large-scale production due to challenging scalability.[15] The implementation of *in situ* liquid-liquid extraction might be a promising alternative. Here, a liquid organic phase, which is exhibiting a miscibility gap with the aqueous fermentation broth, is added to the system. The product transfers into the organic phase, lowering the surfactant concentration in the fermentation broth, and thus prevents foaming. Previously, ethyl acetate was favored for the *ex situ* extraction of RLs from fermentation broth.[16,17]

In situ liquid-liquid extractions have been applied in different setups for biotechnological production processes to resolve product inhibitions. A common setup is spatially separating production and extraction by coupling the fermenter compartment to an extraction compartment removing, e.g., lactic acid,[18] gibberellic acid,[19] butanol,[20] and itaconic acid.[21] An industrial ready process was evaluated by DSM Biotech GmbH, Jülich (Germany), and DSM, Geleen (Netherlands) to produce L-phenylalanine.[22] The sequential coupling of fermentation and liquid-liquid extraction allows independent optimization of respective process parameters like dissolved oxygen, shear stress, temperature, or pH value. However, as the fermentation

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broth is circulated through the compartments, a change of parameters causes the production host to encounter repeatedly changing environments perturbating its metabolism, which can result in an impaired production or even cell death.[23,24] Although submerged membranes can retain the organisms in the fermenter compartment, such a setup requires additional equipment with increased investment and maintenance cost due to biofouling of the membrane.[25,26]

Fermentation and *in situ* liquid-liquid product extraction in a single compartment, i.e., two-phase fermentation, was investigated for many catalyst/product pairs overcoming the drawbacks described above. Examples for two-phase fermentations are the production of phenol,[27] p-hydroxysterene,[28] 3-methyl-1-butanol,[29,30] ethanol,[31] and pullulan[32]. In an approach for the epoxidation of styrene, the toxic substrate was supplied *via* an organic phase simultaneously serving as an extractant for the equally toxic product,[33,34] even predicting an economic advantage compared to conventional chemical production processes.[35] Publications on *in situ* liquid-liquid extractions for industrial fermentations are rare. However, e.g., Isobionics (Geleen, Netherlands) owns a patent for two-phase fermentation to produce isoprenoids.[36]

In contrast to coupled extractions, the operational window for two-phase fermentations is defined by the combination of the respective operational windows for fermentation and liquid-liquid extraction, hence the freedom of design and operation is reduced. Challenging operational constraints can cause detrimental effects on fermentation and extraction performance. Examples are underperformance of whole-cell biocatalysts due to toxicity of the extractants or unfavorable surface properties of organisms resulting in poor phase separation, interphase formation, or high viscosity.[37,38] These interactions, as well as physical properties, depend on the choice of solvent, thus making a comprehensive screening of solvents regarding specifications of the fermentation indispensable.

Previous olvent screenings fortwo-phase fermentations consisted mainly of comparing the extraction efficiencies and biocompatibilities of only up to 25 solvents,[39–44] probably to not exceed a feasible number of experiments. However, since there are hundreds of solvent candidates, finding a highly suitable but simultaneously sustainable and safe solvent for the respective application might be accidental and based on available chemicals. The chemical industry uses model-based predictions of liquid-liquid equilibria,[45] enabling an *in silico* screening of large numbers of solvents for their partition coefficients. While this approach is complicated to transfer to fermentations, some physicochemical properties of the solvent can be considered to evaluate the compatibility for the envisioned two-phase fermentation. Therefore, a first reduction of the number of potential solvent candidates can be performed by screening a database for physicochemical properties in a predefined property target space.[46,47]

In this study, 183 organic solvents were evaluated for liquid-liquid *in situ* extraction of RLs in a multi-step, reductive selection approach. First, a data-based screening of physicochemical and economic properties led to a reduced number of solvent candidates, which was further limited by thresholds for experimentally determined partition coefficients, biocompatibility, and biodegradation by *P. putida* KT2440, simultaneously favoring solvents posing low risk to the user. In more detail, characteristics of the most promising solvent, including phase separation behavior and pH-dependency of the (back-)extraction for product and solvent recovery, were determined. The performance of the final organic solvent was evaluated in two-phase fermentations using stirred-tank reactors.

Experimental

Bacterial strain, product, and media

The HV1 certified reference strain *Pseudomonas putida* KT2440 (DSMZ: 6125) was used as a chassis. [48–50] Previously, *P. putida* KT2440 was engineered for RL production by stable genome integration of *rhlA* and *rhlB* from *Pseudomonas aeruginosa* at the *att*Tn7 site for constitutive mono-RL and HAA production[16] resulting in strain *P. putida* KT2440 *attTn7::*P_{ffg}*-rhlAB* referred to as *P. putida* KT2440 SK4 in the following.

For the initial seed culture, lysogeny broth (LB) was used (5 g/L yeast extract, 10 g/L tryptone, 2 g/L NaCl). For secondary seed cultures and production cultures in shake flasks a mineral salts medium modified from Hartmans *et al.*[51], was used (10 g/L glucose (GLC), 11.64 g/L K_2HPO_4, 4.89 g/L NaH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 0.1 g/L $MgCl_2 \cdot 6\ H_2O$, 10 mg/L EDTA, 2 mg/L $ZnSO_4 \cdot 7\ H_2O$, 1 mg/L $CaCl_2 \cdot 2\ H_2O$, 5 mg/L $FeSO_4 \cdot 7\ H_2O$, 0.2 mg/L $Na_2MoO_4 \cdot 2\ H_2O$, 0.2 mg/L $CuSO_4 \cdot 5\ H_2O$, 0.4 mg/L $CoCl_2 \cdot 6\ H_2O$, 1 mg/L $MnCl_2 \cdot 2\ H_2O$). The medium was highly buffered to counteract a decrease of pH due to gluconate production by *P. putida* KT2440. For bioreactor cultivations, during which the pH can be controlled by acid and base addition, the phosphate buffer (K_2HPO_4/NaH_2PO_4) concentration was lowered threefold. For feeding, a highly concentrated and adjusted mineral salts medium was used (200 g/L glucose, 7.76 g/L K_2HPO_4, 3.26 g/L NaH_2PO_4, 40 g/L $(NH_4)_2SO_4$, 0.33 g/L $MgCl_2 \cdot 6\ H_2O$, 3 mg/L EDTA, 66 mg/L $ZnSO_4 \cdot 7\ H_2O$, 3.3 mg/L $CaCl_2 \cdot 2\ H_2O$, 16.5 mg/L $FeSO_4 \cdot 7\ H_2O$, 0.66 mg/L $Na_2MoO_4 \cdot 2\ H_2O$, 0.66 mg/L $CuSO_4 \cdot 5\ H_2O$, 1.32 mg/L $CoCl_2 \cdot 6\ H_2O$, 3.3 mg/L $MnCl_2 \cdot 2\ H_2O$, also modified from Hartmans *et al.*[51]).

Pre-culture preparation

For all cultivations, a strict pre-culturing protocol was followed. Cryopreserved aliquots of *P. putida* KT2440 SK4 (20% (v/v) glycerol, OD_{600} 5, -80 °C) were streaked onto LB agar plates and incubated at 30 °C for 24 h. A single colony was picked to inoculate a seed culture of 5 mL LB medium, which was incubated at 30 °C and a shaking frequency of 300 rpm on a rotary shaker with an eccentricity of 50 mm. Appropriate volumes of the first seed culture were transferred to the second seed culture. For this, 500 mL shake flasks filled with 50 mL mineral salts medium containing 10 g/L glucose were inoculated and incubated at the conditions mentioned above. Cells were harvested in the mid-exponential phase to inoculate

production cultures either in shake flasks or in stirred-tank bioreactors.

Quantification of rhamnolipids and sample preparation

For RL quantification, reversed-phase HPLC-CAD (Ultimate 3000 with a Corona Veo Charged Aerosol Detector, Thermo Fisher Scientific, Waltham, USA; NUCLEODUR C18 Gravity 150 x 4.6 mm column, particle size: 3 µm, Macherey-Nagel GmbH & Co. KG, Düren, Germany) was used, employing a method described previously.[52] The method enabled the detection and quantification of four HAA congeners and four mono-RL congeners (ESI Data 2†, Figure S1). Before sample preparation, the pH of aqueous samples was adjusted to 7 with 1 M KOH or 1 M HCl, if necessary, to avoid quantification bias. Equal volumes of acetonitrile were added to aqueous samples for protein precipitation. After mixing and incubation at 4 °C for more than 4 h, the samples were centrifuged (21,000 g, 3 min) and filtered (Phenex RC syringe filters, 0.2 µm, d = 4 mm, Phenomenex, Torrance, USA). For solvent samples, the organic phase was evaporated at 20 mbar, 60 °C, and 1,400 rpm (ScanSpeed 40 attached to ScanVac Coolsafe 110-4, both Labogene ApS, Lynge, Denmark, and Chemistry Hybrid Pump RC 6, vacuubrand GmbH + Co. KG, Wertheim, Germany) and dry residuals were resolved in appropriate volumes of a 50 % acetonitrile - double distilled water solution before filtering (Phenex RC syringe filters, 0.2 µm, d = 4 mm, Phenomenex, Torrance, USA).

Glucose quantification

Glucose was quantified using a colorimetric enzymatic assay automated on a liquid handling platform (4BioCompact, 4BioCell GmbH & Co. KG, Bielefeld, Germany). The system was calibrated with defined glucose solutions in the range from 0.1 g/L to 4 g/L. For measurements out of range, the liquid handling platform automatically diluted the respective samples and repeated the assay. Before quantification, the aqueous samples were centrifuged (21,000 g, 3 min) to remove cells.

Reductive multi-step solvent selection

Solvent database

Data for organic solvents of different types and various physicochemical properties were collected and listed in a data spreadsheet. The list encompasses properties relevant for extraction processes, such as density, solubility in water at room temperature, and the boiling point at ambient pressure. Additionally, the octanol/water partition coefficient (log *P*) as an indicator for biocompatibility [53,54] and flash points for assessing fire hazards of the solvents were listed. Main data sources were ChemSpider (Royal Society of Chemistry, London, UK), PubChem (National Center for Biotechnology Information. Rockville Pike, USA), and the documentation of the manufacturer depending on data availability.

Extraction efficiency

The extraction efficiency of solvent candidates was tested in cell-free cultivation broth generated from shake flask cultivations of *P. putida* KT2440 SK4 and subsequent centrifugation (14,000 g, 10 min). Organic solvents were saturated with distilled water overnight in a tempered shaker (Thermo Shaker MHR 23, Hettich Benelux BV, Geldermalsen, Netherlands) in 50 ml tubes at 30 °C and 600 rpm to minimize changes of concentrations due to cross-solubility of the solvent with water. Biphasic systems in fixed phase ratios ϕ of 1:4 (v/v) organic solvent to cultivation broth (pH 6.5) were shaken at 99 rpm in an overhead shaker (Intelli-Mixer RM-2L, ELMI Ltd. laboratory equipment, Riga, Latvia) for 4 h. As the temperature for optimal growth and production for *P. putida* KT2440 was previously determined to be at 30 °C [55] and the solvents are assessed for *in situ* extractions, all extraction experiments were conducted at the mentioned temperature. Samples were taken from the aqueous phase to quantify the RL concentration after extraction C_1, thus determining the reduction of RL concentration relative to the initial RL concentration C_0 (extraction efficiency E, Equation 1) and the corresponding partition coefficient P (Equation 2).

Equation 1

$$E = \frac{C_0 - C_1}{C_0} \cdot 100\%$$

Equation 2

$$P = \frac{C_0 - C_1}{C_1 \cdot \phi}$$

Biocompatibility and biodegradation

For the biocompatibility assays, *P. putida* KT2440 SK4 was cultivated in 50 mL mineral salts medium with 4 % (v/v) of the respective solvent added. As state-of-the-art methods for biomass quantification were not feasible due to the formation of stable emulsions, CO_2 production was used as an indicator for growth. CO_2 was measured in the headspace of shake flasks by BCP-CO_2 sensors, and the data was monitored with BlueVis (both BlueSens, Herten, Germany). The shake flask was closed to avoid any evaporation of the solvent. Additionally, custom-made solvent reservoirs were installed in the headspace to saturate the gaseous phase of the shake flask with the respective solvent. The maximal consumable glucose concentration in the medium was estimated to 6.3 g/L based on the amount of available oxygen in the headspace of the shake flasks. For this, the assumptions of ideal gas law and pure combustion reaction were applied, neglecting biomass and product formation. As *P. putida* KT2440 is known for its versatile carbon metabolism, the solvents may potentially be degraded and used as a carbon source resulting in further production of CO_2 after glucose depletion. To be able to observe potential diauxic shifts, the glucose concentration was set to 3 g/L, well below the maximally consumable concentration estimated above.

Characterization of the selected solvent

Phase separation

To determine the settling behavior of the solvent in a vertical settler setup, 150 mL of solvent were continuously sparged at the height h_d of -5.4 cm with a custom-made disperser plate (18 evenly distributed holes with a diameter of 1 mm) and recirculated through 100 mL fermentation broth containing cells and RLs in a cylinder (40 mm ID). The coherent solvent was withdrawn at a height h_w of 4.5 cm (Figure 2) and recirculated by a peristaltic pump (501U, Watson-Marlow, Marlow, UK) at 211 mL/min. The coalescence curve (height of top-most not coalesced droplet within a layer of densely packed layer of droplets, defined as h_C) was plotted similar to the decay of the batch dispersion of Hartland and Jeelani [56] but depicts the formation of dispersion for the continuous settling behavior until steady state is reached. Data points were recorded, starting with the first droplet reaching the interface of the aqueous and organic phase (t = 0 s, h = 0). A stainless-steel mesh was used as a coalescer to optionally enhance coalescence.

pH-dependent extraction

To assess the influence of the pH on the extraction efficiency, the pH of the cultivation broths was adjusted to seven different values in the range of pH 3.3 to pH 10 by adding respective amounts of 1 M NaOH or 1 M HCl. Fixed phase ratios of 1:4 (v/v) organic solvent to cultivation broth were incubated at 30 °C and 1,400 rpm horizontal shaking (HLC Cooling-ThermoMixer MKR13, DITABIS AG, Pforzheim, Germany) for 4 h. Samples were taken from the aqueous and the organic phase for RL quantification.

Back-extraction

For back-extraction, double-distilled water was buffered using tris acetic acid EDTA (TAE, 40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and adjusted with 1 M NaOH and 1 M HCl to respective pH values in the range of 4.8 to 11.75. TAE was chosen as the buffer as it showed compatibility with analytical methods in the relevant concentrations. Fixed phase ratios of 1:1 (v/v) organic solvent enriched with RLs to pH-adjusted double-distilled water were incubated at 30 °C and 1,400 rpm horizontal shaking (HLC Cooling-ThermoMixer MKR13, DITABIS AG, Pforzheim, Germany) for 4 h. Samples were taken from the aqueous and the organic phases for RL quantification.

Two-phase fermentations in stirred-tank bioreactors

The most promising solvent candidate was tested in two-phase batch fermentations in 1.3 L stirred-tank bioreactors (Eppendorf, Germany) in addition to a cultivation without a solvent. The cultivations were fully controlled by BioFlo120 units and DASware Control Software 5.3.1 (both Eppendorf, Germany). A working volume of 700 mL mineral salts medium (refer to medium composition above) was inoculated from the second seed culture to an initial OD_{600} of 0.2. Due to pH control in the fermenter, the buffer concentration was reduced threefold. The pH was adjusted to the desired value (6, 6.5, or 7), monitored with online pH probes (phferm, Hamilton Company, Bonaduz, Switzerland), and automatically controlled with 4 M H_2SO_4 and 2 M KOH during the cultivation. The medium was overlaid with 100 mL of the solvent, which was neither continuously removed nor replenished during the cultivation. The dissolved oxygen tension (DOT) was maintained at above 30 % by a cascaded agitation (400 - 1,000 rpm) to prevent oxygen limitation. The airflow was kept constant at 0.7 L/min, and the cultivation temperature was set to 30 °C. The CO_2 and the O_2 concentrations in the exhaust gas were measured by BlueInOne Ferm gas analyzers, and data was recorded with BlueVis (both BlueSens, Herten, Germany).

In addition to batch cultivations, a fed-batch cultivation was performed using a DOT-based feeding strategy. When the DOT signal increased, indicating a depletion of carbon source, the feeding pump was activated. At a DOT of 70 %, the pump rate was set to 12 mL/h resulting in an addition of feed solution and a decrease of the DOT. At a DOT of 30 %, feeding was stopped until the carbon source was once again depleted, and a DOT of 70 % was reached. A total of 247 mL concentrated feed solution (refer to feed composition above) was added throughout the fermentation. The agitation was kept constant at 1,000 rpm. The initial volume was set to 400 mL mineral salt medium with an overlay of 100 mL solvent. The setpoint of the pH was manually reduced when foam covered the surface of the fermentation broth. All other parameters were set as described for batch cultivations.

Results

Reductive multi-step solvent selection

A primary selection based on physicochemical properties eliminates inapt solvent candidates

Due to the high number of possible solvents, it is too time-consuming to experimentally determine detailed extraction characteristics for all solvents. A theoretical approach based on solvent properties gathered in a database was applied as a first measure to reduce the number of potential candidates.

The database (ESI Data 1[†]) encompasses properties of 183 potential organic solvent candidates, which were selected based on solvents applied as extractants in literature, with a special focus on applications in extractive biotransformations. Solvents not forming miscibility gaps with water or being solid at standard conditions were not included. Among others, a special focus was set on the log *P* and low solubility in water, which favors the selection of biocompatible and recyclable solvents. Toxicity to humans was assessed in form of the Health Score, which is part of the solvent selection guide developed by Prat *et al.*[57,58] in the scope of the CHEM21 project. For each property, two thresholds (primary and secondary) were defined by knowledge-based assumptions. Thresholds and respective reasons for their determination are listed in Table 1.

By applying the thresholds for each parameter, solvent candidates were allocated to three categories. Solvents infringing one of the secondary thresholds were classified as 'not suitable' to be used here as an *in situ* extraction solvent. This was the case for 153 (83.6 %) of the initially listed solvents,

which were therefore no longer regarded. Notably, this included ethyl acetate, which was previously favored as an *ex situ* extractant for RLs.[16,17] Classified as 'limited' solvents infringing at least one of the primary thresholds were 12 (6.6 %) of the candidates, leaving 18 (9.8 %) classified as 'suitable'. Only the latter group of solvents was preliminarily included for further selection.

Extraction efficiencies and flash points further reduce the number of solvent candidates

The capabilities of the selected 19 solvents to extract RLs from cell-free fermentation broth were examined. The solvents showed extraction efficiencies for RLs from 18.9 % to 99.8 %, corresponding to partition coefficients ranging from 0.93 for n-hexadecane to 2,530 for 1-decanol with respect to the ratio of organic to aqueous phase of 1:4 (v/v) (Figure 3). As a high partition coefficient is advantageous from an economic and ecologic perspective since less solvent needs to be used, a minimum partition coefficient of 8 was chosen. The assumption of 4 h extracting until equilibrium was reached was confirmed by time-resolved extractions of two representative solvents (1-decanol and ethyl decanoate; ESI Data 2[†], Figure S2). Further, the flash point as a measure for flammability of the solvents needs to be considered when balancing the performance of the solvent with the effort for introducing appropriate measures for protection against fire hazards.[57,59] Solvents exhibiting a flash point lower than 100 °C were not further considered in the following selection steps to avoid safety hazards or the requirement of laboratory equipment complying with fire safety regulations (refer to Table 1). In summary, , three of the 18 solvents (1-decanol, methyl decanoate, and ethyl decanoate) fulfill the criteria of a partition coefficient above 8 and a minimum flash point of 100 °C.

Ethyl decanoate shows high biocompatibility and negligible biodegradability

In the initial screening based on the physicochemical properties of the solvents, the octanol/water partition coefficient (log *P*) was regarded as an estimation for biocompatibility. 1-Decanol, methyl decanoate, and ethyl decanoate were further investigated by monitoring the accumulation of CO_2 in the headspace of closed shake flask when the cells were cultivated in the presence of the respective solvent. As state-of-the-art methods for biomass determination were not feasible due to the formation of stable emulsions in the presence of solvents, the CO_2 accumulation serves as a measure for growth.

The influence of the respective solvent on the growth of the cells can be determined regarding the deviation of CO_2 volume concentrations in the headspace of shake flasks with solvent from a solvent-free cultivation (Table 2). Conclusions on the biocompatibility of the solvent in the presence of a preferred carbon source (here glucose) as well as the ability to degrade the solvent after depletion of the preferred carbon source can be drawn. Additional to the listed effects, the solvent can cause a prolonged initial adaptation phase of the microorganism.

The rate of CO_2 accumulation in the headspace of shake flasks in the presence of methyl decanoate (0.42 ± 0.01 h^{-1}) and ethyl decanoate (0.44 ± 0.01 h^{-1}) was very similar to the rate of the control cultivation (0.44 ± 0.01 h^{-1}) until glucose was depleted (Figure 4). This indicates a high compatibility of the solvents to *P. putida* KT2440 SK4 as growth is not impaired. In contrast, the rate of CO_2 accumulation in the presence of 1-decanol (0.35 ± 0.01 h^{-1}) is lowered in comparison to the control, suggesting impeded growth. This is not completely in agreement with the log *P* values as an indicator for biocompatibility. While ethyl decanoate has the highest log *P* (4.71) of the tested solvents, the log *P* of 1-decanol (4.57) is higher than the one of methyl decanoate (4.41) and should thus be less biocompatible. However, membrane interactions due to the cross-solubility of the solvent indicated by log *P* is not the only described phenomenon for solvent toxicity to microorganisms as thoroughly reviewed by Ramos *et al.*[60] and Heipieper *et al*.[61] In this regard, more solvent-tolerant *P. putida* strains like *P. putida* S12 or *P. putida* DOT-T1E could serve as production hosts allowing the use of more disruptive solvents as extractants.[62] Here, increased precautions to meet higher safety levels of the strains might need to be considered. Alternatively, solvent tolerance can be increased by strain engineering, as reviewed by Mukhopadhyay *et al*.[63]

After glucose depletion, displayed by a sudden decrease in CO_2 accumulation rate at a CO_2 volume concentration of around 4.5 %, the volume concentrations for the cultures with added methyl decanoate and 1-decanol increased at moderate rates (0.09 ± 0.00 h^{-1} and 0.14 ± 0.01 h^{-1}, respectively) without delay. In comparison, CO_2 accumulated at a lower rate for the cultures with added ethyl decanoate (0.02 ± 0.01 h^{-1}). This indicates a high metabolization rate of 1-decanol and methyl decanoate by *P. putida* KT2440 SK4. Ethyl decanoate, however, cannot be used as a carbon- and energy source as efficiently as indicated by a slow increase of the CO_2 volume concentration after glucose depletion. According to the categories mentioned in Table 2, 1-decanol can be classified as II C, methyl decanoate as III C, and ethyl decanoate as III B. The ideal *in situ* extraction solvent should neither impede cell growth nor be degraded. The results suggest ethyl decanoate to be closest to an ideal *in situ* extraction solvent.

Characterization of the selected solvent

Ethyl decanoate was selected as the most promising of the tested solvents for the *in situ* extraction of RLs. In addition to the physical properties evaluated above, ethyl decanoate has several advantages regarding work safety, economics, and cost-effectiveness. It does not have any hazardous classification, and due to the high flash point, no additional measures for fire hazard protection need to be established. It occurs as a fermentation ester in wine production[64,65] and is used as a food additive.[66] It can be produced without the usage of petroleum-based raw material by the esterification of the natural products ethanol and decanoate,[67] which can be purified from coconut or palm oil.[68] In regard to an industrial application, the non-purified mixture of esters derived from ethanol and fatty acids

from plant oil as well as other commercially available solvent mixtures could potentially be used for *in situ* RL extraction to lower overall process costs. However, due to intermolecular interactions their respective performances need to be confirmed. Detailed characteristics crucial for the application of ethyl decanoate (Merck, Darmstadt, Germany; purity ≥ 98 %) for *in situ* extractions of RLs were further investigated.

Coalescer accelerates phase separation

For continuous two-phase fermentations, fast phase separation is a crucial solvent-specific characteristic. Distinct differences in coalescence behavior of solvents in the presence of RLs produced by *P. aeruginosa* have been reported.[69] In the experiments for extraction efficiency, first qualitative differences in phase separation kinetics of respective solvents were observed (ESI Data 2†, Figure S3). Here, e.g., alkenes coalesced slower with increasing molecular weights. However, ethyl decanoate showed faster coalescence than other molecules with smaller molecular weights, such as 1-decene. The advantageous settling behavior was confirmed in detailed experiments for phase separation.

For a quantitative evaluation of the coalescence behavior of ethyl decanoate, the solvent was continuously dispersed through fermentation broth and recirculated. To quantify the impact of ingredients, pure water was additionally used as the aqueous phase for comparison. Between the coherent solvent and the aqueous phase, a densely packed layer of solvent droplets formed. The height of the top-most solvent droplet was recorded over time, eventually forming the coalescence curve (Figure 5A).

Shortly after the start, the coalescence curves diverged, demonstrating the influence of the different aqueous phases. The visually evaluated height of the densely packed layer of droplets in the water - ethyl decanoate system approaches only about 0.5 cm corresponding to the diameter of the biggest drops in the layer and reached steady state in approximately 15 s. Using fermentation broth as the aqueous phase revealed a faster increase of the coalescence height. This experiment was aborted after the densely packed layer of droplets reached the level of the outlet for solvent recirculation at 4.5 cm after 103 s, thus not reaching steady state. The low coalescence rate of ethyl decanoate potentially complicates the separation of the phases if a continuous removal of the coherent phase is envisioned. Withdrawing the emulsion would lead to a substantial loss of whole-cell biocatalysts. The fast coalescence of ethyl decanoate in water indicates the negative impact of produced RLs, cells, and proteins, decreasing the coalescence rate of the solvent in the fermentation broth.

When installing a coalescer in form of a stainless-steel mesh spanning from a height of 1.1 cm to 4.5 cm, the coalescence curve diverged from the one recorded in the system without coalescer as soon as first droplets contacted the mesh. However, due to the unstructured surface of the coalescer resulting in an inhomogeneous packing, droplets could rise into the coalescer up to a level of 2.8 cm, where steady-state was reached. For a single long-time experiment, the system stayed in steady-state, recirculating only coherent solvent for 14 h (Figure 5B), after which the experiment was terminated. Therefore, the installation of a coalescer increased the coalescence rate enabling a continuous, long-term solvent recirculation.

Extrapolation of the feasible phase separation in the experiment to the fermentation process is only valid if similar process conditions are implemented, ensuring the same drop size and distribution. In water, monodisperse ethyl decanoate droplets generated solely by sparging could be observed, concluding that a narrow drop size distribution was formed in the fermentation broth, too. In stirred-tank reactors (STRs), stirrers, especially Rushton turbines commonly used for bacterial cultures, induce high shear force, causing small Sauter mean diameter and a broad drop size distribution of a second phase.[70] While being advantageous for extractions due to a high volume-to-surface ratio, the formed emulsions tend to coalesce slower.[71] Nevertheless, continuous two-phase fermentations have been conducted in STRs.[30,72] The RLs produced here lower the interfacial tension of the droplets,[73] resulting in enhanced droplet breakage, thus enhanced emulsification and deteriorated phase separation at given shear stress. Therefore, continuous solvent removal was not pursued, but phase separation was achieved by centrifugation subsequently to (fed-)batch fermentations. Alternatively, for an envisioned continuous fermentation, process designs with reactor setups providing a uniform drop size distribution,[74] including a coalescer in the settler compartment, are favored.

Extraction of rhamnolipids with ethyl decanoate is dependent on pH

The dissociated form of molecules with carboxylic groups dissolves easily in water, whereas the protonated form can pass interfaces into organic phases. This principle for pH-dependent extraction has been described and modeled, e.g., for carboxylic acids[75], to which the produced RLs are similar as they exhibit a carboxyl group (Figure 1), In accordance, the pH was previously shown to have an impact on the extraction of RLs with ethyl acetate as an organic solvent[17]. However, as determined in the preliminary selection based on physicochemical properties, ethyl acetate does not meet the requirements for being applied as an *in situ* extraction solvent in bioprocesses.

In detail, the pH dependency was investigated for the extraction with ethyl decanoate in a broad pH range with a focus on the physiologically feasible range (pH 6-8) for *P. putida* KT2440. The experiments confirmed that the equilibrium concentrations of RL in the respective phases are dependent on the pH (Figure 6A). At acidic conditions, the RLs were almost exclusively present in the organic phase. In contrast, at alkaline pH, a shift of the partition coefficient below 4 at the respective phase ratio of 1:4 (v/v) was observed (Figure 6B). Considering different congeners, the pH for reaching an inversion of the partition coefficient for HAAs in favor of the aqueous phase was shifted to a more alkaline pH value in comparison to mono-RLs (ESI Data 2†, Figure S4). However, as the percentage of mono-RLs in the extracted fermentation broth was 80 % of the total amount of RLs, the sum of all HAA and mono-RL congeners, was used as a measure for extraction.

The transitional range, in which inversion of the partition coefficient is reached, roughly spans from pH 6 to pH 8. Thus, the pK_A-value of the RLs is located around neutral pH. In contrast, pK_A-values for RLs produced by *P. aeruginosa* determined by potentiometry and spectroscopic approaches are ranging from 4.28 to 5.50;[76] however, the congener composition influences the pK_A as indicated when considering the different congeners individually (ESI Data 2†, Figure S4).

RLs are amphiphilic molecules, i.e., they exhibit highly hydrophilic and hydrophobic regions. Therefore, they tend to accumulate at interfaces[77] or form micelles in bulk phases.[78] Since the presence of the RLs in respective phases depends on the pH value, their overall affinity changes most likely by the dissociation degree of the carboxylic group enabling the RLs to pass the phase boundary.

Generally, at higher pH values, more interphase was visually detected. As RL concentrations could only be measured in the coherent phases, the product entrapped in the interphase was not quantified, explaining the inconsistent total mass of RLs at different pH values. Thus, the partition coefficient was calculated from the ratio of concentrations quantified in the coherent phases. The accumulation of RLs in the interphase was previously identified as one of the main reasons for the loss of product.[79] As the formation of the interphase is strongly dependent on the pH and proteins typically present in the fermentation broth, precipitation of the proteins can prevent interphase formation and overcome product loss. However, this is not feasible for an *in situ* extraction as protein precipitation cannot be applied during fermentation. Nevertheless, the recovery of RLs from the interphase should be considered for a higher overall yield of the process.

For comparison, the same pH-dependent extraction experiments were conducted with 1-decanol and methyl decanoate. No pH dependency of the extraction was observed for 1-decanol, whereas distribution of RLs when extracting with methyl decanoate was less sensitive to pH compared to extraction with ethyl decanoate (ESI Data 2†, Figure S5). Therefore, pH dependency of RL extraction cannot be assumed universally, but is dependent on the solvent as well.

Efficient recovery of rhamnolipids and solvent is enabled by pH shift

Although often not considered in studies for two-phase fermentations, subsequent product recovery from the organic phase after *in situ* extraction has to be taken into account, as it economically and ecologically affects the overall process. Here, a thermal separation is not applicable as unreasonable amounts of energy would be required due to the high boiling point of ethyl decanoate (241.5 °C) at normal pressure. Therefore, back-extraction as a non-thermal recovery operation was assessed for pH values in the range from 4.8 to 11.75.

. In general, the back-extraction showed similar results compared to the initial extraction as it was strongly influenced by the pH (Figure 7). While the bulk of the RLs remained in the organic phase at acidic pH values, the partition coefficient shifted to a minimum, close to zero, at only slightly alkaline pH values. Therefore, the RLs can be recovered from ethyl decanoate, allowing the reuse of the solvent for further extractions. Concerning interphase formation, similar phenomena could be observed as described above for extracting RLs from the aqueous phase. Considering process design, the high sensitivity to pH proposes back-extraction of RLs as the product recovery operation subsequent to *in situ* extraction.

Application in two-phase fermentations

Ethyl decanoate was identified as the most promising solvent candidate for *in situ* RL extraction. The promising characteristics were validated in two-phase fermentations in stirred-tank bioreactors.

Ethyl decanoate can prevent foaming in two-phase fermentations

First, fermentations were conducted without *in situ* extraction. Notably, the fermentations started to foam excessively at an RL concentration of merely 18.5 mg/L shortly after inoculation. Foaming could only be controlled by the addition of large amounts of anti-foaming agent. In contrast, in the two-phase batch fermentation at pH 7, the addition of ethyl decanoate initially prevented foaming (Figure 8), thus enabling cultivation for a short time. However, to cultivate longer than 3.8 h, again, extensive amounts (> 40 mL) of anti-foaming agent (Antifoam 204, Sigma-Aldrich) had to be added. The addition of anti-foaming agent resulted in unprecise RL quantifications as indicated by enlarged error bars. Moreover, due to high costs and increased efforts in further downstream processing, the addition of large amounts of anti-foaming agent is not recommended[9,10] and was not further considered.

Foam-free fermentations prolonged by reducing the pH

The addition of ethyl decanoate enabled fermentations in STRs at reference conditions. However, the duration of the fermentation was not prolonged enough resulting in low RL concentrations at the time of foaming. As a strong dependency of the extraction efficiency on the pH value in cell-free fermentation broth was observed, its feasibility was attempted to be transferred to *in situ* extractions in the presence of *P. putida* KT2440 SK4. Here, the impact of a lowered pH on cell growth and productivity was assessed by comparing the previous batch fermentations at pH 7 (standard condition) with fermentations at pH 6.5 and pH 6. The fermentations were terminated when excessive foaming occurred, or glucose was depleted.

As shown above, at pH 7, the fermentation started to foam excessively 3.8 h after inoculation. Here, about 1.5 g/L RLs accumulated in the organic phase, and 26 mg/L remained in the aqueous phase. By reducing the pH to 6.5, foaming caused the fermentation to be terminated after 6.1 h, during which 3.2 g/L RLs were extracted into the organic phase, and 38 mg/L remained in the aqueous phase. At pH 6, no foaming occurred, and 10 g/L glucose was depleted within 10.8 h resulting in 6.8 g/L RLs present in the organic phase and about 84 mg/L in the aqueous phase translating to a yield of 0.106 g_{RL}/g_{GLC} (Figure

9A). After glucose depletion, cells did not show any respiratory activity, indicating an immediate metabolization of ethyl decanoate without adaptation to be infeasible for *P. putida* KT2440 SK4 at the given conditions. Therefore, the characteristics determined in the biocompatibility and biodegradation assay are transferable to fermentations in STRs. Interestingly, comparing the cultivations, the RL concentrations in the aqueous phases differed at the time foaming occurred. Higher concentrations at lower pH values indicate a direct impact on the foaming characteristics of RLs, which has been suggested previously.[80] Here, the undissociated RLs might have a less amphiphilic character at lower pH values resulting in a lower tendency towards foaming. The ratio of produced RLs was composed of approximately 20 % HAAs and 80 % mono-RLs, which did not alter throughout all fermentations.

Since optical density measurements or the determination of the cell dry weight were not possible due to the formation of stable emulsions, the oxygen transfer rate (OTR) was used to estimate growth rates. Assuming an equal oxygen demand for each cell at each point of time and exponential growth, the slope of the logarithmic OTR over time represents the growth rate (Figure 9B). The cultivations at pH 7 showed an OTR-derived growth rate of 0.55 h^{-1}, which is in agreement with published growth rates of *P. putida* KT2440 on glucose as the sole carbon and energy source in the absence of a solvent.[81,82] Therefore, it can be concluded that ethyl decanoate is not detrimental to the viability of *P. putida* KT2440 SK4, confirming its biocompatibility. At lowered pH values of 6.5 and 6, the OTR-derived growth rates decreased to 0.45 h^{-1} and 0.37 h^{-1}, respectively, which can be attributed to the limited acidic stress response of *P. putida* KT2440[83] and indicates non-optimal conditions for the production strain. However, the determined growth rates are still sufficiently high for efficient fermentation, therefore decreasing the pH was shown to be an efficient measure for foam prevention in two-phase fermentations while maintaining a good overall performance of the whole-cell biocatalyst for producing RLs.

Gradual pH reduction prevents foaming at optimal growth in fed-batch fermentation

As a lowered pH is disadvantageous for growth but prevents foaming, a two-phase fermentation strategy with foam-dependent reduction of the pH was developed, ensuring optimal cultivation conditions for the longest possible duration. Here, the initial pH was set to 7 and was gradually reduced every time the surface of the fermentation broth was covered with foam. pH shifts in the course of fermentations are common, however, usually focused on influencing the metabolism of the whole-cell biocatalyst for higher production[84–87] rather than on establishing fermentation stability via ISPR. The previous fermentations demonstrated that foaming could be prevented entirely at pH 6, a phase ratio of 1:7 (v/v) organic to aqueous phase and 10 g/L glucose. Here, the initial aqueous phase was reduced to 400 mL resulting in a ratio of 1:4 (v/v) organic to aqueous phase to enable the addition of feed solution after the initial 10 g/L glucose had been depleted. Thus, over time, the phase ratio decreased to 1:7 (v/v) organic to aqueous phase.

After initial glucose depletion after 8.6 h, a DO-controlled feeding was applied to enable fermentation at glucose-limited conditions, with the exception when starting the feed. Throughout the fermentation, the bulk of the produced RLs was extracted by ethyl decanoate. While the concentration of RLs in the organic phase increased over time until the end of the fermentation, the concentration in the aqueous phase remained between 1.2 g/L and 1.5 g/L after approximately 15 h (Figure 10). The pH had to be reduced in shorter time intervals at the beginning of the cultivation, whereas only one reduction was necessary after the start of the feed, although the bulk of RLs was produced in fed-batch mode. At depletion of the feeding solution, final titers of 29.6 g/L RLs in the organic phase, and 1.2 g/L RLs in the aqueous phase were achieved. This corresponds to 3.8 g produced RLs within 33.2 h (volumetric productivity of 0.16 g/L·h). At this point, a total of 52.7 g glucose was converted, translating to a yield of 0.072 g_{RL}/g_{GLC}. Both performance indicators compare well with the study of Blesken *et al.*[13], while exceeding those of Anic *et al.*[14] and Beuker *et al.*[11], all producing RLs from glucose using recombinant *P. putida* KT2440 and integrating *in situ* RL recovery by foam fractionation, partially coupled to adsorption. The yield of the batch phase, i.e., before the start of the feed, is increased by 11 % to 0.118 g_{RL}/g_{GLC} compared to the yield of the batch fermentation at a constant pH of 6 (0.106 g_{RL}/g_{GLC}) indicating a beneficial effect of the foam-dependent pH reduction. The pH had to be lowered to a minimum of 6.2 to prevent foaming. Therefore, the pH dependency for RL extraction with ethyl decanoate shown *in vitro* could be successfully transferred to fermentations, as a moderate pH shift was sufficient for foam prevention. In particular, the pH represents an easily controllable parameter in fermentation processes, which is standardly assessed and maintained in state-of-the-art fermentations. Thus, no extraordinary technical effort as required for foam fractionation or foam recycling is necessary, allowing better scalability. Increasing the phase ratio by adding more solvent or the implementation of fermentation concepts with integrated continuous solvent circulation[72,74] could enable foam prevention even at elevated pH values, thus potentially improving the yield even further.

Discussion

In this study, we developed and applied a multi-step, reductive solvent selection approach for *in situ* liquid-liquid extractions in bioprocesses. Ethyl decanoate was identified as highly suitable for *in situ* extraction of RLs produced by recombinant *P. putida* KT2440, thus preventing foaming during fermentation. A holistic process assessment considering the solvent characteristics and the resulting implications for the solvent selection approach are discussed in the following.

Holistic process assessment regarding solvent characteristics is crucial

In general, processes cannot be designed only considering a single unit operation because conditions resulting in favorable

performance indicators for one unit operation might be disadvantageous for others. Especially in case of process intensifications, where unit operations are often not separated spatiotemporally, potentially contrary effects need to be assessed carefully. For two-phase fermentations, this encompasses not only the interactions of fermentation and extraction but also further operations for product recovery.

In the presented case of selecting an extraction solvent to recover RLs from the fermentation broth *in situ*, a sole assessment of the partition coefficient and the flash point, ensuring safe processing regarding flammability, would favor the selection of 1-decanol over ethyl decanoate. However, these performance indicators do not assess solvent regeneration. As downstream processing is typically responsible for the majority of the overall process costs[88] and solvent wastage is environmentally critical,[89] a careful assessment early in process development is inevitable. Rectification is commonly applied for solvent regeneration after extraction. However, a premise for its application is a reasonably low boiling point to avoid excessive use of energy. Generally, solvents exhibiting low boiling points typically are low in molecular size. Contrarily, solvents exhibiting high flash points, which are necessary for safe operation, are high in molecular size. Even if measures for protection against fire hazards can be taken, the application of solvents of low molecular size often conflicts with lower biocompatibility due to an increased solubility in the aqueous fermentation broth expressed in a low log *P*.[90] Both solvent candidates for *in situ* RL extraction, 1-decanol and ethyl decanoate, exhibit high boiling points at ambient pressure. Therefore, rectification is economically and ecologically not suitable for further recovery. The energy required for rectification results in an unfavorable Environmental Score according to the CHEM21 solvent selection guide by Prat *et al.*[57] and caused Rosinha Grundtvig *et al.*[47] to rate both solvents as 'problematic' for their application as extractants.

In this study, we present back-extraction by pH shift as an operation for the recovery of RLs from the extraction solvent as alternative to rectification, thus bypassing the requirement of a low boiling point. For back-extraction, ecologic and economic evaluation parameters are the amounts of applied acids and bases as well as the produced salts to adjust the pH, usually further treated as waste.[91] The partition coefficient of RLs in a 1-decanol-water system was shown not to be dependent on the pH. In contrast, in an ethyl decanoate-water system, the partition coefficient could be completely inverted by shifting the pH solely by one unit from neutral to acidic or alkaline. While the pH during fermentation needs to be slightly acidic to prevent foaming and still allow sufficient growth and production, its shift into the alkaline milieu for back-extraction requires only small amounts of base and is thus ecologically and economically favorable. Further, back-extraction as recovery operation regenerates the solvent for reusage, thus minimizing its wastage, which was viewed as an exclusion criterion for the use of solvents for biosurfactant recovery by Najmi *et al*.[92] Therefore, the feasibility of applying back-extraction to recover RLs strongly favors ethyl decanoate over 1-decanol.

Contrary to an evaluation solely based on the partition coefficient, in an overall process consideration, ethyl decanoate clearly outcompetes 1-decanol to be applied as a sustainable extractant for RLs. This supports the importance of a holistic process assessment, which, among others, has previously been highlighted by Kampwerth *et al.*,[93] evaluating the overall solvent performance in a model comprised of extraction and subsequent recovery by rectification.

Reductive multi-step approach enables efficient solvent selection

In the scope of a holistic process evaluation, the solvent for a two-phase fermentation is of central importance. To decide on a suitable solvent, many solvent parameters have to be considered. Here, a selection approach based on thermodynamic models is advantageous as many solvents can be evaluated for specific applications without experimental effort. In studies by Scheffczyk *et al.*[94] and Kruber *et al.*,[95] more than 4,600 solvents were considered to extract γ-valerolactone from an aqueous phase based on the prediction of thermodynamic properties. Solvents were identified, reducing the total annual cost by more than 50 % compared to a benchmark solvent. Although the studies aim for product removal from fermentation broths, they considered a binary component system. However, the complex and undefined nature of fermentation broths might lead to inaccurate predictions of the models as thermodynamic interactions of different metabolites, salts, proteins, and cells influence the extraction performance. This conflict is reflected in simulations by Birajdar *et al.*,[96] who could qualitatively predict distribution coefficients for sequential extraction of 2,3-butanediol from fermentation broth. However, quantitative results could only be obtained after implementing adapted parameters based on experimental data. While the fermentation broth can be clarified before a sequential extraction, e.g., by precipitation and centrifugation, potentially increasing the prediction accuracy of the binary component models, this is not suitable for *in situ* extractions. Additionally, the system is dynamic, as the composition of the broth changes throughout the fermentation.

Next to evaluations of solvents by thermodynamic models, several guides have been published, which base the solvent selection on physicochemical properties.[57,89,97–101] While these mainly regard solvent usage in the chemical industry, Rosinha Grundtvig *et al.*[47] focused on solvent applications in bioprocesses. This screening of organic solvents based on the CHEM21 solvent selection guide[57] addresses environment, health, and safety challenges in early selection stages. Further, Rosinha Grundtvig *et al.*[47] state the vital necessity to experimentally evaluate pre-selected solvents, as the actual suitability of the solvent for the process is dependent on the characteristics of the overall system, including interactions of the organism, the product, and the solvent. However, the experimental effort necessary to collect data increases with the number of considered solvents, rapidly reaching such efforts

where time and resources invested outweigh the benefit of selecting the superior extraction solvent.

In accordance, we here propose a reductive, multi-step solvent selection approach. The objective of this approach is to consider many solvents and nevertheless examine critical parameters in detail by combining theoretical and experimental approaches. After a preceding selection based on physicochemical properties retaining an experimentally manageable number of solvents, further selection steps are structured in ascending order of experimental effort. The eventually chosen solvent is further characterized in detailed experiments, which consider the overall system, and finally tested in fermentations.

Next to focusing on physicochemical properties of the solvents in the data-based preselection, we favored sustainable and low-hazardous solvents. Equal to the approach by Rosinha Grundtvig *et al.* we used the Health Score of the CHEM21 solvent selection guide, but in contrast adjusted the safety and environmental considerations to the studied system. In the presented approach, especially the strong influence of the boiling point on the rating was attenuated to account for solvent recovery methods other than rectification such as back-extraction.

A disadvantage of the reductive approach could be the requirement of potential iterative loops for adjusting primarily chosen thresholds to prevent the exclusion of all solvents due to underperformance. In this case, primary thresholds would need to be adjusted by considering solvent candidates classified here as 'Limited' in the data-based screening. .

In contrast to other published guides, we here demonstrate the feasibility of the proposed selection approach by successfully applying the selected solvent, ethyl decanoate, in an *in situ* extraction of RLs produced by recombinant *P. putida* KT2440 in laboratory-scale STRs. However, we cannot surely claim that ethyl decanoate is the best solvent in terms of overall cost and efficiency for the envisioned process. Due to the reductive nature of the selection, a solvent performing better overall might have been excluded in early stages based on a single rated property. Nevertheless, ethyl decanoate was shown to be highly suitable as its application could not only resolve the initial objective of overcoming the process instability due to extensive foaming, but the pH-dependent distribution coefficient also enables efficient product recovery and sustainable solvent reuse. Further, ethyl decanoate poses little risk in handling due to its low hazardousness.

The list of solvent candidates and their respective physicochemical properties is published as open access‡ to enable a transfer of the approach to other solvent selections, specifically in the field of bioprocessing. The user can edit, extend, and refine the solvent list, as we do not claim completeness. Additionally, the thresholds for the physicochemical properties chosen here, have to be adapted for distinct applications.

Conclusion

We have developed a reductive, multi-step approach for the selection of *in situ* extraction solvents in the field of bioprocessing. Particularly, we demonstrated its applicability by establishing a two-phase fermentation for the foam-free production of rhamnolipids. Although customized to the fermentation and product system, the presented approach will facilitate other solvent selections. Its reductive multi-step character minimizes experimental effort, while still enabling the selection of highly efficient and sustainable solvents for bioprocesses until a theoretical, truly holistic methodology is available.

Author contributions

Property-based solvent screening, solvent settling behavior: PD, MVC. Extractions and back-extractions: PD, MVC, CG. Biological experiments, analytics: PD, CG. Interpreted results: PD, MVC CG, TT, AJ, LMB. Drafted the manuscript: PD, MVC. Revised and edited the manuscript: TT, AJ, LMB. All authors read and commented on the manuscript before publication. All authors approved the final manuscript.

Conflicts of interest

LMB and TT declare that they are inventors of three related patents. 1) L. M. Blank, F. Rosenau, S. Wilhelm, A. Wittgens, T. Tiso, "Means and methods for rhamnolipid production", HHU Düsseldorf University, TU Dortmund University, 2013 (WO 2013/041670 A1), 2) L. M. Blank, B. Küpper, E. M. del Amor Villa, R. Wichmann, C. Nowacki, "Foam adsorption", TU Dortmund University, 2013 (WO 2013/087674 A1), and 3) L. M. Blank, T. Tiso, A. Germer, "Extracellular production of designer hydroxyalkanoyloxy alkanoic acids with recombinant bacteria", RWTH Aachen University, 2015 (WO2017006252A1).

Apart from that, the authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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‡ https://git.rwth-aachen.de/campenhausen/list-of-solvents

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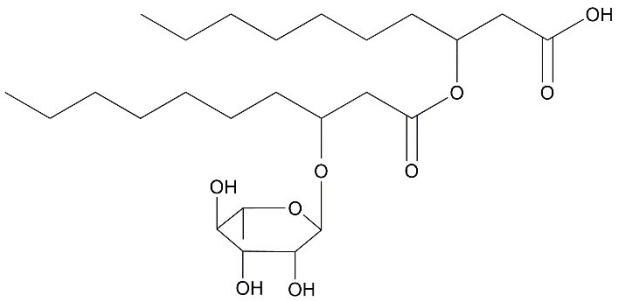

Figure 1 Representative chemical structure of rhamnolipids. The mainly produced mono-RL with two 10-carbon fatty acid tails is depicted. RLs occur as congeners varying in fatty acid tail lengths and saturation degree, as well as the number of rhamnose moieties.

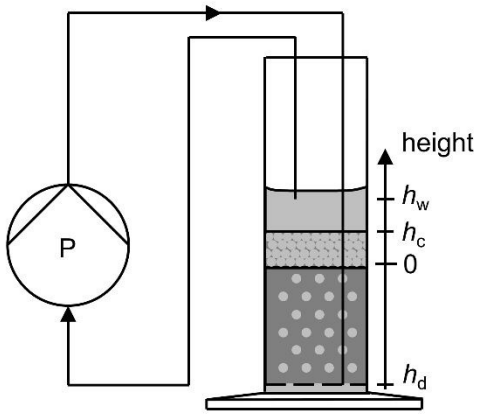

Figure 2 Schematic setup of the experiment for phase separation. The peristaltic pump (P) disperses the solvent at height h_d. The droplets coalesce at height h_c to a coherent phase being withdrawn at height h_w.

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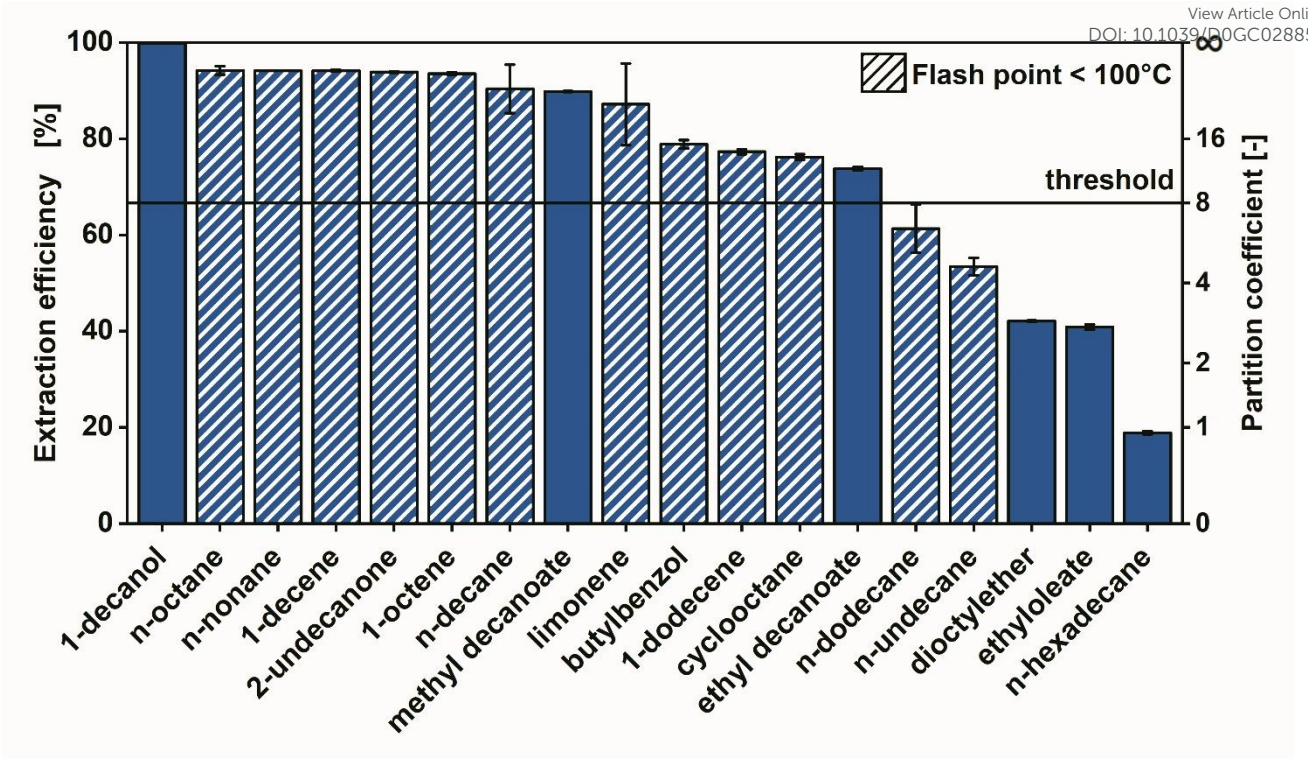

Figure 3 Extraction efficiencies of 18 organic solvents for the extraction of rhamnolipids from fermentation broth at pH 6.5. The extraction efficiency was determined from the remaining RLs in the aqueous phase after extraction relative to non-extracted cultivation broth. The black horizontal line marks the threshold for the partition coefficient of 8. Error bars represent the maximum and minimum values of measurements from two independent experiments.

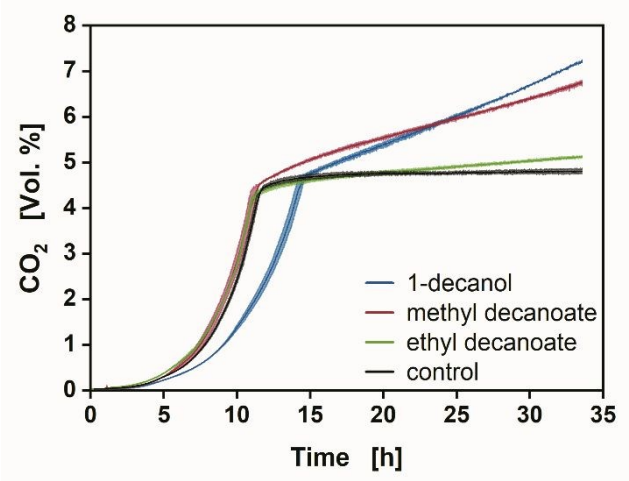

Figure 4 Mean CO_2 volume concentration in the headspace of shake flask cultivations in the presence of 1-decanol (blue), methyl decanoate (red), ethyl decanoate (green), and a control in the absence of solvent (black). Shaded areas represent the range of measurements from two independent experiments.

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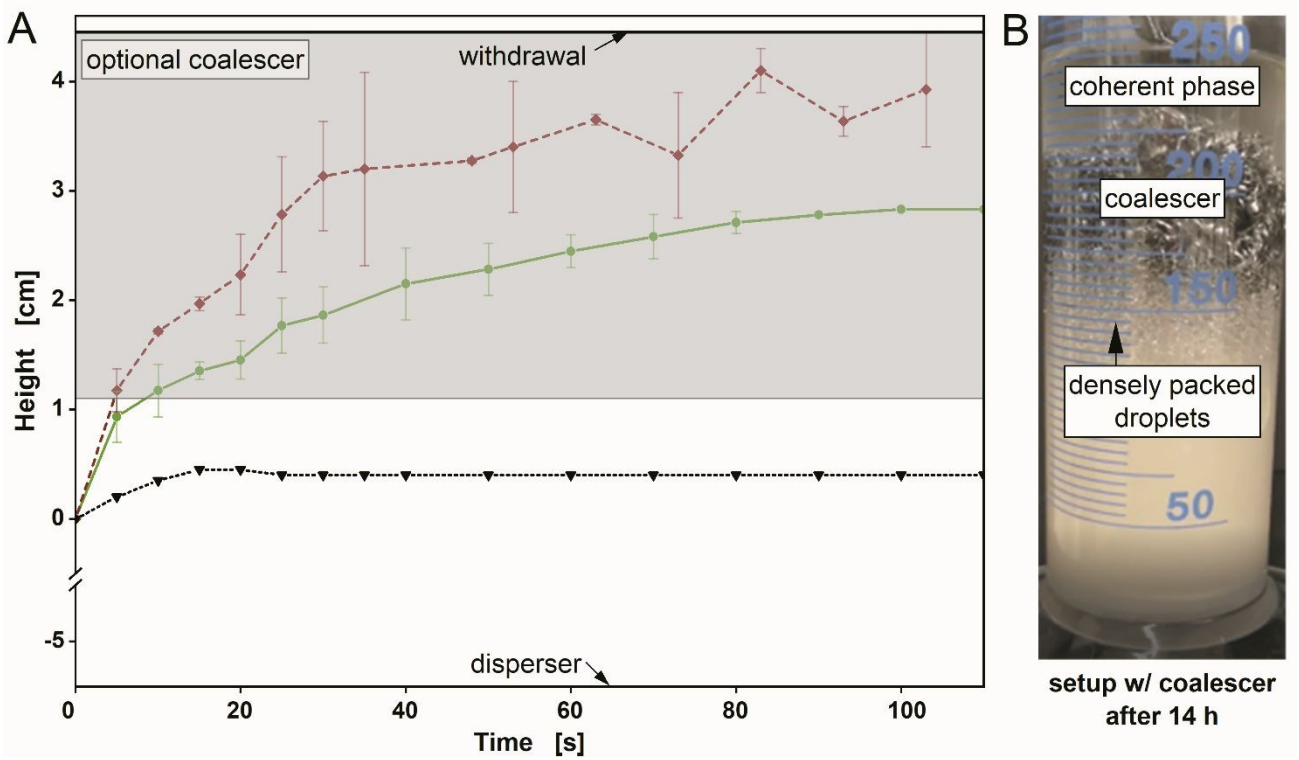

Figure 5 Coalescence curves defined by the clear cut between the densely packed drop layer and the coherent phase of ethyl decanoate. Coalescence curves for two-phase systems of **(A)** ethyl decanoate - water (black) and ethyl decanoate - fermentation broth without (red) and with coalescer (green) until 110 s and **(B)** the system with coalescer after 14 h are shown. The grey shaded area represents the location of the optional coalescer. Error bars represent standard deviations from the mean of measurements from three independent experiments.

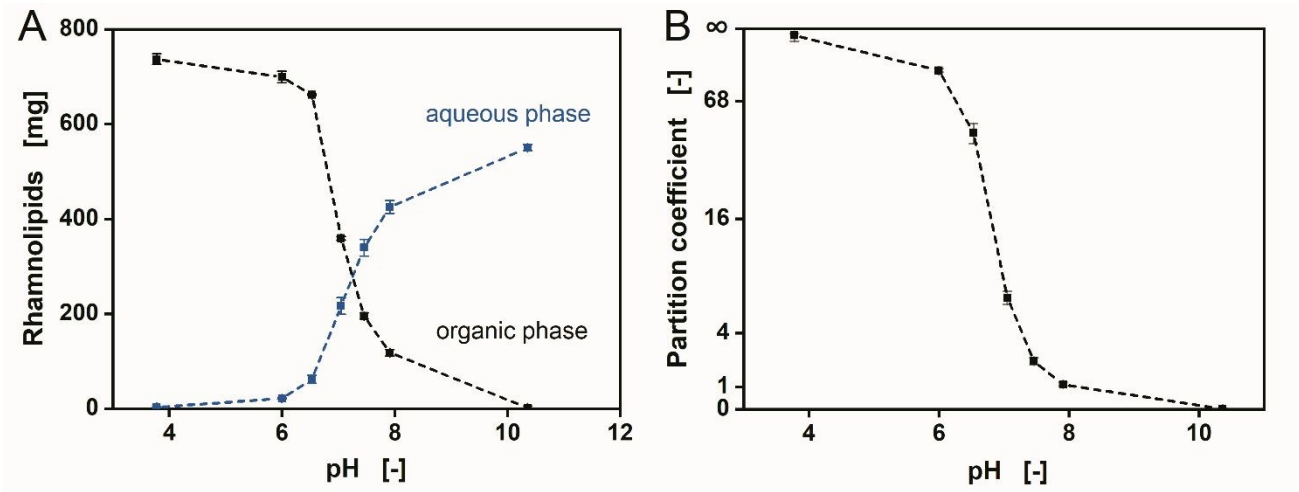

Figure 6 pH-dependency of rhamnolipid extraction with ethyl decanoate. **(A)** RL amount in the coherent ethyl decanoate (black) and aqueous (blue) phase at different pH values, and **(B)** respective partition coefficients for extraction from the aqueous phase to the organic phase. Error bars represent standard deviations from the mean of measurements from three independent experiments.

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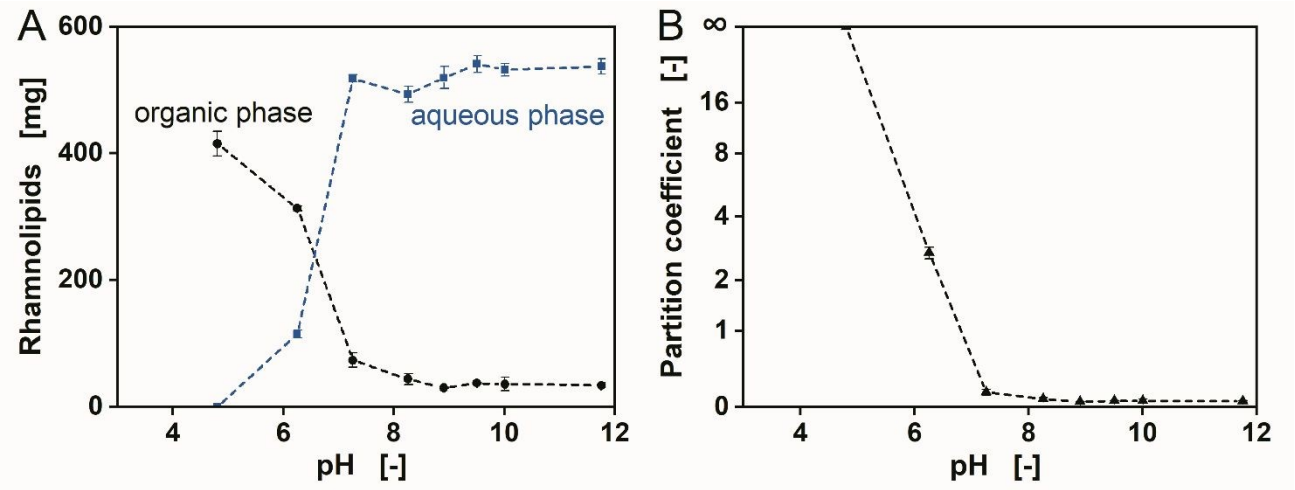

Figure 7 Back-extraction of rhamnolipids from loaded ethyl decanoate. (A) RL amount in coherent ethyl decanoate (black) and aqueous (blue) phase at different pH values, and (B) respective partition coefficients for back-extraction from the organic phase to the aqueous phase. Error bars represent standard deviations from the mean of measurements from three independent experiments.

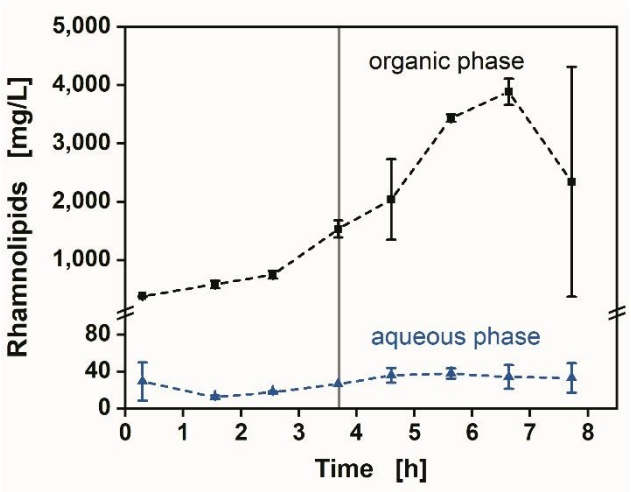

Figure 8 Extractive two-phase fermentation at standard conditions. Data for rhamnolipid concentrations in the organic phase (blue triangles) and the aqueous phase (black squares) are shown. The grey vertical line marks the time foaming occurred and the addition of anti-foaming agent. Error bars represent the maximum and minimum values of measurements from two independent experiments.

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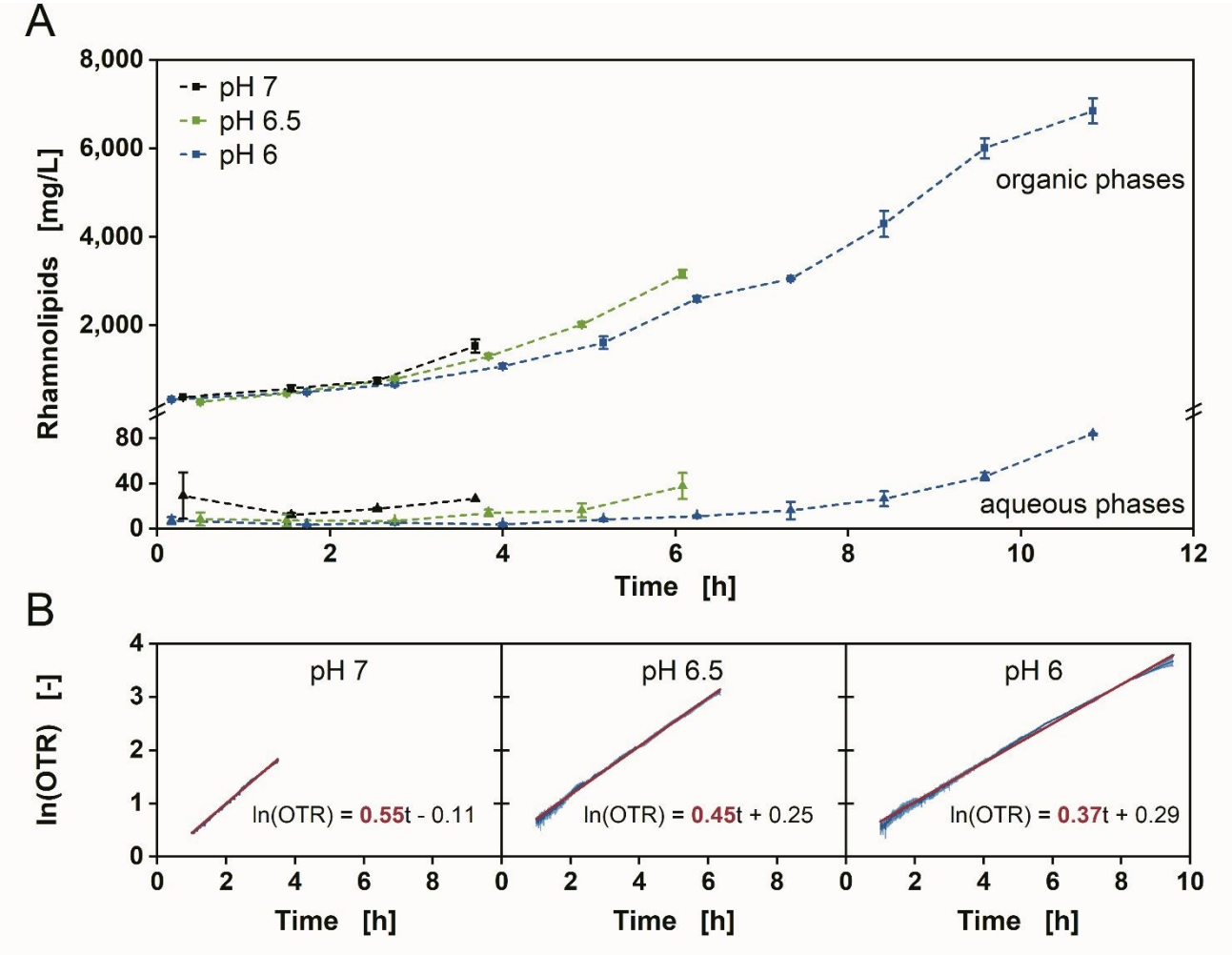

Figure 9 Production of rhamnolipids in extractive two-phase fermentations and OTR-derived growth rates at different pH values. (A) RL titers over the course of the fermentations at pH 7 (black), pH 6.5 (green), and pH 6 (blue) in organic (square) and aqueous (triangle) phases. (B) Determination of OTR-derived growth rates at respective pH values. Fermentations were terminated when foaming occurred. Error bars or borders of shaded areas represent maximum and minimum values of measurements from two independent experiments.

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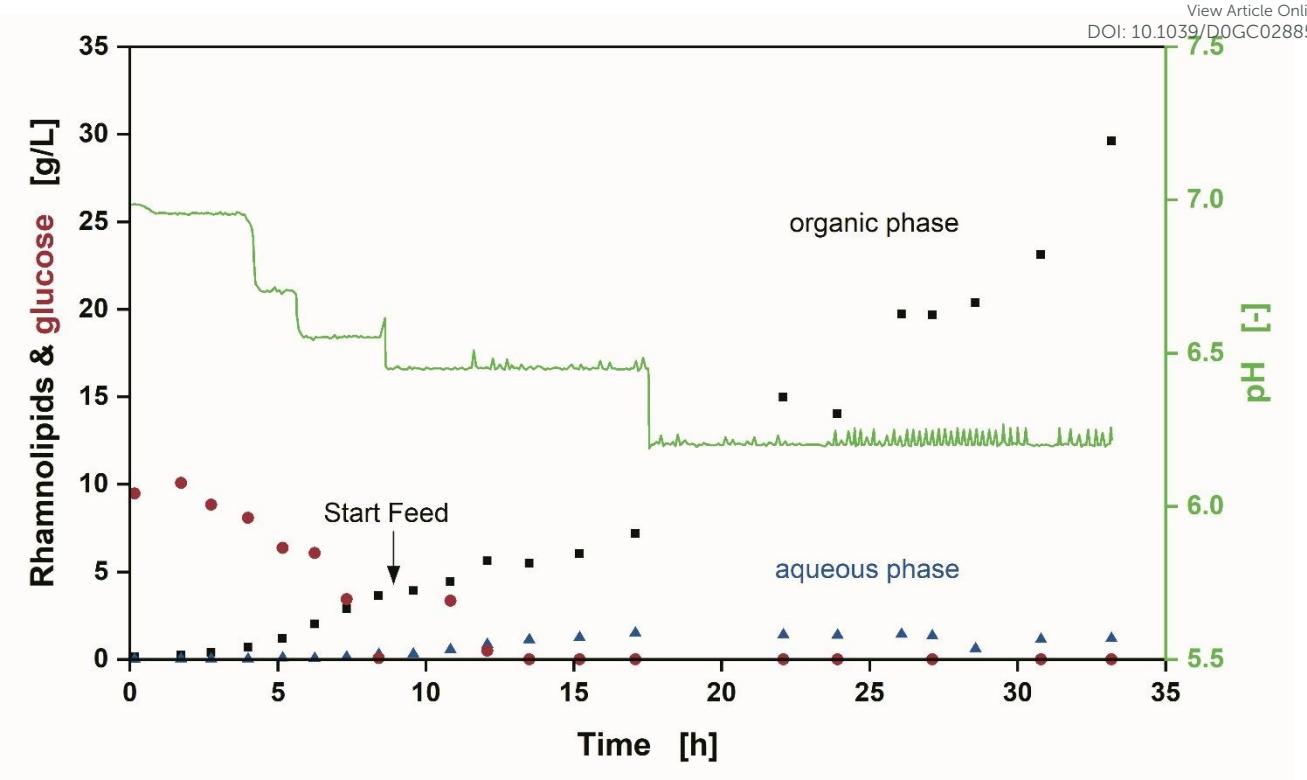

Figure 10 Production of rhamnolipids in a fed-batch fermentation with a foam-based pH reduction. RL titers in the organic (black squares) and the aqueous phase (blue triangles), glucose concentrations (red circles) as well as the pH profile (green line) are shown.

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Table 1 Thresholds and short explanations for performed solvent selection steps

Property	**Thresholds (classification)**		**Reason**
	Primary threshold ('Suitable')	Secondary threshold ('Limited')	
Density	≤ 880 g/L	≤ 920 g/L	Sufficient difference in density to the aqueous phase for gravimetric separation
Boiling point	≥ 100 °C	≥ 80 °C	Limit the loss of solvent to the gaseous phase
Solubility in water	≤ 0.3 g/L	≤ 0.5 g/L	Limit the loss of solvent to the aqueous phase
log *P*	4	3.5	Preselection for biocompatibility of the solvent
Price	≤ 100 €/100 mL	≤ 120 €/100 mL	Economically affordable range
Toxicity	Health Score[a] ≤ 2	Health Score[a] ≤ 4	Limit toxic hazards for operators
Flash point			Flash points above 100 °C avoid reaching flammability thresholds of applied solvents in aqueous fermentation systems [b]

[a] According to Prat *et al.*[57], [b]Solvents have been examined until solvent selection steps were conducted, for which protection against fire hazards could not be guaranteed using available laboratory equipment.

Table 2 Theoretical CO_2 accumulation in the headspace of the shake flask with solvents compared to a cultivation without solvents and the corresponding characteristic of the interaction of solvent and microorganism

	Before depletion of preferred carbon source			**After depletion of preferred carbon source**	
	Development of CO_2 accumulation	**Impact on cell growth**		**Development of CO_2 accumulation**	**Impact on cell metabolism**
I	None	Solvent is bacteriocidal	A	None	Solvent is not metabolized
II	Lower rate	Negative impact of solvent on growth but not bacteriocidal	B	Delayed	Adjustment of metabolism to solvent as carbon source
III	Same rate	No impact of solvent on growth	C	Immediate	Instant metabolization of solvent as carbon source