1 General introduction

1.1 The bioeconomy - Initiating biotechnology for a sustainable economy

Due to an increasing population, the depletion of resources, growing pressure on the environment, and climate change, new strategies must be considered in terms of the production, consumption, processing, storage, and disposal of biological resources. [1] In this context, the term bioeconomy has become established in order to realize a more sustainable economy, which is even referred to as the next industrial revolution. [2] The bioeconomy encompasses the production of renewable biological resources and the conversion of these resources and waste streams into value-added products. [1] The product spectrum includes food, health, fiber, and industrial products, and energy. [3] In the food industry, new supplements are implemented and resources are to be used more efficiently. New bio-based chemicals, polymers, and other materials reveal new functionalities and properties. Furthermore, the production of bioenergy and biofuels is enforced to replace fossil energies. [4] In general, the bioeconomy is regarded as a driving force for the transition from fossil resources to a sustainable carbon cycle. [5] Carbon dioxide is used during photosynthesis by plants or phototrophic/autotrophic microorganisms to produce biobased products. It is released at the end of the product life cycle without increasing the atmospheric CO_2 concentration, while fossil-based products release carbon, which was previously stored underground. [6]

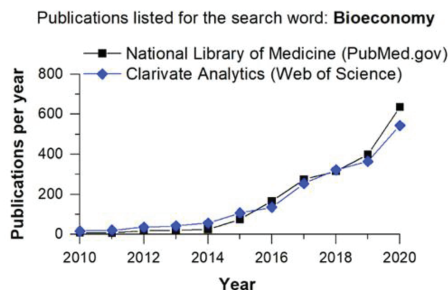

Figure 1: Number of new publications per year listed on central academic online databases when searching for the term Bioeconomy. National Library of Medicines' PubMed.gov [7] (black squares) and the Clarivate Analytics' Web of Science [8] (blue rhombi).

Based on political and economic roadmaps (*e.g.*, the 'European bioeconomy strategy' and the 'Green Deal' of the EU [9, 10], the 'Bioeconomy to 2030 agenda' of the OECD [11], and the 'Paris agreement' of the UN [12]) and corresponding funding programs (*e.g.*, Horizon 2020 of the EU [13]), research on bioeconomy increased tremendously in the last decade, indicated by the number of

new publications found under the search term 'bioeconomy' (**Figure 1**). Furthermore, *e.g.* in Germany, workshops were established at the Bioeconomy Science Center (BioSC) or at the DECHEMA for an enhanced knowledge transfer for academia, industry, and the general public. [14, 15]

Within the bioeconomy, the biotechnology sector is particularly relevant to achieve the proclaimed goals. [16] The yield performance of crops can be enhanced, resistance against outer influences (*e.g.*, pests or aridity), or the composition of this raw material may be altered by molecular biology or breeding methods. [17, 18] Specific biorefineries convert the generated biomass and waste streams into various biochemical and chemical intermediates. [19, 20] Besides biomass and waste streams, renewable carbon may derive from the conversion of carbon dioxide and hydrogen into basic chemicals. This process is particularly sustainable as hydrogen can be produced, *e.g.* by the electrolysis of water using electricity from renewable energies. [21, 22] Industrial biotechnology, using whole-cell biocatalysts or enzymes, realizes the synthesis of biodegradable products at reduced energy consumption and waste generation. Multi-step chemical syntheses are replaced by single-step conversions at low energy input and high selectivity. [17, 23] Based on metabolic engineering or directed evolution, genetically modified organisms (GMOs) are introduced to increase efficiency of existing production processes or the generation of even new pathways in microbial conversions. [24] A classic bioeconomy-success-story is the production of 1,3-propandiol (1,3-PDO, an important raw material of the polymer industry) by recombinant *Escherichia coli* growing on glucose. In this process, 40% less energy is consumed and greenhouse gas emissions are reduced by 20% compared to conventional processes. [25, 26] Another example is the production of polylactic acid (PLA, a bioplastic) based on the synthesis of lactic acid by a yeast strain. The process is already well established since the 1990s, but the biocatalysts still undergo improvements to reduce raw material consumption, side products, and the tolerance of lower pH values. [27, 28] For the sake of the bioeconomy, PLA production gains more importance to replace non-degradable plastic. However, current research, also at the Institute of Applied Microbiology (iAMB), showcases plastic (*e.g.*, polyethylene terephthalate (PET)) degradation with a subsequent synthesis to bioplastics (*e.g.*, polyhydroxyalakanoate (PHA)) by bacteria. [29, 30]

Another relevant bulk chemical derived by microbial conversion are biosurfactants. Biosurfactants' synthesis is a perfect example of how conventional processes to produce industrially important surfactants can be replaced by biotechnological processes, thereby contributing to the envisaged bioeconomy.

1.2 The production, characterization, and utilization of biosurfactants

Since the 1960s, biosurfactants are known as extracellular amphiphilic compounds produced by hydrocarbon fermentations. [31] Since then, several biosurfactants are established in the industry. Significant challenges for a broader implementation are the high production costs for biosurfactants. The efficiency of bioprocessing methods, microbial strains, and downstream processing (DSP) must be increased to lower the price for biosurfactant production. [32, 33] However, by 2019, the surfactants market size of microbially synthesized (*i.e.*, biosurfactants) and plant oil based surfactants exceeded 1.5 billion USD and is projected to rise to a value of 2.5 billion USD by 2026. [34] The growing market is fostered by numerous paramount advantages of biosurfactants compared to chemically synthesized surfactants. Most chemically synthesized surfactants derive from petrochemical feedstocks, while biosurfactants are synthesized exclusively based on a biocatalytic conversion of renewable resources. [35] Besides using refined substrates as sugars or plant oils, the application of waste streams reduces costs and exceedingly contributes to the bioeconomy guidelines. Numerous publications, summarized by Banat *et al.*[36], present biosurfactant synthesis based on waste streams derived from the food and oil industry, agroindustrial waste, dairy and distillery waste, animal fat, and oil industry. Based on the broad range of organisms, substrates, and available tools for genetic modifications, biosurfactants are available in a high structural diversity. [37] In contrast to chemically synthesized surfactants, biosurfactants are generally regarded as environmentally safe and biodegradable. [38, 39] Further benefits are the production at low temperatures, a high selectivity because of specific functional groups, and in certain cases an implementation at a broader range of temperatures, pH, and salt concentrations. [32, 33, 35, 40] Biosurfactants, as all surfactants, lower surface and interfacial tension in liquid-liquid, gas-liquid, and solid-liquid interfaces. Therefore, they promote solubility, wetting ability, and foaming capacity, crucial for materials such as adhesives, flocculating agents, deemulsifiers, and penetrants. [41, 42] Due to their versatility, biosurfactants are discussed for use in the chemical, cosmetic, pharmaceutical, and food industries, as well as for bioremediation of polluted soils and enhanced oil recovery. [39, 43, 44]

Surfactants are amphiphilic substances containing hydrophobic and hydrophilic structures. In general, the hydrophobic part (the tail) contains fatty acids, hydroxyl fatty acids, or fatty alcohols, while the hydrophilic part (the head) may include ester, hydroxyl, phosphate, or carboxyl groups, carbohydrates, peptides, or proteins. [44] Because of their excellent biological degradability, efficiency, production in high yields, and production from renewable resources, glycolipids are among the most industrially relevant biosurfactants. [44, 45] The hydrophilic head of glycolipids consists of a mono or oligosaccharide such as glucose, mannose, or rhamnose. [44] From the more

than 250 different glycolipids that are known [46], rhamnolipids (RLs), sophorolipids (SLs), and mannosylerythriol lipids (MELs) are already produced for economically relevant applications. The characteristics of RLs are presented in detail in section **1.2.1**, as RLs are of central interest in this doctoral thesis. SLs comprise a C_{16} or C_{18} fatty acid tail and a sophorose head. They occur in an acidic form, with increased foaming and solubility, or as lactonic form, with an enhanced surface and antimicrobial activities. [45] The yeast *Candida bombicola* is the most common SL producer, achieving titers of 400 g_{SL}/L. [47] Examples for present industrial applications containing SLs are dishwashing liquids and cosmetics. [48, 49] Due to antimicrobial and even antifungal properties, a broad introduction of SLs as a cleaning agent for vegetables and to prevent hospital-acquired infections is envisaged. [50, 51] MELs are synthesized by smut fungi in a large structural variety, depending on the microorganism and substrate used. The highest titers of 165 g_{MEL}/L are reported by cultivating *Pseudozyma aphidis* on soybean oil and glucose, producing mainly MEL-A, a diacetylated MEL. [52] Applications of MELs can be found in the field of cosmetics, *e.g.* for moisturization of dry skin or the repair of damaged hair. [53, 54] Besides the glycolipids, the cyclic lipopeptide surfactin, produced, *e.g.* by *Bacillus subtilis*, shows a high potential for industrial applications. [37] Surfactin consists of seven amino acids and C_{13} or C_{15} hydroxy-fatty acids. [55] Very unique is the low critical micelle concentration (CMC) of 16 mg/L [56], demonstrating the molecules' high efficiency to lower surface tensions. Surfactin reacts cytotoxic against numerous tumor cell lines and may therefore find applications in cancer therapies. [57] In larger scales, versatile applications of surfactin are possible, *e.g.* in the food industry or in the recovery of oil-contaminated environments. [56, 58] Low titers still hamper an industrial implementation of a surfactin production. [37, 57] However, recent approaches with engineered strains achieved an enhanced titer of 9 $g_{surfactin}$/L. [59]

1.2.1 RLs and derivatives as valuable goods for the bioeconomy

1.2.1.1 RLs and derivates synthesized by whole-cell biocatalysts

RLs are produced natively by the Gram-negative bacteria of the genus *Pseudomonas* and *Burkholderia*. Other identified RL producers are, *e.g.* from the genus *Dickeya*, *Lonsdalea*, *Pantoea*, and *Serratia* as summarized by Germer *et al.*[60] The predominant RL producing strain, achieving titers above 70 g_{RL}/L, is the opportunistic pathogen *Pseudomonas aeruginosa*. [61, 62, 63] It can be found in soil, oil-contaminated ground or water, and causes hospital-acquired infections. [64] *P. aeruginosa* is also the strain that was first reported in 1949 to synthesize RLs. [65] The organism synthesizes RLs for biofilm formation, [66] swarming, [67] and to access water-insoluble hydrocarbons. [68] Due to the pathogenicity of *P. aeruginosa*, efforts for the implementation of an industrial RL production are hampered as costs for safety measures would dramatically increase the overall

production costs. Furthermore, the favored carbon source of *P. aeruginosa* for high-titer RL production are vegetable oils. [69, 70] Besides the fact that the use of vegetable oils competes with the use for food production, the oils introduce a second phase into the liquid culture, leading subsequently to a more difficult RL recovery. Therefore, the genes relevant for RL synthesis were identified and transferred, *e.g.* into non-pathogenic *Pseudomonas putida* KT2440 for heterologous expression. [71] The KT2440 is the only *P. putida* classified as a safety class 1 organism in Germany. *P. putida* KT2440 is a well investigated organism with numerous available tools for genome editing, making it an excellent host for enhanced RL production and therefore to the predominant whole-cell biocatalyst applied in this thesis. [72, 73, 74] Furthermore, the organism is regarded as particularly robust and capable of adapting to changing physical and metabolic conditions as glucose depletion or oxidative stress. [75, 76] Besides RL synthesis, *P. putida* KT2440 is used, *e.g.* for the production of PHA, [77] terpenoids, [78] and phenazines. [79] Another envisaged application is the degradation of a variety of toxic and polluting hydrocarbons (*e.g.*, aromatic compounds as phenol, toluene, and xylene). [80, 81]

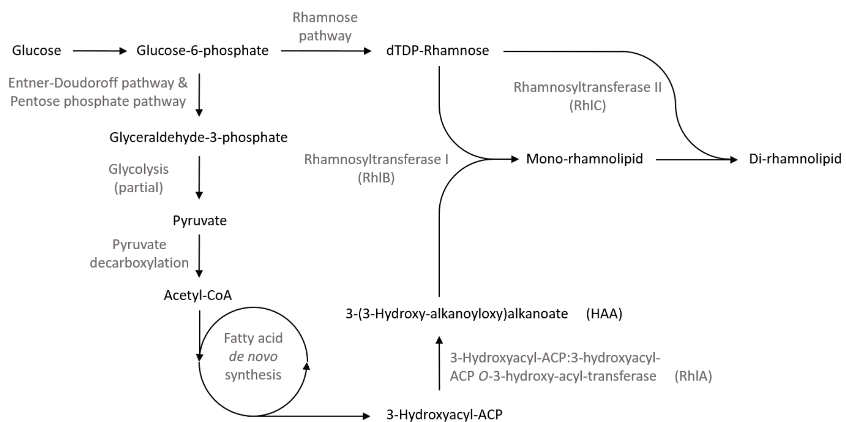

Figure 2: The relevant enzymes RhlA, RhlB, and RhlC originate from *P. aeruginosa* PAO1. Glucose is catabolized in the CCM *via* ED & PP pathway to glyceraldehyde-3-phosphate that is converted by a part of the glycolysis to pyruvate. The pyruvate decarboxylation by the pyruvate dehydrogenase leads to acetyl-CoA that is converted in the fatty acid de novo synthesis to 3-hydroxyacyl-ACP. Two 3-hydroxyacyl-ACP react to HAA by the enzyme RhlA. HAA is linked to a dTDP-rhamnose by the enzyme RhlB, to yield mono-RL. The enzyme RhlC connects a second dTDP-rhamnose to the mono-RL, forming di-RL.

Figure 2 shows the biosynthesis pathway for RL production in a recombinant *P. putida* KT2440, containing the genes encoding the enzymes 3-hydroxyacyl-ACP (acyl carrier protein):3-hydroxyacyl-ACP O-3-hydroxy-acyl-transferase (RhlA), rhamnosyltransferase I (RhlB), and rhamnosyltransferase II (RhlC) from *P. aeruginosa* PAO1. [71] First, glucose is metabolized in the

central carbon metabolism (CCM). It is phosphorylated to glucose-6-phosphate, which is converted *via* the Entner-Doudoroff (ED) pathway and the pentose phosphate (PP) pathway to glyceraldehyde-3-phosphate. The glyceraldehyde-3-phosphate enters a part of the glycolysis to be transformed into pyruvate. Acetyl-CoA (coenzyme A) is formed by pyruvate decarboxylation and enters the fatty acid *de novo* synthesis. In the fatty acid *de novo* synthesis, 3-hydroxyacyl-ACP is synthesized as an intermediate. [82] Two activated hydroxyacyl fatty acids (*e.g.*, 3-hydroxydecanoyl-ACP) are connected *via* esterification to 3-(3-hydroxyalkonoyloxy)alkanoate (HAA,**Figure 3 A**), catalyzed by the enzyme RhlA. [83, 84] The second precursor for RL synthesis is dTDP-rhamnose, which derives from glucose-6-phosphate by five enzymatic steps. [85] In gram-negative bacteria, rhamnose is synthesized for the formation of lipopolysaccharides (LPS), a fundamental part of the outer membrane of the cell. [86] The dTDP (deoxythymidine diphosphate)-rhamnose is fused *via* a glycosidic bond to the HAA by the enzyme RhlB to yield a mono-RL **(Figure 3 B**). [83, 87] A further rhamnose is linked to the mono-RL by the enzyme RhlC, forming a di-RL. [87, 88]

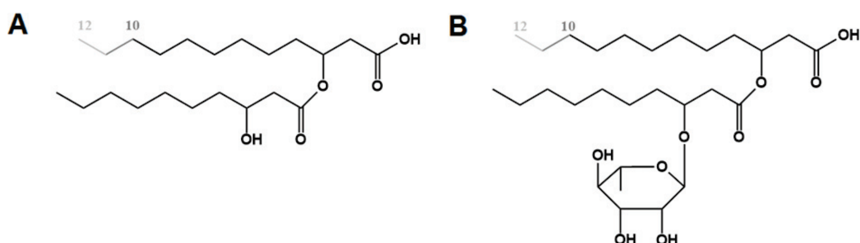

Figure 3: Molecular structure of A) HAA and B) mono-RL. The hydrocarbon chain length varies between C_8 and C_{12} for the applied recombinant *P. putida* KT2440.[adapted from 89]

The amphiphilic structure of a RLs consists of a hydrophobic fatty acid tail and one or two rhamnoses as a hydrophilic head. However, HAAs also show tensioactive properties due to hydrophilic structures as the ester, the carboxyl, and the hydroxy groups. [83, 84] *P. aeruginosa* produces mainly the C_{10}-C_{10} congener of mono-RLs (Rha-C_{10}-C_{10}) and di-RLs (Rha-Rha-C_{10}-C_{10}). [90] Consequently, the *P. putida* KT2440 containing the RL production genes from *P. aeruginosa* (*rhlA*, *rhlB*, and *rhlC*) also synthesizes mostly C_{10}-C_{10} congeners. Other possible congeners are C_{10}-C_8 and C_{10}-C_{12}. The latter congener is formed to an equal share with an unsaturated C-C bond. The latest studies revealed the position of the unsaturation being incorporated in the 5-dodecenoic acid. [91] By a correspondent integration of the specific production genes, recombinant whole-cell biocatalysts were designed that either produce HAAs (including *rhlA*) [84], mainly mono-RL (containing *rhlA* and *rhlB*) [92], or for the most part di-RL (containing *rhlA*, *rhlB*, and *rhlC*) [84].

Furthermore, Abdel-Mawgoud *et al.*[93] claim that β-oxidation is the dominant origin for fatty acids in *P. aeruginosa*, rather than the fatty acid *de novo* synthesis as described by Zhu and Rock[82]. With a correspondent isotopic tracing, CoA-linked fatty acids (3-hydroxyacyl-CoA), derived from β-oxidation, were identified as HAA precursor even if no fatty acid is used as a carbon source. [93] According to this, Gutierrez-Gomez *et al.*[94] suggest that 3-hydroxylacyl-CoA is synthesized to HAA-CoA, as a PHA precursor but not for RL synthesis. Further investigations are required to clarify the importance of β-oxidation for RL synthesis.

Individual whole-cell catalysts broaden the spectrum of available carbon chain length in RLs. For example, *Burkholderia plantari* synthesizes mainly RLs with chain length of C_{14} [95], *Pseudomonas desmolyticum* secretes RLs predominantly with C_6 to C_8 fatty acids [96], and the genus *Thermus* mainly with C_{16} to C_{18} fatty acids. [97] Recent studies focused on the RhlA enzyme in terms of an enlarged product spectrum and productivity. Germer *et al.*[60] integrated *rhlA* genes from several native strains into *E. coli*. In this way, the recombinant bacteria expressed HAA congeners comprising C_4 to C_{18} chain lengths. Dulcey *et al.*[98] achieved an increased catalytic activity and substrate specificity of the RhlA enzyme by homology modeling and structural mutagenesis of the RhlA from *P. aeruginosa* and *Burkholderia glumae*. Furthermore, an increased selectivity towards specific HAA congeners by mutagenized RhlB was reported by Han *et al.*[99] .

Many aspects of the synthesis of RLs and HAAs require further investigations, including the determination of the prevailing biosynthesis pathway for fatty acids and the potential of RhlA and RhlB enzyme modification for more efficient production of specific RLs and HAAs. The availability of specific RLs would be beneficial for various applications. The length of the carbon chain is crucial for specific amphipathic-related properties as the surface activity and wetting ability of the molecule. Specific product compositions are also required for a potential fuel production based on HAAs. For example, kerosene consists of C_8 to C_{13} alkanes to achieve a flat boiling curve (see section **1.6**).

1.2.1.2 Rhamnolipid synthesis by *P.putida* KT2440 on various substrates

As a carbon resource, glucose is the most common feedstock for biotechnological processes. [100] The sugar is obtained from hydrolyzed starch, *e.g.* from sugar cane or beet. [100] Recent research efforts showcase the use of alternative substrates for RL and HAA synthesis. Based on adaptive laboratory evolution (ALE) and molecular engineering, a *P. putida* KT2440 was designed for an enhanced RL and HAA production based on ethanol. Ethanol is metabolized to acetyl-CoA and enters the fatty acid *de novo* synthesis without carbon loss. Remarkably, a higher proportion of HAAs by using ethanol instead of glucose could be detected, as the biosynthesis of

dTDP-rhamnose from ethanol requires further energy. [101] Bio-ethanol is available in large quantities in the fuel industry as a fermentation product of yeasts growing on glucose. So-called 2nd generation substrates that do not compete with the food production were also tested successfully for RL production by *P. putida* KT2440. With glycerol, which occurs as a waste stream in the biodiesel production, as high RL titers as on glucose were achieved. [84] Glycerol enters the CCM at the stage of glyceraldehyde-3-phosphate and therefore avoids the oxidative PP pathway, which frees carbon *via* CO_2 production. [71] Bator *et al.*[102] integrated relevant genes of *E. coli* and *Pseudomonas taiwanensis* into the *P. putida* KT2440 genome to achieve growth and RL and HAA production on xylose. Xylose is a component of lignocellulosic biomass that is available in abundance as agricultural residues or from the paper industry. [103] Besides xylose, small organic acids derived from a pyrolysis of lignocellulosic biomass are reported as a resource for RL synthesis. [104] By growing a recombinant *P. putida* KT2440 on polyurethane (PU) hydrolysates as a carbon source for RL production, Utomo *et al.*[105] demonstrate direct microbial recycling of plastic monomers to a valuable good.

Further recombinant *P. putida* KT2440 are available that were engineered to grow on other relevant substrates, like sucrose (the main sugar in waste molasses) and galactose (from lignocellulose biomass). [106, 107] By a correspondent integration of the RL and HAA production genes into these strains, the substrate spectrum can be broadened accordingly.

1.2.1.3 Commercial applications of rhamnolipids and derivatives

HAAs have been considered only as a RL precursor until the *rhlA* gene was individually integrated into a non-pathogen *P. putida* KT2440 to produce HAAs at relatively high titers (1.5 g_{HAA}/L) as the sole product about 4 years ago. [84, 108] Since then, novel concepts were developed to establish this new product as a platform chemical to promote the bioeconomy. 1-Octene could be synthesized by an ethenolysis of HAAs at the unsaturated C-C bond of the C_{10}-C_{12} congener. [91] 1-Octene is a precursor of versatile chemical syntheses as for polyethylene and usually derives from petroleum. In another approach, a chemo-catalytic polymerization of HAAs yielded bio-PU, a bioplastic. [30, 109] Tiso *et al.*[30] used a recombinant *Pseudomonas* sp. GO16 for HAA production, capable of growing on PET monomers. Like this, a comprehensive conversion of PET to a bioplastic could be demonstrated. Further studies that were initiated by the KERoSyn project (see section **1.6**) focused on the conversion of HAAs, being hydrolyzed into 3-hydroxy-fatty acid, and converted into biofuel. By deoxygenation of 3-hydroxydecanoic acid with a corresponding metal catalyst, a conversion either into secondary alcohols or linear alkanes was achieved. [110] Meyers *et al.*[111] demonstrated an electrochemical conversion of the hydroxy-fatty acid into a drop-in oxygenate diesel fuel. Further applications may also be found in a more conventional application

for biosurfactants as HAAs lower the surface tension in between an aqueous solution and air to 25 mN/m, with a CMC at 113 mg_{HAA}/L, and therefore to even lower values as detected for mono-RLs with a minimal surface tension of 28 mN/m and a CMC at 124 mg_{RL}/L. [84]

RL concentrates are commercialized since 2016 as Rhapynal (Biotensidon GmbH, Karlsruhe, Germany), a mixture of RLs, pyoverdine, and alginate. [112, 113] In 2018, RHEANCE One (Evonik Industries AG, Essen, Germany) was introduced as a highly pure RL concentrate, used as an ingredient in cosmetic and cleaning products. [114, 115] On a smaller scale, RLs are provided by other companies as fine chemical, as AGAE Technologies LLC (Corvallis, OR, USA), GlycoSurf LLC (Salt Lake City, UT, USA), and Jeneil Biosurfactant Inc. (Saukville, WI, USA).

RLs are implemented in various applications for environmental protection, in the agriculture sector, in medicine, in cosmetics, and in the food industry. RLs are used for the demulsification of waste crude oil to gain the oil portion for the refinery process and the separated water for a conventional wastewater treatment. [116] An advanced bioremediation in the presence of RLs for the degradation of hydrocarbons as hexadecane, pristine, tetradecane, and creosote is reported, as summarized by Maier and Soberon-Chavez[117]. In this context, the large-scale use of RLs for the degradation of oil spills in the oceans and shores is discussed. [118] The cultivation of crops benefits from the application of RLs as biopesticide that replaces chemical pesticides. Due to the antimicrobial effects of RLs, zoospore lysis, spore germination, and mycelial inhibition are reported. The RLs intercalate into plasma membranes and therefore alter physicochemical properties or permeabilize the membrane. [119] Plants which seeds were previously treated with RLs developed immune responses against pathogens. [120] Further application may be found in the medical sector. RLs have been shown to prevent microbial colonization on silicone rubber, which is beneficial, *e.g.* for the development of voice prostheses. [121] The treatment of burn wounds with RLs contributes to the healing of the skin by stimulating keratinocyte proliferation and a parallel inhibition of fibroblast proliferation and collagen synthesis. [122] The cosmetic industry also uses RLs because of the antimicrobial properties but also because of the high emulsifying activity, *e.g.* for acne pads and contact lens solutions. [123] As RLs do not irritate the skin, they are used in anti-wrinkle and anti-aging products. [123, 124] Evonik, as a major commercial provider of RL related cosmetic products, regards the exceeding foamability of RLs as a further advantage. The company lists skin, hair and mouth cleansing products, and toothpaste with RLs in the portfolio. [125] RLs are also available in biodegradable household cleaners. [114, 126] In the food industry, RLs are applied as a food additive due to the low toxicity and biodegradability. [127] RLs enhance the conservation of bread due to their antimicrobial activity and improve the stability and texture of the dough. [128] The use of RLs as an emulsifier for vegetable oils showed a particularly efficiency for gingelly oil (a

sesame oil). [129] Due to the rhamnose moiety of RLs, the molecule may also be attractive for the production of high-quality flavor compounds. [127]

1.3 Bioreactor systems for rhamnolipid production

For an enhanced RL production, in terms of process intensification, scalability, and regulation opportunities, bioreactor cultivation systems are studied intensively. The conventional RL production in bioreactors is based on the cultivation of a pathogenic *P. aeruginosa* strains, with the highest titers on water-immiscible carbon sources. Syldatk *et al.*[90] obtained a titer of 13 g_{RL}/L with n-tetradecane as the sole carbon source already in the 1980s. A process with an outstanding RL titer of 120 g_{RL}/L was patented in 1997. [130] However, such a high titer was not verified since then on laboratory scale. [131] Within the last decade, the RL titers of up to 71 g_{RL}/L were reported with a *P. aeruginosa* growing on soybean oil. [131, 132] Oils act as a defoamer in the culture broth so that foam formation is limited to an extend that is controllable, *e.g.* by a mechanical foam breaker in the headspace of the reactor. [131] To lower the production costs, cheaper substrates as glucose or glycerol were introduced for RL production right from the beginning of the process development. In addition, the use of soluble substrates was intended to provide a less complex product separation when compared to a two-phase system. First pilot-plant cultivations of *P. aeruginosa* on glucose yielded 2.3 g_{RL}/L. An excessive foam formation was declared as a major drawback for enhanced production. [133] Due to the amphiphilic structure of the RLs and HAAs and the fact that gas is introduced into the medium by stirring and gassing, the molecules adsorb onto the gas-liquid interface. The interface stabilized by the surfactant thus leads to foam formation in the headspace of the reactor.

With excessive foaming, culture broth that is entrapped in the foam obstructs filters, valves, tubings, and causes sterility problems in the fermenter. [134] Furthermore, the foam may limit substrate and oxygen availability for the biocatalyst. [135, 136] Despite numerous approaches that have been pursued over the past 40 years, massive foaming is still considered one of the biggest challenges in establishing industrial RL production. [137] **Figure 4** provides an overview of techniques used in bioreactor systems for a stable RL production. In a bioreactor, foam formation is either prevented (foam free system) or the formed foam is destroyed. A third approach regards foaming even as beneficial as it already contains highly enriched product and can therefore be directly separated from the process in a so-called foam fractionation. The latter is described in detail in section **1.4**, being the technique for RL and HAA production that is comprehensively examined in this doctoral thesis.

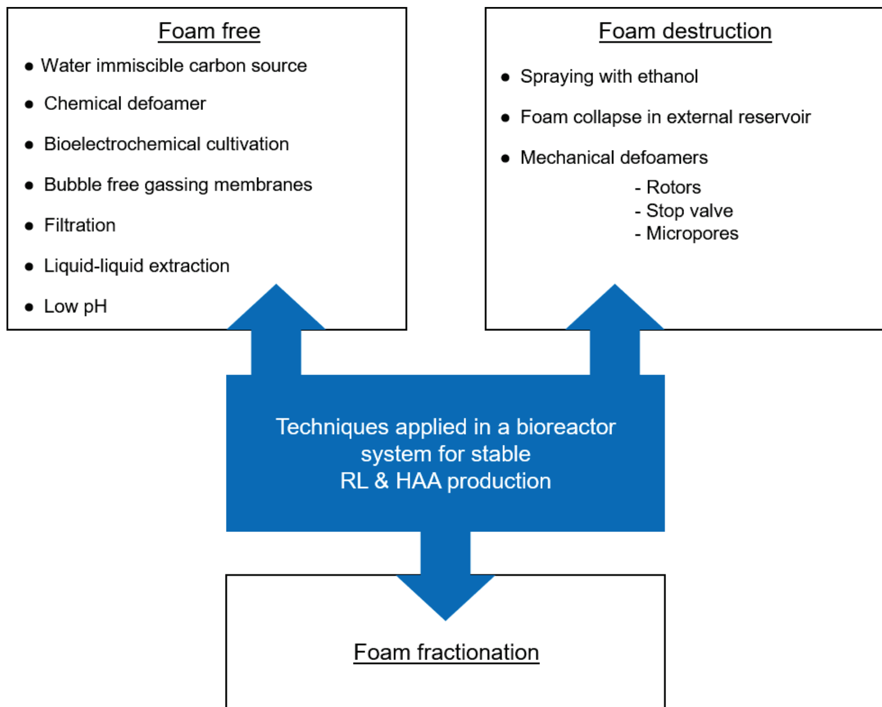

Figure 4: Techniques applied in a bioreactor system for stable RL and HAA production. The processes are differentiated by whether foam formation is prevented in principle (foam free), the foam is destroyed (foam destruction), or it is used for product separation (foam fractionation).

Foam formation can be prevented by the application of chemical defoamers, realizing stable lab-scale production processes with titers of 15 g_{RL}/L. [138] As defoaming agents are expensive and again hamper a subsequent DSP, this technique is not applicable for an intended large-scale RL production. [139] Bioelectrochemical cultivation systems with an anode as the electron acceptor for RL and HAA production are an innovative alternative to aerated bioreactor systems. However, low titers of 30 mg_{RL}/L reached in recent bioelectrochemical cultivations indicate the early stage of the development. [140] Therefore, a sufficient oxygen supply, realized by a correspondent gassing of the liquid culture remains unavoidable for efficient RL and HAA production. Specific gassing membranes were used by Gruber *et al.*[141] and Kronemberger *et al.*[142] to achieve a bubble-free oxygen supply in the culture broth. With a RL productivity of 30 mg_{RL}/L·h, Kronemberger *et al.*[142] achieved less than one tenth of the value reached by Müller *et al.*[131] in a gassed biphasic system. The development of more efficient bubble-free aeration systems for bioreactors is further enforced, *e.g.* by the BioThrust spin-off project of the RWTH. [143] Another approach considers a continuous

RL and HAA separation from the cultivation process. By an integrated product recovery, the cultivation process itself gains stability as foaming is reduced when surfactants do not agglomerate in the broth. Furthermore, the entire biosurfactant production process is intensified if a first DSP operation is established simultaneously with a bioreactor process. Gruber *et al.*[141] designed, next to the previously mentioned gassing membrane, a filtration membrane for an integrated RL separation from the cultivation process. The filtration membrane was described as highly efficient for cell recycling, but largest RL concentrations were detected in an additional bleed stream from the reactor and not in the filtrate. Recently, Demling *et al.*[144] conducted a comprehensive study about the integration of a liquid-liquid extraction into a cultivation process for RL production. Next to a generally high extraction efficiency regarding RLs, a feasible extraction agent applicable directly in the cultivation must fulfill versatile properties. The agents were screened in terms of biocompatibility (*i.e.*, evoking no toxicity against the RL production host), degradability of the solvent by the bacterium itself, phase separation (*i.e.*, forming no interphase), security measures (*i.e.*, low flash points), pH-dependency, and the possibility for a back-extraction to reuse the extraction agent. By considering all these aspects, ethyl decanoate was detected as most feasible. Despite the presence of the organic phase, the pH value in the culture had to be lowered to pH 6.2 to prevent excessive foaming. Lowering the pH value from the optimal culture conditions, which is generally a neutral pH value for *Pseudomonas*, is a simple technique to reduce or even prevent foam formation. However, lowering the pH value for foam prevention was only successfully applied in combination with a second organic phase in the bioreactor. Sodagari *et al.*[145] describe a stable RL production up to 42 g_{RL}/L with a *P. aeruginosa* growing on soybean oil at pH 5.7. Zhu *et al.*[132] reached even higher titers and productivities (71 g_{RL}/L and 0.6 g_{RL}/L·h) by regulating the pH value down to pH 6-6.5 together with the use of a chemical defoamer.

Compared to foam free processes, the techniques applied for foam destruction allow the formation of foam either to a certain level in the bioreactor headspace or to separate the foam through the air exhaust for *ex situ* destabilization. Spraying ethanol on the foam is an exquisite procedure as it promotes foam destruction and provides an additional carbon source for the whole-cell biocatalyst. Sha *et al.*[146] collected the discharged foam of a *P. aeruginosa* culture in an external bottle. The foam was formed after the primary carbon source (rapeseed oil) was almost consumed. The foam was destabilized by an ethanol spray and pumped back into the reactor to promote cell growth and RL production. Bator *et al.*[101] enhanced this technique by applying a non-pathogenic *P. putida* KT2440 and used ethanol as the sole carbon source, obtaining a biomass concentration of 22 g_{CDW}/L and a surfactant concentration of 5.3 g_{RL}/L. The principle of an external foam collection vessel to destabilize the foam to foamate that is pumped back into the reactor was also applied by

Tiso *et al.*[147]. Foam destabilization was achieved with a high filling volume in the reactor and by producing a relatively wet foam at high stirring speeds. The discharged wet foam collapses faster and is transferred more efficiently back into the reactor. Furthermore, systems based on mechanical foam destruction are applied. A conventional technique in bioreactor systems are foam breaker (*e.g.*, foam centrifuges and impeller) that are also used for RL production. [131, 133, 137] Individual designs consider guiding the discharged foam through a stop valve or micropores to separate the liquid from the gas portion of the foam. [148, 149]

1.4 Foam fractionation for an integrated rhamnolipid recovery

In the previous section (**see 1.3**) filtration and extraction were presented as techniques for an integrated recovery of RLs from the culture liquid. To the best of the authors' knowledge, no research has built on the work of Gruber *et al.*[141] in the 1990s, presenting an integrated filtration to gain RLs as cell-free filtrate. Küpper[150] considered an integrated cross-flow filtration, but pretest already showed low recoveries and the filter has clogged. A liquid-liquid extraction to separate RLs and HAAs with the extraction agent from the bioreactor in a continuous operation mode is also not published yet. So far, foam fractionation is the most investigated technique for a RL recovery directly integrated into the bioreactor cultivation process. Foam fractionations do not require complex technical installations and are generally regarded as a rather cost-effective method for product purification. As the DSP accounts for 60-80% of the total biosurfactant production costs, [151] the implementation of a foam fractionation as a primary purification step is particularly interesting to lower the total costs. Furthermore, foam fractionation is a gentle and ecologically compatible process as the application of organic solvents and chemical defoamers is prevented. The foam fractionation is based on the fact that the rising gas bubbles in the foam are stabilized when the surfactants adsorb onto the gas-liquid interface to decrease the Gibbs free energy. [152] The hydrophobic tail of the surfactant is orientated to the gas phase while the hydrophilic head remains in the interstitial liquid in between the bubbles. While the gas bubbles rise, the interstitial liquid drains past the bubbles, through the lamella, in the direction of the gravity force. Consequently, the fractionated foam contains less liquid and gains higher surfactant concentrations. This principle is illustrated in **Figure 5** in the context of an integrated foam fractionation into the cultivation process, considering that also bacterial cells accumulate in the foam and adsorb onto the gas-liquid interface. Microorganisms show a specific cell surface hydrophobicity (CSH) that is beneficial, *e.g.* for *Pseudomonas* to establish a broader solvent tolerance. [153, 154] The bacterial foam adhesion is a particularly critical parameter for a stable cultivation process in combination with foam fractionation. The bioreactor production process loses its biocatalyst and thus productivity. In

parallel, the subsequent DSP of the separated foam requires additional operations to remove the biomass from the product.

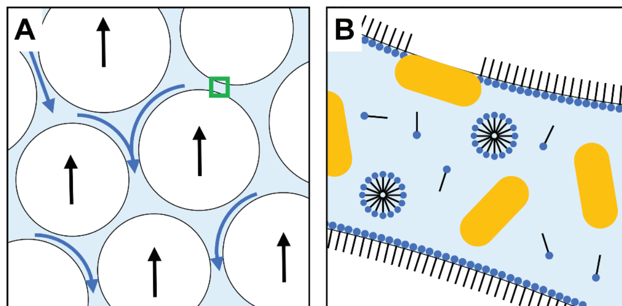

Figure 5: Graphical explanation of foam fractionation and foam stability. A) Rising gas bubbles (white, black arrows indicate flow directions) and draining interstitial liquid (light blue, blue arrows indicate flow directions). The foam lamella marked with a green frame is enlarged in B) with surfactants (black line: hydrophobic tail; blue points: hydrophilic head) either adsorbed on the gas-liquid interface, agglomerated as micelles, or dissolved in the liquid. Pseudomonads (yellow) are suspended in the liquid or adsorbed on the gas-liquid interphase by hydrophobic cell surface structures. [89]

To prevent the loss of biomass, first approaches of an integrated foam fractionation considered the immobilization of a *P. aeruginosa* on an alginate support in a fluidized bed reactor. Only low RL productivities were obtained, caused by limitations in the process (*e.g.*, low oxygen supply). [155] Heyd *et al.*[156] applied a magnetic matrix to immobilize the whole-cell biocatalyst to realize a cultivation in a gassed and stirred bioreactor with a magnetic field to separate the immobilized cells from the discharged foam. However, the productivity of the immobilized cells remained on a low level (23 $mg_{RL}/L{\cdot}h$). Further approaches with a suspended *P. putida* KT2440 demonstrated the ability to reach high RL enrichments in the fractionated foam but only at low gassing rates that also cause a reduced production. [157] A complex dependence of various process variables as the gassing rate and the initial gas bubble size has been evaluated for a cell-free culture broth in a stand-alone foam fractionation column. [158] For non-integrated lab-scale foam fractionations, comprehensive screening strategies for the relevant process variables and their interactions based on a Design of Experiment (DoE) are available as single fractionation performances do not require much time effort. [159] With an integrated foam fractionation into the bioreactor process, multiple additional variables need to be considered under permanently changing conditions (*e.g.*, biomass and surfactant concentration). Furthermore, the variables can only be varied to a certain value (*e.g.*, gassing rate) to maintain optimal conditions for the cultivation of the whole-cell biocatalysts during the fractionation process. **Figure 6 A** shows the reactor design for RL production

considering the bioreactor headspace for foam fractionation. [157, 160] In similar processes for surfactin production by *B. subtilis*, Chen *et al.*[161] used an additional foam fractionation column linked to the gas exhaust. These approaches show foam fractionations highly dependent on process variables for the cultivation process. To gain a higher degree of freedom to adjust optimal variables for the cultivation process as well as for foam fractionation, further bioreactor designs will be evaluated in this doctoral thesis, as suggested in **Figure 6 B&C**. External foam fractionation columns allow individual designs to achieve, *e.g.* a longer residence time of the foam and therefore a more efficient drainage. By destruction of the discharged foam and a subsequent fractionation at optimized conditions, a comprehensive decoupling of both operational steps is envisaged.

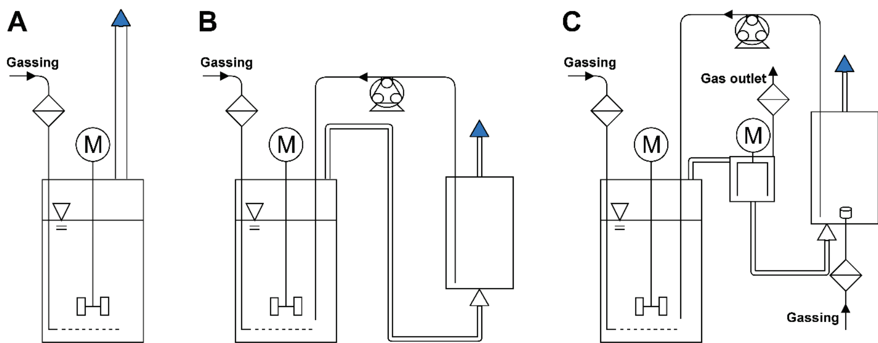

Figure 6: Bioreactor systems for biosurfactant production and an integrated product separation *via* foam fractionation. The blue arrow indicates the exhaust of the fractionated foam. A) Bioreactor with foam fractionation limited to the headspace of the reactor and a fractionation column at the gas exhaust. B) Bioreactor connected to a foam fractionation column with an active recirculation of the drained liquid. C) Bioreactor with a decoupled foam fractionation. Discharged foam is destabilized *via* a foam centrifuge to gain a foamate that is injected into a foam fractionation column equipped with a sparger and an active recirculation of the drained liquid.

1.5 Downstream processing of rhamnolipids and derivatives

Apart from the price for raw materials, the availability of biosurfactants is mainly hampered due to an expensive DSP, *e.g.* requiring a large amount of solvents. [151] Next to the mentioned integrated product recovery, also called *in situ* product recovery (ISPR), in the previous section, numerous lab-scale RL separation and purification procedures were developed. From an aqueous culture broth, bacterial cells are generally separated *via* centrifugation. [133, 162, 163, 164] Supernatants are further processed by acidification/precipitation, extraction, filtration, foam fractionation, and adsorption. The acidification of the cell-free culture broth (pH 2) causes precipitation of the protonated RLs. In a subsequent extraction, RLs are separated from other precipitated medium components. [164] As proteins hinder phase separation during extraction [165], a correspondent

pretreatment of the supernatant *via* anti-solvent precipitation is suggested by Biselli *et al.*[162]. For the separation of extracted RLs, reverse extraction is applied to transfer deprotonated RLs back into an (alkalic) aqueous phase, or the extraction agent is evaporated.[144, 166] Two-step ultrafiltration techniques have been developed as an alternative to extraction, using solvent to prevent micelle formation.[167, 168] Witek-Krowiak *et al.*[168] obtained a RL recovery of 84%, demonstrating filtration techniques as an efficient tool for RL separation in a non-integrated process. Even higher RL recoveries (R_{RL} > 95) are reported by applying foam fractionation.[158, 169] In a subsequent foam adsorption, foam is introduced into an adsorption column to adsorb RLs, being highly enriched in the foam lamellae onto the hydrophobic adsorbent.[170] As the foam collapses during foam adsorption, Anic *et al.*[160] integrated a correspondent adsorption column into the bioreactor process with a recirculation of the broth entrapped in the discharged foam back into the reactor. By combining foam fractionation and foam adsorption, an integrated RL recovery process was presented to yield RLs already separated from the culture broth. In general, adsorption/desorption techniques for RL recovery from a pretreated culture have already been suggested for a pilot-plant production scale in the 1980s.[133] For adsorption, silica-based materials and resins are applied, and the desorption of the surfactant is performed with organic solvents as eluent.[124, 133, 155, 160, 171]

Other chromatography techniques were established for the separation of smaller product quantities. Thin-layer chromatography (TLC) is applied to separate mono- and di-RLs.[172, 173] Wittgens *et al.*[174] developed a preparative TLC to separate specific mono-RL species. High-performance liquid chromatography (HPLC) is applied as ion-exchange[175] and reverse-phase chromatography[92, 176]. The reversed phase chromatography allows the separation of HAAs, mono-RLs, and di-RLs, and correspondent congeners.[84]

1.6 Achieving a renewable kerosene production

Aviation accounts for 3% of the total anthropogenic CO_2 emissions.[177, 178] Only in Europe, the number of flights is expected to increase by 42%, associated with increased CO_2 emissions by 21% between 2017 and 2040.[179] The expected lower incline of emissions compared to flight numbers is based on envisaged technical efforts for emission reduction. These efforts include the construction of more efficient airplanes itself, *e.g.* by improved aerodynamics or novel engine technologies as enforced by the Clean Sky project of the European Union.[180] Potentially more promising for CO_2 reduction in the next decades, as the already existing airplane fleet can still be used, is the intensified utilization of renewable resources for jet-fuel (*i.e.*, kerosene) production. So far, eight different biokerosene production process are approved as blends on commercial airliners (**Table 1**).[181] The Fischer-Tropsch (FT) kerosene production process uses syngas, *e.g.*

from wood waste. The carbon monoxide and hydrogen of the syngas are converted into alkanes. [182] Largest quantities of commercially available biokerosene are produced by the hydroprocessed fatty acid esters and fatty acids (HEFA) process. [183] Purified vegetable oils, like palm oil or rapeseed oil, undergo hydrogenation to gain hydrocarbons that are subsequently cracked and isomerized. [182] Certain processes are already based on the microbial utilization of specific substrates. The synthetic iso-paraffins (SIP) process is based on the synthesis of farnesene by yeasts, growing on sucrose from sugarcane or sugar beet. [179, 182] Another microbial approach is the cultivation of the algae *Botryococcus braunii* to produce oil that is further processed by the established HEFA process. [184]

Table 1: Approved processes to produce blends that can be used as biokerosene on commercial airliners. [179, 181, 185]

No	Process	Resource	Max. blending ratio (v/v)	Year of approval	ASTM[1)] reference
1	Fischer-Tropsch (FT)	wood, agricultural residues & municipal solid waste	50	2009	ASTM D7566 Annex 1
2	Hydroprocessed fatty acid esters and fatty acids (HEFA)	vegetable oils & animal fats	50	2011	ASTM D7566 Annex 2
3	Synthetic Iso-Paraffins (SIP)	biomass used for sugar production	10	2014	ASTM D7566 Annex 3
4	Fischer-Tropsch with aromatics (FT-SKA)	wood, agricultural residues & municipal solid waste	50	2015	ASTM D7566 Annex 4
5	Alcohol to jet fuel synthetic paraffinic kerosene (ATJ-SPK)	biomass for ethanol or isobutanol production	50	2016	ASTM D7566 Annex 5
6	Catalytic hydrothermolysis jet fuel (CHJ)	vegetable oils	50	2020	ASTM D7566 Annex 6
7	Hydroprocessed hydrocarbons synthetic paraffinic kerosene (HC-HEFA-SPK)	oil derived from algae (*Botryococcus braunii*)	10	2020	ASTM D7566 Annex 7
8	Co-processing	fats, oils, and greases added in petroleum refining	5	n.a.	ASTM D1655

1) ASTM: American Society for Testing and Materials

While the production costs for fossil kerosene are 0.3 to 0.6 USD/L, the production cost with the cheapest biokerosene production process (*i.e.*, HEFA) ranges from 0.7 to 1.6 USD/L. The higher production costs are the major drawback to replace non-renewable kerosene on the market. [186] However, according to the just recently (January 2021) launched Carbon Offsetting and Reduction Scheme for International Aviation (CORSIA), more than 88 countries aiming for a carbon neutral growth of the aviation sector. [187, 188] As the availability of fossil resources for kerosene production is limited and environmental consequences by an increased CO_2 emission become more and more apparent, the industrial application of renewable fuel will be unavoidable.

According to the efforts to establish a renewable production of kerosene, the KERoSyn project was initiated by the Institute of Technical and Macromolecular Chemistry (ITMC) and the Institute of Applied Microbiology (iAMB) of the RWTH Aachen University, sponsored by the Fachagentur

Nachwachsende Rohstoffe of the German Federal Ministry of Food and Agriculture (BMEL, No. 22403415). With the production of HAAs using recombinant *Pseudomonas* strains, alkanoates should be provided for a chemical synthesis into biofuel. For the recombinant *P. putida* KT2440 and *P. taiwanensis* VLB120, being available as chassis for HAA production, growth on a broad range of carbon sources can be used (see section **1.2.1.2**). Next to glucose, glycerol and xylose are reported for HAA or RL production. [84, 102] These sugars are available as side products of other production processes, increasing the value-added chain for carbon use. Glycerol is produced in large quantities as a side product in biodiesel production. [189] Lignocellulosic biomass, containing C_5 (xylose) and C_6 sugars, derived from agricultural or forestry residues. [103, 190, 191] In comparison, the already established biokerosene production routes are mainly based on the conversion of vegetable oil derived, *e.g.* from rapeseed or palm oil. The use of these resources is in direct competition with the production of food or promotes deforestation. [192] Using other crops that are not relevant for the food industry, as jatropha and camelina, would require additional agricultural land. Furthermore, to gain oil from the plants, a complex pretreatment, oil separation, and refining must be conducted. [193] As the dominant hydrocarbon chain lengths of the vegetable oils are C_{16} to C_{18}, [194] they do not fulfill the specifications for kerosene. The alkanes must be cracked and isomerized. [182, 193] Kerosene consists primarily of C_8 to C_{13} alkanes. [195] HAAs, as they can be synthesized by *P. putida* KT2440 or *P. taiwanensis* VLB120 occur in C_8 to C_{12} congeners, directly matching kerosene specifications. Another advantage is the secretion of the HAAs from the cell directly into the liquid culture medium instead of a product accumulation in the cell as with algae cultures or plants. [193] This phenomenon generally simplifies the product recovery and even enables the implementation of product recovery techniques directly integrated into the production process (see section **1.5**).

The recovered HAAs are further processed into kerosene by chemical synthesis. The conversion is initiated by the hydrolysis of the HAA ester moiety to gain 3-hydroxy-fatty acid. Several strategies are available to convert the hydroxy-fatty acid into fatty alcohols, fatty acid esters or alkanes, as suggested by Beller *et al.*[196]. Chemo-catalytic deoxygenations to convert vegetable oils (*i.e.*, fatty acids) into desired fuels or chemicals require elevated temperatures and high pressures of hydrogen, [197] and therefore reveal a high energy demand. However, bio-refineries capable of performing chemo-catalytic conversions are versatile in the industry, and a large-scale production can be implemented based on already established technology. In contrast to a chemo-catalytic deoxygenation, moderate temperatures and pressures are applied in electrochemical deoxygenation. To develop a regenerative biofuel production process, electrochemical reactions are particularly attractive as the required electricity may be provided by regenerative resources as

wind and solar energy. [198] One of the most relevant electrochemical conversions of bio-derived acids is the Non-Kolbe electrolysis, including a decarboxylation and subsequent deoxygenation. [199] As a Non-Kolbe electrolysis shows a high tolerance against impurities, a direct application of a cell-free culture medium is envisaged.

1.7 Scope and outline of this thesis

This doctoral thesis presents bioreactor and process configurations for the enhanced production of RLs and HAAs. Parallel to the development of efficient RL and HAA production methodologies, strategies for product separation and purification from the culture broth should be implemented as a generally low-cost biosurfactant production can only be achieved with an efficient DSP. As HAAs have been shown to degrade again in the culture broth, an integrated product separation into the production process was considered as particularly beneficial.

Chapter 1 introduces the reader to the scientific context on which this doctoral thesis is based. The products of interest, *i.e.* RLs and HAAs are described in terms of microbial production, particularly by *P. putida* KT2440. Known bioreactor processes for RL production are showcased, regarding foam fractionation as the preferred method for integrated product recovery. Further DSP techniques are described to separate RLs from impurities. RLs and HAAs derived by microbial conversion of sustainable substrates are presented as value-added products contributing to a vital bioeconomy.

Chapter 2 describes the applied materials and methods.

Chapter 3.1 shows a RL and HAA production *via* recombinant *P. putida* KT2440 and *P. taiwanensis* VLB120. With the establishment of microbial HAA production in a stirred and aerated bioreactor system, a more efficient production was envisaged. First approaches to enable a continuous product separation *via* foam fractionation are presented, revealing critical process parameters for a stable production process. In addition, the use of foam fractionation for RL and HAA separation was studied independently of the bioreactor process in an individually designed foam fractionation column. Filtration and adsorption techniques for product purification from a culture broth were also tested. Furthermore, methods were implemented to gain pure product by applying a preparative HPLC.

Chapter 3.2 provides a characterization of modified *P. putida* KT2440 lacking specific surface structures for a reduced biomass accumulation in the foam. Correspondent test methods were developed to evaluate the hydrophobicity of bacterial cell surfaces. With the implementation of

cell-surface deletion mutants as RL and HAA producers, a reduced biomass agglomeration in the separated and fractionated foam could be realized in a bioreactor system.

In Chapter 3.3, the bioreactor process for RL and HAA production was optimized by decoupling the foam fractionation from the bioreactor cultivation to achieve optimized conditions for both process steps. With such a system, the culture volume scale-up could be realized. In addition, an automated foam adsorption was integrated into the bioreactor process for final separation of the produced RLs and HAAs from the culture broth discharged from the bioreactor with the foam.

Chapter 4 discusses how all results and achievements presented in this thesis contribute to enhanced production and recovery of RLs and HAAs. In addition, potential approaches are outlined for further possible intensifications of a RL and HAA production process. These approaches are discussed in the context of a more cost-efficient and sustainable production together with the discussion of a potential system scale-up.