# Electronic Supplementary Information of

# 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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## Supplementary Text 1. Representative HPLC chromatogram for the detection and quantification of RL congeners

Using HPLC-CAD, eight different RL congeners (4 HAAs and 4 mono-RLs) were detected and quantified. The shown chromatogram (Figure S1) was acquired by measuring a sample originating from the ethyl decanoate phase of the two-phase fed-batch fermentation (Figure 10, main article) at 30.8 h after inoculation. The ethyl decanoate was evaporated and residuals were resolved in an appropriate volume of a 50 % acetonitrile - double distilled water solution to stay within the range of linear correlation.
Notably, the amount of hydrophilic compounds represented by the injection peak at a retention time of 1 min is low, indicating relatively high purity of hydrophobic compounds, mainly RLs.

## Supplementary Text 2. Time-resolved extraction of RLs from fermentation broth

To validate if the duration of 4 h for extracting RLs from fermentation broth applied to determine extraction efficiencies of 19 solvents was sufficient to reach equilibria, time-resolved extractions with two representative solvents, ethyl decanoate and 1-decanol, were performed (Figure S2).
The extraction with both ethyl decanoate and 1-decanol led to a rapid decrease of RLs in the fermentation broth. Equlibrium was reached after approximately 30 s and 120 s when extracting with ethyl decanoate and 1-decanol, respectively. Whereas 1-decanol depeleted RLs in the aqueous phase completely, approximately 200 mg of RLs remained in the aqueous phase when extracting with ethyl decanoate.
Concluding, the 4 h of extraction applied in the experiment for determining the extraction efficiencies was sufficiently high to reach equilibria.

## Supplementary Text 3. Qualitative differences in phase separation kinetics

In the experiments for determining the extraction efficiencies of 19 solvents qualitative differences in phase separation kinetics were observed. Representative phase separations are shown in Figure S3.
While some solvents (e.g., alkenes) formed stable emulsions or indistinct phase boundaries after being mixed with RL-containing fermentation broth for 4 h, ethyl decanoate separated distinctly form the fermentation broth without the need for centrifugation. This indicated substantial differences in phase separation kinetics, which were further characterized for ethyl deacanoate in the phase separation experiments.

## Supplementary Text 4. pH-dependent extraction of RLs with ethyl decanoate resolved for different congeners

As eight different RL congeners were detected and quantified with the presented HPLC measurements, the influence of the pH on the extraction of respective congeners, which are present in the fermentation broth, could be determined (Figure S4).

In general, the distributions of mono-RLs in the aqueous and organic phases are similar for all identified congeners. At pH 6, the bulk of the respective congener is present in the ethy decanoate phase, whereas the distribution is inverted at pH 8. Therefore, the extraction of mono-RLs is highly sensitve to slight pH changes of ±1 unit around neutral pH. In contrast, the distribution of HAA congeners is mostly in favor of the ethyl decanoate phase up to a pH value of up to 8, with exception of the $C_{10}$-$C_8$ congener. However, an inversion of HAA distribution can be achieved at higher pH values, here measured for pH 10.36. As there is a mixed congener distribution present in the fermentation broth as a result of microbial production with *P. putida* KT2440 SK4, the presence of the other congeners might have had an influence on the extraction of the respective single congener. Individual extraction experiments with pure congeners have not been conducted, as ethyl decanoate was intended to be used as an *in situ* extraction solvent, therefore always encountering a mixture of congeners.
It has to be noted that the total amount of HAAs in the fermentation broth is low (20 %) in comparison with the amount of mono-RLs (80 %). Therefore, the sum of all HAA and mono-RL congeners, referred to as RLs, was used as a measure for extraction efficiency (Figure 6 in the main article).
Further, a pH-dependency of the extraction was not observed for methyl decanoate (Figure S5A), but the range for a complete inversion of distribution spanned approximately 4 pH units (pH 6 – pH 10) and was thus shifted to the alkaline milieu when compared to the extraction with ethyl decanoate. In case of 1-decanol used as an extractant, the pH did not influence the distribution of RLs (Figure S5B).

## Supplementary Figures

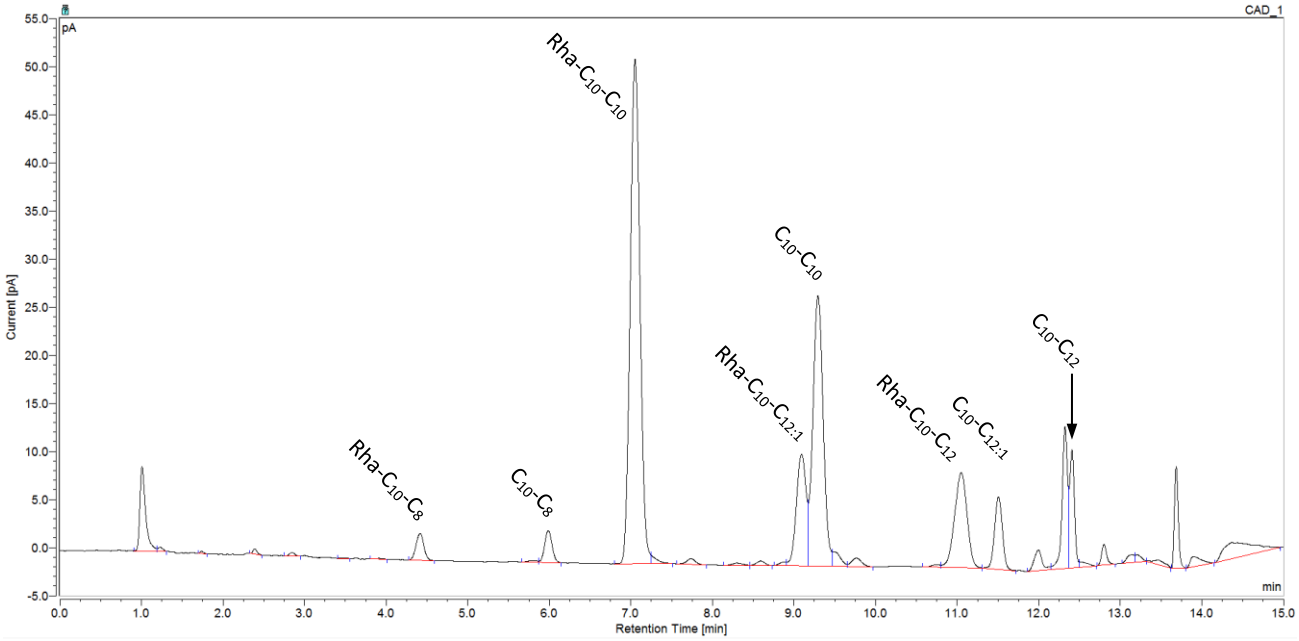


**Figure S1** Representative HPLC-CAD chromatogram. Peaks of all eight identified congeners are annotated.

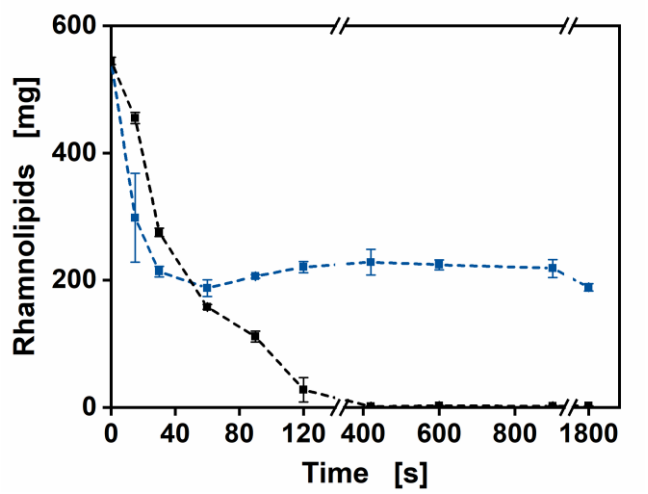


**Figure S2** Time-resolved depletion of rhamnolipids in the aqueous phases. Extractions were performed with ethyl decanoate (blue squares) and 1-decanol (black squares). Error bars represent standard deviations from the mean of measurements from three independent experiments.

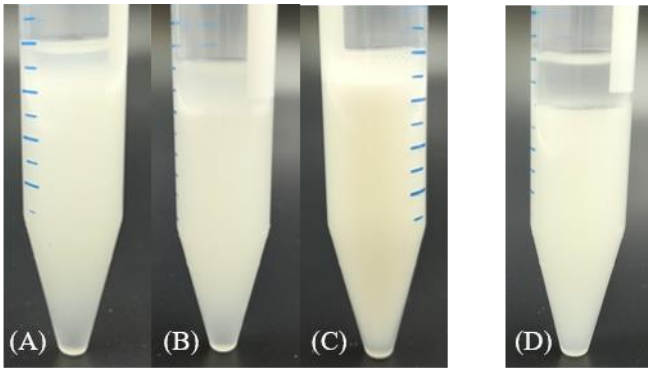


**Figure S3** Selected solvents after shaking for 4 h before centrifugation. Alkenes (A - 1-octene, B - 1-decene, C - 1-dodecene) and ethyl decanoate (D)

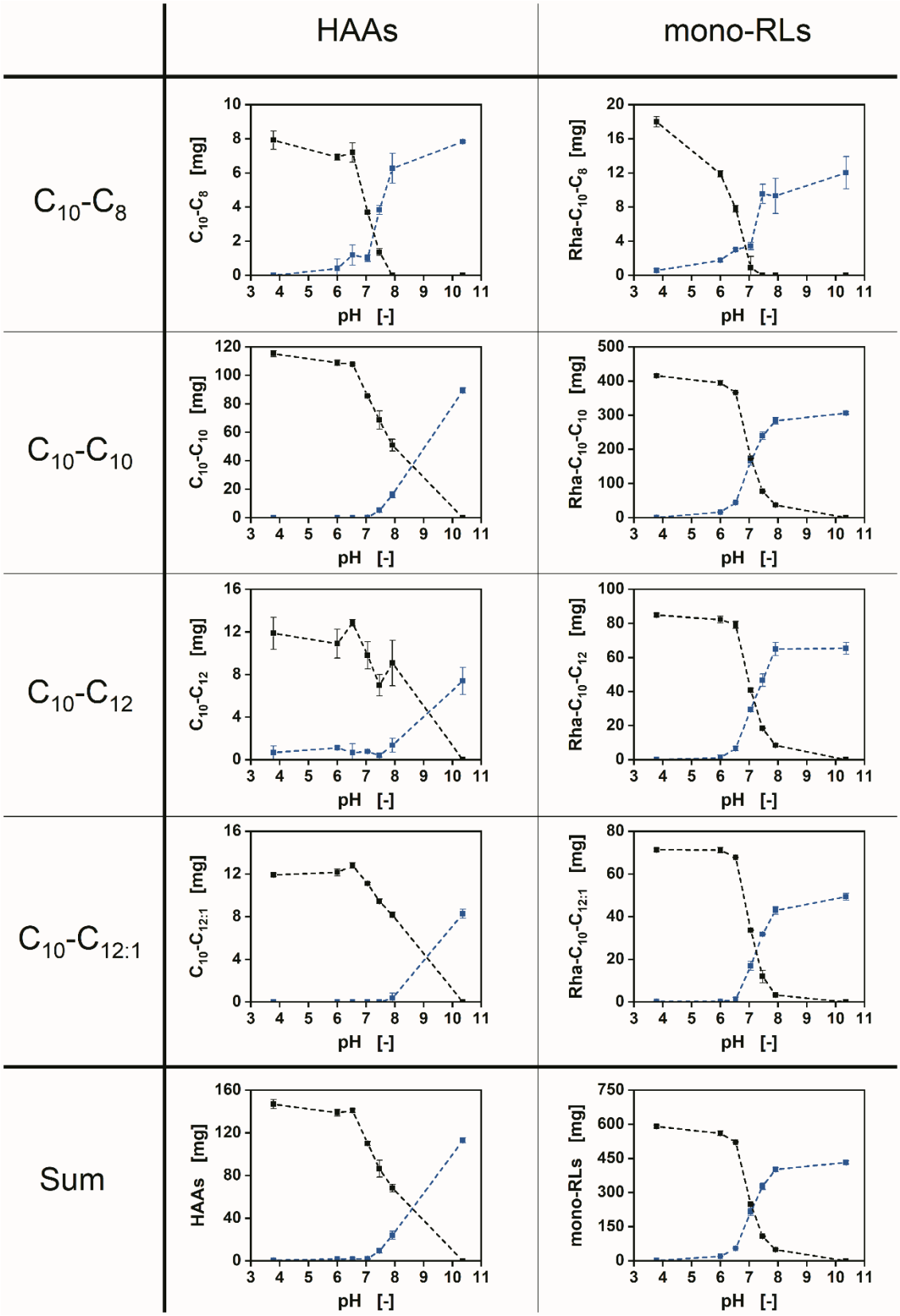


**Figure S4** pH-dependency of rhamnolipid extraction with ethyl decanoate resolved for measured congeners. Rhamnolipid congeners in the coherent ethyl decanoate phase (black) and the aqueous phase (blue) at different pH values. Columns differentiate between HAAs and mono-rhamnolipids (mono-RLs) and lines differentiate between chain length and saturation of the fatty acid groups. Composite masses of all HAAs and all mono-RLs are given in the bottom line. An overall composite, including both HAAs and mono-RLs, is presented in the main article (refer to Figure 6). Error bars represent standard deviations from the mean of measurements from three independent experiments.

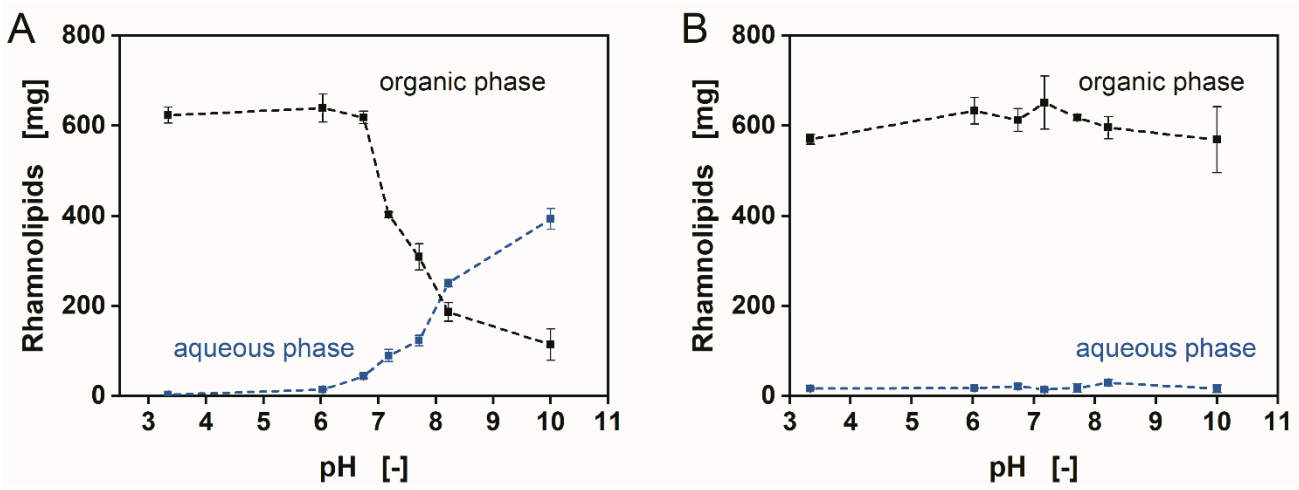


**Figure S5** Present rhamnolipids in coherent organic (black) and aqueous (blue) phases at different pH values. (A) Methyl decanoate, (B) 1-decanol. Error bars represent standard deviations from the mean of measurements from three independent experiments.