1 General Introduction

1.1 The urgency to transfer to a circular bioeconomy

Within the last century, the exploitation of non-renewable fossil resources to generate platform chemicals for the production of fine chemicals, pharmaceuticals, and commodities such as plastics and fuels has led mankind to a critical turning point. The emission of greenhouse gases, most prominently carbon dioxide as a waste product from processing fossil resources and consuming petrochemically derived products, has been causing global warming. Critical consequences for the global ecosystem, *e.g.*, warming of oceans and water bodies, rising sea levels due to the melting of the polar ice caps, desertification, increased frequency of extreme weather anomalies, and ultimately mass extinction have been observed [1–5]. Although fossil resources are finite and thus could be depleted, technological advancement for identifying and exploiting new sources of fossil resources, some of them ironically claimed to be sustainable, will satisfy the demand in the foreseeable future [6, 7]. The demand is predicted to further increase due to a growing world population, however, at a reduced rate [8]. In turn, this causes the climate crisis with all its destructive consequences for the global ecosystem to be intensified, resulting in a shortage of arable and habitable land and, consequently, insecurities in food and water supply [9, 10]. Therefore, critical rethinking and intervention are urgently required to decrease the dependency on products derived from fossil resources [11].

An obvious solution to reduce the demand is limiting the continuous growth of the world population. Although the population in Europe and Asia is predicted to stagnate or decline within the next 30 years, the global population is presumed to grow at least until the end of the century [12]. As this period is too long to avoid irreversible consequences stemming from climate change, sustainable and resource-efficient solutions need to be developed and implemented immediately [13]. The awareness for sustainability has been rising among the population [14], raising pressure on governments to instate stricter regulations for carbon dioxide emission [15] and on companies to transit to ecologically friendly and sustainable production processes [16–18]. However, to truly reach long-term sustainability, global zero-waste resource cycles need to be established and maintained to impact climate change and potentially reverse global warming. Transitioning from a linear economy based on fossil resources to a circular bio-based economy, also referred to as bioeconomy, is crucial to establish closed carbon cycles [19]. In the bioeconomy, renewable resources are utilized to produce chemicals, fuels, and energy [20], which are currently derived from fossil resources, or respective alternatives to replace established production processes. Here, closed carbon cycles cause net-zero carbon emission processes, transforming society to be carbon-

neutral. Reaching this goal by 2050 was declared by the European Union (EU) within The European Green Deal and by other states [21], ultimately paving the way to limit global warming with all its consequences. However, a carbon-negative society might be required to limit or potentially reverse global warming [22].

1.2 Industrial biotechnology as a major driver for the bioeconomy

Industrial biotechnology is a crucial sector to establish the envisaged bioeconomy as carbon from renewable resources, such as biomass, carbon dioxide, and waste, can be used as a feedstock for bioconversions to produce desired, value-added compounds. Bioconversions are catalyzed by prokaryotes or eukaryotes as whole living or dead cells or by cell-free systems such as purified enzymes. In the last decades, substantial progress has been made for the targeted production of valuable compounds, and there is a growing number of promising cases in industrial biotechnology that are expected to be successfully implemented in the market within the next decade [23]. While this positive development is gaining momentum, products are typically highly specific, *i.e.*, pharmaceuticals and fine chemicals. Despite a few exceptions, like the production of ethanol [24] or amino acids such as L-lysine [25], hardly any biotechnological production processes for bulk chemicals or commodities have been able to compete with processes utilizing fossil resources due to several reasons. First, biotechnological production processes cannot compete economically with petrochemical processes in many cases [26]. This is mainly due to the comparably low price of fossil resources and optimized and depreciated production processes. However, with increasing costs for exploitation to satisfy the demand for fossil resources as well as stricter regulations on carbon dioxide emission (*e.g.*, carbon dioxide pricing in the EU and Germany) and, in turn, more cost-efficient biotechnological production, economic competitiveness might be reached relatively soon. Second, the comparably high complexity and dynamic nature of biological systems often result in a limited understanding of the whole-cell biocatalyst and the entire process, which can cause severe losses in productivity and performance, particularly during scale-up from laboratory to industrial scale [27]. Third, the downstream processing (DSP) of biotechnologically produced compounds is generally more complicated due to the high dilution of products and the complex composition of cultivation broth, including cells, cell debris, and byproducts [28]. Cost- and labor-intensive product recovery and purification are unprofitable unless the product has an accordingly high market price, which is usually not the case for bulk chemicals.

While the challenges to establish biotechnological production processes in industry to contribute to a circular bioeconomy are manifold, there are no alternatives to overcome the dependency on fossil resources. Innovative approaches and holistic perspectives, considering the overall production process already at primary stages of development, are required [29]. Thereby,

the transition of biotechnological approaches from research-focused laboratory scale to industrial applications will be achieved as famously demanded for synthetic biology as an integral part of biotechnology already in 2010 [30].

1.3 Integration of process development stages in microbial biotechnology

Microbial bioprocess development comprises several stages ranging from the molecular scale to large-scale production and eventually product recovery and formulation [29, 31]. Different stages are outlined, and crucial interactions are identified in the following paragraphs.

Initially, the demand for a novel compound or an alternative production route for established products is identified. Promising economic or ecologic advantageousness drives the identification and engineering of a metabolic pathway on multiple levels [32–34]. Here, genes encoding for enzymes that catalyze the desired reactions are designed, selected, and potentially modified to increase efficiency and alter the specificity of the enzymes [35]. By interconnecting functional DNA elements, such as promotors or regulatory sequences, the expression levels of genes can be altered [36]. Further modifications can be implemented at the translational level [37]. In recent years, the development of tools in metabolic engineering, synthetic biology, automation, and DNA synthesis have paved the way for rapid and more cost-effective pathway construction [38, 39].

To implement the production pathway, a whole-cell biocatalyst has to serve as a chassis. While the overall type of whole-cell biocatalyst needs to be carefully selected for a specific application, certain characteristics can be engineered, such as enhancing the solvent tolerance [40], modifying the cell surface [41], or restricting motility [42]. Furthermore, carbon fluxes can be re-routed [43], *e.g.*, by gene deletions, and the redox balance can be modified, both enhancing the production capacity of the chassis strain. Similar to metabolic pathway engineering, recent technological developments have accelerated strain engineering. Moreover, systems biology approaches, such as rapid whole-genome sequencing, high-throughput analysis of the transcriptome, proteomics, fluxomics, and metabolomics, enable data collection on multiple cellular levels [29, 34]. Acquired data are integrated into regulatory and metabolic networks organized in predictive and descriptive models, thereby allowing the generation of knowledge on a systems level [44].

Reaction engineering is required to exploit the production capacity of the whole-cell biocatalyst optimally. Here, the configuration of the whole-cell biocatalyst is defined, *i.e.*, if growing or resting cells are employed and if a pure, one-organism culture or a mixed co-culture is utilized [45]. Further, parameters for reaction conditions are defined, such as the pH value, temperature, aeration, and the composition of the cultivation medium. If beneficial, *in situ* product removal strategies for toxic or process-inhibiting products can be explored to push the limitation in maximally producible titers [46]. Suitable strategies to assess biocompatibility, *e.g.*, the toxicity

of an organic solvent in case of an *in situ* extraction, and the feasibility of the technical implementation are defined. While the construction of whole-cell biocatalysts is rapid nowadays, their characterization and the definition of optimal reaction parameters are considered as a bottleneck [47]. However, increasing automation and scalable cultivation devices allow a more reliable transition from multiplexed micro- and small-scale cultivation to production scale without the loss of performance [48–51].

Lastly, the fermentation process needs to be embedded in the process chain. This requires the definition of its mode of operation, *i.e.*, batch, fed-batch, continuous cultivation, or hybrid strategies [52, 53]. Further, the design and scale-up of the reactor need to be considered. Next to conventional stirred-tank reactors (STRs), other types of reactors are conceptualized and constructed, such as bubble columns, air lift reactors, and integrated systems like a multiphase loop reactor (MPLR), allowing *in situ* extraction [54–56]. For reactor design and scale-up, computational fluid dynamic (CFD) models are required to assess mixing, mass transfer, and trajectories of cells through different zones or compartments of the reactor [27]. Further, an efficient strategy for DSP needs to be designed to recover and formulate the product. Typically, DSP is comprised of a sequence of unit operations, varying in length and complexity depending on the product and the required purity, but eventually ending in product polishing and formulation [57, 58]. The operational windows of all process segments are defined and aligned to each other, and the overall process is optimized from an economic and ecologic perspective [28, 59].

What appears to be a linear approach for developing and establishing a bioprocess needs to have a circular and iterative character (Figure 1). Every stage of bioprocess development implies consequences for the other stages, displaying its high requirement for interaction, especially as people of different disciplines, namely biotechnology, chemical engineering, and economics, have to work hand in hand. While there are numerous interactions, selected ones are highlighted in the following paragraph.

Products and processing routes need to be identified, which have added value and have a chance to compete economically and ecologically with existing alternatives when introduced to the market. Here, tools such as techno-economic assessment (TEA) based on mass balances, the process scheme, and economic data, as well as life-cycle assessment (LCA) introducing ecological weighing factors, are applied [60–62]. These tools define objectives for the overall process, such as productivity, product titer, yields, robustness, selectivity, and product localization [29]. The defined objectives directly impact the design and conception of a molecular production pathway and the selection of a suitable chassis strain to meet the required objectives. Only the overlap in products of economic and ecologic interest and biotechnological feasibility can be considered attractive for production. Further, the metabolic pathway requires a suitable chassis to construct

an efficient whole-cell biocatalyst, heavily influencing reaction conditions and the mode of cultivation. Required resources and produced waste streams at cultivation can, in turn, have economically and ecologically unfavorable impacts, conflicting set objectives. Besides, the type of product, type of whole-cell biocatalyst, and type of upstream processing (USP) also have direct implications on the design of DSP, which typically accounts for a large fraction of the overall production costs due to usually high demands in energy and resources but depends on the type of product and purity [28]. A TEA can *a priori* assess if the highly diluted produced molecule can be recovered from the cultivation broth while still being economically and ecologically beneficial. Further, the necessary production volume can be determined with consequences for scaling or numbering the overall production process [26]. Concluding, for establishing a bioprocess with the most beneficial outcome, all disciplines have to constantly interact, be governed, and be monitored. Thereby, the acquired performance is validated to match the superordinated objectives, and adjustment and iteration can be prompted if required.

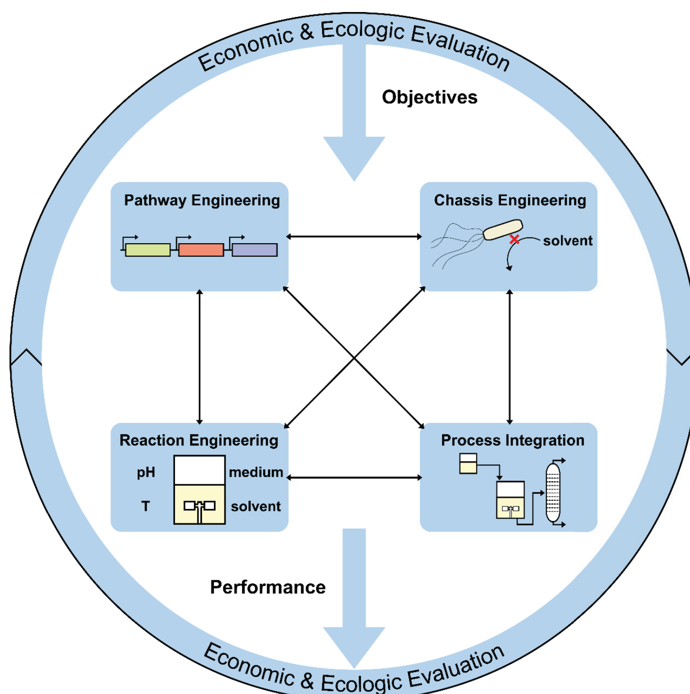

Figure 1 Integrated stages of bioprocess development. All stages comprising pathway engineering, chassis engineering, reaction engineering, and process integration need to be integrated and constantly re-evaluated for economic and ecologic efficiency. The overall process design requires iterative optimization to achieve the performance matching the objectives.

1.4 Bioprocess intensification

The concept of process intensification (PI) has been introduced into bioprocess development several decades ago but has attracted increasing attention in recent years. Remarkably, or perhaps even caused by its rising popularity and its resulting application to many fields, there is a lack of a clear definition of PI [63]. In a broader sense, PI is enhancing the efficiency of a process at a level ranging from transport phenomena and energy conversion on the molecular scale to the miniaturization and integration of apparatuses for unit operations to save time and space, to improve functionality, or to increase sustainability [63, 64]. For bioprocesses, Woodley [65] defined four complementary strategies for PI. The first two, (i) reactor compartmentalization and (ii) hybridization, are similar to their counterparts in traditional chemical engineering, although performed for different purposes. The other two, (iii) metabolic engineering and (iv) enzyme engineering, are unique to bioprocesses and can yield more degrees of freedom. Considering the mentioned definitions, holistic bioprocess development itself can be defined as PI.

The two PI strategies (i-ii) common to conventional chemical engineering are further introduced in a biotechnological setting. Here, integrating multiple unit operations, usually spatiotemporally separated, into a single stage of the process allows the unit operations to be performed simultaneously in one, optionally compartmented, apparatus [66]. Thereby, the efficiency of the overall process can be increased, ultimately making it economically more beneficial, as the production cycle can be shortened as well as the size of the production plant and ideally operational expenditures are reduced [67]. A drawback of these strategies is that all integrated unit operations have to be functional and efficient at the same conditions applied to the intensified stage of the process. Therefore, a joint operational window allowing optimal functionality has to be defined.

The definition of the operational window is driven by constraints in a multivariate parameter space of the single unit operations (Figure 2). Determining a joint operational window is not trivial and often requires trade-offs, especially if a microbial cultivation for producing a value-added compound is subject to be intensified [68]. Whole-cell biocatalysts often have narrow optima for different process parameters, such as pH value and temperature. Deviations from these optima might be possible within certain narrow limits. However, large deviations are typically detrimental to growth and production. As conditions of unit operations of chemical processes can be harsh in terms of temperature and pressure, matching the rather non-flexible boundaries of whole-cell biocatalysts might be challenging.

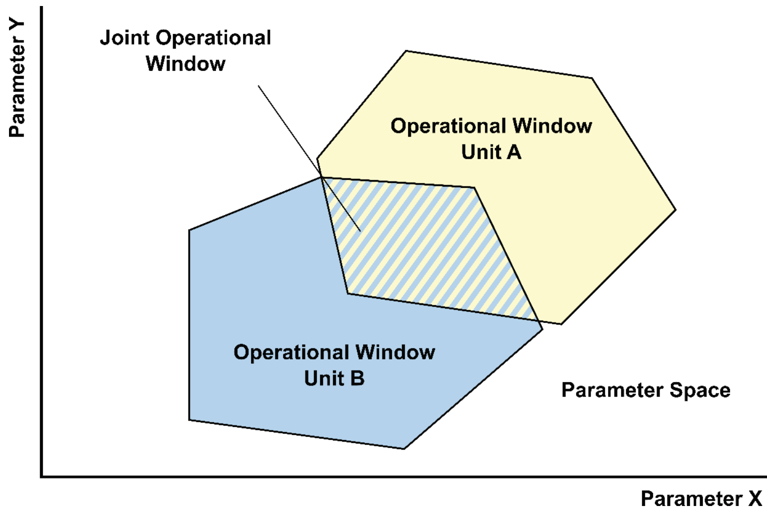

Figure 2 Definition of a joint operational window in bioprocess intensification. The operational window of each unit operation is multivariate and restricted by several parameters. For illustration, only two parameters are displayed, however, the operational window is defined in a parameter space. The joint operational window for the intensified bioprocess (shaded area) is defined by the intersection of the individual operational windows.

1.4.1 Strategies for *in situ* product removal

Despite the challenges, the integration of unit operations has been successfully applied in microbial bioprocesses. Thus, defining a joint operational window has been demonstrated to be feasible. A prominent example is the application of *in situ* product removal (ISPR) in fermentations. Depending on the physicochemical properties of the product, such as the molecular weight, volatility, hydrophobicity, charge, and reactivity, different types and strategies for ISPR have been explored [46, 69, 70]. The most common techniques are briefly described in the following paragraphs.

In situ liquid-liquid extraction (LLE) is one of the most extensively studied methods for ISPR in bioprocesses. Here, the product is extracted into a second liquid phase, driven by a concentration gradient. The partition coefficient, *i.e.*, the ratio of product concentrations in the two liquid phases, is used as a measure for the efficiency of extraction at given conditions [71]. In bioprocesses, LLEs have been most prominently implemented by the direct addition of an organic solvent exhibiting a miscibility gap to the aqueous cultivation broth. Few strategies for selecting a suitable solvent have been developed, targeting efficient extraction in terms of capacity and selectivity, simultaneously exhibiting no detrimental effects on the whole-cell biocatalysts [72, 73]. The latter has been identified as one of the most exclusive parameters in solvent selections for bioprocesses

due to the lack of biocompatibility of many solvents [74]. However, although case studies highlighted economic benefits [75, 76], holistic approaches and their implementations in industry are hardly reported. Another challenge is the emulsification occurring when the two liquid phases are in direct contact. However, membrane-supported approaches and other methods promise remediation [77]. Further, aqueous two-phase systems have been used in bioprocesses [78].

A product can be removed from the cultivation broth by evaporation. Here, the product needs to be more volatile than water, as water is the main component of a cultivation broth. Different technical implementations have been assessed, ranging from simple gas stripping [79, 80] to flash or vacuum fermentations [81, 82] and continuous product distillations with cell retention [83]. For gas stripping, the conventionally applied gas stream in aerated bioreactors can be used to recover the product. Although flash and vacuum fermentations can significantly enhance productivity [69, 76], these approaches appeared to be economically unfavorable. In contrast, continuous distillation with cell retention by centrifugation was previously successfully applied and transferred to industrial scale more than three decades ago [83].

Another method for ISPR is immobilization in the form of adsorption. Here, the product is adsorbed onto a resin, such as a polymeric matrix, and is thereby withdrawn from the cultivation broth. The characteristics and the type of the polymeric matrix need to be tailored to the product. In this regard, the interaction between the matrix and the product is most prominently based on hydrophobic or ionic interactions [69]. As the surface of the matrix is decisive for the capacity of product removal, mesoporous materials with engineered pore structures and functionalities have been applied [84, 85]. With higher capacity, the cost for applied material can be reduced, resulting in economic viability.

The molecular size of the product can be utilized for its separation in bioprocesses, similar to established methods in conventional DSP. Here, selective membranes allow the permeation of the product while retaining other components of the cultivation broth in the reactor compartment. The main driving force is either the concentration gradient of the product or the pressure gradient across the membrane [86]. The product needs to be removed from the downstream side of the membrane to maintain the gradient. Therefore, membranes are most effectively combined with other methods of ISPR, such as evaporation (resulting in pervaporation) and LLE (resulting in perstraction). Further, the introduction of a membrane either for selective permeation of the product or for cell retention prevents the direct contact of the whole-cell biocatalyst and the extractant, which is beneficial in terms of biocompatibility or biodegradability [70]. However, fouling and obstruction of the membrane need to be considered.

Other methods exploit the solubility and reactivity of the product for ISPR. Here, the product is precipitated or crystallized [87], or the solubility of the product is adjusted by reversible

complexation of the product. Precipitation can occur spontaneously if the product concentration exceeds the saturation concentration [87, 88] or is, among others, mediated electrochemically [89]. The complexation or other binding of the product involving a chemical reaction is referred to as reactive extraction [90, 91] and is typically combined with other ISPR techniques.

1.4.2 *In situ* liquid-liquid extraction in the scope of bioprocess intensification

Considering ISPR as a method for bioprocess intensification, the importance of holistic process perspectives and the definition of a common operational window as described in previous sections become obvious. Here, this is demonstrated at the example of *in situ* LLE for the recovery of a microbially produced compound.

By utilizing molecular biology, the whole-cell biocatalysts can be engineered to enhance production and tailor the strain to specific reaction conditions, here, the presence of an organic solvent. In turn, regarding reaction engineering, the solvent has to guarantee efficient extraction and likewise sufficient biocompatibility to sustain growth and production. Thus, the conditions for optimal extraction have to match cultivation conditions. Similarly, considerations for further DSP are required in early stages of process development to recover the product from the extractant efficiently. Further, the economic and ecologic impact of the used solvent has to be assessed. Concluding, all mentioned disciplines are dependent on each other for successfully implementing the most efficient production process.

1.5 Integrated bioprocessing for the production of rhamnolipids

This thesis focuses on the interactions of different stages of bioprocess development and aims to advocate holistic perspectives of bioprocess chains. Practically, this was demonstrated at the example of intensifying a biosurfactant production process with recombinant *Pseudomonas putida* KT2440 serving as the whole-cell biocatalyst. To fully comprehend the studied system, short introductions into the topics of *P. putida*, biosurfactants, and the challenges for production are given in the following paragraphs.

1.5.1 Biosurfactants

A group of products that have recently drawn increased attention for biotechnological production are biosurfactants as a replacement for conventional surfactants [92]. Surfactants are amphiphilic molecules and are therefore utilized in several commodities, such as cleaning agents, cosmetics, and food, due to their emulsifying properties. While conventional surfactants are based on fossil resources, typically requiring harsh conditions and elevated demands for energy for production [93], biosurfactants are produced from renewable feedstocks. Next to a replacement of

conventional surfactants, biosurfactants have distinct properties, which allow novel applications. Exemplary, as biosurfactants are typically biodegradable, they can be utilized in open environments, such as in agriculture and for remediation purposes, while having a smaller ecological footprint than their conventional counterparts [94]. Many other applications ranging from generic utilization in cleaning agents to oil recovery and highly specialized applications in medicine have been reported, which have been extensively described and reviewed [94, 95]. However, most proposed applications have not been established beyond the level of proof of concept or are predicted based on their physicochemical properties, with few exceptions, mainly in the detergent industry. Nevertheless, the economic interest in biosurfactants is rising, expressed by a generated revenue expected to increase from 4.5 billion USD in 2020 to 6.5 billion USD by 2027 [96]. However, due to the broad range of proposed applications and resulting requirements for the final product, the market price of biosurfactants is varying strongly.

Multiple microorganisms are capable of naturally producing biosurfactants [97]. The synthesis of biosurfactants typically gives the host an advantage in specific environments. For example, biosurfactants are used by its producer to make carbon sources, specifically hydrophobic hydrocarbons, accessible as a nutrient and energy source [98]. Depending on the microorganism and its specific environmental niche, different classes of biosurfactants have been discovered and classified in the groups of glycolipids, phospholipids, lipopeptides, as well as particulate and polymeric biosurfactants [97]. For biotechnological production, either the native producers are utilized, or metabolic production pathways are transferred to established chassis as reported by many studies [92]. In this thesis, the main focus is on the production of rhamnolipids (RLs). Therefore, a more detailed insight into this type of biosurfactant is given.

1.5.2 Rhamnolipids as prominent representatives of biosurfactants

Among biosurfactants, RLs are one of the most studied class of glycolipids [99]. As a surfactant, the molecular structure of RLs exhibits a hydrophilic and a hydrophobic moiety (Figure 3). The hydrophobic part typically comprises two β-hydroxy fatty acids, which are esterified by the acyltransferase RhlA to form a 3-(hydroxyalkanoyloxy)alkanoic acid (HAA). The hydrophilic part is formed by the free or esterified carboxylic groups of the HAA, which can be enhanced by the fusion of rhamnose moieties catalyzed by the rhamnosyltransferases RhlB and RhlC [100]. The class of RLs itself covers more than 50 congeners [101]. Depending on the homolog of RhlA [102], the chain length and degree of saturation of the esterified β-hydroxy fatty acids can typically vary from 8 to 16 carbon atoms each [101], while chain lengths of up to 24 carbon atoms have been reported [103]. Further, the number of rhamnose moieties can vary from zero to two, depending on the synthesis of RhlB (adding the primary rhamnose moiety resulting

in a mono-RL) and RhlC (adding the secondary rhamnose moiety resulting in a di-RL). Additionally, other variants, such as RLs with one fatty acid chain or a double bond have been reported [102, 104].

RLs are highly promising to replace surfactants based on fossil resources as they can be efficiently produced by various microorganisms [102]. Its commercial interest has recently been highlighted by the successful introduction of a microbial RL production process by Evonik Industries AG and the subsequent application in commodities [105]. In previous studies, *Pseudomonas aeruginosa* has been used as the most common whole-cell biocatalyst for RL production. However, as *P. aeruginosa* is an opportunistic human pathogen, a transfer of the genetic cassette encoding enzymes responsible for RL production to non-pathogenic chassis strains has been adapted. In this thesis, recombinant *P. putida* KT2440, previously constructed and established to enable RL production [106, 107], has been used. In short, *P. putida* is regarded as a promising whole-cell biocatalyst for industrial applications due to several reasons. Its versatile metabolism allows great substrate flexibility [108, 109], and *P. putida* shows a robust phenotype at various environmental conditions. Its capability in regenerating redox cofactors [110, 111] results in high resistance against oxidative stress [112]. Detailed information on the mentioned characteristics and the application of *P. putida* for the production of various value-added compounds have been extensively reviewed [113–115], and relevant aspects are described in respective sections of this thesis.

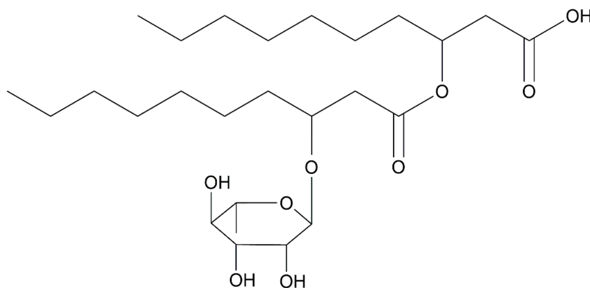

Figure 3 Representative structure of a rhamnolipid. The displayed RL is a mono-RL-C_{10}-C_{10} (one rhamnose moiety linked to a HAA with two carbon chains of 10 carbon atoms). Multiple different variants of RLs, so-called congeners, have been reported, varying in number of rhamnose moieties, carbon chain length, and saturation. This figure was previously published [125] and is reprinted with permission from Green Chemistry. Copyright The Royal Society of Chemistry 2020.

1.5.3 Challenges in rhamnolipid production and remedial approaches

The amphiphilic character of RLs is a desired trait for its application in multiple different products, as outlined above. However, the same characteristic imposes particular challenges for bioprocessing. The presence of RLs in the cultivation broth causes excessive foaming in conventional bioreactors due to the aeration and agitation applied to ensure sufficient oxygen transfer into the liquid for cellular respiration and to enable thorough mixing [116]. Titers in the lower range of mg L^{-1} of RLs in the cultivation broth already induce foaming at given conditions. This causes a rapid build-up of foam in the headspace of the reactor, eventually leading to an overflow of the reactor. The whole-cell biocatalyst is typically entrapped in the foam as it is physically equivalent to a particle in the liquid cultivation broth and attaches to the phase boundary due to hydrophobic regions of the cell surface [41, 117]. Therefore, the whole-cell biocatalyst is washed out of the reactor and is lost for catalyzing the reaction desired for production, resulting in an inefficient process.

To maintain efficiency, approaches for enabling bioprocessing were developed, which either can manage extensive foaming or prevent foaming in the first place. Intuitively, antifoaming agents have been added to the cultivations [118]. Although foam could be avoided and high titers of RLs were produced, a comparably high amount of antifoam was required. However, antifoam is typically expensive and has severe effects on the cultivation and particularly on DSP due to more complicated product recovery [119, 120]. Thus, the use of antifoam is regarded as economically unfavorable. In cultivations of *P. aeruginosa,* plant oil has been used as a substrate [121], simultaneously preventing foam formation. However, the same difficulties for DSP as described above occur.

Instead of preventing foam formation, the foam has been used as a method for ISPR. Here, the foam exits the reactor in a controlled manner and is subsequently fractionated [41, 122]. Promising approaches, *e.g.*, combined with integrated adsorption of RLs, have been established [117, 123]. However, the biocatalyst can still be washed out of the reactor as described above. Cell retention and cell recycling have been applied to mitigate the loss of whole-cell biocatalyst [117]. Further, Blesken *et al.* [41] engineered the cell surface to reduce the enrichment of *P. putida* KT2440 in the foam. While foam fractionation has been established as demonstrations of functionality in laboratory scale [124], a transferability to industrial-scale production is questionable. Another ISPR strategy could be the application of *in situ* liquid-liquid extraction, limiting the concentration of RLs in the cultivation broth below critical levels, thereby preventing foam formation.

The challenges to position renewable carbon-based chemicals in the market are specific to the respective product. Here, the challenges are outlined for RLs, for which their favorable traits cause difficulties in USP and DSP.

1.6 Scope and outline of this thesis

In the presented thesis, an integrated approach of USP and DSP development for the biochemical production of RLs with recombinant *P. putida* KT2440 as a whole-cell biocatalyst is pursued. USP development includes rational and untargeted strain engineering, the characterization of *P. putida* KT2440 to be suitable for industrial-scale production, and the development of novel fermentation and reactor concepts. Rather unconventional to common objectives in chassis design and metabolic engineering, strain engineering is here not mainly targeted to maximize product yields and titers, but it is process-guided, *i.e.*, the aim of strain engineering is to enable specific fermentation concepts. All approaches in USP development consider integrating DSP development and an overall process chain, with a focus on PI in a holistic perspective.

In Chapter 1, the thesis is embedded in the overall scope of the emerging bioeconomy. Strategies of integrated USP and DSP development and the intensification of fermentation processes are presented. In this regard, the concept of integrated and iterative adaptation of both the whole-cell biocatalyst and the reactor in the scope of holistic process development is introduced. As an exemplary holistic process development, the production of RLs with recombinant *P. putida* KT2440 is elucidated, building the structure of the presented thesis.

Chapter 2 details the experimental approaches, which have been performed to obtain and reproduce the results described in Chapter 3.

In Chapter 3.1, a methodology for selecting an *in situ* liquid-liquid extraction solvent for bioprocesses is presented and applied for a foam-free production of RLs in a two-liquid phase fermentation. A data-based selection preceded experimentally acquired performance parameters, such as extraction efficiency, biocompatibility, phase separation, and pH-dependency of extraction and back-extraction, to reduce the number of potentially suitable candidates, thus limiting the experimental effort to a reasonable level. The performance of the selected solvent was confirmed in laboratory-scale (fed-)batch fermentations, thereby defining a mutual operational window for integrated USP and DSP.

In Chapter 3.2, the approaches for both targeted and untargeted engineering of *P. putida* KT2440 to enhance its tolerance towards organic solvents, potentially used as extractants in two-liquid phase fermentations, are described. Targeted approaches included the expression of genes encoding for an efflux pump, a chaperone complex, and a cold-shock protein, all viewed as promising candidates for increasing or enabling solvent tolerance. Further, adaptive laboratory evolution (ALE) as an untargeted approach was applied to enhance tolerance to 1-octanol. Strains generated by introducing the unveiled point mutations into wild-type *P. putida* KT2440 *via* reverse

genome engineering were assessed for increased tolerance. The improved phenotypes of the strains were further characterized, and their capacities to produce RLs and 1-octanol were evaluated.

Chapter 3.3 further explores fed-batch cultivation strategies beyond the one developed in Chapter 3.1. The different feeding strategies comprise altering feeding profiles and compositions of the feeding solution. Their interactions with the implemented *in situ* extractions are evaluated. Further, catastrophic phase inversion (CPI) as a measure for breaking emulsions, particularly prominent in two-liquid phase fed-batch cultivations with high biomass titers, is assessed.

Chapter 3.4 focuses on the capability of *P. putida* KT2440, conventionally viewed as an obligate aerobic microorganism, to withstand repeated, short-term oxygen limitation. Thereby, it is assessed if the strain is suited for production in large-scale fermenters, in which gradients can occur due to non-ideal mixing, or in specialized compartmented reactors, such as the multiphase loop reactor described in the following chapter. Oscillation of dissolved oxygen tensions (DOTs) was induced across different cultivation scales and setups. Controlled scale-down experiments were performed to subject *P. putida* KT2440 to a defined duration of oxygen starvation, enabling comparison of growth at given conditions with well-aerated fermentations. Further, the impact of oscillating DOT values on the production of RLs was evaluated. First insights into coping mechanisms were gained by untargeted proteomics and the quantification of intracellular nucleotides.

In Chapter 3.5, the developed fermentation process for foam-free production of RLs is transferred from STRs to a novel multiphase loop reactor (MPLR). After the design and construction of the reactor, a first cultivation revealed that the installed aeration system could not satisfy the oxygen demand of the growing cells. Therefore, a redesigned, highly porous sparger enabling microbubble formation was constructed and integrated into the MPLR. A subsequent batch cultivation of RL-producing *P. putida* KT2440 could be supported until carbon depletion. Simultaneously, the implemented counter-current *in situ* LLE of RLs combined with specific bioprocess design prevented excessive foaming, and an *in situ* phase separation enabled continuous solvent recirculation. The newly established fermentation process was compared with STR cultivations of previous chapters.

In Chapter 4, the approaches for a holistic perspective on an integrated bioprocess design are discussed. Further considerations and general aspects of establishing microbial production processes in the industrial landscape to eventually contribute to the envisaged bioeconomy are presented.