Genetic Cell-Surface Modification for Optimized Foam Fractionation

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Rhamnolipids are among the glycolipids that have been investigated intensively in the last decades, mostly produced by the facultative pathogen Pseudomonas aeruginosa using plant oils as carbon source and antifoam agent. Simplification of downstream processing is envisaged using hydrophilic carbon sources, such as glucose, employing recombinant non-pathogenic Pseudomonas putida KT2440 for rhamnolipid or 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA, i.e., rhamnolipid precursors) production. However, during scale-up of the cultivation from shake flask to bioreactor, excessive foam formation hinders the use of standard fermentation protocols. In this study, the foam was guided from the reactor to a foam fractionation column to separate biosurfactants from medium and bacterial cells. Applying this integrated unit operation, the space-time yield (STY) for rhamnolipid synthesis could be increased by a factor of 2.8 (STY = 0.17 gRL/L·h) compared to the production in shake flasks. The accumulation of bacteria at the gas-liquid interface of the foam resulted in removal of whole-cell biocatalyst from the reactor with the strong consequence of reduced rhamnolipid production. To diminish the accumulation of bacteria at the gas-liquid interface, we deleted genes encoding cell-surface structures, focusing on hydrophobic proteins present on P. putida KT2440. Strains lacking, e.g., the flagellum, fimbriae, exopolysaccharides, and specific surface proteins, were tested for cell surface hydrophobicity and foam adsorption. Without flagellum or the large adhesion protein F (LapF), foam enrichment of these modified P. putida KT2440 was reduced by 23 and 51%, respectively. In a bioreactor cultivation of the non-motile strain with integrated rhamnolipid production genes, biomass enrichment in the foam was reduced by 46% compared to the reference strain. The intensification of rhamnolipid production from hydrophilic carbon sources presented here is an example for integrated strain and process engineering. This approach will become routine in the development of whole-cell catalysts for the envisaged bioeconomy. The results are discussed in the context of the importance of interacting strain and process engineering early in the development of bioprocesses.

Keywords: rhamnolipid, 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA), integrated product recovery, foam fractionation, cell surface hydrophobicity, large adhesion protein, flagellum, metabolic engineering
INTRODUCTION

Bio-based materials such as biosurfactants are in high demand (Müller et al., 2012), as their use potentially lowers the carbon footprint compared to fossil-based surfactants. Biosurfactants like rhamnolipids and derivatives can be utilized for a wide range of industrial applications, e.g., in the chemical, cosmetic, pharmaceutical, and food industries, as well as for bioremediation of polluted soils and enhanced oil recovery (Banat et al., 2000; Singh et al., 2007; Kosaric and Vardar-Sukan, 2015). Latest publications discuss the use of hydroxalkanoyloxy alkanoates (HAA s), representing the hydrophobic moiety of rhamnolipids (RLs), for the conversion to biofuel (Mukherjee et al., 2006; Winterburn et al., 2011; Winterburn and Martin, 2012). However, foaming can also be an in situ product removal technique. In a foam fractionation column, the denser culture medium drains through the foam back into the reactor. It is only retarded by the shear force experienced at the gas-liquid interface (Stevenson and Li, 2014). For microbial rhamnolipid production, foam fractionation is reported (Siemann and Wagner, 1993; Heyd et al., 2011; Beuker et al., 2016b; Anic et al., 2018). Foam fractionation is a cost-effective technology that requires only simple technical installations. However, for efficient ex or in situ foam fractionation, the loss of biocatalyst from the reactor due to biomass accumulation in the foam is a challenge. Foam adhesion of cells might be influenced by the cell surface hydrophobicity (CSH).

So far, the CSH of Pseudomonas strains is mainly discussed in the context of biofilm formation and general stress adaptation (Heipieper et al., 2007; Baumgarten et al., 2012a; Eberlein et al., 2018). In Pseudomonas, several cell surface molecules contributing to changes in CSH have been identified, such as the lipopolysaccharide layer (LPS) (Makin and Beveridge, 1996; Kobayashi et al., 2000). Pseudomonas putida’s large adhesive protein A (Lap A) and particularly F (LapF) increase cell surface hydrophobicity (Lahesaare et al., 2016). In P. putida KT2440, LapA is the largest surface protein and required for cell-to-cell as well as for abiotic surface interactions (Hinsa et al., 2003; Fuqua, 2010). LapF is the second largest surface protein with a key role in the development of a mature biofilm (Martinez-Gil et al., 2010). Additionally, it was shown that the release of outer membrane vesicles (OMV) as a general stress response mechanism increases CSH in P. putida (Baumgarten et al., 2012b). For the non-flagellated P. putida KT2440, a significantly lowered surface hydrophobicity was determined (Martinez-Garcia et al., 2014).

We present rhamnolipid and HAA production with recombinant P. putida KT2440 in a bioreactor equipped with a foam fractionation column. The challenge of cell loss during foam discharge was tackled by strain engineering. More than ten different cell surface structure deletion mutants were tested for lowered CSH. In a newly established experimental setup, the correlation of a reduced CSH with a lower tendency for cell enrichment in the foam was confirmed. Here, especially the deletion of the flagellar machinery, LapF, and LapF in combination with LapA reduced biomass adhesion to the foam. Indeed, cell surface engineered strains allowed stable rhamnolipid and HAA production using foam fractionation for integrated product removal from the bioreactor.
### TABLE 1 | Bacterial strains and plasmids used in this study.

| Strains and plasmids | Characteristics | References or sources |
|----------------------|-----------------|----------------------|
| **E. coli**          |                 |                      |
| Pir2                 | F−, Δlac169, rapS(Am), robB1, creC510, hsdR514, endA, recF1994, recA1, strR514ΔM(h)1::pir, pir::oriV(R6K) vectors | ThermoFisher Scientific |
| Pir2 pBG14fgf        | Pir2 harboring Tn7 delivery vector pBG14fgf; containing BCD2-msgfip fusion | Köbbing et al., 2020 |
| HB101 pRK2013        | SmR, hsdR-M′, proA2, leuB6, thi-1, recA; harboring plasmid pRK2013 | Ditta et al., 1980 |
| Pir2 pKS03           | Pir2 harboring Tn7 delivery vector pKS03 for chromosomal integration; containing riaA from P. aeruginosa PA01; pBG derivative | This study |
| Pir2 pEMG-pvd         | Pir2 harboring plasmid pEMG-pvd | This study |
| Pir2 pEMG-flag1       | Pir2 harboring plasmid pEMG-flag1 | This study |
| Pir2 pEMG-flag2       | Pir2 harboring plasmid pEMG-flag2 | This study |
| Pir2 pEMG-alg         | Pir2 harboring plasmid pEMG-alg | This study |
| Pir2 pEMG-bcs         | Pir2 harboring plasmid pEMG-bcs | This study |
| Pir2 pEMG-pee         | Pir2 harboring plasmid pEMG-pee | This study |
| Pir2 pEVS12S-peb      | Pir2 harboring plasmid pEVS12S-peb | This study |
| Pir2 pEMG-lapA        | Pir2 harboring plasmid pEMG-lapA | This study |
| Pir2 pEMG-lapF        | Pir2 harboring plasmid pEMG-lapF | This study |
| DH5α-Δpir pαpha       | DH5α-Δpir harboring plasmid pαpha | Mato Aguirre, 2019 |
| DH5α pSW-2            | DH5α harboring plasmid pSW-2 encoding I-SceI nuclease, tool for genomic deletion | Martinez-Garcia and de Lorenzo, 2011 |
| DH5α pTNS1            | DH5α-Δpir harboring plasmid pTNS1 | Choi et al., 2005 |
| DH5α pTNS1 pSK02       | DH5α-Δpir harboring Tn7 delivery vector pSK02 for chromosomal integration; containing riaAB genes from P. aeruginosa PA01 | Bator et al., 2020 |
| **P. taiwanensis**   |                 |                      |
| VLB120               | wild type       | Panke et al., 1998   |
| P. putida DTT1E       | wild type       | Ramos et al., 1998   |
| S12                  | wild type       | Hartmans et al., 1990 |
| KT2440               | wild type       | Bagdasarian et al., 1981 |
| KT2440 Δflag          | ΔPP_4328-4344, ΔPP_4351-4397 deletion of flagellum operon | This study |
| KT2440 Δalg           | ΔPP_1277-1288 deletion of alginate operon | This study |
| KT2440 Δabc           | ΔPP_2634-2638 deletion of cellulose operon | This study |
| KT2440 Δape           | ΔPP_2312-2314 deletion of exopolysaccharide a operon | This study |
| KT2440 Δapeb          | ΔPP_1795-1798 deletion of exopolysaccharide b operon | This study |
| KT2440 ΔlpaA          | ΔPP_0168 deletion of large adhesion protein A operon | This study |
| KT2440 ΔlpaF          | ΔPP_0806 deletion of large adhesion protein F operon | This study |
| KT2440 ΔlpaAΔlpaF      | ΔPP_0168, ΔPP_0806 cumulative deletion of lapA and lapF | This study |
| KT2440 Δpha           | ΔPP_5003-5008 deletion of polyhydroxyalkanoate operon | This study |
| KT2440 ΔfimbriaeΔpili | ΔPP_1887-1891, ΔPP_2357-2363, ΔPP_4986-4992, ΔPP_5080-5083, ΔPP_6007-6011 deletion of fimbriae and pili operon | BacMine S. L., unpublished |
| KT2440 ΔfimbriaeΔpiliΔcurli | ΔPP_1887-1891, ΔPP_2357-2363, ΔPP_4986-4992, ΔPP_5080-5083, ΔPP_6007-6011, ΔPP_1993-1993 deletion of fimbriae, pili and curli operon | BacMine S. L., unpublished |
| KT2440 GR20           | ΔPP_4219-4221, ΔPP_4328-4344, ΔPP_4351-4397, ΔPP_1277-1288, ΔPP_2634-2638, ΔPP_2312-2314, ΔPP_1795-1798, ΔPP_0168, ΔPP_0806, ΔPP_5003-5008 cumulative deletion of lapA and lapF, pyoverdine, flagellum, alginate, cellulose, exopolysaccharide a & b, polyhydroxyalkanoate operon | This study |
| KT2440 KS3            | attTn7::Pγ3-riaA | This study |
| KT2440 SK4            | attTn7::Pγ3-riaAB | This study |
| KT2440 ΔlpaF_HAA       | P. putida KT2440 ΔlpaF with attTn7::Pγ3-riaA | This study |
| KT2440 ΔlpaF_RL         | P. putida KT2440 ΔlpaF with attTn7::Pγ3-riaAB | This study |
| KT2440 ΔlpaAΔlpaF_RL    | P. putida KT2440 ΔlpaAΔlpaF with attTn7::Pγ3-riaAB | This study |
| KT2440 ΔlpaF_RL         | P. putida KT2440 ΔlpaF with attTn7::Pγ3-riaA | This study |
| KT2440 GR20_RL         | KT2440 GR20 with attTn7::Pγ3-riaAB | This study |
MATERIALS AND METHODS

Bacterial Strains and Plasmids

All bacterial strains and plasmids used in this study are listed in Table 1. The deletion mutants were constructed by using the I-SceI-system described by Martinez-Garcia and de Lorenzo (2011). Briefly, 500–800 bp upstream and downstream flanking regions of the target sites were amplified from the genomic DNA of P. putida KT2440 and cloned into the non-replicative pEMG (Km\(^R\)) or pSEVA512S (Tc\(^R\)) vector. The resulting plasmids were transferred into *Escherichia coli* PIR2 and conjugated into *Pseuodomnas* strains via triparental mating. The plasmid pSW-2, encoding for the I-SceI restriction enzyme, was transformed to allow for the deletion of the gene locus of interest. Positive colonies sensitive for kanamycin or tetracycline were verified for targeted deletion by colony polymerase chain reaction (PCR). To obtain marker-free clones, the recombinant strains were cured of pSW-2 plasmid by re-inoculation in lysogeny broth (LB) medium without gentamycin and verified again by colony PCR (Supplementary Figure 1). After verification via colony PCR, the single deletion strains were sequenced by Eurofins Genomics (Ebersberg, Germany) to exclude mutations. In this study, twelve knock-out mutants were engineered (Table 1).

The construction of the rhamnolipid production strains was performed by using the mini-Tn7 delivery transposon vector pSK02 as described previously (Bator et al., 2020). Mono-rhamnolipid producing clones were identified using cetrimide-blood agar plates (7.5% (v/v) sheep blood, Fiebig-Nährstofftechnik, Idstein-Niederauroff, Germany). The HAA production strain was constructed using vector pKS03, which was generated based on pSK02. Using primers KS08 and KS02 a DNA fragment (4,393 bp) was obtained for the construction of plasmids and verification of deletion assembly. The resulting mini-Tn7 vector pKS03 was transferred to a streamlined method (KT2440 and cloned into the non-replicative pEMG (NH\(_4\))\(_2\)SO\(_4\) and the trace elements 10 mg EDTA, 0.1 mg MgCl\(_2\)·6 H\(_2\)O, 2 mg ZnSO\(_4\)·7 H\(_2\)O, 1 mg CaCl\(_2\)·2 H\(_2\)O, 5 mg FeSO\(_4\)·7 H\(_2\)O, 0.2 mg Na\(_2\)MoO\(_4\)·2 H\(_2\)O, 0.2 mg CuSO\(_4\)·5 H\(_2\)O, 0.4 mg CoCl\(_2\)·6 H\(_2\)O, and 1 mg MnCl\(_2\)·2 H\(_2\)O.

Bacterial Foam Adhesion

In order to determine biomass flotation characteristics in foam, cells were cultivated in shake flasks (start OD\(_{600}\) = 0.01, MSM, 10 g/L glucose) and harvested in the late exponential phase, at optical densities in between OD\(_{600}\) 4 and 6. A defined amount of biomass was washed twice with 25 mL 0.9% (w/v) NaCl. The pellet was resuspended in 37 mL 0.9% (w/v) NaCl to reach an OD\(_{600}\) of about 2. Subsequently, 3 mL of a purified aqueous rhamnolipid solution was added to the suspension to reach a final rhamnolipid concentration of 0.6 gRL/L. Before the fractionation, the pH values of all suspensions ranged from 6.2 to 6.6. The used foam fractionation glass column (O\(_{inner}\) = 32 mm, h = 600 mm) was fixed in an upright position. The sparger mounted on the column bottom had a pore size of 20 μm (bibi-biotech GmbH, Berlin, Germany). As soon as the cell suspension was filled into the column, the air flow \(V_g\) was set to 5 L/h (corresponding to a gas superficial velocity \(j_G\) = 10.36 cm/min) by a rotameter (RGC2422, Yokogawa GmbH, Ratingen, Germany) at an overpressure of 0.5 bar. The foam raised to the upper column opening where it dropped into a funnel connected to a flask with a thin tube (O\(_{inner}\) = 1 mm) to collapse the foam. The collection flask was set under low pressure (0.5 bar) using a vacuum pump (Type 115053, ILMVAC GmbH, Ilmenau, Germany). The fractionation was terminated after 30 min by turning off the gas flow. When the foam in the collection vessel (foamate) collapsed completely, samples were taken for OD\(_{600}\) measurement. The whole procedure is illustrated in Figure 1.

Contact Angle Measurement

The evaluation of the surface hydrophobicity was carried out by the water contact angle measurement technique, as developed by Neumann et al. (2006). The strains were cultivated in shake flasks (start OD\(_{600}\) = 0.1, MSM, 10 g/L glucose) until an OD\(_{600}\) > 0.5 was reached. The cells were harvested by centrifugation and resuspended in 1.8 mL 0.9% (w/v) NaCl. This washing step was repeated twice before 500 μL of the suspension was added to 19.5 mL 0.9% (w/v) NaCl solution. The suspension was vacuum filtered to obtain a cell lawn on the membrane filter (O\(_{pores}\) = 0.45 μm, Labsolute Th. Geyer GmbH & Co. KG, Renningen, Germany). The filter with the bacterial lawn was dried for 2 h at room temperature before the contact angle measurement was performed via the automated analysis system DSA100 with a
A 3 µL water droplet was placed on the bacterial lawn and the angle was measured after 80 ms.

**Fermentation Setup**

The fermentation was performed using a BioFlo 120 bioreactor system with a DASWare control (Version 5.0) software package (both Eppendorf AG, Hamburg, Germany). The applied vessel with a total volume of 3 L was filled with 2 L minimal medium containing 20 g/L glucose. The conducted fermentation procedure was separated into two phases: 1. the growth phase to reach a defined biomass concentration and 2. the harvest phase. The stirred reactor (800 rpm) was inoculated with a preculture to an OD\(_{600}\) of 0.2. The here applied preculture was previously incubated for 12 h (start OD\(_{600}\) = 0.1, MSM, 20 g/L glucose) to gain an OD\(_{600}\) > 6. When the bioreactor culture reached an OD\(_{600}\) > 0.5, the gassing rate through a ring sparger was turned on (0.25 vvm) to prevent oxygen limitation. The dissolved oxygen (DO) was maintained at 30% by the addition of pure oxygen. The appearing foam left the reactor through the air exhaust into a foam centrifuge (Foamex 5 LS, Heinrich Frings GmbH & Co. KG, Rheinbach, Germany) collapsing the foam at 4,000 rpm. The foamate was pumped back into the reactor with 235 mL/min. After the foam formation exceeded the foamate reflux, the fractionation column (\(O_{\text{inner}} = 135\) mm, \(h = 190\) mm) with a drainage pump (\(V_{\text{drainage}} = 50\) mL/min) was introduced between the reactor exhaust and the foam centrifuge. The stirring speed in the fermenter was reduced from 800 to 500 rpm. After 10 h of cultivation, 40 g glucose and trace elements (20 mg EDTA, 0.2 mg MgCl\(_2\) · 6 H\(_2\)O, 4 mg ZnSO\(_4\) · 7 H\(_2\)O, 2 mg CaCl\(_2\) · 2 H\(_2\)O, 10 mg FeSO\(_4\) · 7 H\(_2\)O, 0.4 mg Na\(_2\)MoO\(_4\) · 2 H\(_2\)O, 0.4 mg CuSO\(_4\) · 5 H\(_2\)O, 0.8 mg CoCl\(_2\) · 6 H\(_2\)O, and 2 mg MnCl\(_2\) · 2 H\(_2\)O) were added to the broth. A second glucose feed was applied throughout the harvest period to prevent limitations. When a biomass concentration in the reactor of 5 g\(_{\text{CDW/L}}\) was reached, the process was moved from the growth phase to the harvest phase. The surfactant harvest was initiated by stopping the foamate reflux into the reactor (Figure 2). Instead, the foamate leaving the system was collected and weighed. A gas superficial velocity of \(j_g = 3.49\) cm/min was reached in the fractionation column. The filling volume in the reactor was maintained at 2 L every 2 h by addition of fresh medium.

**Sampling and Processing**

From shake flask cultivations, less than 750 µL sample were taken per sampling. From bioreactor cultivations, samples were taken from the reactor broth and the foamate. The OD\(_{600}\) was measured using an Ultrospec 10 cell density meter (Biochrom, Cambridge, UK). An OD\(_{600}\) of 1.0 corresponds with a determined cell dry weight, as listed in Supplementary Table 2. Glucose was analyzed as described previously (Hosseinpour Tehran et al., 2019) in a Dionex Ultimate 3000 HPLC system, composed of the pump ISO-3100, the autosampler WPS-3000, and the column oven TCC-3000, connected to a DIONEX UltiMate 3000 Variable Wavelength Detector set to 210 nm (Thermo Fisher Scientific Inc., Waltham, MA, USA) and an RI detector SHODEX RI-101 (Showa Denko Europe GmbH, Munich, Germany) equipped with an ISERA Metab AAC 300 x 7.8 mm column (particle size: 10 µm, ISERA GmbH, Düren, Germany). The ammonium concentration in the culture supernatant was measured by a colorimetric method according to Willis et al. (1996), using salicylate and nitroprusside. For the determination of rhamnolipid and HAA concentrations, analytical methods and sample preparations were performed according to Bator et al. (2020), based on a method developed previously (Behrens et al., 2016; Tiso et al., 2016). Briefly, a RP-HPLC Ultimate 3000 HPLC system, composed of the pump LPG-3400, the autosampler WPS-3000, and the column oven TCC-3000, connected to a Corona Veo charged aerosol detector (CAD) (all Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a NUCLEODUR C18 Gravity 150 × 4.6 mm column (particle size: 3 µm, Macherey-Nagel GmbH & Co. KG, Düren, Germany) was used. All components were identified via the retention time and quantified via the peak area compared to corresponding standards.

**Data Analysis**

To define process parameters, the following equations were used: \(X\) is biomass, \(S\) is glucose, \(P\) is product, \(t\) is time, \(V\) is volume flow, \(V\) is volume, \(A\) is area, and \(m\) is mass. The yields of biomass from glucose (\(Y_{X/S}\)) from ammonium (\(Y_{X/\text{NH}_4}\)), and the product yields from biomass (\(Y_{P/X}\)) were determined for the shake flask cultivations for every defined time \(t\) by taking starting conditions as reference. The yields of product from glucose \(Y_{P/S}\) were always calculated for the total cultivation time. For the bioreactor applications, \(m_P\) is the sum of the mass of product in the reactor and in the separated foamate. \(m_S\) is defined as the mass of glucose remaining in the reactor and \(m_{\text{feed}}\) the glucose added during cultivation.
The biomass enrichment factors $E$ are defined individually for the stand-alone bacterial foam adhesion experiments and the bioreactor experiments with foam fractionation. For the stand-alone experiments, the optical densities were multiplied with the correspondent foamate and initial volume to account for evaporation losses. For bioreactor experiments, enrichment factors were defined for each sampling point.

**Equation 4**

$$
E_{OD_{600}\cdot V} = \frac{OD_{foamate/initial}}{OD_{foamate}} \quad [-]
$$

$$
E_{biomass}(t_i) = \frac{OD_{foamate}(t_i)}{OD_{reactor}(t_i)} \quad [-]
$$

$$
E_{surfactant}(t_i) = \frac{c_{surfactant, foamate}(t_i)}{c_{surfactant, reactor}(t_i)} \quad [-]
$$

**Rhamnolipid Purification**

The purification of the rhamnolipids in the supernatant was performed by adsorption of the surfactants from a cell-free solution with a C18 derivatized silica-based adsorbent (AA12SA5, YMC Europe GmbH, Dinslaken, Germany). For desorption, pure ethanol was used as eluent. The eluate was evaporated and chromatographically separated using a preparative HPLC system consisting of an AZURA analytical pump P 6.1L, an AZURA autosampler 3950 (both Knauer GmbH, Berlin, Germany) connected to a SEDEX 58 LT-ELSD detector (SEDEX, Olivet, France), the fraction collector Foxy R1 (Teledyne ISCO Inc., Lincoln, NE, USA) and equipped with a VP250/21 NUCLEODUR C18 HTec column (particle size: 5 µm, Macherey-Nagel GmbH & Co. KG, Düren, Germany). The flow rate was set to 10 mL/min and 3 mL sample were injected. As eluent, acetonitrile and ultra-pure water supplemented with 0.2% (v/v) formic acid were used. The gradient was increased from 70 to 76% between 5 and 10 min,
from 76 to 80% between 10 and 25 min, and to 100% until 35 min. It was decreased back to 70% between 45 and 50 min. Rhamnolipids were fractionated in between 25 and 47 min retention time. These fractions were evaporated to obtain pure, solvent-free rhamnolipids.

RESULTS

Low Biomass Concentrations in Flask Cultivations Limits Production

In shake flask cultivations, the rhamnolipid production strain \( P. \) putida KT2440 SK4 and the HAA production strain \( P. \) putida KT2440 KS3 reached final titers of 0.91 ± 0.14 g RL/L and 0.94 ± 0.07 g HAA/L, respectively (Figure 4A). For \( P. \) putida KT2440 SK4, as for all rhamnolipid producers in this study, the produced rhamnolipid concentration is defined as the sum of synthesized HAAs and mono-rhamnolipids. The congener compositions of synthesized rhamnolipids and HAAs are depicted in Figure 3. For both strains, the C_{10}-C_{10} dimer is dominant with a share of over 60% (w/w).

In the applied shake flask cultivations, foam formation occurred only marginally due to laminar fluid motion, ensuring homogeneous conditions. For \( P. \) putida KT2440 SK4 and KS3, the maximal growth rates were 0.46 ± 0.02 h^{-1} and 0.48 ± 0.003 h^{-1}, respectively (Table 2). The highest surfactant production rate was detected in the late exponential phase after 6 h (Figure 4). Until carbon depletion, total space-time yields of \( STY_{SK4,flask} = 0.06 \pm 0.01 \text{ g RL/L-h} \) and \( STY_{KS3,flask} = 0.07 \pm 0.01 \text{ g HAA/L-h} \) were reached. By referencing product formation to substrate, final yields were similar at \( Y_{P/S} = 0.1 \text{ gP/gS} \) (Table 2). For increased space-time yield for HAA and rhamnolipid production, higher biomass concentrations in the culture are however necessary. A bioreactor process is designed according to the identified need for carbon and nitrogen.

Latest published HAA syntheses with a plasmid-based production host reached space-time yields of 0.06 g HAA/L-h (Germer et al., 2020) and 0.07 g HAA/L-h (Tiso et al., 2017) in complex medium. Now, by integrating the production cassette into the genome for the construction of \( P. \) putida KT2440 KS3, a stable HAA producing strain is available, which does not require the addition of antibiotics. Especially concerning applications in larger scales, the renunciation of antibiotics reduces costs significantly. With the same STY as the previously mentioned strains even on minimal medium (0.07 g HAA/L-h), \( P. \) putida KT2440 KS3 has great potential for further applications regarding HAA synthesis.
Bioreactor Cultivations Lead to Increased Space-Time Yield

As higher biomass concentrations, and consequently higher concentrations of biocatalysts are mandatory for an improved HAA and rhamnolipid production without any limitations in substrates and oxygen, a bioreactor cultivation process was designed. In the bioreactor setup (Figure 2), the process was divided into a growth and a harvest phase: 1. In the growth phase, the foam leaving the gas exhaust of the reactor was collapsed and continuously recirculated into the reactor. This prevented the loss of biomass by cells entrapped in the foam and resulted in biomass concentrations of 5 g CDW/L and growth rates of $\mu = 0.44 \, \text{h}^{-1}$ for both producer strains. 2. In the harvest phase conducted for 10 h, the fractionated foam was collected as foamate. Here, biomass concentration in the reactor could be maintained for *P. putida* KT2440 SK4, while the biomass concentration of *P. putida* KT2440 KS3 increased continuously (Figures 5A,D). The biomass concentrations in
with a concentration of 6 gHAA/L were harvested and 2.5 gHAA/L remained in the reactor, leading to a total process STY<sub>KS3, reactor</sub> = 0.12 gHAA/L·h. Compared to the rhamnolipid and HAA synthesis in the shake flasks, the STY was increased by 2.8-fold and by 1.7-fold, respectively. This improvement is achieved despite the loss of cells from the reactor into the fractionated foam.

### Surface Structures Have a Significant Influence on CSH and Thus Bacterial Foam Adhesion

In the previous fermentation, high biomass loss due to foam adhesion of cells was observed. In addition to reduced productivity, higher biomass concentrations in the foamate result in a more complex downstream processing (DSP). Consequently, *P. putida* KT2440 was modified to identify cell surface structures responsible for cell surface hydrophobicity (CSH), thereby contributing to foam adhesion. A broad range of surface structures was removed by genetic engineering. By foaming a rhamnolipid solution with added surface-modified strains vertically through a column, foam adhesion of these strains was quantified. The introduced enrichment factor E<sub>OD<sub>600</sub>V</sub> was used here to assess the foam adhesion tendency of the individual strains. More than half of the investigated strains had an E<sub>OD<sub>600</sub>V</sub> of 0.65 to 0.85, corresponding to the highest observed values (Figure 6). With E<sub>OD<sub>600</sub>V</sub> = 0.77, the enrichment factor of the *P. putida* KT2440 wild-type strain is located in this range together with strains with a deleted synthesis of exopolysaccharide (Δfime) and pili (ΔfimbriaeΔpili). Therefore, these surface structures seemed to have no significant impact on bacterial foam adhesion. *P. putida* KT2440 without the flagellum (Δflag), the adhesin LapF (ΔlapF), and LapA and LapF combined (ΔlapAΔlapF) depict E<sub>OD<sub>600</sub>V</sub> values of below 0.65. With the multi-deletion-strain *P. putida* KT2440 GR20, the lowest tendency for foam adhesion for knock-out mutants was reached with an E<sub>OD<sub>600</sub>V</sub>GR20 of 0.33. *P. putida* KT2440 GR20 contains among other deletions, no flagellum and no LapA and LapF. Notably, the minimal
cell enrichment in the foam was reached with a *P. putida* S12 wild-type strain.

In order to validate the impact of the applied deletions on the CSH, water contact angles of the correspondent bacterial lawn were recorded. To evaluate if a correlation between CSH and foam adhesion existed, the CSH values were plotted against the biomass enrichment in the foam (Figure 6). In general, the same trend was observed as seen for the enrichment test. Many mutations did not influence the CSH having contact angles between 60 and 72° like *P. putida* KT2440. *P. putida* KT2440 Δflag, a strain among those with a reduced $E_{OD_{600}}$ value, was also within the water contact angle range of the wild-type strain. A reason for this phenomenon is probably the relatively low size of the flagellum compared to the total cell surface and therefore a low influence on CSH. The impact on enrichment might be caused by its long hydrophobic tail, acting like an anchor in the hydrophobic air bubbles. Apart from the flagellum deletion, mutants featuring a lower foam adhesion tendency also demonstrated a lower surface hydrophobicity based on a water contact angle of 40 to 50°. These results indicate that the factor $E_{OD_{600}}$ correlates to the CSH. The knock-out mutants standing out (i.e., which are in the lower left quadrant of the graph) were *P. putida* KT2440 ΔlapF, *P. putida* KT2440 ΔlapAΔlapF, and the cumulative deletion-strain *P. putida* KT2440 GR20. Additionally, wild-type strains *P. putida* DOT-T1E and S12 are among the best performing strains. According to a BLAST analysis (Altschul et al., 1990), *P. putida* DOT-T1E, and S12 genomes contain no gene encoding for the adhesin LapF. These strain-to-strain differences explain why many *P. putida* strains are in use and still new isolates with additional phenotypes are reported.

Enhanced Product Separation From Biomass With Cell Surface-Modified Biocatalysts

The strains that featured a lower foam adhesion were equipped with rhamnolipid and HAA production genes and used for fermentation. The aim was to show that a low biomass accumulation in the foam could be achieved not only in stand-alone tests but also in the actual production process. To focus on the agglomeration tendency of strains in the foam, all process conditions were kept exactly as applied for the non-modified production strains earlier. The biomass concentrations of surface-modified strains exclusively for the harvest phase, are depicted in Figures 7A,F, with the non-modified strains as reference. Except for *P. putida* KT2440 ΔlapF, all biomass concentrations in the reactor and the rhamnolipid concentration in the foamate rose. *P. putida* KT2440 Δflag_RL and *P. putida* KT2440 GR20_RL cultures reached biomass concentrations higher than 9 g CDW/L and caused the highest rhamnolipid concentrations in the separated foamate, with values over 7 g RL/L. For all surface-modified HAA and rhamnolipid production hosts, biomass enrichment factors $E_{biomass}$ were on average lower than the enrichments measured for *P. putida* KT2440 Δflag_RL ($E_{biomass} = 1.36 \pm 0.2$) and *P. putida* KT2440 KS3 ($E_{biomass} = 1.1\pm 0.6$). *P. putida* KT2440 Δflag_RL and *P. putida* KT2440 GR20_RL had minimal average biomass flotation tendencies with $E_{biomass} = 0.74 \pm 0.23$ and $E_{biomass} = 0.82 \pm 0.3$, respectively (Figure 7C, Table 3). With *P. putida* KT2440 Δflag_RL, the bacterial foam adhesion could be reduced by 46%. In contrast to the stand-alone bacterial foam adhesion tests, the strain without flagellum enriched less in the foam than strains with deleted genes encoding for LapA and LapF.
In terms of surfactant enrichment, the average rhamnolipid enrichment by fractionation lay between \( E_{\text{surfactant}} = 1.69 \pm 0.3 \) and \( 2.68 \pm 1 \) except for the cultivation of \( P. \text{putida} \) KT2440 \( \Delta \text{lapF}_R \) (Table 3). The rhamnolipid enrichment dropped throughout the harvest phase in all experiments. As already shown with the HAA production host without surface modifications, biomass foam adhesion was generally lower and product enrichment was higher than in rhamnolipid synthesizing processes. These characteristics, which promote the separation process, were confirmed with the HAA production strain without...
LapF. *P. putida* KT2440 ΔlapF_HAA continuously grew in the harvest phase, reaching a final concentration of 19 g<sub>CDW</sub>/L (Figure 7F). The HAA concentration trend in the foamate showed the same curve as the HAA concentration trend of the non-modified strain, reaching 8 g<sub>HAA</sub>/L (Figure 7G). With the *lapF* deletion, the biomass enrichment in the foamate was on average lower and the surfactant enrichment higher. A product enrichment factor of $E_{\text{surfactant}} = 4.98 \pm 1.6$ made *P. putida* KT2440 ΔlapF_HAA the strain with the highest $E_{\text{surfactant}}$ value.

Besides the investigated biomass agglomerations and product enrichments in the foam, the optimized bioreactor process had to be analyzed concerning biosurfactant productivity and product separation efficiency. With 10 g produced rhamnolipids, the cumulative deletion-strain *P. putida* KT2440 GR20_RL was the most efficient producer (Table 3). Consequently, the reached $STY_{GR20\_RL} = 0.24$ g<sub>R</sub>/L·h was 1.4-fold improved compared to the space-time yield that has been achieved with the rhamnolipid producer without surface modifications. Higher surfactant concentrations provoked a fortified foam formation. *P. putida* KT2440 GR20_RL produced 1.3-fold more foamate in the collection bottle than using the second-best rhamnolipid producer *P. putida* KT2440 Δflag_RL (Figure 7E). In general, 28 to 36% of the total produced rhamnolipids were separated via foam fractionation. Again, higher separation efficiencies could be realized in the HAA production process. For both applied strains, *P. putida* KT2440 KS3 and *P. putida* KT2440 ΔlapF_HAA, about half of the secreted HAAs were transferred into the foamate collection bottle. Despite a lowered biomass enrichment of 34% in the foamate with a LapF negative strain, no higher productivity could be obtained in comparison to *P. putida* KT2440 KS3.

**DISCUSSION**

**Integrated Foam Fractionation as a Trade-Off Between Productivity and Separation Efficiency**

The applied bioreactor setup with an integrated foam fractionation achieved high STYs for rhamnolipid and HAA production. However, this high productivity was achieved at the expense of lower product separation efficiency, as at least half of the total produced surfactant remained in the culture broth. Beuker et al. (2016b) used a setup with integrated foam fractionation similar to the one presented in this study. A lower biomass concentration of 3.3 g<sub>CDW</sub>/L, compared to 5 g<sub>CDW</sub>/L in our experiments was reached after a cultivation time of 10 h. A lower growth is most likely caused by the lower gassing rate of 0.067 vvm compared to the applied gassing rate of 0.25 vvm in our experiments, as growth of the aerobic *P. putida* is impaired when not enough oxygen is available. Furthermore, foam separation was conducted right from the beginning of the fermentation, most likely reducing biomass amounts in the liquid in the study of Beuker et al. (2016b). In a similar setup Anic et al. (2018) applied a gassing rate of 0.1 vvm and installed an additional foamate reflux, reaching a biomass concentration of more than 5 g<sub>CDW</sub>/L after 40 h. Foam destabilization was carried out by an integrated rhamnolipid adsorption. In our study, in the harvest phase, the fractionation efficiency declines after 5 h. With *P. putida* KT2440 SK4, the rhamnolipid concentration in the reactor broth rose, resulting in a lowered enrichment factor. Higher rhamnolipid concentrations in the broth led to wetter foam. This is underlined by an increased rate of foamate formation over time while surfactant concentrations in the foamate declined (Figure 7), a phenomenon also reported by Anic et al. (2018). The operation window for optimal foam fractionation is a trade-off. On the one hand, higher gassing rates omit oxygen limitations in the broth promoting rapid microbial growth and surfactant production, whereas an efficient product separation is achieved by reducing gassing rates. With the here developed multi-stage process (divided into growth and harvest phase), a 4.5 and a 2.3-fold higher STY could be achieved for rhamnolipid production by *P. putida* KT2440 SK4 with continuous foam fractionation compared to Beuker et al. (2016b) and Anic et al. (2018), respectively. Even though the product recovery in the foam of 97% reported from Beuker et al. (2016b) is much higher than in this study. Here, only 32% of the produced rhamnolipids were separated by foam fractionation. Probably during higher aeration, the liquid content in the foam increases, changing also the content of biosurfactant in the foamate. To avoid the dependence of separation efficiency on the gassing rate, immobilized cells can be applied, e.g., by entrapment of microbial cell factories in polymers (Siemann and Wagner, 1993; Heyd et al., 2011). With no cells leaving the reactor, foam fractionation conditions can be optimized, e.g., by an increased residence time in the fractionation column with larger column dimensions (Sarachat et al., 2010). As the quantitative surfactant secretion into the medium depends on culture conditions as cell vitality, growth, and density, the regulation of these conditions is of central importance. However, as already discussed for the gassing rate, process variables have a direct impact on the subsequent fractionation.

**Abiotic Parameters Reveal Potential for Increasing the Process Efficiency**

In the applied setup, technical adjustments for enhanced process efficiency are numerous (Figure 8). They are briefly outlined here in the context of studies focused on individual process variables. (A) The gas-liquid surface area is dependent on the bubble size, which can be adjusted by altering the diameters of the pores in the sparger while maintaining the gassing rate (Khanchezar et al., 2019). (B) The stirring speed influences the water content in the foam (Long et al., 2016). (C) Medium components, such as multivalent anionic ions (e.g., Mg<sup>2+</sup>) are discussed to reduce bacterial floation (Somasundaran, 1975; Beuker et al., 2016b). (D) With a lowered pH, rhamnolipids form less foam (Özdemir et al., 2004). (E) In this work, a rather small headspace volume was chosen to guarantee stable foaming through the reactor outlet, even at low surfactant concentrations. (F) For the headspace, as for the connected foam fractionation column, the vertical flow behavior is intended to be as homogeneous as possible, which is especially challenging at the in- and outlets. (G) With increased column dimensions at a constant height to diameter ratio, separation efficiencies increase due to a lower
impact of wall effects (Merz, 2012). Again, the conditions in the reactor are subjected to change by a correspondent (H) return of medium and biocatalysts impacting the cultivation. Despite the many parameters influencing the process performance, the here developed setup facilitated the identification of a suitable set of parameters for stable process operation.

Cell Surface-Modified Strains for Enhanced Production

While metabolic traits are often targets for strain improvement, cell surface properties are rarely engineered. Anic et al. (2017) suggested that P. putida without the flagellar machinery has a reduced tendency to agglomerate in foam, which was confirmed here. Despite that, we did not observe a lowered CSH. However, Martinez-Garcia et al. (2014) measured a lower CSH of a P. putida KT2440 flagellum deletion strain in comparison to the wild type via a microbial adherence to hydrocarbon (MATH) test (Rosenberg et al., 1980). Furthermore, the same study showed that non-flagellated cells form more biofilm than wild-type cells, fostered by a de-repression of exopolysaccharide production (Martinez-Garcia et al., 2014). This finding could be the reason why the lowest biomass agglomeration in foam was detected for the cumulative deletion-strain P. putida KT2440 GR20_RL with the lowest enrichment in the biomass flotation tests had a higher enrichment than P. putida KT2440 Δflag_RL in the foamate during bioreactor cultivation (Table 3). In other studies is was reported that flagellum deletion improves biomass yield on substrate (Martinez-Garcia et al., 2014) as well as the rhamnolipid production performance of the microbial cell factory (Tiso et al., 2020a). Higher biosurfactant production influences foaming and therefore the biomass adhesion tendency as well. The assessment of the biomass agglomeration promoted by certain surface structures with the performed stand-alone biomass flotation experiments that were always conducted with the same surfactant concentration therefore provides a better insight into the monocausal relation between CSH and cell foam adhesion. However, the results from the fermentation experiments allow for a better assessment and selection of the strain best suited for the here developed process. Overall, P. putida KT2440 GR20_RL is the best producer, reaching almost 10 g rhamnolipids within 20 h. With P. putida KT2440 Δflag_RL, 8.7 g rhamnolipids were produced in total, resulting in a 1.2 times higher production than P. putida KT2440 SK4.

In summary, we could show that genetic modifications of the bacterial cell surface reduced foam adhesion. This reduction in cell adhesion allowed stable rhamnolipid and HAA production in aerated bioreactors without the need of, e.g., antifoam addition. The expected benefits are not only lower operation cost of biosurfactant production, but especially reduced cost in the subsequent DSP. The integration of strain and process engineering, as discussed by Kuhn et al. (2010), clearly opens new possibilities for tailored process designs (Singh et al., 2019).

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.
AUTHOR CONTRIBUTIONS

CCB conducted the tests for biomass agglomeration in the foam and contact angle measurements for cell surface hydrophobicity detection. Furthermore, CCB performed all shake flask and bioreactor experiments. CCB analyzed the data, prepared the figures and wrote the manuscript. IB generated all P. putida KT2440 deletion-strains and most of the rhamnolipid producers. HIH and CE advised on contact angle measurements, gave support in all questions concerning cell surface hydrophobicity and revised the manuscript. TT and LMB initiated the project, advised on all experiments, analyzed and discussed data, and edited the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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The remaining 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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