

Surfactants are valuable products with many potential applications due to their amphiphilic structure. The majority of surfactants are produced partially or completely from fossil feedstocks, having negative environmental impacts as a consequence. Biosurfactants can be produced as an alternative and have the potential to replace fossil-based surfactants. Moreover, the production of biosurfactants, such as rhamnolipids, with microbes using renewable substrates contributes to the establishment of a sustainable bioeconomy. However, the production with wild-type strains is challenging in many ways and alternative hosts are investigated, such as *Pseudomonas* strains. *Pseudomonads* are promising candidates for bioprocesses as they can be applied for redox-intensive reactions and due to their intrinsic endurance toward stressors.

In this thesis, the non-pathogenic *Pseudomonas putida* KT2440 was engineered for the synthesis of rhamnolipids from renewable feedstocks. Lignocellulose-derived sugars and organic acids, such as glucose, xylose, and galacturonic acid, were used. Furthermore, ethanol was used, which can be produced from lignocellulosic biomass by microbes. Synthetic biology tools were used to enable efficient substrate utilization. The resulting strains were characterized regarding their growth and rhamnolipid production. Different biomass yields, growth rates, product titers, and product yields were observed for all substrates, which allowed new insights into the respective metabolic pathways. In addition, a unique biosurfactant production process was developed by using ethanol as carbon source and defoamer simultaneously, tackling the challenge of excessive foam formation.

Moreover, metabolic engineering was performed to enhance rhamnolipid production. *P. putida* KT2440 was modified by targeted deletion of dispensable genomic regions coding for energy-intensive processes or the synthesis of competitive metabolites. With this, rhamnolipid titers could be increased by about 70%. In addition, the overexpression of heterologous genes was performed to overcome a bottleneck in precursor supply. The combination of these strategies further increased the rhamnolipid production up to 94%.

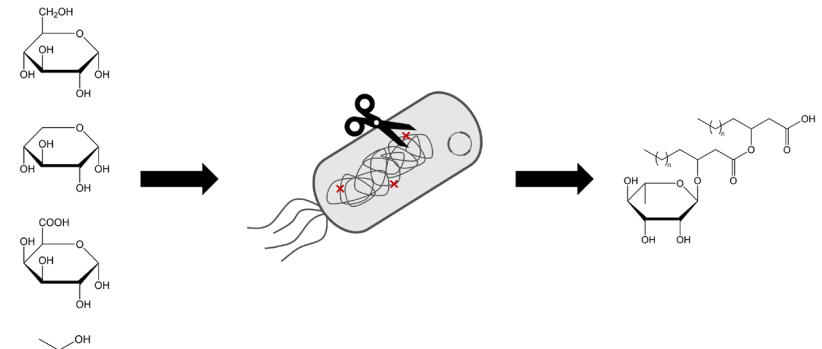
Altogether, this thesis demonstrates that *P. putida* KT2440 is a suitable host for sustainable biosurfactant production with improved yields enabled by metabolic engineering using renewable substrates, thereby contributing to the realization of efficient and industrial bioprocesses.

Deep genome editing of *Pseudomonas putida* for rhamnolipid production using non-conventional substrates

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Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der
RWTH Aachen University zur Erlangung des akademischen Grades einer Doktorin
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Summary

Biosurfactants, such as rhamnolipids, are valuable products with many potential applications. Their amphiphilic structure allows their use in different fields. The overall aim of this thesis was to produce rhamnolipids from renewable substrates using the non-pathogenic *Pseudomonas putida* KT2440, which would contribute to a sustainable bioeconomy. Various substrates were implemented, which allowed new insights into the respective metabolism.

In order to reduce the costs for rhamnolipid production, lignocellulose-derived substrates, such as xylose and galacturonic acid, can be used. While *P. putida* can natively utilize galacturonic acid, the use of xylose does not lead to growth. Three bacterial xylose utilization pathways were introduced and the engineered strains were characterized regarding their growth and production. While the implementation of the oxidative xylose pathways resulted in higher biomass production and rhamnolipid titers, the product yield was higher for the Isomerase pathway caused by the different stoichiometries of the pathways. Rhamnolipid production from galacturonic acid was immediately efficient, however the production rate was improved by laboratory evolution.

Ethanol is another sustainable carbon source as it can be produced out of lignocellulosic biomass by microbes. Laboratory evolution was applied to obtain a strain with a high growth rate on ethanol at elevated concentrations. Genome re-sequencing and gene expression studies revealed a rerouting of the metabolism for carbon-efficient ethanol utilization. The resulting biosurfactant producer was used in a fed-batch process, where the water-soluble ethanol served as carbon source and defoamer. This unique setup enabled a biosurfactant production of over 5 g L⁻¹ in one day with a great space time yield of 0.23 g L⁻¹ h⁻¹.

In addition, *P. putida* KT2440 was modified to generate a genome-reduced chassis by targeted deletion of dispensable elements or processes. Several deletions led to a 66% improved rhamnolipid production. As precursor supply seemed to be a bottleneck, the overexpression of the phosphoglucotransferase and rhamnose-operon was performed. The combination of a reduced genome and the overexpression further increased the rhamnolipid production up to 94%.

In conclusion, this thesis clearly illustrates that rhamnolipid production from renewable substrates is feasible. Further, the application of *P. putida* KT2440 as microbial cell factory in industrial processes is demonstrated. A combination of the improved chassis and the use of any renewable lignocellulose-derived substrate will truly contribute to establish a competitive production process.

Zusammenfassung

Biotenside, wie z.B. Rhamnolipide, sind wertvolle Produkte mit vielen möglichen Anwendungen. Aufgrund ihrer amphiphilen Struktur können sie in verschiedenen Bereichen eingesetzt werden. Das Gesamtziel dieser Arbeit war die Produktion von Rhamnolipiden aus nachwachsenden Substraten unter der Verwendung des nicht-pathogenen *Pseudomonas putida* KT2440, was zu einer nachhaltigen Bioökonomie beitragen würde. Es wurden verschiedene Substrate eingesetzt, die neue Einblicke in den jeweiligen Metabolismus ermöglichten.

Um die Produktionskosten zu reduzieren, können lignocellulose-abgeleitete Substrate, wie Xylose und Galacturonsäure, genutzt werden. Während *P. putida* Galacturonsäure natürlich verwerten kann, führt das Nutzen von Xylose zu keinem Zellwachstum. Es wurden drei bakterielle Xylose-Stoffwechselwege eingebracht und die modifizierten Stämme wurden bezüglich ihres Wachstums und ihrer Produktion charakterisiert. Während der Einsatz der oxidativen Xylose-Wege zu einer höheren Biomasseproduktion und höheren Rhamnolipid-Titern führte, war die Produktausbeute für den Isomerase-Weg besser aufgrund der unterschiedlichen Stöchiometrien der Wege. Die Rhamnolipidproduktion aus Galacturonsäure war direkt effizient. Die Produktionsrate wurde jedoch durch Laborevolution gesteigert.

Ethanol ist eine weitere nachhaltige Kohlenstoffquelle, da es mittels Mikroben aus lignocellulosehaltiger Biomasse hergestellt werden kann. Eine Laborevolution wurde durchgeführt, um einen Stamm mit hoher Wachstumsrate auf Ethanol bei erhöhten Konzentrationen zu erhalten. Genom-Resequenzierung und Genexpressions-Studien offenbarten ein Umsteuern des Metabolismus für eine kohlenstoffeffiziente Ethanolverstoffwechselung. Der resultierende Biotensidproduzent wurde in einem Fed-Batch Prozess eingesetzt, bei dem das wasserlösliche Ethanol als Kohlenstoffquelle und Entschäumer diente. Dieser einzigartige Aufbau ermöglichte eine Biotensidproduktion von über 5 g L^{-1} innerhalb eines Tages mit einer großartigen Raum-Zeit-Ausbeute von $0.23 \text{ g L}^{-1} \text{ h}^{-1}$.

Zusätzlich wurde *P. putida* KT2440 modifiziert, um genomreduzierte Chassis durch die gezielte Entfernung von überflüssigen Elementen oder Prozessen zu generieren. Einige Deletionen führten zu einer um 66% gesteigerten Rhamnolipidproduktion. Da die Zufuhr von Vorläufermolekülen als möglicher Engpass galt, wurde die Überexpression der Phosphoglucomutase und des Rhamnose-Operons durchgeführt. Die Kombination der reduzierten Chassis und der Überexpression konnte die Rhamnolipidproduktion weiter auf bis zu 94% steigern.

Zusammenfassend zeigt diese Arbeit deutlich, dass die Rhamnolipidproduktion aus nachwachsenden Substraten realisierbar ist. Des Weiteren wird der Einsatz von *P. putida* KT2440 als mikrobielle Zellfabrik in industriellen Prozessen demonstriert. Eine Kombination der verbesserten Chassis und der Verwendung eines beliebigen nachwachsenden Lignocellulose-abgeleiteten Substrat wird wahrhaftig dazu beitragen einen wettbewerbsfähigen Produktionsprozess zu etablieren.

List of abbreviations

%	percent
#	number
Ø	diameter
°C	degree Celsius
Ω	ohm
μ	growth rate
μF	microfarad
μg	microgram
μL	microliter
μRAMOS	microtiter plate-based respiration activity monitoring system
<i>A. calcoaceticus</i>	<i>Acinetobacter calcoaceticus</i>
<i>A. tumefaciens</i>	<i>Agrobacterium tumefaciens</i>
ACP	acyl carrier protein
ADP	adenosine diphosphate
ALE	adaptive laboratory evolution
alg	alginate
ATP	adenosine triphosphate
<i>B. glumae</i>	<i>Burkholderia glumae</i>
<i>B. plantarii</i>	<i>Burkholderia plantarii</i>
<i>B. pseudomallei</i>	<i>Burkholderia pseudomallei</i>
<i>B. thailandensis</i>	<i>Burkholderia thailandensis</i>
bcs	bacterial cellulose (synthesis)
BS	biosurfactant
BLAST	basic local alignment search tool
bp	base pairs
<i>C. crescentus</i>	<i>Caulobacter crescentus</i>
<i>C. glutamicum</i>	<i>Corynebacterium glutamicum</i>
Ca	calcium
CaCl ₂	calcium chloride
Cas	CRISPR protein
CCM	central carbon metabolism
cDNA	complementary DNA
CDW	cell dry weight
cm	centimeter
CMC	critical micelle concentration
Cmol	carbon mole
CO	carbon monoxide
CO ₂	carbon dioxide
CoA	coenzyme A
Cre	cyclization recombination
CRISPR	clustered regularly interspaced short palindromic repeats
CuCl ₂	copper(II) chloride
DNA	deoxyribonucleic acid

List of abbreviations

DNase	deoxyribonuclease
DO	dissolved oxygen
dTDP	deoxythymidine diphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
e.g.	exempli gratia
ED	Entner-Doudoroff
EDEMP	Entner-Doudoroff-Embden-Meyerhof-Parnas
EPS	exopolysaccharides
EtOH	ethanol
eV	electron volt
FAD ⁺	flavin adenine dinucleotide
FBA	flux balance analysis
FeSO ₄	iron(II) sulfate
flag	flagellar
Flp	flippase
FRT	flippase recognition target
g	gram
g	g-force
GC	gas chromatography
GFP	green fluorescent protein
Glc	glucose
Gm ^R	gentamycin resistant
h	hour(s)
H ₂ O	water
H ₂ SO ₄	sulfuric acid
H ₃ BO ₃	boric acid
HAA	3-(3-hydroxyalkanoyloxy)alkanoic acid
HPLC	High performance liquid chromatography
i.e.	id est
iAMB	Institute of Applied Microbiology
InDel	insertion-deletion polymorphisms
I-SceI	<i>Saccharomyces cerevisiae</i> endonuclease
kb	kilobase pairs
KEGG	Kyoto encyclopedia of genes and genomes
KH ₂ PO ₄	potassium dihydrogen phosphate
Km ^R	kanamycin resistant
kV	kilovolt
L	liter
LapA/F	large adhesive protein A/F
LB	lysogeny broth
<i>loxP</i>	locus of X-over P1
LPS	lipopolysaccharides
m	meter
mg	milligram
Mg	magnesium
MgSO ₄	magnesium sulfate

MHETase	mono-(2-hydroxyethyl)terephthalic acid hydrolase
min	minutes
mL	milliliter
mm	millimeter
mM/mmol	millimolar
mN	millinewton
MnCl ₂	manganese(II) chloride
MS	mass spectrometry
n.d.	not determined
Na ₂ EDTA	ethylenediaminetetraacetic acid disodium salt
Na ₂ HPO ₄	disodium hydrogen phosphate
Na ₂ MoO ₄	sodium molybdate
NaCl	sodium chloride
NAD(P) ⁺	nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	reduced nicotinamide adenine dinucleotide (phosphate)
NH ₄ Cl	ammonium chloride
nm	nanometer
OD ₆₀₀	optical density at 600 nm
ori	origin of replication
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
<i>P. chlororaphis</i>	<i>Pseudomonas chlororaphis</i>
<i>P. fluorescens</i>	<i>Pseudomonas fluorescens</i>
<i>P. fragi</i>	<i>Pseudomonas fragi</i>
<i>P. plecoglossicida</i>	<i>Pseudomonas plecoglossicida</i>
<i>P. putida</i>	<i>Pseudomonas putida</i>
<i>P. stutzeri</i>	<i>Pseudomonas stutzeri</i>
<i>P. taiwanensis</i>	<i>Pseudomonas taiwanensis</i>
PCR	polymerase chain reaction
Pea	<i>putida</i> EPS a
Peb	<i>putida</i> EPS b
PETase	polyethylene terephthalate hydrolase
pgm	phosphoglucomutase
pH	potentia hydrogenii
PHA	polyhydroxyalkanoate
PQQ	pyrroloquinoline quinone
PQQH ₂	reduced pyrroloquinoline quinone
pvd	pyoverdine
qRT-PCR	quantitative real-time PCR
RL	rhamnolipids
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
RWTH	Rheinisch-Westfälische Technische Hochschule
S	substrate
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SDS	sodium dodecyl sulfate

List of abbreviations

SEVA	Standard european vector database
Sm ^R	streptomycin resistant
SNP	single nucleotide polymorphisms
sp.	species
syngas	synthesis gas
TCA cycle	tricarboxylic acid cycle
Tc ^R	tetracyclin resistant
Tn7/ <i>att</i> Tn7	transposon 7/attachment site
TS	target sequence
USD	United States dollar
UV	ultraviolet
v/v	volume per volume
vvm	volume flow per unit of liquid volume per minute
w/v	weight per volume
ZnSO ₄	zinc sulfate

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Chapter 1

General introduction

Contributions:

This chapter was written by Isabel Bator and reviewed by Lars M. Blank.

1 General introduction

1.1 Bioeconomy – a sustainable bio-based production of chemicals

The world population is growing and the demand for food, platform chemicals, electricity, and fuels is constantly increasing. Most of the production processes for commodities of everyday life rely on fossil resources. This is displayed by a constant increase of the production and demand of crude oil, natural gas, and coal in the last decades [1,2]. The finite resources and the ever-increasing growth of the population will lead to a shortage in availability in the future. When the demand exceeds the supply, increasing prices and conflicts due to dependency on oil-exporting countries are a consequence. Further, the use of fossil resources causes enormous emission of pollutants and greenhouse gases, including CO₂ in the atmosphere, contributing to global warming [3], which is already a critical issue nowadays. Thus, a transition from a fossil-based to a sustainable bio-based economy is necessary, the latter also known as bioeconomy.

The bioeconomy represents parts of the economy in which renewable resources, such as crops, forests, fish, animals, and microorganisms, are used to produce food, chemicals, energy, and fuels [4]. Renewable or bio-based resources have a closed carbon cycle in contrast to fossil resources [5,6], addressing sustainability. Besides, renewable resources can grow in various climates and thus, local resources can be used, whereby the dependency from other countries is reduced. The overall goal is to create a circular bioeconomy, where the emitted CO₂ is fixed by plants and in the future also microbes to produce biomass, which can be used as feedstock for the production of fuels and chemicals [5,7] (Figure 1).

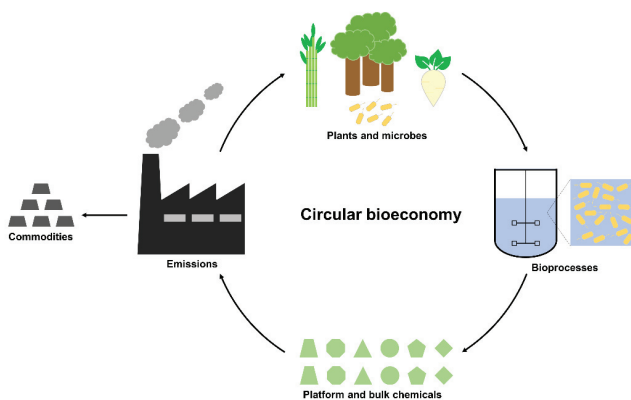


Figure 1: Circular bioeconomy. Emissions are fixed by plants and microbes, which are converted into usable substrates for microorganisms. The microorganisms produce chemicals in bioreactors, which can then be used as building blocks for commodities, resulting at the end-of-life of the commodities to new emissions.

1.1.1 Renewable resources

The use of renewable substrates as carbon and energy sources in industrial biotechnology is one of the main objectives to create cost-efficient and ecologically friendly bioprocesses. Microbes are used to produce platform and bulk chemicals as well as biofuels from bio-based resources. The resources can be divided into first and second generation feedstocks.

First generation feedstocks include vegetable oil, corn, sugarcane, and starch and sucrose fractions of plant carbohydrates [5,8]. One major drawback of using first generation feedstocks in bioprocesses is the competition with food and feed production. This provokes the food versus fuel debate and could lead to increasing prices or a shortage in the availability of these resources [9]. Further, using only the readily fermentable fractions and neglecting the remaining fractions of plants lowers the yields and does not contribute to a sustainable bioeconomy. However, many of the current biotechnological production processes still rely mainly on glucose as carbon source [10].

Thus, second generation feedstocks are considered in bioprocesses to bypass the challenges, especially because they are abundant. Non-edible biomass such as agricultural residues, wood and forest residues, and grasses, which all represent examples of lignocellulosic biomass, belong to second generation feedstocks [5,8,11]. For example, wheat straw and corn stover remaining after harvest and black liquor from the paper industry are residues that can be used as second generation feedstock. Lignocellulose is part of the plant cell wall and contains cellulose (33-51%), hemicellulose (19-34%), and lignin (20-30%), and depending on the feedstock pectin (2-20%). Several monomers can be derived from these fractions, such as glucose, arabinose, galactose, xylose, aromatics, and galacturonic acid [12]. The composition (including the monomers) is dependent on the plant source.

The utilization of these feedstocks is challenging in several ways. On the one hand, second generation feedstocks are not accessible in its untreated form and have to be pretreated and hydrolyzed (enzymatically and/or chemically) prior to use [10]. Hydrolysis of lignocellulosic biomass results in a mixture of pentoses and hexoses, but also microbial inhibitors, such as weak acids, furans, and phenolic residues [13]. To eliminate the inhibitory effect of the hydrolysate, detoxification is adapted, which increases effort and costs. Biological, physical, and chemical detoxification methods can be employed, such as enzyme treatment with peroxidases or laccases [14], rotary evaporation [15], extraction with solvents [15], or treatment with sulfite [16]. On the other hand, many commonly used microbes in bioprocesses can even not utilize the monomers of the hydrolysates [17]. Hence, these microbes have to be engineered to be able to use the carbon sources or new microbes have to

be selected for bioprocesses. One example is the engineering of *Saccharomyces cerevisiae* to utilize xylose [18], arabinose [19], and cellulose [20].

The interest of using lignocellulosic biomass for the production of bulk and fine chemicals as well as biofuel has increased in the last decades due to its abundance and low cost [21]. However, the application of second generation feedstocks for the production of platform and bulk chemicals is still challenging and the processes are not competitive with fossil-based or first generation feedstock-based processes [22]. Most processes are still on the experimental scale, but some processes have been taken one step further and are on pilot scale, e.g., lactic acid production from miscanthus grass [23]. Whereas the production of bioethanol from lignocellulosic biomass is a prominent example of bio-based processes. Various bioethanol plants are already in operation [21,24].

Since many microbes are also capable of utilizing alcohols, ethanol can not only be used as biofuel and platform chemical but also as carbon source for the production of valuable products. This concept would truly contribute to a circular bioeconomy, however, requiring a further value increase.

To minimize land use, CO₂ can be directly used as carbon source for microbes. A high rate of acetate production was achieved with *Moorella thermoacetica* using CO₂, H₂, and glucose co-utilization [25]. In industry, the use of synthesis gas (syngas), CO₂, CO, and H₂, is followed for example by LanzaTech [26]. Here, syngas from steel plants is converted to ethanol. Hence, for the largest industrial microbiology product with a market above 40 million tonnes per year, two alternative sustainable synthesis routes, from lignocellulosic biomass and syngas, exist.

1.2 Biosurfactants as promising alternative

Several chemically synthesized surfactants are known, such as anionic, cationic, nonionic, and amphoteric surfactants, and used in everyday life commodities. They are produced via chemical reactions and most of them are made partially or completely from fossil feedstocks. For the synthesis, harsh conditions are needed, e.g., high temperature or high pressure [27]. These conditions also bring along a high energy demand for production. Biologically produced surfactants, the so-called microbial surfactants or biosurfactants, instead are made from renewable feedstocks under softer conditions using microbes. The production is conducted in a liquid system using fermentation vessels at temperatures around 30-37°C and a neutral to slight acidic pH (4.5-7) according to the specific optimum of the microbe [28,29]. The demand for green solutions is increasing and the global biosurfactant market is estimated at 4.20 billion USD in 2017 with an expected increase to 5.52 billion USD by 2022 [30].

The comparison of chemically synthesized surfactants and biosurfactants is difficult due to the differences that emerge from their origin. Although both surfactant classes have an amphiphilic character, their properties differ significantly in terms of, e.g., biodegradability and ecotoxicity. In general, the soft conditions under which biosurfactants are produced and the nature of their bonds (e.g., esters) lead to a less stable molecule [27], resulting in a better biodegradability. Further, biosurfactants are less toxic to the aquatic environment than chemically synthesized biosurfactants [31,32]. Their unique structures entail new properties that conventional chemical surfactants lack, which is primarily the reason why they gained attention as a valuable product. Furthermore, all surfactants are known to lower surface and interfacial tension [33].

Several biosurfactants were identified and classified based on their structure, as reviewed by Rosenberg and Ron [34]. Five main biosurfactant classes exist: glycolipids, lipopeptides, phospholipids, particulate biosurfactants, and polymeric biosurfactants [33,34]. These biosurfactants have all different structures and properties and are produced by a variety of microorganisms, of which some are listed in Table 1. Out of these classes, sophorolipids, rhamnolipids, and surfactin are the most reported and studied biosurfactants.

Table 1: Selection of biosurfactants and producing microorganisms.

Biosurfactants	Microorganisms	References
Glycolipids		
Mannosylerythritol lipids	<i>Pseudozyma antarctica</i>	[35]
	<i>Ustilago zeae</i>	[36]
Rhamnolipids	<i>Pseudomonas aeruginosa</i>	[37]
	<i>Burkholderia thailandensis</i>	[38]
Sophorolipids	<i>Sarmerella bombicola</i> (former <i>Torulopsis bombicola</i>)	[39]
	<i>Candida batistae</i>	[40]
Lipopeptides		
Gramicidin S	<i>Bacillus brevis</i>	[41]
Serrawettin	<i>Serratia marcescens</i>	[42]
Surfactin	<i>Bacillus subtilis</i>	[43]
Particulate biosurfactants	<i>Acinetobacter</i> species	[44]
Polymeric biosurfactants		
Emulsan	<i>Acinetobacter calcoaceticus</i>	[45]
Liposan	<i>Candida lipolytica</i>	[46]
Mannan-lipid-protein	<i>Candida tropicalis</i>	[47]
Phospholipids		
	<i>Thiobacillus thiooxidans</i>	[48]
	<i>Acinetobacter radioresistens</i>	[49]

1.2.1 Applications of biosurfactants

In the last decades, the pursuit of finding environmentally friendly solutions and replacing non-biodegradable substances increased. Biosurfactants have the potential of replacing chemical surfactants theoretically in a great number of processes or niches. Currently, the use

of biosurfactants in enhanced oil recovery and bioremediation is considered to be the main application due to their effectiveness based on their low critical micelle concentration and biodegradability [50].

Organic chemical hazards, such as pesticides, organic liquids, and oils, and heavy metals are contaminants of the soil and aquatic environment, which are normally difficult to remove. The remediation of those polluted sites is usually done via soil washing or bioremediation. The properties (hydrophobicity) of some contaminants lead to an interaction with non-aqueous phases and thus reduced availability for microbial degradation, since microbes rather degrade chemicals that are dissolved in water [51]. Hence, the efficiency of bioremediation is dependent on the availability of microbes, accessibility of contaminants, and success of degradation of toxic substances in less toxic substances [52,53]. Biosurfactants or microorganisms, which produce biosurfactants, can promote the desorption of the contaminants and thus improve the bioavailability of the contaminants resulting in efficient remediation or degradation [54–56]. Further, the remediation of metal-contaminated soil using biosurfactants was shown to be efficient [57]. However, this was only shown on experimental scale, where rhamnolipid foam was channeled through a column filled with contaminated soil, and feasibility and effectiveness of this technology in field applications has to be further investigated. In general, the application of biosurfactants in bioremediation is enabled due to their emulsifying properties.

Biosurfactants can also potentially be used for the extraction of crude oil when primary and secondary oil recovery methods are reaching their limits, which is called enhanced oil recovery. This technique is especially used in reservoirs with low permeability or for crude oil with high viscosity [52]. There, the insertion of microbes, which produce biosurfactants, or the purified biosurfactant itself is possible. The formation of oilfield emulsions is one of the major problems during oil recovery [58]. To extract the oil thereof, a de-emulsification process is required, which can potentially be fulfilled by using biosurfactants [52]. In enhanced oil recovery, the role of the biosurfactants is generally, e.g., de-emulsification, viscosity reduction, or reduction of the interfacial tension [59]. Glycolipids and lipopeptides (e.g., rhamnolipids and surfactin) were shown to be effective and many field trials were performed since the 1990s [60].

However, biosurfactants have further potential applications in fields, such as food and food-related industries, cosmetics, detergent industry, biomedicine and therapeutics, and agricultural industry. A short overview of applications of the mentioned fields and already available products are given in the next paragraphs.

In the food industry, biosurfactants are discussed as food-formulation ingredients, which can replace the chemical substances and meet the requirements for use in food. Apart from the obvious role of biosurfactants for stabilization and formation of emulsions, they can have other functions in food. They can also increase the shelf life of starch-containing products, modify rheological properties of wheat dough, solubilize flavor oils, and delay staling [61]. For example, the dough stability, texture, and conservation of bakery products was improved by the use of rhamnolipids [62]. One study even showed the replacement of egg yolk by biosurfactants and could maintain the properties of the standard formulation [63]. An essential step in food production is the maintenance of hygiene. Biofilms are encountered on food-contact surfaces, which may lead to food spoilage. The use of biosurfactants (e.g., surfactin) for control of microbial adhesion and especially detachment from surfaces and reduction of colonization is another potential application [64].

Biosurfactants can be deployed in health care and cosmetic products as an emulsifying agent, which is beneficial for the texture of cosmetic products [65]. They can also be applied for the stimulation of dermis cells, which is important in anti-aging, repair, and restructuring products [59,66]. For example, mannosylerythritol lipids act as anti-aging agent and recovery agent after cell damage [67]. These glycolipids are currently produced by the company Toyobo and used in moisturizers [68].

Biosurfactants can also be used as detergents and in cleaning products due to their amphiphilic structure. The replacement of chemical surfactants by biosurfactants as detergents is advisable because they often find their way into the environment and biosurfactants are less toxic and better biodegradable [69]. They can be applied in formulations for washing powder or cleaning soap mixtures for example [69,70]. A sophorolipid-containing product line (Ecover), including household cleaners, hand soap, laundry liquids, and dishwashing liquids, is already available for consumers [71]. Recently, Unilever launched their dishwashing liquid containing rhamnolipids produced by Evonik [72].

Several biosurfactants have shown antimicrobial activity and inhibition of biofilms and can thus be used in therapeutic or biomedical applications. This antimicrobial and -viral activity of different biosurfactants is effective against (pathogenic) bacteria, fungi, algae, and viruses [73–76]. Further, mannosylerythritol lipids were shown to induce growth arrest, apoptosis, and differentiation of mouse malignant melanoma cell cultures and induce cell differentiation instead of cell proliferation in a human promyelocytic leukaemia cell line [77,78], indicating the use of biosurfactants as reagents for treatment of cancer cells. Moreover, some biosurfactants were demonstrated to have anti-inflammatory activity and applied to produce microemulsions for drug delivery [79,80].

In agriculture, biosurfactants can fulfill different functions. They can be applied on the surface of plants, seeds, or on the soil. Biosurfactants can improve the overall quality of the soil due to their antimicrobial activity, resulting in a decrease of plant-pathogens and an increase of nutrient availability [81,82]. But also because of their remediation ability for contaminants in soils as described before. Furthermore, biosurfactants can increase the solubility of pesticides, resulting in better protection, and wettability of soils, which leads to a better distribution of fertilizers [81,83]. Another benefit of biosurfactants is the inhibition of zoospore-forming plant pathogens that have acquired resistance to commercial chemical pesticides [82,84]. Zonix, an released biofungicide from Jeneil Biosurfactant Company, contains rhamnolipids and provides protection against plant pathogens and promotes plant health [85]. Further, the treatment of seeds with biosurfactants can increase the germination rate and seedling development and stimulate the plant immune system [86,87].

1.3 Rhamnolipids as example

Glycolipids are one of the best studied and most patented biosurfactants [88]. Rhamnolipids belong to the class of glycolipids and are already produced on an industrial scale [89] and used in commercial products (e.g., cleansing agents, fungicides, and cosmetics [88,90]). Several companies, including EcoChem Organics Company (Canada), Pendagron Holdings Ltd. (United Kingdom), and Jeneil Biotech Inc. (USA) [52,88], have commercialized rhamnolipid-based products and more products are expected for the future.

Rhamnolipids contain a hydrophobic part, which is made up generally of two β -hydroxy fatty acids forming a 3-(hydroxyalkanoyloxy)alkanoic acid (HAA), which is connected via a glycosidic linkage to a hydrophilic part, consisting of one or two rhamnose molecules [37,91] (Figure 2A). Biosurfactants are always produced as a mixture of several congeners, meaning different molecules of one class are present. In the case of rhamnolipids, varying chain lengths and degree of saturation of the fatty acid chains and different numbers of rhamnose molecules were determined. Fatty acid chain lengths between 8 and 16 carbon atoms were reported [92]. Furthermore, rhamnolipids with only one fatty acid chain, the so-called mono- or di-rhamno-mono-lipids, were reported [93]. A detailed overview of the reported congeners is given by Abdel-Mawgoud *et al.* [92]. Thus, the characterization of the class itself is not obvious and primarily a variety of molecules define the reported characteristics. Further, the reported characteristics and activities for rhamnolipids vary due to different production processes and purification steps resulting in a different composition of these mixtures.

Surfactants are described by physicochemical properties, such as surface tension activity, critical micelle concentration (CMC), and emulsification activity. Water has a surface tension of 72 mN m^{-1} at 25°C . Rhamnolipids from *P. aeruginosa* can reduce the surface tension of

water below 30 mN m^{-1} [94,95]. Compared to a conventional and effective surfactant (sodium dodecyl sulfate (SDS)), the surface tension reduction is in a similar range.

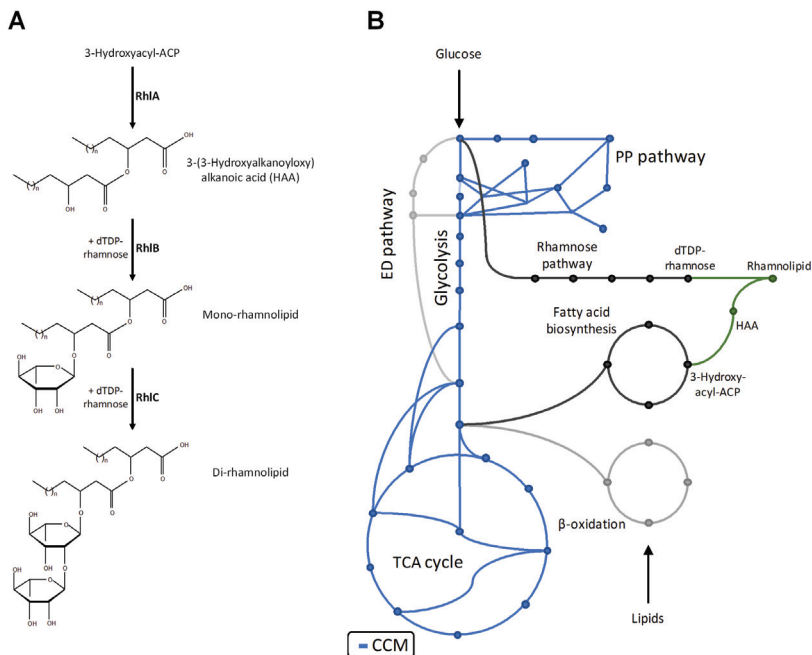


Figure 2: Rhamnolipid biosynthesis pathway with the respective enzymes. (A) Shown are the enzymes RhlA, RhlB, and RhlC, which catalyze the formation of the precursor 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA), mono-rhamnolipids, and di-rhamnolipids. The molecule structure indicates that different chain lengths of the fatty acid chains exist. (B) Schematic illustration of rhamnolipid biosynthesis with the central carbon metabolism (CCM) structure and the rhamnolipid precursor-providing pathways (dark grey): the rhamnose pathway and fatty acid biosynthesis. The blue lines indicate the CCM based on Sudarsan *et al.* [96] and the light grey lines indicate the β -oxidation and Entner-Doudoroff (ED) pathway. The structure of the central carbon metabolism varies between different producer strains. The green lines show the rhamnolipid synthesis pathway.

The CMC is a parameter that determines the concentration above which micelles are formed and monomers and micelles exist in a dynamic equilibrium [97]. A lower CMC results in a higher efficiency for the biosurfactant and thus, lower amounts are needed for a reduction of the surface tension. It was observed that the CMC strongly depends on the producing organism and the mixture of the congeners (i.e., saturation degree, chain lengths, rhamnose molecules). For rhamnolipids, varying CMC values between 5 and 234 mg L^{-1} are reported [98–101]. The CMC of SDS in water is multiple times higher with a value of $2,100 \text{ mg L}^{-1}$ compared to rhamnolipids [69].

Another factor for the characterization of biosurfactants is the emulsification activity, which is normally determined for oil-in-water emulsions. This parameter is strongly dependent on the experimental setup and the used organic phase. Nevertheless, it gives a rough estimation of the properties and emulsification activities. Activities of 60 to 100% for rhamnolipids are reported [95,102].

1.3.1 Biosynthesis of rhamnolipids

The biosynthesis proceeds in three consecutive enzymatic steps in microbes (Figure 2A). The first step is the synthesis of HAA via esterification of two β -hydroxyacyl-ACP molecules, catalyzed by the acyltransferase RhlA [103,104]. Then, the rhamnosyltransferase RhlB adds a dTDP-rhamnose to the HAA molecule to form a mono-rhamnolipid [103]. The third and last step is the synthesis of a di-rhamnolipid by adding another dTDP-rhamnose molecule to the mono-rhamnolipid, catalyzed by the rhamnosyltransferase RhlC [105]. The biosynthesis of mono- or di-rhamno-mono-lipids is still unknown. However, hydrolysis of one fatty acid chain from the dimer of esterified fatty acids from mono- or di-rhamnolipids is speculated [106].

Both precursor molecules, dTDP-rhamnose and β -hydroxyacyl-ACP, are derived from primary metabolism of the cells (Figure 2B). Rhamnose is a molecule, which is common in Gram-negative bacteria because it is introduced in lipopolysaccharides (LPS), which are components of the outer membrane. The synthesis of dTDP-rhamnose is catalyzed by five enzymes starting from glucose-6-phosphate. A phosphoglucosyltransferase (pgm) and four enzymes called RmlA, RmlB, RmlC, and RmlD catalyze the consecutive reactions [107]. The activated β -hydroxy fatty acids are derived from the general fatty acid metabolism, namely the fatty acid *de novo* synthesis or β -oxidation. The stereochemistry of the HAA fatty acid chains resembles the conformation of the fatty acids from *de novo* synthesis [108]. Thus, it is suggested that RhlA competes with FabA and FabI for the intermediate and is removing the β -hydroxyacyl-ACP from the fatty acid *de novo* synthesis [109]. On the other hand, it was shown that fatty acids derived from the β -oxidation are involved in rhamnolipid synthesis [110,111]. Hence, the involvement of both pathways for the provision of the fatty acids for HAA synthesis is assumed.

1.3.2 Microbial production of rhamnolipids

The microbial production of rhamnolipids was first found in the opportunistic human pathogen *Pseudomonas aeruginosa* [37]. Until today, the production of rhamnolipids is best studied and characterized in this organism and the highest titers are also reached with *P. aeruginosa* [112,113]. Rhamnolipids are involved in biofilm formation and swarming

motility in *P. aeruginosa* [114,115]. Rhamnolipid production was also discovered in other bacteria, which belong to the *Pseudomonadales*, including *P. chlororaphis* [116], *P. fluorescens* [117], and *A. calcoaceticus* [118]. Several gammaproteobacteria, e.g., *Enterobacter asburiae*, *Pantoea stewartii* [119], and *Thermus aquaticus* [120], are also capable of rhamnolipid production. Another genus with many representatives, which harbor rhamnolipid biosynthesis genes, is *Burkholderia*. Rhamnolipid production was detected in, e.g., *B. pseudomallei* [121], *B. thailandensis* [38], and *B. plantarii* [122]. Among these species, the first is a human and animal pathogen. Natural biosynthesis is regulated by a complex regulatory network, including quorum sensing and described for various species, e.g., *P. aeruginosa* or *B. glumae* [123–126].

A different genetic organization of rhamnolipid genes comes along with the existence of rhamnolipid production in several microorganisms. In *P. aeruginosa*, the genes *rhlA* and *rhlB* are located in a bicistronic operon [103]. The gene *rhlC* located elsewhere in the genome is also organized in a bicistronic operon with a gene of unknown function [105]. Further, strains that harbor only *rhlA* and *rhlB* and solely produce mono-rhamnolipids, like *P. chlororaphis* or a taxonomic outlier *P. aeruginosa*, are described [116,127]. In *Burkholderia* species, the *rhl* genes are organized in an operon [38,128]. Additionally, two identical copies of these *rhl* operons exist in several *Burkholderia* species, e.g., *B. thailandensis* and *B. pseudomallei*, and both contribute to rhamnolipid synthesis [38].

1.3.3 Challenges of microbial large-scale production

Although the production is studied in detail, their commercial use compared to chemical surfactants is still relatively low. This is because of the high process costs and thus, rhamnolipid production processes cannot compete with chemical surfactants economically. Rhamnolipids are usually produced using hydrocarbons as carbon source, which are typically expensive substrates and thus contributing to high raw material costs. In addition, oily substrates can increase the downstream processing effort and costs. It is estimated that the raw material costs (such as carbon and nitrogen sources) in biosurfactant processes account for up to 50% of the total costs [17]. Furthermore, low productivity, excessive foam formation, and downstream costs are challenges that have to be addressed for an economically feasible production [129]. Several strategies are applied to achieve more economic production.

A first attempt is the optimization of the medium composition involving renewable substrates. Different waste substrates can be used for rhamnolipid production, such as fatty acids from soybean oil refinery wastes [98], olive oil production effluents [130], and whey wastes [131]. Waste substrates probably become more important in the future since they do not directly compete with food and are usually less expensive [17]. Rhamnolipid production

was also achieved using water-soluble substrates, such as glucose, glycerol [132], ethanol [133], and molasses [134]. However, not only the carbon source plays an important role, the nitrogen source is also of relevance. Contrary to carbon sources, nitrogen sources are cheaper in its pure form (nitrate, ammonia, urea) [17]. Out of different nitrogen sources, nitrate is the best source for rhamnolipid production, but also the carbon/nitrogen ratio is a crucial factor to be included [135,136]. Further, growth-limiting conditions and limiting concentrations of several ions (e.g., Mg and Ca) are beneficial for rhamnolipid production [52,135].

The cultivation strategy is another parameter, which is addressed for improved biosurfactant production. The use of bioreactors allows the control of oxygen transfer and pH and can thus create optimal conditions for rhamnolipid production. Both continuous and fed-batch fermentation strategies are proposed to be more economically viable compared to batch fermentations due to the reduction of time-consuming and expensive steps, such as inoculum preparation and sterilization [52,137]. One major problem arises using aerated bioreactors for biosurfactant production with hydrophilic substrates, which is intensive foaming. The foaming facilitates product and biomass loss, while the loss of the producer reduces the production efficiency [138]. Several strategies can be applied to control the foaming, such as antifoam agents or mechanical foam breakers. However, they have a negative impact on the process, such as reduced oxygen transfer rates and additional energy demand, leading to more laborious downstream processes and complex setups [139,140]. To overcome this limitation, alternative strategies can be applied, e.g., adsorption of cell-containing foam [141], bubble-free membrane reactors [139], and foam fractionation [142].

Another step of a production process is the downstream processing, which has a main impact on the economic feasibility due to its high costs. However, the impact of this parameter is dependent on the used strain, produced rhamnolipid mixtures, and importantly the application of the product since a high purity is not always mandatory. Conventional downstream techniques were used for biosurfactant recovery, such as acid precipitation, solvent extraction, crystallization, and centrifugation [33]. In addition, methods that take advantage of the properties of biosurfactants and are applicable for continuous recovery from the fermentation broth, are reported. These methods include foam fractionation, ultrafiltration, ion exchange chromatography, and adsorption-desorption [143].

To achieve a higher productivity, mutants and recombinant strains are established in biosurfactant processes. In general, a process cannot be viable and profitable, if the product to substrate yield is low and thus would not be commercialized. Rational and non-rational engineering strategies can be applied to improve strain performance and consequently the

rhamnolipid production, such as transposons [144], chemical mutagens [145], or targeted gene overexpression [146]. Another factor that has to be taken into account is the pathogenicity of various producer strains and the regulated rhamnolipid production. These features have a considerable negative impact on product permission and productivity. In order to circumvent regulation and safety concerns, recombinant strains, which are non-pathogenic, are used for heterologous rhamnolipid production. Another benefit of this strategy is that the synthesis is uncoupled from growth, resulting in a constant-specific rhamnolipid production rate [147]. Recombinant rhamnolipid production by expression of *rhlAB* genes from *P. aeruginosa* is achieved in several strains. Some examples are given in Table 2.

Table 2: Rhamnolipid production of recombinant strains.

Microorganism	Substrate	Rhamnolipids (g L ⁻¹)	References
<i>P. putida</i> KT2440	glucose	0.22	[147]
<i>Escherichia coli</i>	tryptone	-	[148]
<i>P. fluorescens</i>	glycerol	0.25	[149]
<i>P. taiwanensis</i> VLB120	glucose/xylose	~ 0.7	[150]
<i>Pantoea</i> sp. P37	glycerol	0.4	[151]
<i>P. putida</i> KT2440	acetate	0.25	[152]
<i>P. stutzeri</i> DQ1	glycerol	0.87	[153]
<i>S. cerevisiae</i>	sucrose	-	[154]

1.4 *Pseudomonas* as microbial cell factory

Members of the genus *Pseudomonas* are ubiquitous Gram-negative bacteria belonging to the class of gammaproteobacteria. Almost 200 different species are described, which belong to this genus. They are found in different habitats, including soil, water, plants, and animals [155]. Their ubiquitous incidence already implies that they harbor a versatile metabolism. Some *Pseudomonas* strains are able to utilize more than 100 different carbon and energy sources, including toxic organics such as aliphatic and aromatic hydrocarbons [156,157]. They fulfill important metabolic activities in the environment, such as degradation and recycling of biogenic and xenobiotic organic compounds and growth promotion and protection of plants [156]. Hence, they are deployed in biotechnological applications, e.g., in bioremediation [158] and as biocontrol agents in plant protection [159]. Pseudomonads are also renowned for their solvent tolerance, have simple nutritional requirements, feature fast growth, and synthesize low amounts of by-products [156,160,161]. In addition, pseudomonads are able to support high redox cofactor regeneration rates. *Pseudomonas* species belong to the minority of microbes bearing both the soluble, non-energy-dependent transhydrogenase and the membrane-bound, proton-translocating transhydrogenase allowing

for tremendous flexibility in redox cofactor balancing [162]. For this reason, *Pseudomonas* was recognized as a promising host for redox-intensive reactions [163,164].

Due to the described features, pseudomonads seem to be ideal candidates for biotechnological purposes. This is reflected by the huge number of publications dealing with the characterization and use of *Pseudomonas* species, especially *P. putida*, *P. fluorescens*, *P. taiwanensis*, and *P. aeruginosa* [155,164–166]. Many of them are used as microbial cell factories for the recombinant synthesis of various products. Some examples of products are listed in Table 3. Pathogenic strains exist among them, which likely evoke safety concerns. *P. putida* strains are one of the most promising and frequently applied strains in biocatalysis. However, this class was reclassified from safety class 1 to safety class 2 in 2011 in Germany due to reports stating their pathogenicity [155]. However, one commonly used strain of this class, *P. putida* KT2440, remained in safety class 1. This is likely based on the questionable derivation since *P. putida* KT2440 has a higher 16S rRNA gene sequence similarity to *P. plecoglossicida* than to *P. putida* strains [167].

Table 3: Products synthesized in *P. putida*

Strain	Product	References
<i>P. putida</i> S12	phenol	[168]
<i>P. putida</i> S12	<i>p</i> -hydroxybenzoate	[169]
<i>P. putida</i> S12	<i>t</i> -cinnamate	[170]
<i>P. putida</i> S12	<i>p</i> -hydroxystyrene	[171]
<i>P. putida</i> S12	<i>p</i> -coumarate	[172]
<i>P. taiwanensis</i> VLB120	phenol	[173]
<i>P. taiwanensis</i> VLB120	<i>t</i> -cinnamate	[174]
<i>P. taiwanensis</i> VLB120	<i>p</i> -hydroxybenzoate	[175]
<i>P. taiwanensis</i> VLB120	isobutyric acid	[176]
<i>P. taiwanensis</i> VLB120	<i>n</i> -octanol	[177]
<i>P. putida</i> DOT-T1E	2-phenylethanol	[178]
<i>P. putida</i> DOT-T1E	L-phenylalanine	[178]
<i>P. putida</i> KT2440	zeaxanthin	[179]
<i>P. putida</i> KT2440	rhamnolipids	[147]
<i>P. putida</i> KT2440	flaviolin	[180]
<i>P. putida</i> KT2440	prodigiosin	[181]
<i>P. putida</i> KT2440	pyocyanin	[182]
<i>P. putida</i> KT2440	<i>N</i> -methylglutamate	[183]

Thus, *P. putida* KT2440 seems to be an ideal candidate for biotechnology, particularly in Germany. The genome of this strain is sequenced and well-annotated [184–186], allowing for genomic engineering. Further, the metabolic network structure of *P. putida* KT2440 and its growth behavior on different substrates is studied in detail [96,187,188].

The metabolic network structure for utilization of glycolytic substrates, e.g., the frequently used glucose, is unusual and thus characteristic for *P. putida*. Glucose is converted

to glucose-6-phosphate by phosphorylation or to gluconate-6-phosphate via gluconate or 2-ketogluconate by oxidation [189]. Due to the lack of a 6-phosphofructokinase, the linear Embden-Meyerhof-Parnas pathway is incomplete, which usually yields pyruvate. Instead, glucose-6-phosphate is mainly metabolized using the Entner-Doudoroff pathway yielding glyceraldehyde-3-phosphate and pyruvate. Further, a minor part of 6-phosphate-gluconate is introduced in the pentose phosphate pathway. The glyceraldehyde-3-phosphate resulting from the Entner-Doudoroff pathway is either converted into pyruvate or channeled in the Embden-Meyerhof-Parnas pathway in a gluconeogenic fashion forming a cyclic metabolic architecture, the EDEMP cycle [187]. The Embden-Meyerhof-Parnas pathway is superior for ATP generation and the Entner-Doudoroff pathway and EDEMP cycle play an important role in the provision of reducing power in the form of NADPH [187,190,191]. The increased NADPH regeneration rates are beneficial for counteracting oxidative stress, facilitating the application of *P. putida* for redox-intensive reactions [191].

Pseudomonas species offer further benefits for biotechnological applications compared to other species. Generally, many of them have a distinct tolerance against solvents or other toxic compounds [192], with *P. putida* S12 and *P. putida* DOT-T1E as prominent representatives. In contrast, intermediates of biosynthesis pathways for fuels or other valuable products or the product itself are often toxic for established industrial workhorses, such as *E. coli* [193,194]. In the case of the here chosen product, rhamnolipids, three industrially established strains showed significant changes in the growth rate or lag phase in the presence of up to 90 g L⁻¹ di-rhamnolipids, while *P. putida* KT2440 showed only little changes [147]. Further, the solvent tolerance is an advantage in the establishment of biphasic fermentation processes [163]. Although the production of, e.g., *p*-coumaric acid with *P. putida* KT2440 was shown [195], this strain only exhibits a moderate solvent tolerance and other pseudomonads might be better suited for the production of highly toxic products due to presence of several RND-type efflux pumps [196,197]. But not only the tolerance against toxic compounds is advantageous, also the indicated versatile metabolism plays a key role in “green” processes. Over 80 genes were found in the chromosome of *P. putida* KT2440, which allow the growth on various aromatic compounds [198,199]. The combination of the tolerance and ability to degrade aromatics and organic acids facilitates the valorization of lignocellulosic biomass in bioprocesses catalyzed by *P. putida* [152,200,201], where other microbes would fail. As described in 1.1.1, hydrolysates of lignocellulose contain the monomeric sugars, but also growth-inhibiting compounds for a number of industrial workhorses [202,203].

1.5 Synthetic biology-guided engineering of *Pseudomonas*

The production of bulk and fine chemicals in industrial biotechnology for commodities of everyday life requires efficient microbial cell factories. Since wild-type organisms are mostly not efficient enough for commercial processes, there is a need for synthetic biology tools to modify microbes, which also enable the implementation of non-traditional host organisms [204]. As a result, e.g., pseudomonads (especially *P. putida*) developed from a non-traditional host to a frequently used host in biotechnological applications in recent years [193,204]. Today, many tools are available to genetically engineer *Pseudomonas*, facilitating the efficient synthesis of various compounds and sustainable production by implementing renewable resources.

The expression of heterologous genes can easily be achieved by endowing cells with a plasmid. Plasmids can be transferred in *Pseudomonas* via electroporation or bacterial conjugation using mating procedures [205,206]. Many applied vectors originate from natural systems, but synthetic expression vectors are also commonly used [207]. The utilization of well-established plasmids in other organisms, including *Pseudomonas* sp., which are mostly characterized for *E. coli*, is not trivial as different copy numbers can result, which engenders impaired transmissibility [208]. The ‘Standard European Vector Architecture’ database was developed to address this issue and to assist in choosing the optimal expression plasmid for prokaryotes, especially *Pseudomonas*. These standardized vectors have restriction sites between the cargo, origin of replication, and antibiotic resistance marker allowing an easy exchange [209]. The antibiotic resistance marker is needed for selective pressure to prevent plasmid loss. Hence, the maintenance of the plasmid is achieved by the addition of a respective antibiotic in the medium. However, the use of antibiotics in industry is not appropriate because of regulatory effects, increased material costs, and growth defects of *Pseudomonas* [210]. Further, plasmids can be unequally distributed in a cell culture, resulting in varying plasmid copy numbers and thus, in impaired heterologous production and reproducibility [211,212].

Another option for the expression of heterologous genetic elements is the integration into the chromosome, which is considered as stable and thus preferred. The integration is achieved by homologous recombination or transposition [213]. Several methods were developed for the integration of genetic elements, of which some are listed in the following. The Tn5-based transposition can be used to integrate gene cassettes at random sites in the chromosome [214]. However, this method requires laborious screening to detect clones with sufficient protein expression, since the integration at different loci in the chromosome entails different expression strength. Instead, the application of Tn7-based transposition results in the

integration of a gene cassette at a specific locus called *attTn7* site, which is located downstream of the *glmS* gene [215,216]. One further example is the integration of genetic elements in rRNA operons of *Pseudomonas* species via homologous recombination. This tool allows the integration of multiple copies of gene cassettes due to the presence of multiple rRNA operons [217].

For effective and strong expression of genetic elements, either genomically integrated or plasmid-based, a suitable promoter has to be utilized. In some cases, e.g., Tn5- and rRNA-based integration, native promoters of the respective integration site are exploited [217,218]. Apart from that, promoters, both natural or synthetic, can be integrated into the gene cassette [195,216]. Recently it was shown that stacking of strong synthetic promoters results in even stronger expression [219]. Further, genetic elements, such as the ribosomal binding site, play an important role in tuning protein expression, and thus several ribosomal binding sites were characterized in *P. putida* [220].

Moreover, various tools for genome editing of *Pseudomonas* were developed, which are based on homologous recombination for deletion and exchange of genetic elements. Tools, such as the FLP-*FRT* or the Cre/*loxP* recombination system [221–224], where the antibiotic resistance cassette can be recycled, were used for editing *Pseudomonas* strains. However, these recombination systems leave a scar in the genome. One prominent tool that allows scarless deletions and clean replacement of DNA segments is the I-SceI-based system [225]. This tool is applied in many studies to delete large genomic fragments [226,227]. Furthermore, it features low mutation rates in the flanking regions and shows higher efficiencies than other knockout-methods [161,225].

However, most of the genome editing techniques are laborious and slow. Hence, a lot of research is done regarding the development of faster (and automated) genome editing techniques. The well-known CRISPR/Cas9-based system was adapted and merged with ssDNA recombineering, which led to an increased recombineering efficiency [228]. Also, attempts were made to establish a method for multiplex genomic manipulations in *P. putida* [229], which can be implemented in an automated manner, which is known as multiplex automated genomic manipulations.

1.6 Scope and outline of this thesis

The aim of this thesis was the genetic engineering of the non-pathogenic *P. putida* KT2440 for efficient production of rhamnolipids using glucose as well as non-conventional substrates. Further, the potential of using *P. putida* KT2440 as microbial cell factory should be demonstrated.

Chapter 1 provides a general introduction of the topic. The necessary switch from a fossil-based to a bio-based economy using renewable substrates is outlined. In particular, the use of microbial cell factories, especially *P. putida*, for biotechnological production of platform chemicals is stated, including the currently available tools for genetic engineering. As rhamnolipids are the product of choice in this study, an overview of biosurfactants, their applications, and their microbial production and the challenges to overcome are outlined.

In Chapter 2, the materials and methods used in this thesis are described.

Chapter 3.1 deals with the engineering of *P. putida* KT2440 to enable xylose utilization, which is one of the most abundant sugars in nature. Three bacterial xylose utilization pathways are known and were individually introduced in the strain. These strains were characterized regarding their growth, revealing a more efficient growth using the oxidative pathways. Production capacities of different valuable products were investigated *in silico* and confirmed *in vivo* using heterologous production of rhamnolipids and phenazines, allowing the concept of engineering tailored cell factories for valuable product synthesis.

In Chapter 3.2, the use of ethanol was aimed for, which is a carbon source that enables the establishment of a circular bioeconomy due to use of biomass or greenhouse gases for bioethanol production. *P. putida* KT2440 is able to use ethanol natively. However, adaptive laboratory evolution (ALE) was applied to optimize growth and withstand elevated concentrations. Emerged mutations after evolution were identified and allocated to the improved phenotypic changes. Based on the strain engineering, a unique fermentation process could be developed, where ethanol simultaneously functions as carbon source and defoamer and thus enabling control of the foaming, which is one of the main challenges in biosurfactant production.

Chapter 3.3 describes the streamlining of the genome of *P. putida* KT2440. Energy- and resource-demanding cellular components or synthesis cluster for by-product formation were deleted, which are not needed for the targeted synthesis of valuable products. This includes the flagellar-machinery, pyoverdines, polyhydroxyalkanoates, and biofilm-related clusters. Genome reduction is a technique aiming for better overall performance and potential energy charge increase of a microbial cell factory. Several strains were engineered featuring different characteristics in terms of substrate uptake and biofilm formation as well as increased production capacity of rhamnolipids, i.e., higher product yields.

In Chapter 3.4, the efficient use of xylose and galacturonic acid was aimed for. Emerged mutations after laboratory evolution on xylose were identified and reverse engineered to repeat the beneficial outcomes of an ALE experiment. Moreover, stable integration into the chromosome of the genes coding for the Weimberg pathway was conducted. The growth of

P. putida KT2440 based on galacturonic acid was improved by ALE. Rhamnolipid production capacities from these two substrates were investigated in lab-scale bioreactors.

In Chapter 4, all results and achievements are discussed and placed in a larger context. Improvements and ideas for further research for even more efficient and sustainable rhamnolipid production to reach the overall goal of large-scale production are elucidated.

Chapter 2

Material and methods

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Some of the deletion mutants and rhamnolipid producers were published in:

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Blesken, C. C., Bator, I., Eberlein, C., Heipieper, H. J., Tiso, T., Blank, L. M. (2020) Genetic cell-surface modification for optimized foam fractionation. *Frontiers in Bioengineering and Biotechnology*, 8, 1-16, <https://doi.org/10.3389/fbioe.2020.572892>

Contributions:

This chapter was written by Isabel Bator and reviewed by Till Tiso and Lars M. Blank. Strains and plasmids were engineered by Isabel Bator, Marie Dielentheis-Frenken, Elena Recker, Hans M. Böhne, and Jan Brinkmann and accordingly denoted in the strain list.

2 Material and methods

2.1 Chemicals

The chemicals used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA), Carl Roth (Karlsruhe, Germany), or Merck (Darmstadt, Germany) unless stated otherwise.

2.2 Bacterial strains, media, and growth conditions

All used strains in this study are listed in Table 4. *E. coli* strains, *P. putida* KT2440 (DSM6125, ATCC47054), and engineered *P. putida* strains were routinely cultivated in lysogeny broth (LB) medium containing 10 g L⁻¹ peptone, 5 g L⁻¹ yeast extract, and 10 g L⁻¹ NaCl. If solid medium was needed, 2% (w/v) agar was added. *P. putida* was cultivated at 30°C and *E. coli* at 37°C. If required, 50 µg mL⁻¹ kanamycin, 30 µg mL⁻¹ gentamycin, 15 µg mL⁻¹ tetracycline, and 50 µg mL⁻¹ streptomycin were added to the medium to avoid loss of plasmid. After mating procedures, *P. putida* strains were selected on cetrimide agar (Sigma-Aldrich, St. Louis, MO, USA). Growth and production experiments were performed using M9 minimal medium with a final composition (per L) of 8.5 g Na₂HPO₄•2H₂O, 3 g KH₂PO₄, 0.5 g NaCl, 1 g NH₄Cl, 2 mM MgSO₄, 4.87 mg FeSO₄•7H₂O, 4.12 mg CaCl₂•2H₂O, 1.5 mg MnCl₂•4H₂O, 1.87 mg ZnSO₄•7H₂O, 0.3 mg H₃BO₃, 0.25 mg Na₂MoO₄•2H₂O, 0.15 mg CuCl₂•2H₂O, 0.84 mg Na₂EDTA•2H₂O [230], and 10 g glucose for pre-cultures or different concentrations of various substrates for main cultures as indicated. The main culture was inoculated to start cultivation with an optical density (OD₆₀₀) of 0.1.

Liquid cultivations were performed in 500 mL shake flasks with 10% filling volume at 200 rpm and a throw of 25 mm or in the case of rhamnolipid production experiments at 300 rpm and a throw of 50 mm and in 24-deep well plates (SystemDuetz; EnzyScreen B.V., Heemstede, The Netherlands) with 1 mL filling volume at 300 rpm and a throw of 50 mm. A Growth Profiler 960 (EnzyScreen B.V., Heemstede, The Netherlands) was used for online analysis of growth without sampling. 96-well plates with 200 µL filling volume at 225 rpm and a throw of 50 mm were incubated and the density was determined by image analysis.

In Chapter 3.3, determination of growth rates without sampling was performed in a µRAMOS (Respiration Activity MONitoring System) device (Aachener Verfahrenstechnik, Biochemical Engineering, RWTH Aachen University, Germany) using 96-deep well plates (based on [231]) with 400 µL filling volume at 300 rpm with a throw of 50 mm. To prevent the cells from entering the stationary phase, a 48-well glucose feed plate (prototype of SMFP20704, Kuhner Shaker GmbH, Herzogenrath, Germany) was used for the pre-culture.

In the μ RAMOS device, the oxygen transfer rate is monitored, from which the growth rate can be calculated [232].

2.2.1 ^{13}C -labeling experiments

Strains for the isotope labeling experiments were grown under the conditions stated above. The medium contained 50% of $1\text{-}^{13}\text{C}$ labeled xylose (99% purity, Sigma-Aldrich, St. Louis, MO, USA). When the culture reached pseudo-steady state, samples with defined amounts of biomass (0.3 mg) were taken, hydrolyzed, and derivatized according to Schmitz *et al.* [233]. The mass spectrometry (MS) data were processed with iMS2Flux [234], which enables the correction of the data for the presence of naturally occurring isotopes and the determination of the fractional labeling of selected amino acids. The fractional labeling indicates the fraction of ^{13}C -labeled carbon atoms.

2.3 Plasmid and strain construction

Plasmid construction was planned with NEBuilder Assembly online tool and performed with NEBuilder HiFi DNA Assembly (New England Biolabs, Ipswich, MA, USA) [235]. DNA fragments for Gibson Assembly were amplified using Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) according to the manual. The genes for xylose utilization were amplified from genomic DNA of *E. coli* DH5 α and *P. taiwanensis* VLB120 (isolated with High Pure PCR Template Preparation Kit, Roche Holding, Basel, Switzerland). The expression vector pBT including the constitutive P_{tac} promoter [236] was linearized with XbaI (New England Biolabs, Ipswich, MA, USA) prior to assembly. All used primers for construction of expression plasmids are listed with their nucleotide sequences in Table 5. For pBT-Isomerase, the operon *xylAB* (b3565 and b3564 from *E. coli* DH5 α) was amplified using primers IB-27 and IB-28 and cloned into the linearized pBT vector by Gibson Assembly. To generate pBT-Weimberg, an operon from *P. taiwanensis* VLB120 was used. The first two genes (PVLB_18555 and PVLB_18550), originally on antisense strand, were amplified using primers IB-63 and IB-64 and the following two genes (PVLB_18560 and PVLB_18565) were amplified using primers IB-65 and IB-66. Afterwards, the linearized pBT vector and both amplified fragments were assembled in one reaction. After that, pBT-Weimberg was used as template for pBT-Dahms. The backbone including PVLB_18555 and PVLB_18550 was amplified from pBT-Weimberg with primers IB-67 and IB-74. Amplification of gene PVLB_18565 from *P. taiwanensis* VLB120 was performed using primers IB-68 and IB-124 and the gene for the aldolase *yagE* (b0268 from *E. coli* DH5 α) was amplified using the primers IB-118 and IB-125 to create a synthetic operon. All fragments were assembled via Gibson reactions. In a final step to obtain pBT-Dahms, the putative

dehydratase PVLB_18550 was deleted from the assembled vector. Therefore, the vector was amplified with primers IB-93 and IB-94. Subsequently, the amplified vector was self-ligated using T4 Polynucleotide Kinase, T4 DNA Ligase, and T4 DNA Ligase Reaction Buffer (all New England Biolabs GmbH, Ipswich, MA, USA) according to the manual.

For pBT-*araBAD*, the operon *araBAD* (b0063 – b0061 from *E. coli* DH5 α) was amplified using primers IB-209 and IB-210 and cloned into the XbaI-linearized pBT vector by Gibson Assembly.

To generate pBT-*fabG*, the gene *fabG* (b1093) from *E. coli* DH5 α was amplified using primers IB-249 and IB-250 and cloned into the XbaI-linearized pBT vector by Gibson Assembly.

The resulting plasmids, pBT-Isomerase, pBT-Weimberg, pBT-Dahms, pBT-*araBAD*, and pBT-*fabG* were transferred individually into chemically competent *E. coli* PIR2 or DH5 α cells using heat shock according to Hanahan [237]. Transformants were selected on LB agar with kanamycin. Positive colonies were verified via colony PCR using OneTaq 2x Master Mix with Standard Buffer (New England Biolabs, Ipswich, MA, USA). The efficiency of colony PCR was increased by lysis of cell material with alkaline polyethylene glycol according to Chomczynski and Rymaszewski [238]. Plasmids were validated by Sanger sequencing performed by Eurofins Genomics (Ebersberg, Germany). Subsequently, plasmids were isolated with Monarch Plasmid Miniprep Kit (New England Biolabs, Ipswich, MA, USA) and transferred via electroporation in competent *P. putida* cells according to Choi *et al.* [205]. Electroporation was performed using a GenePulser Xcell (Biorad, Hercules, CA, USA) using a cuvette with a 2 mm gap and the settings 2.5 kV, 200 Ω , and 25 μ F. Plasmid pBT-Isomerase was transferred individually in *P. putida* KT2440 and *P. putida* KT2440 Δ *gcd*. Plasmid pBT-Weimberg was transferred individually in *P. putida* KT2440, *P. putida* KT2440 Δ *gcd*, *P. putida* KT2440 Δ *ptxS*, *P. putida* KT2440 Δ *ptxS* PP_3377_A117G, *P. putida* KT2440 PP_3377_A117G, and *P. putida* KT2440 Δ *ptxS* Δ *kgu*. Plasmid pBT-Dahms was transferred individually in *P. putida* KT2440, *P. putida* KT2440 Δ PP_2836, *P. putida* KT2440 Δ *gclR* Δ PP_2836, and *P. putida* KT2440 Δ *gclR* Δ PP_2836 Δ *ptxS*. Plasmid pBT-*araBAD* was transferred in *P. putida* KT2440. Harboring of the plasmids was verified via colony PCR using primers IB-5 and IB-35 (as above).

Deletion mutants were obtained using the I-SceI-based system developed by Martínez-García and de Lorenzo [225]. Briefly, 500-1,000 bp upstream and downstream flanking regions of the target sites, named TS1 and TS2 regions, were amplified from the genomic DNA of *P. putida* KT2440 and cloned into the non-replicative pEMG or pSEVA512S vector (kindly provided by Victor de Lorenzo) by Gibson Assembly. All used primers for

construction of pEMG- or pSEVA512S-derived plasmids are listed in Table 5. The resulting pEMG- or pSEVA512S-derived plasmids were transferred individually into chemically competent *E. coli* PIR2 cells using heat shock as described above. Afterwards, the plasmids were verified by Sanger sequencing and transferred from the *E. coli* PIR2 strains into the required *Pseudomonas* strains via conjugational transfer. Therefore, triparental mating was performed using a streamlined protocol according to Wynands *et al.* [173]. The I-SceI-encoding plasmid pSW-2 was used and no 3-methylbenzoate for induction of I-SceI expression was needed. Positive colonies with loss of kanamycin resistance were verified for targeted deletion by colony PCR using OneTaq 2x Master Mix with Standard Buffer. To obtain marker-free and pure clones, the recombinant strains were cured of pSW-2 plasmid by re-inoculation in LB medium without gentamycin and verified again by colony PCR.

For the amino acid substitution in *kguT* in *P. putida* KT2440 and *P. putida* KT2440 Δ *ptxS*, the respective base was integrated between TS1 and TS2 into the pEMG-PP_3377_A117G substitution vector. The primer IB-225 was designed to contain the point mutation in the TS2 region. All further steps were performed according to the usual pEMG protocol. Base exchange was verified by Sanger sequencing performed by Eurofins Genomics (Ebersberg, Germany).

Integration of the Weimberg pathway into the genome of *P. putida* KT2440 and *P. putida* KT2440 Δ *ptxS* was performed using the mini-Tn7 delivery transposon vector (pBG) developed by Zobel *et al.* [216], which inserts an expression/synthesis module in a single genomic locus of the chromosome. Plasmid pBG14f_80i_14g [219] with the strong constitutive P_{fig} promoter was used as backbone and amplified with primers IB-260 and IB-261. The genes PVLB_18550-PVLB_18565 enabling growth on xylose were amplified from plasmid pBT-Weimberg with primers IB-259 and IB-262 and cloned into the amplified backbone via Gibson Assembly. The correct assembly was verified performing a colony PCR with the primers IB-260 and IB-93a and Sanger sequencing (Eurofins Genomics, Ebersberg, Germany). For integration of only one xylose gene (PVLB_18565), the pBG backbone was amplified with primers IB-257 and IB-260. The xylose gene was amplified from plasmid pBT-Dahms with the primers IB-258 and IB-259 and cloned into the amplified backbone via Gibson Assembly. The correct assembly was verified via colony PCR with the primers IB-184 and IB-257 and Sanger sequencing (Eurofins Genomics, Ebersberg, Germany). The resulting mini-Tn7 vectors pBG_PVLB_18550-18565 and pBG_PVLB18565 were integrated into the genome of *P. putida* KT2440 and *P. putida* KT2440 Δ *ptxS*, respectively, according to Zobel *et al.* [216]. The integration of the xylose module into the *attTn7* site was verified by colony PCR using primers 4_glmS and 5_glmS.

To demonstrate the production of secondary metabolites, two target molecules, rhamnolipids and phenazines, were chosen. For the production of mono-rhamnolipids, the mini-Tn7 delivery transposon vector pSK02 was used [239]. This plasmid contains a strong constitutive, synthetic promoter (14f_80i_14f_80i_14g) [219] and the genes *rhlAB* from *P. aeruginosa*, enabling rhamnolipid production. The mini-Tn7 vector pSK02 was integrated into the genome of glucose, xylose, ethanol, or galacturonic acid consuming strains via transposition according to Zobel *et al.* [216]. The integration of the rhamnolipid synthesis module into the *attTn7* site was verified by colony PCR as described above. Mono-rhamnolipid producing clones were identified using cetrimide-blood agar plates (7.5% (v/v) sheep blood, Fiebig-Nachstofftechnik, Idstein-Niederauroff, Germany). If rhamnolipid synthesis occurred, a halo around the colony was visible, because of their hemolytic activity. The ten clones showing the largest halos, were selected for experiments in minimal medium with the respective carbon source (10 g L⁻¹ glucose, 10 g L⁻¹ xylose, 7.6 g L⁻¹ ethanol, or 10.8 g L⁻¹ galacturonic acid) to quantitatively examine rhamnolipid production. Subsequently, the best three producers of each strain were subjected to replicate experiments and out of these the best was chosen for further characterization and is described in this study.

Phenazine production was achieved by plasmid-based expression. Plasmid pJNN_ *phzA1-G1,M,S* (modified from Schmitz *et al.* [182]) was transferred into competent xylose consuming strains via electroporation according to Choi *et al.* [205]. Harboring of the plasmid was confirmed via colony PCR as described above. Based on Schmitz *et al.* [182], five clones were used to inoculate 1 mL minimal medium containing antibiotics and 10 g L⁻¹ xylose at a starting OD₆₀₀ of 0.1 and induced with 0.1 mM salicylate to induce phenazine synthesis. If phenazine synthesis occurred, a blue color, which is typical for pyocyanin production, was observed. The best three producers of each strain were subjected to replicate experiments and out of these the best was chosen for further characterization and is described in this study.

In the case of overexpression studies, rhamnolipid producers with a salicylate inducible promoter in the rhamnolipid cassette [240] were used and kindly provided by Sonja Kubicki (Heinrich Heine University, Düsseldorf, Germany). The phosphoglucomutase-overexpression plasmids pLO03, pLO04, and pWJ06 [241,242], and the empty vector pSEVA441 [209] were isolated with a Monarch Plasmid Miniprep Kit (New England Biolabs, Ipswich, MA, USA) and transferred via electroporation in competent *P. putida* cells as described before. Plasmids were transferred individually in *P. putida* KTsalmlRL, *P. putida* GRsalmlRL, *P. putida* KTsalmlRL+Rha, and *P. putida* GRsalmlRL+Rha. The fatty acid-overexpression plasmid pBT-*fabG* was isolated with a Monarch Plasmid Miniprep Kit (New England Biolabs, Ipswich, MA, USA) and transferred via electroporation in competent *P. putida* KTsalmlRL

cells. After transformation, transformants were selected on LB agar containing streptomycin (pSEVA441) or kanamycin (pBT) and cetrимide-blood agar containing gentamycin, streptomycin or kanamycin, and 2 mM salicylate to confirm rhamnolipid production. Then, five colonies were tested for rhamnolipid production. Subsequently, up to three producers of each strain, which produced the most rhamnolipids, were subjected to replicate experiments and out of these the best was chosen for further characterization and is described in this study. Harboring of the plasmids was verified via colony PCR using primers MM05 and SK62 for pSEVA441-derived plasmids and IB-5 and IB-35 for pBT-*fabG*.

Table 4: Bacterial strains used in this study.

Strains and plasmids	Characteristics	Reference or source	Strain# iAMB
<i>E. coli</i>			
DH5α	<i>supE44, ΔlacU169 (Φ80lacZΔM15), hsdR17 (r⁻ m⁺ c⁺), recA1, endA1, thi-1, gyrA96, relA1</i>	[243]	2382
DH5αpir	<i>λpir</i> lysogen of DH5α; host for <i>oriV</i> (R6K) vectors	[244]	2077
PIR2	F ⁺ , <i>ΔlacI69, rpoS(Am), robA1, creC510, hsdR514, endA, recA1 uidA(ΔMluI)::pir</i> ; host for <i>oriV</i> (R6K) vectors	Thermo Fisher Scientific	3222
HB101 pRK2013	Sm ^R , <i>hsdR-M⁺, proA2, leuB6, thi-1, recA</i> ; harboring plasmid pRK2013: Km ^R , <i>oriV</i> (RK2/ColE1), <i>mob⁺, tra⁺</i>	[245]	2037
DH5α pSW-2	DH5α harboring plasmid pSW-2: Gm ^R , <i>oriRK2, xylS</i> , P _m → I-SceI (transcriptional fusion of I-SceI to P _m), tool for genomic deletion	[225]	2404
DH5αpir pEMG	DH5αpir harboring plasmid pEMG: Km ^R , <i>oriR6K, lacZα</i> with two flanking I-SceI sites	[225]	2075
PIR2 pEMG-PP_2836	PIR2 harboring plasmid pEMG-PP_2836	This study	4870
PIR2 pEMG- <i>gcd</i>	PIR2 harboring plasmid pEMG- <i>gcd</i>	This study	4436
PIR2 pEMG- <i>fleQ</i>	PIR2 harboring plasmid pEMG- <i>fleQ</i>	This study	6180
PIR2 pEMG-pvd	PIR2 harboring plasmid pEMG-pvd	This study	4266
PIR2 pEMG-flag1	PIR2 harboring plasmid pEMG-flag1	This study	4393
PIR2 pEMG-flag2	PIR2 harboring plasmid pEMG-flag2	This study	4394
PIR2 pEMG-alg	PIR2 harboring plasmid pEMG-alg	This study	4392
PIR2 pEMG-bcs	PIR2 harboring plasmid pEMG-bcs	This study, [246]	4547
PIR2 pEMG-pea	PIR2 harboring plasmid pEMG-pea	This study, [246]	4554
DH5αpir pSEVA512S	DH5αpir harboring plasmid pSEVA512S: Tc ^R , <i>oriR6K, lacZα</i> with two flanking I-SceI sites	[209]	-
PIR2 pSEVA512S-peb	PIR2 harboring plasmid pSEVA512S-peb	This study, [246]	4597
PIR2 pEMG- <i>lapA</i>	PIR2 harboring plasmid pEMG- <i>lapA</i>	This study, [246]	4606
PIR2 pEMG- <i>lapF</i>	PIR2 harboring plasmid pEMG- <i>lapF</i>	This study, [246]	4607
DH5αpir pEMG-pha	PIR2 harboring plasmid pEMG-pha	[247]	6663
DH5αpir pEMG-PP_1887-PP_1891	DH5αpir harboring plasmid pEMG-PP_1887-PP_1891	BacMine S. L., unpublished	5545
DH5αpir pEMG-PP_2357-PP_2363	DH5αpir harboring plasmid pEMG-PP_2357-PP_2363	BacMine S. L., unpublished	5546

DH5 α pir pEMG-PP_4986-PP_4992	DH5 α pir harboring plasmid pEMG-PP_4986-PP_4992	BacMine S. L., unpublished	5547
DH5 α pir pEMG-PP_5080-PP_5083	DH5 α pir harboring plasmid pEMG-PP_5080-PP_5083	BacMine S. L., unpublished	5548
DH5 α pir pEMG-PP_0607-PP_0611	DH5 α pir harboring plasmid pEMG-PP_0607-PP_0611	BacMine S. L., unpublished	5549
DH5 α pir pEMG-PP_3396-PP_3399	DH5 α pir harboring plasmid pEMG-PP_3396-PP_3399	BacMine S. L., unpublished	5551
DH5 α pir pEMG-PP_1993	DH5 α pir harboring plasmid pEMG-PP_1993	BacMine S. L., unpublished	5552
DH5 α pir pEMG-PP_5093	DH5 α pir harboring plasmid pEMG-PP_5093	BacMine S. L., unpublished	5553
DH5 α pir pTNS1	DH5 α pir harboring plasmid pTNS1: Ap ^R , ori <i>R6K</i> , <i>TnSABC+D</i> operon	[248]	3221
DH5 α pBT	DH5 α harboring plasmid pBT: Km ^R , P _{tac} (constitutive), <i>ori</i> pBBR1, expression vector	[236]	2049
DH5 α pBT-Isomerase	DH5 α harboring plasmid pBT; containing the <i>xyLAB</i> genes from <i>E. coli</i> DH5 α	This study	4435
DH5 α pBT-Weimberg	DH5 α harboring plasmid pBT; containing the PVLB18550/18555/18560/18565 genes from <i>P. taiwanensis</i> VLB120	This study	4559
DH5 α pBT-Dahms	DH5 α harboring plasmid pBT; containing the PVLB18555/18565 genes from <i>P. taiwanensis</i> VLB120 and <i>yagE</i> gene from <i>E. coli</i> DH5 α	This study	4871
PIR2	PIR2 harboring Tn7 delivery vector	[219]	6460
pBG14f_80i_14f_80i_14g	pBG14f_80i_14f_80i_14g: Km ^R , Gm ^R , <i>ori</i> R6K, stacked promoter 14f_14f_14g, <i>msfGFP</i> , inverted spacer with a length of 80 bp		
DH5 α pir pSK02	DH5 α pir harboring Tn7 delivery vector pSK02 (pBG14f_80i_14f_80i_14g-derived) for chromosomal integration; containing <i>rhlAB</i> genes from <i>P. aeruginosa</i> PA01	[239]	5234
DH5 α pJNN_ <i>phzA1-G1,M,S</i>	DH5 α harboring plasmid pJNN_ <i>phzA1-G1,M,S</i> ; containing <i>phzA1-G1,M,S</i> genes from <i>P. aeruginosa</i> PA01	Simone Schmitz	6662
DH5 α pYTSK10K_0G7	DH5 α harboring plasmid pYTSK10K_0G7 (<i>ori</i> T, Tn7-L, CIS I, <i>PnagAa/nagR</i> +BCD2+PA- <i>rhlAB</i> , CIS II, Gm ^R , <i>tns7A-D</i> , Tn7-R)	[249]	-
DH5 α pYTSK51K_0G7	DH5 α harboring plasmid pYTSK51K_0G7 (<i>ori</i> T, Tn7-L, CIS I, <i>PnagAa/nagR</i> +BCD2+PA- <i>rhlAB</i> +PA- <i>rmlBDAC</i> , CIS II, Gm ^R , <i>tns7A-D</i> , Tn7-R)	[240]	-
CC118 λ pir pSEVA441	CC118 λ pir harboring plasmid pSEVA441: Sm ^R , <i>ori</i> T	[209]	2876
PIR2 pLO03	PIR2 harboring plasmid pLO03 (pSEVA441 derived); containing <i>algC</i> from <i>P. aeruginosa</i> PA01	[241]	5264
PIR2 pLO04	PIR2 harboring plasmid pLO04 (pSEVA441 derived); containing <i>algC</i> from <i>P. putida</i> KT2440	[241]	5259

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DH5α pWJ06	DH5α harboring plasmid pWJ06 (pSEVA441 derived); containing <i>xanA</i> from <i>P. putida</i> KT2440	[242]	5126
PIR2 pEMG- <i>fabA</i>	PIR2 harboring plasmid pEMG- <i>fabA</i>	This study	5080
PIR2 pBT- <i>fabG</i>	PIR2 harboring plasmid pBT- <i>fabG</i> ; containing <i>fabG</i> from <i>E. coli</i> DH5α	This study, [250]	-
PIR2 pEMG-PP_3377_A117G	PIR2 harboring pEMG-PP_3377_A117G; for amino acid exchange in <i>kguT</i> (base G to C)	This study, [251]	6664
PIR2 pEMG- <i>ptxS</i>	PIR2 harboring plasmid pEMG- <i>ptxS</i>	This study, [251]	6665
PIR2 pEMG- <i>kgu</i>	PIR2 harboring plasmid pEMG- <i>kgu</i> (for deletion of <i>kgu</i> operon)	This study, [251]	6666
PIR2 pBG14f_80i_14g	PIR2 harboring Tn7 delivery vector pBG14f_80i_14g for chromosomal integration: Km ^R , Gm ^R , <i>oriR6K</i> , pBG-derived, stacked promoter 14f_14g, <i>msfGFP</i> , inverted spacer with a length of 80 bp	[219]	6458
PIR2 pBG_PVLB_18565	PIR2 harboring Tn7 delivery vector pBG_PVLB_18565 (pBG14f_80i_14g derived) for chromosomal integration; containing PVLB_18565 from <i>P. taiwanensis</i> VLB120	This study, [251]	6667
PIR2 pBG_PVLB_18550-18565	PIR2 harboring Tn7 delivery vector pBG_PVLB_18550-18565 (pBG14f_80i_14g derived) for chromosomal integration; containing PVLB_18550 – PVLB_18565 from <i>P. taiwanensis</i> VLB120	This study, [251]	6668
DH5α pBT- <i>araBAD</i>	DH5α harboring plasmid pBT; containing the <i>araBAD</i> genes from <i>E. coli</i> DH5α	This study	-
<i>P. taiwanensis</i>			
VLB120	wild type	[252]	2060
<i>P. putida</i>			
KT2440	wild type	[253]	2058
KT2440 Δ <i>gcd</i>	Δ <i>gcd</i>	This study	4462
KT2440 Δ <i>gclR</i>	Δ <i>gclR</i>	[254]	4017
KT2440 ΔPP_2836	ΔPP_2836	This study	4876
KT2440 Δ <i>gclR</i> ΔPP_2836	Δ <i>gclR</i> , ΔPP_2836	This study	6669
KT2440 pIsO	<i>P. putida</i> KT2440 harboring plasmid pBT-Isomerase	This study	6670
KT2440 Δ <i>gcd</i> pIsO	Δ <i>gcd</i> harboring plasmid pBT-Isomerase	This study	6671
KT2440 pWeim	<i>P. putida</i> KT2440 harboring plasmid pBT-Weimberg	This study	6672
KT2440 Δ <i>gcd</i> pWeim	Δ <i>gcd</i> harboring plasmid pBT-Weimberg	This study	6673
KT2440 pDahms	<i>P. putida</i> KT2440 harboring plasmid pBT-Dahms	This study	6674
KT2440 ΔPP_2836 pDahms	ΔPP_2836 harboring plasmid pBT-Dahms	This study	6675
KT2440 Δ <i>gclR</i> pDahms	Δ <i>gclR</i> harboring plasmid pBT-Dahms	This study	6676
KT2440ΔΔ pDahms	Δ <i>gclR</i> , ΔPP_2836 harboring plasmid pBT-Dahms	This study	6677
EM42 Δ <i>gcd</i>	EM42 Δ <i>gcd</i> harboring plasmid pSEVA2213_ <i>xylABE</i>	[255]	6678
pSEVA2213_ <i>xylABE</i>			
KT2440 pWeim2	Isolate of <i>P. putida</i> KT2440 pWeim after 17 th transfer from laboratory evolution on xylose	This study	6679
KT2440ΔΔ pDahms2	Isolate of <i>P. putida</i> KT2440ΔΔpDahms after 26 th transfer from laboratory evolution on xylose	This study	6680

KT2440 plso_RL	<i>P. putida</i> KT2440 plso with <i>attTn7::Pffg-rhlAB</i>	This study, [256]	RLX1
KT2440 plso2_RL	<i>P. putida</i> EM42 Δ <i>gcd</i> pSEVA2213_ <i>xylABE</i> with <i>attTn7::Pffg-rhlAB</i>	This study	RLX4
KT2440 pWeim_RL	<i>P. putida</i> KT2440 pWeim with <i>attTn7::Pffg-rhlAB</i>	This study, [256]	RLX7
KT2440 pWeim2_RL	<i>P. putida</i> KT2440 pWeim2 with <i>attTn7::Pffg-rhlAB</i>	This study, [256]	RLX10
KT2440 $\Delta\Delta$ pDahms_RL	<i>P. putida</i> KT2440 $\Delta\Delta$ pDahms with <i>attTn7::Pffg-rhlAB</i>	This study, [256]	RLX13
KT2440 $\Delta\Delta$ pDahms2_RL	<i>P. putida</i> KT2440 $\Delta\Delta$ pDahms2 with <i>attTn7::Pffg-rhlAB</i>	This study, [256]	RLX16
KT2440 plso_PZ	<i>P. putida</i> KT2440 plso harboring plasmid pJNN_ <i>phzA1-G1,M,S</i>	This study	6681
KT2440 plso2_PZ	<i>P. putida</i> EM42 Δ <i>gcd</i> pSEVA2213_ <i>xylABE</i> harboring plasmid pJNN_ <i>phzA1-G1,M,S</i>	This study	6682
KT2440 pWeim_PZ	<i>P. putida</i> KT2440 pWeim harboring plasmid pJNN_ <i>phzA1-G1,M,S</i>	This study	6683
KT2440 pWeim2_PZ	<i>P. putida</i> KT2440 pWeim2 harboring plasmid pJNN_ <i>phzA1-G1,M,S</i>	This study	6684
KT2440 $\Delta\Delta$ pDahms_PZ	<i>P. putida</i> KT2440 $\Delta\Delta$ pDahms harboring plasmid pJNN_ <i>phzA1-G1,M,S</i>	This study	6685
KT2440 $\Delta\Delta$ pDahms2_PZ	<i>P. putida</i> KT2440 $\Delta\Delta$ pDahms2 harboring plasmid pJNN_ <i>phzA1-G1,M,S</i>	This study	6686
KT2440 E1	Isolate of <i>P. putida</i> KT2440 after 23rd transfer from laboratory evolution on ethanol	This study, [256]	6687
KT2440 E1.1	Isolate of <i>P. putida</i> KT2440 after 33rd transfer from laboratory evolution on ethanol	This study, [256]	6688
KT2440 Δ <i>fleQ</i>	Δ <i>fleQ</i>	This study	6183
KT2440 SK4	<i>P. putida</i> KT2440 with <i>attTn7::P_{flg}-rhlAB</i>	This study	RLW1
KT2440 E1.1_RL	KT2440 E1.1 with <i>attTn7::P_{flg}-rhlAB</i>	This study, [256]	RLE1
KT2440 Δ pvd	Δ PP_4219-PP_4221	This study	4395
KT2440 Δ flag	Δ PP_4328-PP_4344, Δ PP_4351-P_4397	This study	4458
KT2440 Δ alg	Δ PP_1277-PP_1288	This study	4474
KT2440 Δ bcs	Δ PP_2634-PP_2638	This study, [246]	4555
KT2440 Δ pea	Δ PP_3132-PP_3142	This study, [246]	4598
KT2440 Δ peb	Δ PP_1795-PP_1788	This study, [246]	4608
KT2440 Δ lapA	Δ PP_0168	This study, [246]	4668
KT2440 Δ lapF	Δ PP_0806	This study, [246]	4672
KT2440 Δ pha	Δ PP_5003-PP_5008	This study	5081
KT2440 Δ pili1	Δ PP_1887-PP_1891	This study, [250]	5671
KT2440 Δ pili2	Δ PP_2357-PP_2363	This study, [250]	5675
KT2440 Δ pili3	Δ PP_4986-PP_4992	This study, [250]	5683
KT2440 Δ pili4	Δ PP_5080-PP_5083	This study, [250]	5780
KT2440 Δ pili5	Δ PP_0607-PP_0611	This study	5942
KT2440 Δ curl1	Δ PP_3396-PP_3399	This study	5948
KT2440 Δ pili6	Δ PP_1993	This study	5944
KT2440 Δ pili7	Δ PP_5093	This study	5946
KT2440 GR18	Δ pvd Δ flag Δ alg Δ bcs Δ pea Δ peb Δ lapA Δ lapF	This study, [246]	4674
KT2440 GR41	Δ pvd Δ flag Δ alg Δ bcs Δ pea Δ lapA Δ lapF Δ pha Δ pili1 Δ pili3 Δ pili4 Δ pili5	This study	6642

KT2440 Δpvd_RL	<i>P. putida</i> KT2440 Δpvd with <i>attTn7::P_{fig}-rhlAB</i>	This study	RL1
KT2440 Δflag_RL	<i>P. putida</i> KT2440 Δflag with <i>attTn7::P_{fig}-rhlAB</i>	This study	RL10
KT2440 Δalg_RL	<i>P. putida</i> KT2440 Δalg with <i>attTn7::P_{fig}-rhlAB</i>	This study	RL19
KT2440 Δbcs_RL	<i>P. putida</i> KT2440 Δbcs with <i>attTn7::P_{fig}-rhlAB</i>	This study, [246]	RL28
KT2440 Δpea_RL	<i>P. putida</i> KT2440 Δpea with <i>attTn7::P_{fig}-rhlAB</i>	This study, [246]	RL34
KT2440 Δpeb_RL	<i>P. putida</i> KT2440 Δpeb with <i>attTn7::P_{fig}-rhlAB</i>	This study, [246]	RL40
KT2440 ΔlapA_RL	<i>P. putida</i> KT2440 ΔlapA with <i>attTn7::P_{fig}-rhlAB</i>	This study, [246]	RL46
KT2440 ΔlapF_RL	<i>P. putida</i> KT2440 ΔlapF with <i>attTn7::P_{fig}-rhlAB</i>	This study, [246]	RL52
KT2440 Δpha_RL	<i>P. putida</i> KT2440 Δpha with <i>attTn7::P_{fig}-rhlAB</i>	This study	RL58
KT2440 Δpili1_RL	<i>P. putida</i> KT2440 Δpili1 with <i>attTn7::P_{fig}-rhlAB</i>	This study, [250]	RL91
KT2440 Δpili2_RL	<i>P. putida</i> KT2440 Δpili2 with <i>attTn7::P_{fig}-rhlAB</i>	This study, [250]	RL97
KT2440 Δpili3_RL	<i>P. putida</i> KT2440 Δpili3 with <i>attTn7::P_{fig}-rhlAB</i>	This study, [250]	RL103
KT2440 Δpili4_RL	<i>P. putida</i> KT2440 Δpili4 with <i>attTn7::P_{fig}-rhlAB</i>	This study, [250]	RL109
KT2440 Δpili5_RL	<i>P. putida</i> KT2440 Δpili5 with <i>attTn7::P_{fig}-rhlAB</i>	This study	RL115
KT2440 Δcurli_RL	<i>P. putida</i> KT2440 Δcurli with <i>attTn7::P_{fig}-rhlAB</i>	This study	RL118
KT2440 Δpili6_RL	<i>P. putida</i> KT2440 Δfim1 with <i>attTn7::P_{fig}-rhlAB</i>	This study	RL121
KT2440 Δpili7_RL	<i>P. putida</i> KT2440 Δfim2 with <i>attTn7::P_{fig}-rhlAB</i>	This study	RL124
KT2440 GR18_RL	<i>P. putida</i> KT2440 GR18 with <i>attTn7::P_{fig}-rhlAB</i>	This study, [246]	RL55
KT2440 GR41_RL	<i>P. putida</i> KT2440 GR41 with <i>attTn7::P_{fig}-rhlAB</i>	This study	RL127
KTsalmRL	<i>P. putida</i> KT2440 with <i>attTn7::PnagAa/ nagR+BCD2+PA-rhlAB</i>	[240]	6689
KTsalmRL + pSEVA441	<i>P. putida</i> KT2440 with <i>attTn7::PnagAa/ nagR+BCD2+PA-rhlAB</i> harboring pSEVA441	This study, [257]	6690
KTsalmRL + pLO03	<i>P. putida</i> KT2440 with <i>attTn7::PnagAa/ nagR+BCD2+PA-rhlAB</i> harboring pLO03	This study, [257]	6691
KTsalmRL + pLO04	<i>P. putida</i> KT2440 with <i>attTn7::PnagAa/ nagR+BCD2+PA-rhlAB</i> harboring pLO04	This study, [257]	6692
KTsalmRL + pWJ06	<i>P. putida</i> KT2440 with <i>attTn7::PnagAa/ nagR+BCD2+PA-rhlAB</i> harboring pWJ06	This study, [257]	6693
KTsalmRL+Rha	<i>P. putida</i> KT2440 with <i>attTn7::PnagAa/ nagR+BCD2+PA-rhlAB+PA-rmlBDAC</i>	[240]	6694
KTsalmRL+Rha + pSEVA441	<i>P. putida</i> KT2440 with <i>attTn7::PnagAa/ nagR+BCD2+PA-rhlAB+PA-rmlBDAC</i> harboring pSEVA441	This study, [257]	6695
KTsalmRL+Rha + pLO03	<i>P. putida</i> KT2440 with <i>attTn7::PnagAa/ nagR+BCD2+PA-rhlAB+PA-rmlBDAC</i> harboring pLO03	This study, [257]	6696
KTsalmRL+Rha + pLO04	<i>P. putida</i> KT2440 with <i>attTn7::PnagAa/ nagR+BCD2+PA-rhlAB+PA-rmlBDAC</i> harboring pLO04	This study, [257]	6697
KTsalmRL+Rha + pWJ06	<i>P. putida</i> KT2440 with <i>attTn7::PnagAa/ nagR+BCD2+PA-rhlAB+PA-rmlBDAC</i> harboring pWJ06	This study, [257]	6698
GRsalmRL	<i>P. putida</i> KT2440 GR18 with <i>attTn7::PnagAa/ nagR+BCD2+PA-rhlAB</i>	[240]	6699
GRsalmRL + pSEVA441	<i>P. putida</i> KT2440 GR18 with <i>attTn7::PnagAa/ nagR+BCD2+PA-rhlAB</i> harboring pSEVA441	This study, [257]	6700
GRsalmRL + pLO03	<i>P. putida</i> KT2440 GR18 with <i>attTn7::PnagAa/ nagR+BCD2+PA-rhlAB</i> harboring pLO03	This study, [257]	6701

GRsalmRL + pLO04	<i>P. putida</i> KT2440 GR18 with <i>attTn7::PnagAa/nagR</i> +BCD2+PA- <i>rhlAB</i> harboring pLO04	This study, [257]	6702
GRsalmRL + pWJ06	<i>P. putida</i> KT2440 GR18 with <i>attTn7::PnagAa/nagR</i> +BCD2+PA- <i>rhlAB</i> harboring pWJ06	This study, [257]	6703
GRsalmRL+Rha	<i>P. putida</i> KT2440 GR18 with <i>attTn7::PnagAa/nagR</i> +BCD2+PA- <i>rhlAB</i> +PA- <i>rmlBDAC</i>	Sonja Kubicki	6704
GRsalmRL+Rha + pSEVA441	<i>P. putida</i> KT2440 GR18 with <i>attTn7::PnagAa/nagR</i> +BCD2+PA- <i>rhlAB</i> +PA- <i>rmlBDAC</i> harboring pSEVA441	This study, [257]	6705
GRsalmRL+Rha + pLO03	<i>P. putida</i> KT2440 GR18 with <i>attTn7::PnagAa/nagR</i> +BCD2+PA- <i>rhlAB</i> +PA- <i>rmlBDAC</i> harboring pLO03	This study, [257]	6706
GRsalmRL+Rha + pLO04	<i>P. putida</i> KT2440 GR18 with <i>attTn7::PnagAa/nagR</i> +BCD2+PA- <i>rhlAB</i> +PA- <i>rmlBDAC</i> harboring pLO04	This study, [257]	6707
GRsalmRL+Rha + pWJ06	<i>P. putida</i> KT2440 GR18 with <i>attTn7::PnagAa/nagR</i> +BCD2+PA- <i>rhlAB</i> +PA- <i>rmlBDAC</i> harboring pWJ06	This study, [257]	6708
KTsalmRL + pBT- <i>fabG</i>	<i>P. putida</i> KT2440 with <i>attTn7::PnagAa/nagR</i> +BCD2+PA- <i>rhlAB</i> harboring pBT- <i>fabG</i>	This study, [250]	-
KT2440 PP_3377_A117G	<i>P. putida</i> KT2440 with <i>kguT</i> ^{A117G}	This study, [251]	6709
KT2440 PP_3377_A117G pWeim	<i>P. putida</i> KT2440 PP_3377_A117G harboring plasmid pBT-Weimberg	This study, [251]	6710
KT2440 Δ <i>ptxS</i>	Δ <i>ptxS</i>	This study, [251]	6711
KT2440 Δ <i>ptxS</i> pWeim	<i>P. putida</i> KT2440 Δ <i>ptxS</i> harboring plasmid pBT-Weimberg	This study, [251]	6712
KT2440 $\Delta\Delta$ Δ <i>ptxS</i>	Δ <i>gclR</i> , Δ PP_2836, Δ <i>ptxS</i>	This study, [251]	6713
KT2440 $\Delta\Delta$ Δ <i>ptxS</i> pDahms	<i>P. putida</i> KT2440 $\Delta\Delta$ Δ <i>ptxS</i> harboring plasmid pBT-Dahms	This study, [251]	6714
KT2440 Δ <i>ptxS</i> Δ <i>kgu</i>	Δ <i>ptxS</i> Δ <i>kguEKTptxD</i>	This study, [251]	6715
KT2440 Δ <i>ptxS</i> Δ <i>kgu</i> pWeim	<i>P. putida</i> KT2440 Δ <i>ptxS</i> Δ <i>kgu</i> harboring plasmid pWeim	This study, [251]	6716
KT2440 Δ <i>ptxS</i> PP_3377_A117G	Δ <i>ptxS</i> with <i>kguT</i> ^{A117G}	This study, [251]	6717
KT2440 Δ <i>ptxS</i> PP_3377_A117G pWeim	<i>P. putida</i> KT2440 Δ <i>ptxS</i> PP_3377_A117G harboring plasmid pBT-Weimberg	This study, [251]	6718
KT2440 Tn7_PVLB_18550-18565	<i>P. putida</i> KT2440 with <i>attTn7::Pfg_PVLB_18550-PVLB_18565</i>	This study, [251]	6719
KT2440 Δ <i>ptxS</i> Tn7_PVLB_18565	<i>P. putida</i> KT2440 with <i>attTn7::Pfg_PVLB_18565</i>	This study, [251]	6720
KT2440 GA1	Isolate of <i>P. putida</i> KT2440 after 48 th transfer from laboratory evolution on galacturonic acid	This study	6721
KT2440 GA1_RL	<i>P. putida</i> KT2440 GA1 with <i>attTn7::P_{ng}-rhlAB</i>	This study	RLGA1

Table 5: Oligonucleotides used in this study.

Name	Direction	Used for	Sequence
4_glmS	fwd	Tn7 integration	AGTCAGAGTTACGGAATTGTAGG
5_glmS	rev	Tn7 integration	GTCGAGAAAAATGCCGAGCT
IB-5	fwd	pBT/pEMG-plasmids	CAAGGCGATTAAAGTTGGG
IB-6	rev	pEMG-plasmids	TCCGGCTCGTATGTTGTG
IB-27	fwd	<i>xylAB</i> amplification	caggtagcgaattcctcgagaggaggtgtcaaatGCAAGCCTATTTTGAC
IB-28	rev	<i>xylAB</i> amplification	ggcccgacgtcgcgatgctccTTACGCCATTAATGGCAG
IB-29	fwd	<i>gcd</i> -TS1 amplification	taacagggtaatctgaattcAGCGCCATGCCGTAGGCTTTG
IB-30	rev	<i>gcd</i> -TS1 amplification	agaacctacgGCGACACCGCTCCCGCAG
IB-31	fwd	<i>gcd</i> -TS2 amplification	gcggtgtcgcCGTAGGTCTCCGTCAGG
IB-32	rev	<i>gcd</i> -TS2 amplification	ttgcatgctgcaggtagcgcTTC AACCTCGAATCCGG
IB-33	fwd	<i>gcd</i> -KO verification	GAGCACTTCTGCAACTG
IB-34	rev	<i>gcd</i> -KO verification	GGTGGCATCTGGTACAAC
IB-35	rev	pBT-plasmids	ATACGCAAACCGCCTCTC
IB-63	fwd	Weim amplification	caggtagcgaattcctcgagaggaggtGGATCACATGTCGGACCTG
IB-64	rev	Weim amplification	catggcatacaacctcctTCAGCGAATCGGTTACAG
IB-65	fwd	Weim amplification	gattcgctgaaggaggtTGATGCCATGCCGATAAC
IB-66	rev	Weim amplification	ggcccgacgtcgcgatgctccTCAATGAGAATGCCGTGG
IB-67	rev	pBT-Weimberg	ACCTCCTTCAGCGAATCG
IB-74	fwd	pBT-Weimberg	GGAGCATGCCAGCTCGGG
IB-68	fwd	PVLB18565 amplifi.	accgattcgctgaaggaggtAATGACATGACCGATACC
IB-124	rev	PVLB18565 amplifi.	acctcctTCAATGAGAATGCCGTGG
IB-87	fwd	PP2836-TS1 amplifi.	taacagggtaatctgaattcCCTACCTCAAGCCATACAC
IB-88	rev	PP2836-TS1 amplifi.	agcgagccgtCTGTGTTCCCTTGCTTTG
IB-89	fwd	PP2836-TS2 amplifi.	gggaacacagACGGCTCGCTGCGTCAGG
IB-90	rev	PP2836-TS2 amplifi.	ttgcatgctgcaggtagcgcTCGGCAGCGGTTTGCCCTTC
IB-91	fwd	PP2836-KO verifi.	GGTGTTCACGCTGTTC
IB-92	rev	PP2836-KO verifi.	GTA CTGCGGTGCTTGAAC
IB-93	rev	pBT-Dahms	TCAATCCTCCGAAACGCGAC
IB-94	fwd	pBT-Dahms	AGGAGGTAATGACATGACCGATACC
IB-118	rev	<i>yagE</i> amplification	ggcccgacgtcgcgatgctccTCAGCAAAGCTTGAGCTG
IB-125	fwd	<i>yagE</i> amplification	tgccacggcattctcattgaaggaggtGATCTCATGCCGAGTGCC
IB-278	fwd	<i>fleQ</i> -TS1 amplification	taacagggtaatctgaattcCACCCGCCAGGCAGCAA
IB-279	rev	<i>fleQ</i> -TS1 amplification	ttgctattgcTGTTTGGGCTGGGTGTTCCG
IB-280	fwd	<i>fleQ</i> -TS2 amplification	agcccaacaGCAATAGCAACTCCCTAGTCATATC
IB-281	rev	<i>fleQ</i> -TS2 amplification	ttgcatgctgcaggtagcgcGCTCCGCCGAGCAATAATAC
IB-282	fwd	<i>fleQ</i> -KO verification	CAATTGGCCGATCAGAGG
IB-283	rev	<i>fleQ</i> -KO verification	GAAGTCGCGCAGTTGATG
IB-311	fwd	PP_2679 (cDNA)	GGACATCTGGAACGAAGGCA
IB-312	rev	PP_2679 (cDNA)	AGCACGCCGATGTAGTCTTC
IB-313	fwd	PP_2680 (cDNA)	GTT CATGGCCGAGGTGATGA
IB-314	rev	PP_2680 (cDNA)	TGTCGTACTGTTGCTCGGAC
IB-315	fwd	PP_2681 (cDNA)	GGTCCTCGAGCTGCTGGA
IB-316	rev	PP_2681 (cDNA)	CAGTACGTCCTCTTCGAGCC
rpoB f	fwd	<i>rpoB</i> (cDNA)	ATACGGCGCGGCATACA

rpoB	rev	<i>rpoB</i> (cDNA)	CGTTCACGTCGTCGACTT
IB-1	fwd	pvd-TS1 amplification	taacagggtaatctgaatteGCAGGTGCACTGTTGCTGGC
IB-2	rev	pvd-TS1 amplification	gaccccgagcGCTTTCGGGGCCGCCAGC
IB-3	fwd	pvd-TS2 amplification	ccccgaaagcGCTCGGGGTCCTTACTGATC
IB-4	rev	pvd-TS2 amplification	ttgcatgctgcaggtcgacTACAGCCTCGACTGGCCG
IB-7	fwd	pvd-KO verification	ACCCATACGCATGAAGTC
IB-8	rev	pvd-KO verification	TACTGCTGCGTGGTTTCG
IB-9	fwd	alg-TS1 amplification	taacagggtaatctgaatteCACCAGCCAGGTGAGGATC
IB-10	rev	alg-TS1 amplification	ggatgtagcgATGCTGACTCGCCCCTGG
IB-11	fwd	alg-TS2 amplification	gagtcagcatCGCTATCACCTCATGTGTTTATC
IB-12	rev	alg-TS2 amplification	ttgcatgctgcaggtcgacCGAATCCGACGCCAAAAATC
IB-13	fwd	alg-KO verification	TCTTGCCAGACCACGAAC
IB-14	rev	alg-KO verification	TACTACAGTGCCGAGCAG
IB-15	fwd	flag1-TS1 amplification	taacagggtaatctgaatteGCTGCCAGGTGCGCTTCA
IB-16	rev	flag1-TS1 amplification	aggactcgcgGATTGTATACAACCTGTCGAGCCCG
IB-17	fwd	flag1-TS2 amplification	gtatacaateCGCGAGTCTCTTGTATGC
IB-18	rev	flag1-TS2 amplification	ttgcatgctgcaggtcgacTATCATAGATCTCGACAATGTCTTCAATC
IB-19	fwd	flag1-KO verification	ATGGCGAAGAACACCAAC
IB-20	rev	flag1-KO verification	TCCACCGAGTCATGAAGG
IB-21	fwd	flag2-TS1 amplification	taacagggtaatctgaatteCGTTTGTGTTTGAAGAGATC
IB-22	rev	flag2-TS1 amplification	cgaccgacgaCAGTTGAAAGTTTCTTGAC
IB-23	fwd	flag2-TS2 amplification	cttcaactgTCGTCGGTCGGTAAAAACAGAAAAAC
IB-24	rev	flag2-TS2 amplification	ttgcatgctgcaggtcgacTTTTCGGCGCTGCTGGT
IB-25	fwd	flag2-KO verification	AAACGGGATGGCAACAGC
IB-26	rev	flag2-KO verification	GAGCCGAAGTTCTTTCATC
IB-43	fwd	bcs-TS1 amplification	taacagggtaatctgaatteGCCGACGCGGTACCCGC
IB-44	rev	bcs-TS1 amplification	gcgattgccgGGCGCGACCCTCTCGCGC
IB-45	fwd	bcs-TS2 amplification	gggtcgcgccCGGCAATCGCTGCGTTGC
IB-46	rev	bcs-TS2 amplification	ttgcatgctgcaggtcgacGGTGCGAAGAAGGCTTC
IB-47	fwd	bcs-KO verification	AGTGACCTGGATGTCTTG
IB-48	rev	bcs-KO verification	TCACCGCCACAGTCTTTC
IB-49	fwd	pea-TS1 amplification	taacagggtaatctgaatteAAAGAGGCCAGCACGAAG
IB-50	rev	pea-TS1 amplification	agcgcttcgcGACTTCAATCTCTGACTGATTGG
IB-51	fwd	pea-TS2 amplification	gattgaagtcGCGAAGCGCTCCTGCTTC
IB-52	rev	pea-TS2 amplification	ttgcatgctgcaggtcgacGAAATCCGCGGCACATTGC
IB-53	fwd	pea-KO verification	CGTAACCCAGTGCAATCG
IB-54	rev	pea-KO verification	ATGCGCCAAGTGTGAAGAG
IB-55	fwd	peb-TS1 amplification	ttgcatgctgcaggtcgacAGTCAAAAAACAAAGTTTGAC
IB-56	rev	peb-TS1 amplification	taacaacgccATACATCACAAAGCCTG
IB-57	fwd	peb-TS2 amplification	tgatgtgatGGCGTTGTTAAGCTCCTTTTAAAC
IB-58	rev	peb-TS2 amplification	taacagggtaatctgaatteCAATGTTGACGTGCTGCC
IB-59	fwd	peb-KO verification	GCGCCATAATCAATGCTG
IB-60	rev	peb-KO verification	TACACATCCCTGCTCAAC
IB-61	fwd	pha-KO verification	AGCGTTTGCTCGAAGAAGTG
IB-154	rev	pha-KO verification	GCAATAGATCCGGTAGGG
IB-75	fwd	<i>lapA</i> -TS1 amplification	taacagggtaatctgaatteATGCAAGGGCTTTGGGTTTC

IB-76	rev	<i>lapA</i> -TS1 amplification	cggtaggtctgTGGACTCTCCGTGTGACC
IB-77	fwd	<i>lapA</i> -TS2 amplification	ggagagtccaCAGACCACCGGGGCCGCA
IB-78	rev	<i>lapA</i> -TS2 amplification	ttgcatgcctgcaggtcgacAAGGCGGCTGACATTTTCACTCCC
IB-79	fwd	<i>lapA</i> -KO verification	CCTTGAATCGGTGTTGAG
IB-80	rev	<i>lapA</i> -KO verification	GTCCAGGCCAAGATCTC
IB-81	fwd	<i>lapF</i> -TS1 amplification	taacagggtaatctgaattcTGCGCCCCATGTCGCTGC
IB-82	rev	<i>lapF</i> -TS1 amplification	gggagcatccGGCCGCTGTAAACCCGTCG
IB-83	fwd	<i>lapF</i> -TS2 amplification	tacagcggccGGATGCTCCCTGCTGATATC
IB-84	rev	<i>lapF</i> -TS2 amplification	ttgcatgcctgcaggtcgacTTCTATCACCGCCTCAGC
IB-85	fwd	<i>lapF</i> -KO verification	GTA CTGCGACTGGTACTC
IB-86	rev	<i>lapF</i> -KO verification	CTGTTCCCTCGACGAAGTG
IB-215	fwd	pili1-KO verification	TGATCGATGGCGTCGAAC
IB-216	rev	pili1-KO verification	GTCAAGGAAGGCAAAAGTC
IB-217	fwd	pili2-KO verification	ATCACGCGGATACGGTTG
IB-218	rev	pili2-KO verification	TGGTTCGCCTACTTCGAG
IB-246	fwd	pili3-KO verification	CCCAGGCCATTACATTG
IB-220	rev	pili3-KO verification	TGTCGCCGTCCTTGATTG
IB-221	fwd	pili4-KO verification	CGAAGATCAGCTGCAATG
IB-222	rev	pili4-KO verification	TGCCCAGCTTGATCATGC
IB-238	fwd	pili5-KO verification	GAAGATGTGGCCAACCTG
IB-239	rev	pili5-KO verification	GGTGATCTGGCAGTGTTG
IB-255	fwd	curli-KO verification	TTCTCGAAGGCAATCTG
IB-256	rev	curli-KO verification	AACGGCTCGAAGAACCAC
IB-242	fwd	pili6-KO verification	AAAGGCCTGCTGGACATC
IB-243	rev	pili6-KO verification	TGACTTGGCCTGACACTG
IB-244	fwd	pili7-KO verification	CATGTCGCCTAAGCAAAG
IB-245	rev	pili7-KO verification	TTCCGGGATTGCCATCAG
MM05	fwd	pSEVA441 plasmids	GGCCCAGTCTTTCGACTGAG
SK62	rev	pSEVA441 plasmids	TAGTCGCCAGGGTTTTCC
IB-144	fwd	<i>fabA</i> -TS1 amplification	taacagggtaatctgaattcAATGATCAGGTACTGCTG
IB-145	rev	<i>fabA</i> -TS1 amplification	cgaataacccGCGAGCTCCTCAATAACC
IB-146	fwd	<i>fabA</i> -TS2 amplification	aggagctcgcGGGTTATTCGCATGCGCC
IB-147	rev	<i>fabA</i> -TS2 amplification	ttgcatgcctgcaggtcgacACGACTGGCTCCAGTGCTC
IB-148	fwd	<i>fabA</i> -KO verification	ACCTTCACCTGGAGATG
IB-149	rev	<i>fabA</i> -KO verification	CGATGACGAAGCCATCAC
IB-249	fwd	<i>fabG</i> amplification	caggtaccgaattcctcgagaggaggtGAAAATCATGAATTTTGAAGGAA
IB-250	rev	<i>fabG</i> amplification	ggcccgcagtcgcatgctccTCAGACCATGTACATCCC
IB-209	fwd	<i>araBAD</i> amplification	caggtaccgaattcctcgagaggaggtTGAAACGATGGCGATTGC
IB-210	rev	<i>araBAD</i> amplification	ggcccgcagtcgcatgctccTTACTGCCCGTAATATGC
IB-249	fwd	<i>fabG</i> amplification	caggtaccgaattcctcgagaggaggtGAAAATCATGAATTTTGAAGGAA
IB-250	rev	<i>fabG</i> amplification	ggcccgcagtcgcatgctccTCAGACCATGTACATCCC
IB-223	fwd	<i>kguT</i> -TS1 amplification	taacagggtaatctgaattcTTGCCAGCACC GCCGCCA
IB-224	rev	<i>kguT</i> -TS1 amplification	gggggtggtggagggTGCGGTATGCCGCCATG
IB-225	fwd	<i>kguT</i> -TS2 amplification	catgaccgcacCCTCCACCACCCCGAGCAG
IB-226	rev	<i>kguT</i> -TS2 amplification	ttgcatgcctgcaggtcgacTGGTGCGGCAGCCGTGCC
IB-228	fwd	<i>kguT</i> -A117G verifi.	GGCAGTGAGTACTTTTCG

IB-229	rev	<i>kguT</i> -A117G verifi.	CGCCAGTCGTAATGCTTG
IB-260	fwd	pBG14f_80i_14g	GAATTCGAGCTCGGTACC
IB-261	rev	pBG14f_80i_14g	acctcctTCGTGCAATTATACCTGG
IB-262	fwd	pBT-Weimberg	ggccagggtataattgcacgaaggaggtGGATCACATGTCGGACCTG
IB-259	rev	pBT-Weimberg	cgggtaccgagctcgaattcTCAATGAGAATGCCGTGG
IB-93a	rev	pBG_PVLB18550-...	TCAATCCTCCGAAACGCGAC
IB-257	rev	pBG14f_80i_14g	TCGTGCAATTATACCTGG
IB-260	fwd	pBG14f_80i_14g	GAATTCGAGCTCGGTACC
IB-258	fwd	pBT-Dahms	ggccagggtataattgcacgaAGGAGGTAATGACATGAC
IB-259	rev	pBT-Dahms	cgggtaccgagctcgaattcTCAATGAGAATGCCGTGG
IB-184	fwd	pBG_PVLB_18565	CGCGTCGACCTTCTGGTG

2.4 Adaptive laboratory evolution

For adaptation to xylose, the different xylose-consuming strains were grown in M9 minimal medium containing 10 g L⁻¹ xylose. For adaptation to ethanol, *P. putida* KT2440 was grown in M9 minimal medium containing 2 g L⁻¹ glucose and 4% (v/v) ethanol. For adaptation to galacturonic acid, *P. putida* KT2440 was grown in M9 minimal medium containing 10 g L⁻¹ galacturonic acid. The OD₆₀₀ was measured daily and the cells were sequentially transferred to fresh medium with a starting OD₆₀₀ of 0.1. The sub-culturing was carried out 30 times for the Isomerase-strain, 17 times for the Weimberg-strain, and 26 times for the Dahms-strain. In the case of ethanol, after 30 days (23 transfers), the medium composition was changed to 4% (v/v) ethanol and ALE was carried on for twelve more days (10 transfers). For adaptation to galacturonic acid, the sub-culturing was carried out 48 times. The inhomogeneous culture was streaked out on LB-agar to obtain single isolates, which were subsequently tested for adaption to xylose, ethanol, or galacturonic acid in a 96-well plate in a Growth Profiler 960 (EnzyScreen B.V., Heemstede, The Netherlands). Each experiment was performed in two biological replicates.

2.5 DNA techniques

Genome re-sequencing of the wild type, *P. putida* KT2440 E1, and *P. putida* KT2440 E1.1, *P. putida* KT2440 pWeim2, *P. putida* KT2440ΔΔ pDahms2, and *P. putida* KT2440 GA1 was performed to identify mutations obtained by ALE on ethanol, xylose, or galacturonic acid. Therefore, genomic DNA was isolated with the High Pure PCR Template Preparation Kit (Roche Holding, Basel, Switzerland). The sequencing was done by Eurofins Genomics (Ebersberg, Germany) using Illumina technology as paired-end reads of 150 base pairs. Single nucleotide polymorphisms (SNPs) and insertions or deletions (InDels), which were analyzed by GATK's Haplotype Caller [258,259], were visualized with the Integrative Genomics Viewer [260]. The sequences of the wild type, *P. putida* KT2440 E1, and *P. putida*

KT2440 E1.1 were deposited in the Sequence Read Archive with the accession number (PRJNA642834).

Expression levels of genes were determined by quantitative real-time PCR (qRT-PCR). RNA was extracted from the wild type, *P. putida* KT2440 E1, and *P. putida* KT2440 E1.1 when growing in M9 minimal medium containing 0.96% (v/v) ethanol. The medium was inoculated at a starting OD₆₀₀ of 0.1 and the cells were cultivated until they reached an OD₆₀₀ of 1. 1 mL of the cultures was centrifuged at 16,000 g for 2 min, the supernatant was discarded, and the cells were resuspended in 1 mL RNeasy Lysis Solution (Qiagen, Crawley, UK). All further steps for RNA extraction were performed using the RNeasy Total RNA Miniprep Kit (Qiagen, Crawley, UK) following the manual. The RNA concentration was measured with the NanoDrop One (Thermo Fisher Scientific, Waltham, MA, USA) at 260 nm. All samples were adjusted to a concentration of 6 ng μL^{-1} in a total volume of 40 μL . After RNA isolation, an additional DNase digestion was performed for 1 h at 37°C using 5 μL DNase I and 5 μL DNase I reaction buffer (Qiagen, Crawley, UK). The DNase I was inactivated at 75°C for 10 min. 4 μL of the RNA template were used for cDNA synthesis, which was performed using the ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA) following the manual. Subsequently, PCR was performed in white 96-well plates using the Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) in a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). For qRT-PCR, specific oligonucleotides with annealing temperatures of 60°C were designed using the Primer3 software (<http://primer3.ut.ee>). Standard curves for all primer pairs were performed by using twofold dilution series of genomic DNA of the wild type with seven data points. Afterwards, the primer efficiencies were determined and reached values between 2 and 2.13. Nuclease-free water for every primer pair and DNase treated RNA templates were used as negative controls. Each cDNA sample was diluted 1:10 and analyzed as technical triplicate. Data analysis was performed using the Bio-Rad CFX Manager Software. The resulting Ct values were used to calculate the gene expression level via the $2^{-\Delta\text{Ct}}$ method by normalizing to the house keeping gene *rpoB*.

2.6 Fermentation conditions

Batch and fed-batch fermentations for rhamnolipid production were performed as duplicate using modified M9 minimal medium with a final composition (per L) of 1.21 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.43 g KH_2PO_4 , 0.5 g NaCl, 1.9 g NH_4Cl , 2 mM MgSO_4 , 9.74 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 41.2 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.5 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1.87 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 mg H_3BO_3 , 0.25 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.15 mg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, and 13.11 mg $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$

[261]. As carbon sources 7.6 g L⁻¹ ethanol, 10 g L⁻¹ xylose, or 10.8 g L⁻¹ galacturonic acid (each 0.33 Cmol L⁻¹) were used. The fermentations were conducted in a stirred tank reactor (BioFlo 120 glass bioreactor; Eppendorf, Hamburg, Germany) with a working volume of 1 L and a nominal volume of 1.3 L. The fermentations were controlled by a BioFlo120 unit and DASware control software (v5.5.0; Eppendorf, Hamburg, Germany). The setup is illustrated in Figure 3. The bioreactor was equipped with a pH electrode (EasyFerm plus PHI 225, Hamilton, Reno, NV, USA), dissolved oxygen (DO) electrode (InPro 6800, Mettler-Toledo, Columbus, OH, USA), sparger, pH control agent inlets, foam outlet, foam recirculation inlet, septum, Pt100 temperature sensor, hose connection for the inoculation bottle, sampling system, and cooling loop. The agitator shaft was equipped with two Rushton turbines (Ø 5.3 cm). The temperature was held constant at 30°C. The pH was adjusted to 7 via automatic addition of 2 M KOH and 2 M H₂SO₄ for batch fermentations and 30% (v/v) NH₄OH and 2 M H₂SO₄ for fed-batch fermentations. NH₄OH simultaneously served as nitrogen source during fed-batch. The aeration rate was 0.1 L min⁻¹ (0.1 vvm), whereas evaporation was reduced by sparging the supply of sterile air through a sterile water bottle. The DO was kept above 30% by an automatic increase of the agitation speed until 1,200 rpm. The foam produced during the fermentation was directed through the foam outlet via a hose into a downstream foam collection bottle. The reactor was inoculated with cells from a preculture to a final OD₆₀₀ of 0.5. One hour after inoculation, the peristaltic pump for foam recirculation was turned on at 220 rpm (Ø of the inner hose = 1 cm) and the stirrer speed was manually increased from 300 to 500 rpm to obtain wet foam.

For fed-batch fermentations, the feeding phase was initiated after batch phase. The feed was realized with an automated dissolved oxygen (DO)-based feeding strategy via a peristaltic pump. The feed contained 100% (v/v) ethanol (Th. Geyer GmbH & Co. KG, Renningen, Germany) and was activated at DO-levels higher than 40% and deactivated at DO-levels lower than 30%. In one feeding event, 3 mL ethanol were pumped to avoid toxic concentrations. However, the feed did not enter the reactor directly but was sprayed into the foam tank to collapse the collected foam (Figure 3). The collapsed foam is pumped back into the reactor by a peristaltic pump. The stirrer speed was constant at 1,200 rpm and up to 90% pure oxygen was manually mixed to the supply air. When the growth rate decreased, medium components were added to maintain growth and avoid nutrition limitation at higher cell densities. At 20 h the last supplementation was done and the medium contained in total (per L) 7.2 g Na₂HPO₄•2H₂O, 2.58 g KH₂PO₄, 0.5 g NaCl, 1.9 g NH₄Cl, 9.51 g NH₄OH, 26 mM MgSO₄, 48.78 mg FeSO₄•7H₂O, 0.22 g CaCl₂•2H₂O, 48.7 mg MnCl₂•4H₂O, 10.85 mg

$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 3.37 mg H_3BO_3 , 1.55 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 1 mg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, and 65.7 mg $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$.

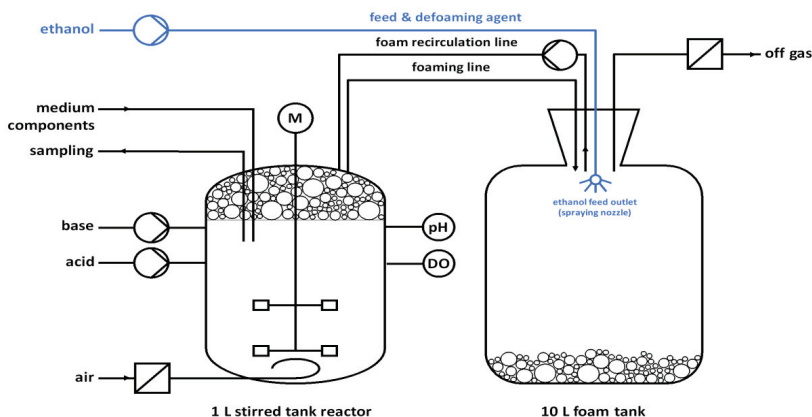


Figure 3: Schematic setup for batch and fed-batch fermentation in a bioreactor with foam recirculation. Foam is produced during the fermentation process and collected in a foam tank. The (collapsed) foam in the tank was continuously pumped back in the reactor. For fed-batch fermentation, pure ethanol as feed and defoaming agent was used (depicted in blue). Ethanol was automatically sprayed inside the foam tank based on a DO-based feeding protocol. After the addition of ethanol, the collected foam collapsed in the tank and was continuously pumped back as a liquid.

To characterize the fed-batch fermentation results, the values of biomass, biosurfactants, and ethanol (during batch phase) were fitted using a logistic equation with four parameters as described by Wittgens *et al.* [147]. An independent fit was used to illustrate the development of all three fermentation parameters.

2.7 Swimming assay

For investigation of the swimming behavior, M9 minimal medium plates containing 10 g L^{-1} glucose and 0.3% (w/v) bacto-agar were used. Precultures were grown in M9 minimal medium with 10 g L^{-1} glucose and were diluted with 0.9% (w/v) NaCl to obtain cell suspensions with an OD_{600} of 0.05. $2 \mu\text{L}$ of the suspension was spotted into the middle of the semi-solid agar plate. Swimming ability was documented after incubation at 30°C for 48 h by taking pictures.

2.8 Biofilm formation assay

The biofilm formation assay was performed based on a protocol from O'Toole [262]. Precultures of various strains (*P. putida* KT2440 and deletion mutants) were grown in LB medium and used to inoculate M9 minimal medium containing 10 g L^{-1} glucose to reach an OD_{600} of 0.1. $100 \mu\text{L}$ of the cell suspensions were transferred to a polyvinyl chloride 96 well

plate (Corning, Kennebunk, ME, USA) that was covered with a gas-permeable membrane. The plate was incubated at 30°C for 13 h. After incubation, the medium was discarded and the wells were washed two times with water. The wells were stained with 0.1% (w/v) crystal violet and washed again two times with water. After drying at room temperature, the biofilm formation was documented by taking pictures. For quantification of the biofilm, the crystal violet was resolubilized in 30% (v/v) acetic acid (125 μ L) and the absorbance was measured at a wavelength of 550 nm in a Synergy Mx microplate reader (BioTek Instruments, Winooski, VT, USA).

2.9 Analytical methods

2.9.1 Analysis of bacterial growth

The optical density was measured at 600 nm using an Ultrospec 10 cell density meter (Biochrom, Cambridge, UK). A correlation with five to six datapoints between OD₆₀₀ and cell dry weight (CDW) was established. Therefore, a defined volume was filtrated through a cellulose-nitrate filter and the filter was weighed before and after filtration and drying at 100°C. An OD₆₀₀ of 1.0 corresponds with a CDW of 310 mg L⁻¹ for medium containing glucose, with a CDW of 369 mg L⁻¹ for medium containing xylose, with a CDW of 313 mg L⁻¹ for medium containing ethanol, and with a CDW of 251 mg L⁻¹ for medium containing galacturonic acid.

2.9.2 Analysis of metabolites

For measurement of substrates and intermediates (glucose, gluconate, 2-ketogluconate, xylose, xylonate, ethanol, and acetate), samples were taken from liquid cultivation and centrifuged at 16,500 g for 1 min. The supernatant was analyzed in a Beckmann Coulter System Gold High Performance Liquid Chromatography (HPLC) (Beckmann Coulter, Brea, CA, USA) with a Metab-AAC 300 x 7.8 mm separation column (particle size: 10 μ m, ISERA GmbH, Düren, Germany), a UV detector 166 (Beckmann Coulter, Brea, CA, USA) at 210 nm and a refractory index detector (RI 2300, Knauer GmbH, Berlin, Germany). Elution was performed with 5 mM H₂SO₄ at a flow rate of 0.5 mL min⁻¹ at 40°C. Glucose, 2-ketogluconate, xylose, ethanol, acetate, and galacturonic acid were analyzed using the RI detector and gluconate and xylonate were analyzed with the UV detector. Glucose and gluconate and xylose and xylonate elute at the same retention time and thus the glucose and xylose concentrations were calculated by subtracting the determined gluconate and xylonate concentration in the RI.

2.9.3 Analysis of rhamnolipids

Reversed-phase chromatography was performed for analyzing HAA and mono-rhamnolipid concentrations based on a method developed earlier [263,264]. For sample preparation, the supernatant was mixed 1:1 with acetonitrile and stored at 4°C overnight. Subsequently, the mixture was centrifuged at 16,500 g for two minutes. All samples were filtered with Phenex RC syringe filters (0.2 µm, Ø 4 mm, Phenomenex, Torrance, USA). The HPLC system Ultimate 3000 with a Corona Veo Charged Aerosol Detector (Thermo Fisher Scientific, Waltham, MA, USA) was used. For separation, a NUCLEODUR C18 Gravity 150 x 4.6 mm column (particle size: 3 µm, Macherey-Nagel GmbH & Co. KG, Düren, Germany) was used. The flow rate was set to 1 mL min⁻¹ and the column oven temperature was set to 40°C. Acetonitrile (A) and 0.2% (v/v) formic acid in ultra-pure water (B) were used as running buffers. The method started with a ratio of 70% buffer A:30% buffer B and a linear gradient was applied to reach a ratio of 80%:20% in 8 min. The acetonitrile fraction was increased linearly from 80 to 100% between 9 min and 10 min and decreased linearly to 70% between 11 min and 12.5 min. The measurement was stopped after 15 min.

2.9.4 Analysis of phenazines

The concentration of phenazines was determined in a spectrophotometer Cary 60 (Agilent, Santa Clara, CA, USA) by analyzing the blue phenazine derivate pyocyanin. Samples were centrifuged at 16,500 g for two minutes. Afterwards, the supernatant was vortexed and pyocyanin was measured at 691 nm. For quantification, Lambert-Beer's law with an extinction coefficient of 4.31 mM⁻¹ cm⁻¹ for pyocyanin was used [265].

2.9.5 Analysis of proteinogenic amino acids

¹³C-labeling pattern of proteinogenic amino acids were determined by gas chromatography coupled with mass spectrometry (GC-MS) as described in Schmitz *et al.* [233]. A Trace GC Ultra coupled to an ISQ single quadrupole mass spectrometer with a TriPlus RSH autosampler (all Thermo Fisher Scientific, Waltham, MA, USA) was used. For separation, a TraceGOLD TG-5SilMS capillary column (length: 30 mm, inner diameter: 0.25 mm, film thickness: 0.25 µm, Thermo Fisher Scientific, Waltham, MA, USA) was used. A sample volume of 2 µL was injected at 270°C with a split ratio of 1:50. The flow rate of the carrier gas helium was set to 1 mL min⁻¹ and the oven temperature was kept constant for one minute at 140°C. The temperature was increased with a gradient of 10°C min⁻¹ to 310°C and then kept constant for one minute. The temperature of the transfer line and the ion source were both set to 280°C. Ionization was performed by electron impact ionization at -70 eV. All raw data were analyzed using Xcalibur (Thermo Fisher Scientific, Waltham, MA, USA).

2.10 Flux Balance Analysis

For the prediction of product yields and rates, the genome-scale models of *P. putida* KT2440, *iJN1411* [266] or *iJN1463* [185], were used. All simulations were carried out in MATLAB (version R2017b or R2019b, The Mathworks, Inc., Natick, MA, USA) using the COBRA toolbox [267], with the linear programming solver of Gurobi (www.gurobi.com). First, the existing model network was extended by the xylose utilization pathways as well as the biosynthesis routes to the desired products, e.g., mono-rhamnolipids, pyocyanin, and ethylene glycol, using reaction information from KEGG [268]. In the case of the xylose study, the evaluation of the respective product yields for the usage of the three xylose utilization pathways and their combination was performed. Here, the uptake of xylose for all investigated cases was set to $10 \text{ mmol g}_{\text{CDW}}^{-1} \text{ h}^{-1}$ and the uptake of other carbon sources (e.g., glucose) was set to zero. The maximization of production of the target molecule was used as objective function in the extended model. In the case of the ethanol study, the uptake of ethanol was set to $10 \text{ mmol g}_{\text{CDW}}^{-1} \text{ h}^{-1}$ and the uptake of other carbon sources was set to zero. The maximization of production of mono-rhamnolipids or biomass was used as objective function in the extended model.

Chapter 3

Results

Chapter 3.1

Comparison of three xylose pathways in *Pseudomonas putida* KT2440 for the synthesis of valuable products

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Contributions:

Isabel Bator performed all experiments, analyzed the data, prepared figures, and wrote this chapter. Strains and plasmids were constructed by Isabel Bator and Hans M. Böhne. Till Tiso provided guidance on *P. putida* biotechnology and reviewed the chapter. Till Tiso, Andreas Wittgens, and Frank Rosenau discussed the data and critically revised the chapter. Lars M. Blank advised on all experiments, analyzed and discussed data, and reviewed the chapter.

We thank Prof. de Lorenzo for providing *P. putida* EM42 Δ *gcd* pSEVA2213_*xylABE*, Dr. Simone Schmitz and Prof. Agler-Rosenbaum for providing plasmid pJNN_*phzA1-G1,M,S*, Dr. Sebastian Köbbing for providing plasmid pBG14f_80i_14f_80i_14g, and Dr. Wing-Jin Li for providing *P. putida* KT2440 Δ *gclR*.

3 Results

3.1 Comparison of three xylose pathways in *Pseudomonas putida* KT2440 for the synthesis of valuable products

3.1.1 Abstract

P. putida KT2440 is a well-established chassis in industrial biotechnology. To increase the substrate spectrum, we implemented three alternative xylose utilization pathways, namely the Isomerase, Weimberg, and Dahms pathways. The synthetic operons contain genes from *E. coli* and *P. taiwanensis*. For isolating the Dahms pathway in *P. putida* KT2440 two genes (PP_2836 and PP_4283), encoding an endogenous enzyme of the Weimberg pathway and a regulator for glycolaldehyde degradation, were deleted. Before and after ALE, these strains were characterized in terms of growth and synthesis of mono-rhamnolipids and pyocyanin. The engineered strain using the Weimberg pathway reached the highest maximal growth rate of 0.30 h^{-1} . After ALE the lag phase was reduced significantly. The highest titers of 720 mg L^{-1} mono-rhamnolipids and 30 mg L^{-1} pyocyanin were reached by the evolved strain using the Weimberg or an engineered strain using the Isomerase pathway, respectively. The different stoichiometries of the three xylose utilization pathways may allow engineering of tailored chassis for valuable bioproduct synthesis.

3.1.2 Introduction

For the establishment of a circular bioeconomy, the chemical industry has to overcome the massive and ever-increasing use of fossil resources and the concomitant production of environmental pollutions including greenhouse gases. The alternative is CO_2 as carbon source, either directly or fixed via chemocatalysis or plants [269,270]. CO_2 -fixation though cannot only proceed under natural conditions, there are also many synthetic approaches to convert CO_2 to valuable products. The conversion of CO_2 mainly results in the production of biomass in microbes and is well studied. Hence, it immediately becomes clear that the knowledge about CO_2 -fixation pathways can be utilized to redirect the metabolic flow into the production of chemicals using the synthetic biology arsenal. On the one hand cell-free systems are used to fix CO_2 via multienzyme cascades [271,272], on the other hand engineered autotrophic microbes are used for the production of chemicals based on CO_2 [273,274]. If the usage of non-autotrophic microbes is desired for the production of multi-carbon compounds via CO_2 -fixation, either existing pathways (e.g., the Calvin cycle) or newly designed pathways have to be introduced into the cells [275–278], with the outcome of alternative stoichiometries [279]. CO_2 -fixation via plants is a common choice as

lignocellulosic biomass is abundant and does not compete immediately with food applications. Lignocellulosic biomass contains about 25-50% hemicellulose [280]. In addition to glucose, the pentose xylose is a dominant building block in hemicellulose. For example, bagasse fibers consist of approximately 23% xylose [281]. While wheat straw and corn stover are industrially used as carbon source for bioethanol production [282–284], exemplifying the feasibility of the alternatives, most other microbes than yeast are still on the experimental scale.

The well-characterized chassis *P. putida* KT2440 is known for its versatile metabolism, the richness of cofactors, and high solvent tolerance [155,160,162,186]. Further, the genome is sequenced and well annotated [184,185,285]. The structure of the central carbon metabolism and the fluxes on different carbon sources are well studied for *P. putida* KT2440 [96]. Further, many tools for genomic engineering are available [164,225,286]. Besides, its non-pathogenicity contributes to it being a chassis relevant for production of valuable products [155,166,186,287].

However, *P. putida* KT2440 lacks the capability to metabolize xylose. Microbial assimilation of xylose has so far been observed by three different metabolic pathways: An isomerase and two oxidative pathways called Weimberg and Dahms. The Isomerase pathway is characterized by the presence of a xylose isomerase and xylulokinase. The resulting intermediate xylulose is subsequently phosphorylated to xylulose-5-phosphate and introduced into the pentose phosphate pathway. The Isomerase pathway is originally found in prokaryotes, such as *E. coli* and *B. subtilis* [288,289]. The oxidative pathways start with the oxidation of xylose to xylonate. This step is often catalyzed by a periplasmic dehydrogenase. A xylonate dehydratase then converts xylonate into 2-keto-3-deoxy-xylonate. The resulting intermediate is dehydrated to α -ketoglutaric semialdehyde in the case of the Weimberg pathway. In the case of the Dahms pathway, α -ketoglutaric semialdehyde is split by an aldolase into pyruvate and glycolaldehyde. While pyruvate is directly converted in the central carbon metabolism and converted to acetyl-CoA, glycolaldehyde is further metabolized in several steps to 2-phosphoglycerate [290]. 2-Phosphoglycerate is then converted in glycolysis. In the Weimberg pathway, an additional oxidation step from α -ketoglutaric semialdehyde to 2-oxoglutarate is present. The latter metabolite is converted in the tricarboxylic acid (TCA) cycle (Figure 4). The Weimberg pathway was reported for example to be present in *P. fragi*, *Haloferax volcanii*, and *Caulobacter crescentus* [291–293]. The genes of the latter species were already heterologously expressed in different organisms to establish the Weimberg pathway [294–296]. Recently, also *P. taiwanensis* VLB120 was found to be a native xylose-consumer using the Weimberg pathway [297]. The Dahms pathway was first found to be

present in an unclassified *Pseudomonas* strain [298]. Some of the genes of *C. crescentus* were also heterologously expressed to establish the Dahms pathway in *E. coli*, because *E. coli* harbors a gene coding for an aldolase, which catalyzes the last step of the Dahms pathway [299,300].

P. putida KT2440 has already been engineered to utilize xylose via the Isomerase pathway [255,301]. Furthermore, *P. putida* S12 was equipped successfully with xylose metabolism genes to establish the Isomerase and the Weimberg pathway [294,302]. In the studies mentioned above, growth via the Isomerase pathway was rather slow. This phenomenon occurred because most of the substrate was oxidized to the dead-end product xylonate. Further, xylose transport was reported to be inefficient in *P. putida* KT2440. By implementing ALE growth on xylose could be improved [302]. Using a different approach, Dvořák and de Lorenzo additionally inserted the proton-coupled symporter XylE, also achieving improved growth [255].

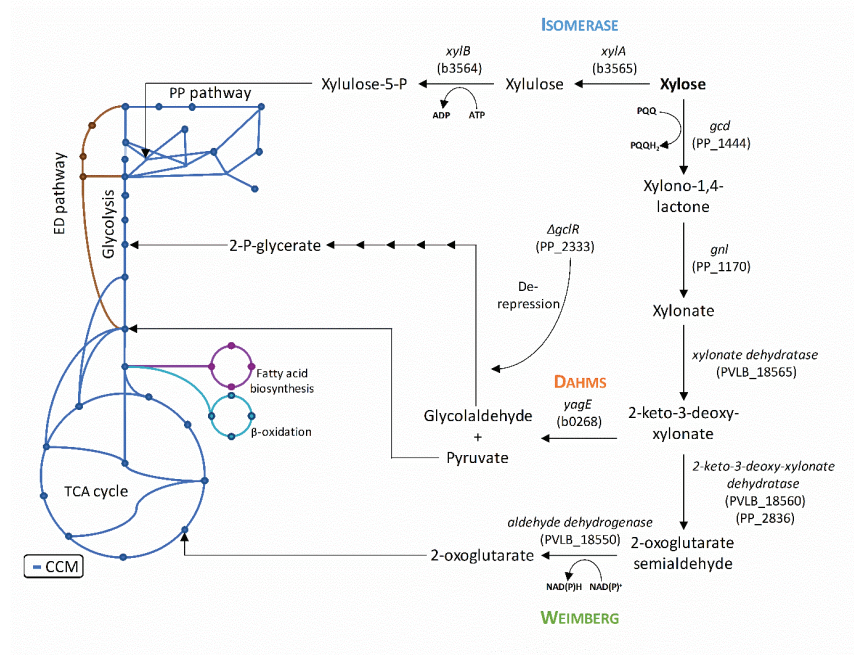


Figure 4: Introduction of xylose metabolism pathways into the central carbon metabolism of *P. putida* KT2440 with the presumed endogenous enzymes and heterologous enzymes from *E. coli* DH5a and *P. taiwanensis* VLB120. The blue lines indicate the central carbon metabolism based on Sudarsan *et al.* [96]. The PP numbers represent the locus tag in *P. putida* KT2440, the PVLB numbers represent the locus tag in *P. taiwanensis* VLB120, and the b numbers represent the locus tag in *E. coli* K12, of which *E. coli* DH5a is derived from. ADP, adenosine diphosphate; ATP, adenosine triphosphate; NAD⁺, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; PQQ, pyrroloquinoline quinone; PQQH₂, reduced pyrroloquinoline quinone.

So far, metabolic engineering approaches were limited to metabolic alterations in order to synthesize a target compound based on a previously determined substrate. We here propose to exploit the metabolic potential of microbes for the production of chemicals by electing for the substrate of choice the most suited degradation pathway. Pseudomonads are suitable hosts for the heterologous production of several products, e.g., surfactants, aromatics, terpenoids, and phenazines besides others [147,150,166,173,182,303]. However, the usage of xylose for the synthesis of valuable chemicals with *P. putida* KT2440 has not been reported so far.

In this work, we aimed at engineering a chassis, which can be used for the production of desired secondary metabolites using xylose. First, we investigated the theoretical potential for the synthesis of valuable products using the different xylose pathways via flux balance analysis (FBA). Second, we implemented the three different xylose pathways into *P. putida* KT2440 and optimized growth by ALE. Third, we used these strains for the synthesis of mono-rhamnolipids and pyocyanin, a derivative of phenazines. We demonstrate efficient growth on xylose via the oxidative pathways. The results show that using xylose pathways having different stoichiometries leads to differences in substrate consumption, growth, and production rates, as well as in biomass and product yields. We propose to use different xylose degradation pathways in dependence of product needs.

3.1.3 Results

3.1.3.1 Alternative xylose utilization pathways for maximal product yield

With FBA one can compute the maximal product yield in different scenarios (e.g., growth, maintenance coefficients, aeration). Here, the genome-scale model *P. putida* KT2440 *i*JN1411 [266] was modified by introducing the alternative xylose utilization pathways. In addition, various synthesis reactions for valuable products according to Werpy and Petersen [304] and chemicals we are working on were introduced into *i*JN1411.

The maximal product yields of 12 of the 14 metabolites computed on xylose were produced by the Isomerase pathway (Table 6). The exceptions of maximal yields of metabolites are such products that are directly synthesized from intermediates of the Weimberg and Dahms pathway. For example, the intermediate 2-oxoglutarate of the Weimberg pathway is an interesting metabolite, because it is introduced into the TCA cycle without carbon loss. The direct conversion of 2-oxoglutarate into glutamate (or proline, OH-proline, etc.) would thus benefit from the Weimberg pathway. This was also computed by FBA with a maximal product yield of 1 mmol mmol⁻¹ for the Weimberg pathway, and only 0.83 and 0.75 mmol mmol⁻¹ for the Isomerase and the Dahms pathway, respectively. The intermediate glycolaldehyde, using the Dahms pathway for xylose degradation, itself is a

relevant chemical for industrial applications and a precursor for ethylene glycol synthesis. Ethylene glycol can neither be synthesized via the Isomerase nor the Weimberg pathway, while the maximal product yield of the Dahms pathway is 1 mmol mmol⁻¹. Consequently, the Dahms pathway is a favorable pathway to produce glycols out of xylose. In the case of the Isomerase pathway, xylulose-5-phosphate is part of the pentose phosphate pathway and the intermediate erythrose-4-phosphate is a starting metabolite for aromatics synthesis via the shikimate pathway. This explains that the Isomerase pathway has the highest yield for the production of the here synthesized aromatic compound pyocyanin (0.27 mmol mmol⁻¹). Moreover, the highest maximal mono-rhamnolipid yield is also possible via the Isomerase pathway (0.14 mmol mmol⁻¹) and lower yields for the Weimberg and the Dahms pathway (0.08 and 0.12 mmol mmol⁻¹) were computed. This is due to CO₂ production using the TCA cycle for the Weimberg pathway and converting glycolaldehyde in several steps to 2-phosphoglycerate for the Dahms pathway, also releasing one CO₂.

Table 6: FBA computed maximal product yield of valuable products for three different xylose utilization pathways (Isomerase, Weimberg, and Dahms) and the combination of the Weimberg (W) and the Dahms (D) pathway. For the combined pathways, also the percentage of the used pathway is indicated. The bold values indicate the highest maximal product yield. ^aDue to constraints in the model, the maximal yield for glycerol cannot be computed for the combination of the Weimberg and the Dahms pathway.

Products	Maximal product yield (mmol _{product} / mmol _{xylose})				
	Isomerase	Weimberg	Dahms	Weimberg + Dahms	
Acetoin	0.83	0.5	0.75	0.75	100% D
Di-rhamnolipids	0.12	0.07	0.1	0.1	19% W, 81% D
Ethylene glycol	0	0	1	1	100% D
Fumaric acid	1.67	1	1.32	1.38	25% W, 75% D
Gluconic acid	0.83	0.5	0.64	0.68	27% W, 73% D
Glutamate	0.83	1	0.75	1	100% W
Glycerol	0.81	0.56	0.47	n.d. ^a	-
HAA	0.17	0.1	0.15	0.15	6% W, 94% D
Malic acid	1.67	1	1.32	1.38	25% W, 75% D
Succinic acid	1.04	1	1	1	100% W
Sorbitol	0.77	0.5	0.56	0.63	50% W, 50% D
Threonine	0.83	0.5	0.75	0.75	100% D
Mono-rhamnolipids	0.14	0.08	0.12	0.12	14% W, 86% D
Pyocyanin	0.27	0.19	0.17	0.23	63% W, 37% D

In addition, the maximal product yields for all possible combinations of the xylose pathways (Isomerase + Weimberg, Isomerase + Dahms, Weimberg + Dahms, and Isomerase + Weimberg + Dahms) were computed. In general, it was observed that always one of the pathways in the combinations is favored and is thus exclusively used. Interestingly, and contrary to the above, the combination of the Weimberg and the Dahms pathways resulted in higher maximal product yields for nine of the 14 metabolites (Table 6) compared to the single pathway strains. Thus, in Table 6 only the combined activities of the latter pathways are also

listed. In summary, the *in silico* study indicates that the strain equipped with the Isomerase pathway is best-suited for a range of chemicals including the production of the here chosen metabolites mono-rhamnolipids and pyocyanin. The Weimberg and Dahms pathways are favored in niche applications (Table 6), when xylose is the sole carbon and energy source.

3.1.3.2 The oxidative pathways enable efficient xylose utilization

A genome analysis using BLAST [305] showed that *P. putida* KT2440 possesses genes, which might encode enzymes needed in xylose utilization, but a complete pathway seems to be absent, indeed correlating with no-growth on this sugar (Figure 5A). All genes required for the Isomerase, Weimberg, and Dahms pathways were compared to the genome of *P. putida* KT2440. No homologs for the genes *xylA* and *xylB* from *E. coli* for the Isomerase pathway are present. Additionally, no homologs for the genes PVLB_18565 from *P. taiwanensis* and *yagE* from *E. coli* for the Weimberg pathway and the Dahms pathway, respectively, are present. In contrast, homologous genes of the Weimberg pathway from *P. taiwanensis* are present (homologues to PVLB_18550, PVLB_18555, and PVLB_18560 with sequence identities of 68 - 72%). HPLC analysis revealed that xylose was consumed by non-growing *P. putida* KT2440, however xylose was converted into the dead-end product xylonate (Figure 5A). Xylonate synthesis proceeds likely via the gene products of *gcd* (PP_1444) and *gnl* (PP_1170) that are besides for glucose also active for xylose and its lactone derivative (Figure 4).

In order to engineer *P. putida* for the utilization of xylose, *P. putida* was equipped with a plasmid for the Isomerase pathway and designated *P. putida* KT2440 pIso. After inoculation, strain *P. putida* KT2440 pIso showed little growth (Figure 5B) with a maximal growth rate of 0.02 h⁻¹ and substrate consumption rate of 0.47 mmol L⁻¹ h⁻¹ (Table 7). In addition, pBT-Isomerase was introduced into *P. putida* KT2440 Δgcd . This strain is unable to produce the dead-end product xylonate and thus more substrate should be available for biomass formation, as it was reported previously in a study with *P. putida* S12 [302]. The resulting *P. putida* KT2440 Δgcd pIso showed no improved growth compared to *P. putida* KT2440 pIso (data not shown). Nevertheless, these results suggest that the cloned genes *xylAB* from *E. coli* are functionally expressed in *P. putida* KT2440 and allow slow growth of the recombinant strain.

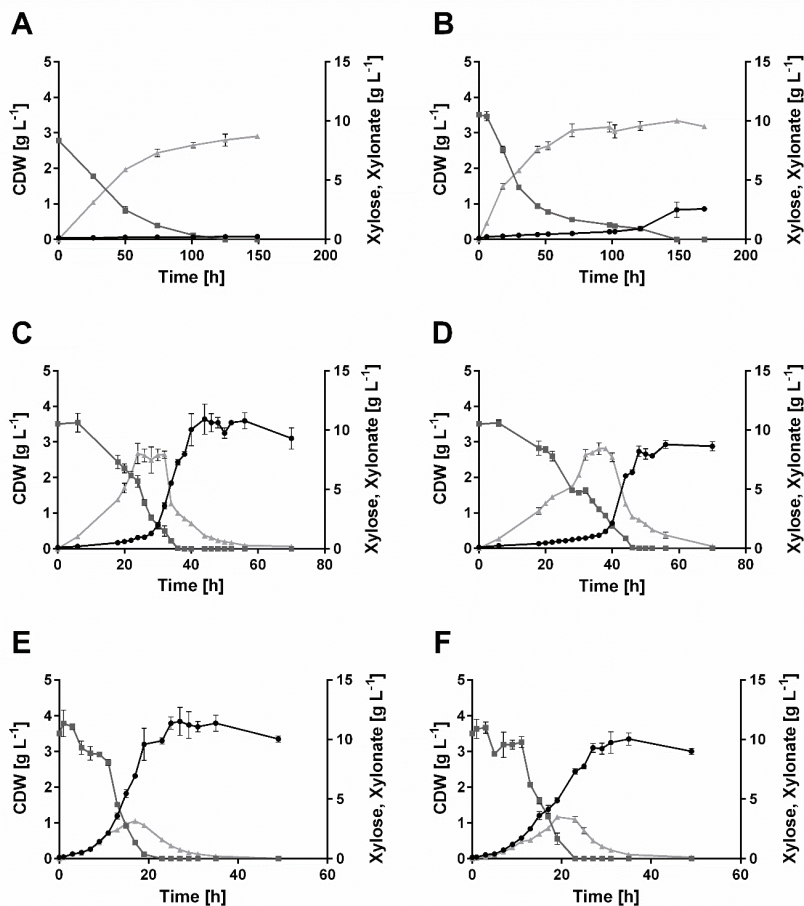


Figure 5: Growth of *P. putida* KT2440, its derivative xylose consuming strains, and the xylose-adapted strains in minimal medium containing 10 g L⁻¹ xylose. Consumption of xylose (■, dark grey), formation of xylonate (▲, light grey), and cell dry weight (●, black) of (A) *P. putida* KT2440, (B) *P. putida* KT2440 pIslo, (C) *P. putida* KT2440 pWeim, (D) *P. putida* KT2440ΔΔ pDahms, (E) *P. putida* KT2440 pWeim2, and (F) *P. putida* KT2440ΔΔ pDahms2. Error bars indicate deviation from the mean (n=3).

In order to establish the oxidative Weimberg pathway in *P. putida* KT2440, the plasmid pBT-Weimberg was transferred into *P. putida* KT2440 resulting in *P. putida* KT2440 pWeim. Interestingly, *P. putida* KT2440 pWeim showed efficient growth with a maximal growth rate of 0.30 h⁻¹ and a strong accumulation of xylonate (Figure 5C). This led to a long lag phase of approximately 24 hours (Table 7). We define the lag phase as a phase before growth was observed due to homogenous adaptation. The growth of a small phenotypic subpopulation on

the new carbon source due to heterogenous adaptation described by Kotte *et al.* [306] is not considered. The observed lag phase indicates a bottleneck in the conversion of xylonate to 2-keto-3-deoxy-xylonate or in the transport from the periplasm to the cytoplasm. Further, the substrate consumption rate was about 4-fold higher ($1.94 \text{ mmol L}^{-1} \text{ h}^{-1}$) in comparison to *P. putida* KT2440 pIso (Table 7). These results show that the xylose operon from *P. taiwanensis* is functionally expressed in *P. putida* KT2440 and results in efficient growth. To verify that the glucose dehydrogenase (Gcd) features a side-activity for xylose in *P. putida* KT2440, the plasmid pBT-Weimberg was introduced into *P. putida* KT2440 Δgcd yielding *P. putida* KT2440 Δgcd pWeim. After inoculation, no growth was observed (data not shown). This behavior confirms the assumption that Gcd is active for xylose because xylose needs to be converted to xylonate to establish the Weimberg pathway (Figure 4).

Table 7: Growth characteristics of wild type, recombinant, and evolved *P. putida* KT2440 strains on xylose. Values are the arithmetic mean of biological triplicates. The deviation indicates the standard deviation from the mean ($n=3$). ^aThe growth rate was determined during the exponential phase. ^bThe substrate consumption rate was determined until xylose is depleted. For the wild type, the whole cultivation time was used as reference. ^cFor the wild type, the substrate consumption rate is considered as substrate conversion rate, because it is not able to grow on xylose.

Strain	Growth rate μ (h^{-1}) ^a	Lag phase (h)	Substrate consumption rate ($\text{mmol L}^{-1} \text{ h}^{-1}$) ^b
<i>P. putida</i> KT2440	-	-	0.45 ± 0.00^c
<i>P. putida</i> KT2440 pIso	0.02 ± 0.00	100 ± 0	0.47 ± 0.00
<i>P. putida</i> KT2440 pWeim	0.30 ± 0.02	24 ± 0	1.94 ± 0.00
<i>P. putida</i> KT2440 $\Delta\Delta$ pDahms	0.21 ± 0.02	34 ± 0	1.46 ± 0.00
<i>P. putida</i> KT2440 pWeim2	0.21 ± 0.00	3 ± 0	3.05 ± 0.00
<i>P. putida</i> KT2440 $\Delta\Delta$ pDahms2	0.21 ± 0.01	3 ± 0	3.05 ± 0.00

Moreover, the expression of genes encoding the Dahms pathway was targeted. Therefore, the plasmid for the Dahms pathway was introduced into wild type yielding *P. putida* KT2440 pDahms. As described before, genome analysis revealed that a homologous gene of PVLB_18560 is present (PP_2836). By comparison with genes expressing xylose-metabolizing enzymes from *C. crescentus*, PVLB_18560 is assumed to encode a dehydratase, which converts 2-keto-3-deoxy-xylonate to α -ketoglutaric semialdehyde (Figure 4). As a result, it is assumed that the product of the homologous gene PP_2836 in *P. putida* KT2440 is likely to catalyze this reaction. To verify this hypothesis, the plasmid pBT-Dahms was also transferred into *P. putida* KT2440 ΔPP_2836 yielding *P. putida* KT2440 ΔPP_2836 pDahms. *P. putida* KT2440 pDahms reached an OD_{600} of 8.2 after 74 hours, while *P. putida* KT2440 ΔPP_2836 pDahms stopped growing after 48 hours (final OD_{600} of 2.0). The latter strain showed cell clumping after 74 hours, which implies stress. We speculated that low optical density and cell-clumping are caused by an accumulation of the toxic intermediate

glycolaldehyde. These findings strongly indicate that PP_2836 encodes a 2-keto-3-deoxy-xylonate dehydratase. In order to overcome the toxic effect of glycolaldehyde, strain *P. putida* KT2440 $\Delta gclR$ was used. The glyoxylate carboligase oxidase pathway is active in this mutant, thus avoiding glycolaldehyde accumulation [254]. The plasmid pBT-Dahms was introduced into *P. putida* KT2440 $\Delta gclR$ ΔPP_{2836} resulting in *P. putida* KT2440 $\Delta\Delta$ pDahms. In comparison to *P. putida* KT2440 ΔPP_{2836} pDahms, strain *P. putida* KT2440 $\Delta\Delta$ pDahms indeed grew to higher cell densities, which led to the conclusion that the genes encoding the enzymes for the Dahms pathway are successfully expressed and the prevention of glycolaldehyde accumulation leads to efficient growth. The growth of *P. putida* KT2440 $\Delta\Delta$ pDahms was further examined and a maximal growth rate of 0.21 h^{-1} and a substrate consumption rate of $1.46\text{ mmol L}^{-1}\text{ h}^{-1}$ was observed (Table 7). Further, accumulation of xylonate occurred as in the case of the Weimberg strain. Thereby, a lag phase of approximately 34 hours results (Figure 5D).

As stated above, it was observed that not only a long lag phase occurs when the Isomerase pathway is used, but also when the oxidative pathways are used. Regulatory mechanisms are assumed as a cause, but in the case of the oxidative pathways, the used route for metabolization is different. There, the xylose gets oxidized to xylonate via xylono-1,4-lactone by the enzymes encoded by *gcd* and *gnl*. It is assumed that these enzymes are active for xylose, but with a lower specificity and thus, the conversion is slower than for glucose and a long lag phase is the result. Further, accumulation of xylonate was observed, which indicates a bottleneck in xylonate conversion or transport.

3.1.3.3 Adaptive laboratory evolution improves growth on xylose

It was shown that all xylose metabolism plasmids are functionally expressed resulting in weak growth for the Isomerase pathway and efficient growth for the Weimberg and Dahms pathways, respectively. However, the lag phase of all strains was relatively long. To optimize growth, ALE was applied. For each of the strains, *P. putida* KT2440 pIso, *P. putida* KT2440 pWeim, and *P. putida* KT2440 $\Delta\Delta$ pDahms, two parallel cultures were sequentially transferred to fresh minimal medium with xylose as the sole carbon source (Figure 6A). The performance of *P. putida* KT2440 pWeim and *P. putida* KT2440 $\Delta\Delta$ pDahms was improved after 17 and 26 transfers corresponding to approximately 115 and 160 generations, respectively. The number of generations was calculated based on the starting OD₆₀₀ and the end OD₆₀₀ of every transfer. Afterwards, 24 isolates were obtained from the culture broth and up to six from each culture were randomly selected and tested for growth on xylose in comparison to the initial strains. In Figure 6B five isolates in comparison to the initial strain *P. putida* KT2440 pWeim are

shown. The adaptation towards xylose is not similar for all isolates, as some isolates still had a longer lag phase, suggesting that an isolation step to obtain the best growing strain is required. Hereafter, the best isolates were designated as *P. putida* KT2440 pWeim2 and *P. putida* KT2440 $\Delta\Delta$ pDahms2. The adaptation of *P. putida* KT2440 pIso to xylose was problematic because the strain did not grow anymore after several transfers.

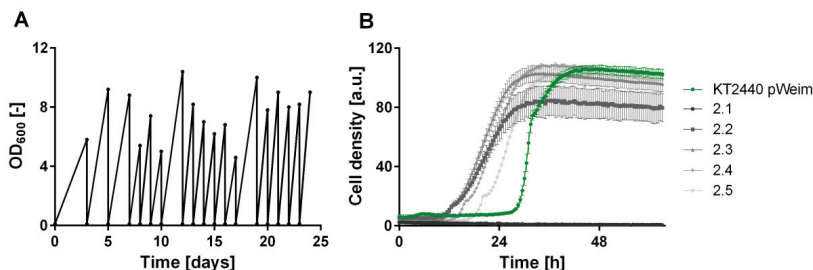


Figure 6: ALE and growth characteristics of isolates of *P. putida* KT2440 pWeim on xylose. (A) ALE of *P. putida* KT2440 pWeim on xylose as sole carbon source. A single representative culture is shown, (B) Comparison of growth for *P. putida* KT2440 pWeim (●, green) and five isolates from the second ALE culture on xylose. Error bars indicate deviation from the mean ($n=3$).

The colonies showing the best performance (*P. putida* KT2440 pWeim2 and *P. putida* KT2440 $\Delta\Delta$ pDahms2) were characterized in detail regarding their growth behavior. Both showed improved growth compared to the initial strains. The lag phase of *P. putida* KT2440 pWeim2 was shortened from approximately 24 hours to 3 hours (Figure 5E). However, the maximal growth rate of the strain was 0.21 h^{-1} . Hence, the growth rate was 1.5-fold reduced compared to the initial strain (Table 7). For *P. putida* KT2440 $\Delta\Delta$ pDahms2, the lag phase was shortened from approximately 34 hours to 3 hours (Figure 5F). The growth rate remained the same with a value of 0.21 h^{-1} (Table 7). Further, the substrate consumption rate was $3 \text{ mmol L}^{-1} \text{ h}^{-1}$ for both evolved strains, which is about 1.6-fold and twofold higher than the consumption rates of their initial strains.

To identify possible mutations, which are responsible for the reduced lag phase and xylonate accumulation, the plasmids of *P. putida* KT2440 pWeim2 and *P. putida* KT2440 $\Delta\Delta$ pDahms2 were isolated and sequenced. Mutations in the promoter, xylose genes, replication initiator protein, origin of replication, and antibiotic resistance of the plasmids carried by strains *P. putida* KT2440 pWeim2 and *P. putida* KT2440 $\Delta\Delta$ pDahms2 could be excluded. Hence, mutations in coding regions in the genome are expected and will be analyzed in the future.

3.1.3.4 The deletion of PP_2836 enables the exclusive usage of the Dahms pathway

To demonstrate that the isolated Weimberg or Dahms pathway are present in the engineered strains, growth experiments with 1-¹³C labeled xylose as substrate were performed. Afterwards, the labeling patterns of the proteinogenic amino acids were analyzed. Since xylose is metabolized via different routes using the Weimberg or the Dahms pathway, the labeling pattern is well distinguishable (Figure 7). Xylose is converted in several steps to 2-oxoglutarate via the Weimberg pathway, which is a direct precursor of glutamate, glutamine, and proline. This would lead to 1-¹³C labeled glutamate, glutamine and proline. In the central carbon metabolism, 2-oxoglutarate is further processed via the TCA cycle and the labeling is removed via CO₂ generation. Furthermore, in the case of the Dahms pathway, xylose is converted into pyruvate and glycolaldehyde. Since pyruvate is a precursor for the amino acids alanine, lysine, and valine, they are fractionally labeled. Pyruvate is also converted to acetyl-CoA in the central carbon metabolism, removing the labeled carbon atom by CO₂ generation.

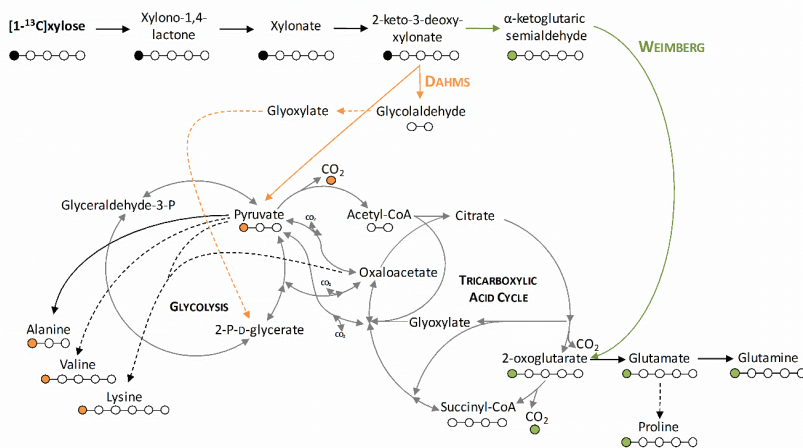


Figure 7: Depiction of the labeling of metabolites resulting from labeled xylose. Shown is the stable isotope introduction into the central carbon metabolism of *P. putida* KT2440. Orange lines indicate the Dahms pathway, green lines indicate the Weimberg pathway, and dashed lines indicate several enzymatic steps. Orange dots indicate the labeling resulting from the Dahms pathway and green dots indicate the labeling resulting from the Weimberg pathway.

For these experiments, *P. putida* KT2440 pWeim und *P. putida* KT2440ΔΔ pDahms were used. As seen before, deletion of the gene PP_2836 entails a completely different growth behavior, which indicates that the deletion of PP_2836 is necessary to establish the Dahms pathway. It is assumed that xylose is utilized via both pathways simultaneously (Weimberg

and Dahms) in a strain that still harbors PP_2836 and is deficient in *gclR* (to activate degradation of the intermediate glycolaldehyde, see Figure 4). This assumption was experimentally tested by introducing the plasmid pBT-Dahms into *P. putida* $\Delta gclR$. The resulting *P. putida* $\Delta gclR$ pDahms was also used in the labeling experiments.

Significant distinctions of the fractional labeling with labeled xylose were observed for all three strains (Figure 8). For the Weimberg-strain, alanine, lysine, and valine were almost not labeled at all while glutamate and proline showed a labeling about 0.1. This meets the expectations since 50% of $1\text{-}^{13}\text{C}$ -labeled xylose was used (one atom out of ten carbon atoms from two xylose molecules should be labeled). For the Dahms-strain, the fractional labeling of the amino acids, which are derived from pyruvate (alanine, lysine, and valine), is about 0.13, 0.06, and 0.07, respectively, and for glutamate and proline below 0.05. Theoretically, a fractional labeling of about 0.17 for alanine, 0.08 for lysine, and 0.1 for valine would be assumed because one out of six, ten, and twelve carbon atoms from two xylose molecules should be labeled, respectively. The observed labeling is a little bit lower than expected, but the distribution meets the expectation since again 50% of $1\text{-}^{13}\text{C}$ -labeled xylose was used. For more quantitative statements, metabolic flux analysis has to be performed in order to involve fluxes through the central carbon metabolism. Further, as described above the labeling is removed when CO_2 is generated. However, via anaplerotic reactions the labeled CO_2 can be reincorporated and ultimately result in labeled glutamate and proline. Nevertheless, both strains showed a distinct labeling pattern, which indicates that the Weimberg and Dahms pathways are truly used in isolation.

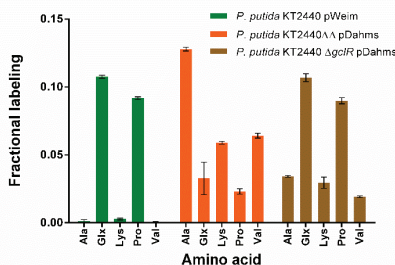


Figure 8: Fractional labeling of amino acids during growth in minimal medium containing 50% $1\text{-}^{13}\text{C}$ -xylose. Error bars indicate deviation from the mean of three technical replicates from one growth experiment ($n=3$). Ala, alanine; Glu, glutamate and deaminated glutamine; Lys, lysine; Pro, proline; Val, valine.

Moreover, for *P. putida* KT2440 $\Delta gclR$ pDahms it was determined that the fractional labeling of glutamate and proline was about 0.1, as in the case of the Weimberg-strain and

that the labeling of alanine, lysine, and valine was in between the values of the Weimberg- and the Dahms-strain. These results show that the fractional labeling is a combination of the single fractional labelings of the Weimberg and the Dahms pathway. Thus, it is suggested that the Weimberg and the Dahms pathway are active simultaneously in this strain. Consequently, the above stated declaration that PP_2836 encodes a dehydratase is verified. The deletion of this gene is necessary to obtain a strain, which solely uses the Dahms pathway.

3.1.3.5 Variations in xylose metabolism networks lead to specialized microbial cell factories

Different stoichiometries of the pathways have an influence on the production yield of specific metabolites. In order to show this dependency, two secondary metabolites (rhamnolipids and phenazines) were chosen for heterologous synthesis in efficient xylose metabolizers. Exemplarily, strains with the individual pathways (Isomerase, Weimberg, and Dahms) were chosen and the production of the initial strains and the adapted strains was investigated. For the production of rhamnolipids, the rhamnolipid synthesis module was integrated as single copy into the genome of all xylose utilizing strains. *P. putida* KT2440 EM42 Δ *gcd* pSEVA2213_*xylABE* [255] was used as optimized strain using the Isomerase pathway because no evolved *P. putida* strain using the Isomerase pathway was generated. The rhamnolipid producers were designated *P. putida* KT2440 pIso_RL, *P. putida* KT2440 pWeim_RL, *P. putida* KT2440 $\Delta\Delta$ pDahms_RL, *P. putida* KT2440 pIso2_RL, *P. putida* KT2440 pWeim2_RL, and *P. putida* KT2440 $\Delta\Delta$ pDahms2_RL.

P. putida KT2440 pIso_RL, utilizing the Isomerase pathway for xylose degradation, showed only little rhamnolipid production (47 mg L⁻¹) after 96 hours, because most of the xylose was converted to the dead-end product xylonate. While *P. putida* KT2440 pIso2_RL produced seven times more mono-rhamnolipids after 54 hours (Figure 9A), although the cells were clumped and did not reach high optical densities. On the contrary, the strains with the Weimberg and Dahms pathways reached higher rhamnolipid titers. *P. putida* KT2440 pWeim_RL produced 13 times more mono-rhamnolipids than *P. putida* KT2440 pIso_RL in less time (54 hours) and the evolved strain with the rhamnolipid production module *P. putida* KT2440 pWeim2_RL produced even more mono-rhamnolipids after 30 hours (720 mg L⁻¹). Further, *P. putida* KT2440 $\Delta\Delta$ pDahms_RL and *P. putida* KT2440 $\Delta\Delta$ pDahms2_RL produced six times more mono-rhamnolipids after 54 hours (290 mg L⁻¹) and 13 times more mono-rhamnolipids after 30 hours (620 mg L⁻¹), respectively. Besides, differences in the substrate consumption were observed. While *P. putida* KT2440 pWeim2_RL and *P. putida* KT2440 $\Delta\Delta$ pDahms_RL consumed all of the provided xylose, xylose and xylonate in the cultures of the other rhamnolipid-producing strains was detected after growth stopped (Figure A 1). Hence,

the ratio of the amount of product synthesized and the amount of substrate consumed (yield) is considered. *P. putida* KT2440 pIso2_RL had the highest yield (78 mg g⁻¹), while *P. putida* KT2440 pWeim2_RL and *P. putida* KT2440ΔΔ pDahms2_RL reached comparable yields, however, 1.4-fold lower than *P. putida* KT2440 pIso2_RL (Figure 9C). As expected, the evolved and optimized strains showed a higher rhamnolipid concentration and yield than their initial xylose utilizing strains (Figure 9A,C).

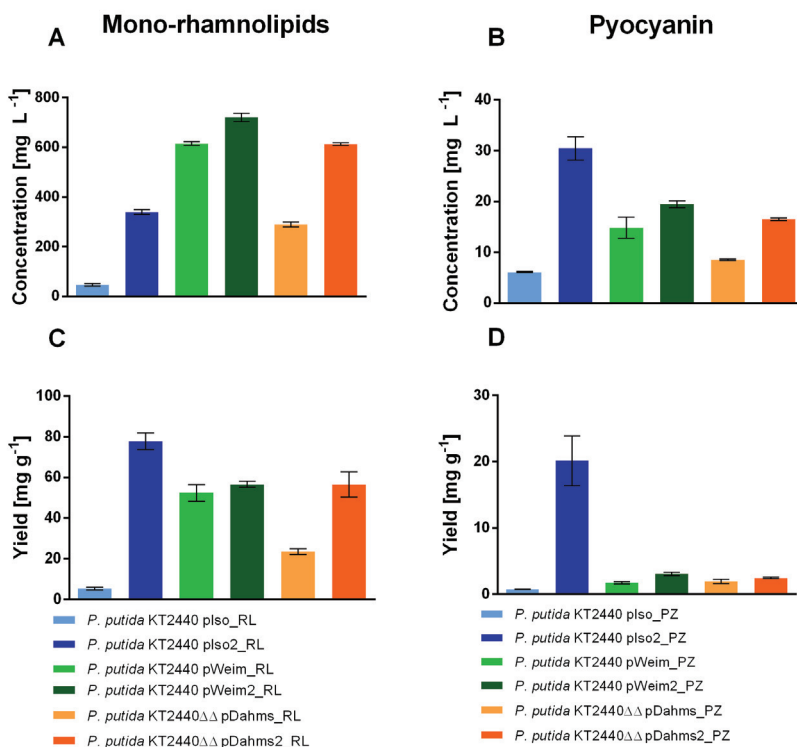


Figure 9: Mono-rhamnolipid and pyocyanin production from xylose by engineered *P. putida* strains. The minimal medium contained 10 g L⁻¹ xylose. Shown are the product titers of mono-rhamnolipids (A), the product titers of pyocyanin (B), the yield of mono-rhamnolipids on xylose (C), and the yield of pyocyanin on xylose (D). Error bars indicate deviation from the mean (n=3).

The synthesis of phenazines was achieved by transferring plasmid pJNN_ *phzA1-G1,S,M* into all initial xylose utilizing strains and the optimized or evolved strains. The pyocyanin producers were named *P. putida* KT2440 pIso_PZ, *P. putida* KT2440 pWeim_PZ, *P. putida* KT2440ΔΔ pDahms_PZ, *P. putida* KT2440 pIso2_PZ, *P. putida* KT2440 pWeim2_PZ, and

P. putida KT2440 $\Delta\Delta$ pDahms2_PZ. All strains produced pyocyanin in a similar range (approx. 15 to 20 mg L⁻¹, Figure 9B), but in a different period, except the strains using the Isomerase pathway. *P. putida* KT2440 pIso_PZ produced around three times less (6 mg L⁻¹) pyocyanin and *P. putida* KT2440 pIso2_PZ produced roughly two times more (30 mg L⁻¹) pyocyanin in comparison to the evolved strain using the Dahms pathway. Pyocyanin was harvested at the same time points as mono-rhamnolipids. Furthermore, as observed in rhamnolipid production experiments, differences in substrate consumption were recognized. *P. putida* KT2440 pIso_PZ converted even more xylose to xylonate than in the rhamnolipid production experiments, which could then not be used for pyocyanin production. Further, less xylose was consumed than in the rhamnolipid production experiment (Figure A 1). Consequently, the yield was determined and *P. putida* KT2440 pIso2_PZ showed the best yield (20 mg g⁻¹, Figure 9D). Furthermore, the evolved strains reached higher yields than their initial strains (*P. putida* KT2440 pWeim2_PZ: 3.2 mg g⁻¹, *P. putida* KT2440 $\Delta\Delta$ pDahms2_PZ: 2.5 mg g⁻¹). It was confirmed that the evolved and optimized strains could reach higher product titers and yields than their initial strains, also in the case of pyocyanin production (Figure 9B,D). This can be explained by the adaptation towards the novel substrate xylose for *P. putida* KT2440 pWeim2 and *P. putida* KT2440 $\Delta\Delta$ pDahms2. In the case of *P. putida* KT2440 pIso2 the production of xylonate is prevented by deletion of the glucose dehydrogenase in comparison to *P. putida* KT2440 pIso and consequently more carbon is available for the production of pyocyanin. Conspicuously, growth was slower for the phenazine-producing strains compared to the rhamnolipid-producing strains and consequently the total consumption of xylose was lower. This might be due to the metabolic burden through plasmid maintenance and the usage of kanamycin to avoid loss of the plasmid harboring the phenazine synthesis genes during cultivation. Further, product toxicity might be a cause for the xylose leftovers. In accordance to the computed data, *P. putida* KT2440 pIso2 harboring the rhamnolipid or phenazine genes showed in both scenarios the best yield (Figure 9).

3.1.4 Discussion

In this study, we integrated three bacterial xylose utilization pathways - Isomerase, Weimberg, and Dahms - in *P. putida* KT2440, to compare *in silico* and *in vivo* the synthesis capacities of these alternative degradation pathways. While other studies focus on heterologous production with *P. putida* KT2440, we aimed at showing the production of various products (i.e., mono-rhamnolipids and pyocyanin) from xylose. Further, we wanted to emphasize that computational analysis can guide strain design, reaching higher yields.

FBA indicates strong preferences for alternative pathways depending on the product of choice due to different stoichiometries. It was determined that mainly the Isomerase pathway is preferred, however the Weimberg and Dahms pathways are favored in niche applications, when for example intermediates of the pathways are precursor for the products. To show this dependency, the xylose pathways and synthesis pathways were integrated in *P. putida* KT2440. In the course of isolating the Dahms pathway, two genes (PP_2836 and PP_4283) were deleted. After successful pathway implementation, the synthesis capacities for mono-rhamnolipids and pyocyanin were investigated. The resulting data matched the computed data. The approach of *in silico* metabolic network design driven by the product of choice, adds another degree of freedom for metabolic engineering.

The computed maximal product yield for both synthesized metabolites, mono-rhamnolipids and pyocyanin, was produced by the Isomerase pathway. In both cases, this was confirmed *in vivo* (Figure 9). While the highest rhamnolipid titer (720 mg L⁻¹) was reached by the evolved strain using the Weimberg pathway, the highest yield (78 mg g⁻¹) was reached by the strain using the Isomerase pathway. The highest pyocyanin titer (30 mg L⁻¹) and yield (20 mg g⁻¹) were also reached by the strain using the Isomerase pathway. Taken together, it was shown that the reached yields are dependent on the metabolization route and hence the stoichiometries of the pathway, which can be determined *in silico*.

Many studies deal with the heterologous production by *P. putida* KT2440. Production of biosurfactants, such as HAAs, mono-rhamnolipids, and di-rhamnolipids [106,150,303], terpenoids (zeaxanthin and β -carotene) [179,307], amino acid-derived compounds (e.g., phenazines) [182], polyketides/non-ribosomal peptides (e.g., flaviolin, prodigiosin) [180,181,307], and *N*-methylglutamate [183] was demonstrated. These examples show how diverse the production spectrum in *P. putida* KT2440 can be. To create sustainable production processes in times of high environmental pollution, one searches for alternative substrates [308]. The concept of using xylose or other C₅ sugars for platform chemicals was already considered before [304]. For example, HAA and terpenoids are benefitting from the Isomerase pathway as acetyl-CoA is the precursor (Table 2). Prodigiosin synthesis involves the precursors pyruvate, proline, and malonyl-CoA [309], favoring a combination of the Isomerase and the Weimberg pathway. The Weimberg pathway would be beneficial for synthesis of *N*-methylglutamate. The attempt of using xylose as renewable source for the production of metabolites was already considered in several studies, where *P. taiwanensis* VLB120 was used for the synthesis of mono-rhamnolipids, phenol, and 4-hydroxybenzoate [150,173,175]. *P. taiwanensis* VLB120 utilizes xylose via the Weimberg pathway natively

[297], but engineering of the strain and enabling xylose utilization via the Isomerase pathway could enhance product yield on substrate due to the superior stoichiometry.

While the integration of the xylose pathways worked *in vivo*, we observed strong differences between the oxidative and the Isomerase pathway operations. *Pseudomonades* using the three xylose pathways natively were reported [297,298,310], indicating that these pathways are compatible with the *Pseudomonades* metabolic network. Notably, we did not find any report suggesting the presence of two of the xylose pathways. The implementation of the Isomerase pathway in *P. putida* was shown before [255,301,302,311]. In accordance to our results, only weak growth was observed in two studies [255,302], which was improved by rational or non-rational engineering. In another study, growth on xylose via the Weimberg pathway in *P. putida* was shown to be immediately efficient [294], matching our results. As already considered by Wang *et al.* [311], xylose metabolization seems to be metabolically demanding for *P. putida* KT2440 using the isomerase route and might be the reason for the discrepancies. But how can this difference between the Isomerase pathway and the oxidative pathways be explained? The consideration of other carbon sources indicates that the usage of oxidative pathways is likely to have an advantage for *Pseudomonas* species. The metabolization of the C₅ sugar arabinose proceeds in *E. coli* via the Isomerase pathway [312], whereas in other *Pseudomonas* species the utilization of arabinose happens via oxidative steps to form the intermediate pentonic acid [313,314]. In the case of galacturonic acid the utilization starts with an isomerase reaction in *E. coli* [315]. Whereas in *Pseudomonas* species the first enzyme is a dehydrogenase, which catalyzes an oxidation reaction [316,317]. There might be two explanations why *Pseudomonas* favors oxidative pathways. First, the conversion of the substrate into an intermediate acid prevents other microbes from using this substrate, which is an advantage in terms of survival. Consequently, the environment is acidified due to the resulting intermediate acid, which creates a further advantage for propagation. *P. putida* KT2440 is a soil bacterium and is able to cope with extreme conditions, such as nutrient limitation, temperature shifts, and pH changes, in contrast to enterobacteria that occur in nutrient-rich niches [199,318]. Second, the energy metabolism is more flexible, because the dehydrogenases have different redox cofactors. This can be explained using glucose as an example. In general, the redox metabolism is balanced and therefore, the rates of reduction and oxidation of the redox cofactors have to be highly similar [162]. The electrons, which are released during oxidation of glucose and gluconate, are used to reduce PQQ and FAD⁺ and are feeding directly the electron transport chain. There, PQQ is directly reoxidized by transferring the electrons to ubiquinone in the inner membrane [155,319]. The transport of glucose over the membrane costs two ATP per

molecule, while only one proton and one sodium ion are necessary for the transport of gluconate and ketogluconate, respectively. Therefore, *P. putida* KT2440 saves energy using the oxidative pathway and does not require additional cofactor regeneration systems.

However, a prolonged lag phase was observed for all three engineered strains. *P. putida* KT2440 also had the longest lag phase with 100 hours, *P. putida* KT2440 pWeim had a lag phase of 24 hours, and *P. putida* KT2440 $\Delta\Delta$ pDahms had a lag phase of 34 hours (Table 3). To identify possible bottlenecks, the plasmids were sequenced after ALE. Interestingly, no mutations in the xylose utilization genes and other encoding areas of the vectors could be detected. Different studies showed that mutations in the replication initiation protein or in the antibiotic resistance cassette could enhance growth by lowering the plasmid copy number, and subsequently reducing the need of resources for the synthesis of kanamycin resistance [210,320]. A second possible bottleneck could be the transport of xylonate from the periplasm to the cytoplasm. *P. putida* KT2440 harbors the transporter GntT (PP_3417), enabling the transport of gluconate from the periplasm into the cytoplasm [321]. Such a transporter is characterized for *E. coli*. A conceivable possibility would be, that this transporter might be active for xylonate, but due to its high affinity towards gluconate it might operate slower for xylonate. Another option could be that a slow, low-affinity transporter is used instead.

To improve the growth performance and reduce the lag phase, ALE was implemented. Interestingly, the lag phase was reduced significantly, while the growth rates did not increase and in one case even decreased during ALE. This can be explained by positive selection for a reduced lag phase and negative selection for the growth rate. The fast-adapting cells does not have stringently the highest growth rate. After reduction of the lag phase, it can be selected for increased growth rates now. While laboratory evolution is an easy method to increase the overall fitness of a population in laboratory scale, the design of the ALE approach for adaptation of the population is not trivial. Conditions such as time point of transfer, passage size, and growth phase can alter in ALE studies [322,323]. Batch cultivation and continuous (chemostat) cultivation are the mostly used ALE techniques [324–326]. Of these, regularly transferred batch cultures are more popular because effort and costs are rather low. However, this method has several limitations due to varying conditions [326]. Further, this method is slower than an automated ALE because the transfer is usually done on a daily basis. While in automated ALE processes, several parameters, including the optical density and growth rate, are monitored online. If the growth rate increases over the course of the ALE, the passage frequency can automatically be increased. Additionally, cultures probably reach the stationary phase in batch cultivations, which then results in an improved survival in stationary phase or

decreased lag phase [327]. Thus, it is not surprising that the batch cultivations for adaptation used in this study resulted in a shortage of the lag phase.

The here presented alternative pathways for xylose utilization open another degree of freedom in the design and metabolic engineering of the production strain. Dependent on the product of interest, the experimenter can compute the best network design considering three different stoichiometries for xylose use. The general applicability of this approach is outlined here, while as an outlook the detailed single strain optimization and the co-consumption of carbon sources *in silico* and *in vivo* should be worked on.

Chapter 3.2

Killing two birds with one stone – strain engineering facilitates the development of a unique rhamnolipid production process

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Contributions:

Isabel Bator performed all engineering and characterization experiments, prepared figures, and wrote the chapter. Strains and plasmids were constructed by Isabel Bator and Hans M. Böhne. Tobias Karmainski performed the fermentations with the help of Isabel Bator. Till Tiso and Lars M. Blank conceived the study, advised on all experiments, discussed data, and reviewed the chapter.

3.2 Killing two birds with one stone – strain engineering facilitates the development of a unique rhamnolipid production process

3.2.1 Abstract

High-titer biosurfactant production in aerated fermenters using hydrophilic substrates is often hampered by excessive foaming. Ethanol has been shown to efficiently destabilize foam of rhamnolipids, a popular group of biosurfactants. To exploit this feature, we used ethanol as carbon source and defoamer, without introducing novel challenges for rhamnolipid purification. In detail, we engineered the non-pathogenic *P. putida* KT2440 for heterologous rhamnolipid production from ethanol. To obtain a strain with high growth rate on ethanol as sole carbon source at elevated ethanol concentrations, ALE was performed. Genome re-sequencing allowed to allocate the phenotypic changes to emerged mutations. Several genes were affected and differentially expressed including alcohol and aldehyde dehydrogenases, potentially contributing to the increased growth rate on ethanol of 0.51 h⁻¹ after ALE. Further, mutations in genes were found, which possibly led to increased ethanol tolerance. The engineered rhamnolipid producer was used in a fed-batch fermentation with automated ethanol addition over 23 hours, which resulted in a HAA and mono-rhamnolipids concentration of about 5 g L⁻¹. The ethanol concomitantly served as carbon source and defoamer with the advantage of increased rhamnolipid and biomass production. In summary, we present a unique combination of strain and process engineering that facilitated the development of a stable fed-batch fermentation for rhamnolipid production, circumventing mechanical or chemical foam disruption.

3.2.2 Introduction

Biosurfactants are surface-active molecules with high industrial potential due to their wide range of applications. They can be used in, e.g., bioremediation, enhanced oil recovery, plant protection, cosmetics, and medicine [50,65,89,328]. In contrast to chemically produced surfactants, biosurfactants can be produced from renewable resources and have a low ecotoxicological potential [17,32]. While the number of biosurfactants described is still increasing, they usually belong to the described classes of glycolipids, phospholipids, lipopeptides, or polymeric biosurfactants [33].

Rhamnolipids were first reported in 1949 by Jarvis and Johnson and belong to the glycolipids [37]. The production of rhamnolipids is mainly a trait of the bacterial genera *Pseudomonas* and *Burkholderia* [329], where the opportunistic pathogen *P. aeruginosa* is one of the most reported species. Rhamnolipids consist of a hydrophobic moiety, one or two β -hydroxy fatty acids, and a hydrophilic moiety, one or two rhamnose molecules, which are

linked through a β -glycosidic bond to the fatty acid chains [91,93]. More than 20 different congeners are produced by *P. aeruginosa*, while the most abundant congener contains a C₁₀-C₁₀ β -hydroxy fatty acid [330]. For the synthesis, two pathways for the precursor molecules are needed, which exist in *Pseudomonas* species. The synthesis of dTDP-rhamnose is catalyzed by five enzymes, a phosphoglucomutase and four enzymes of the *rml*-operon, starting from D-glucose-6-phosphate [107]. The β -hydroxyacyl-ACP is synthesized in the fatty acid *de novo* synthesis. The genes *rhlA* and *rhlB*, which are organized in an operon, are involved in mono-rhamnolipid synthesis [103,104]. The acyltransferase RhlA links two β -hydroxyacyl-ACP molecules to the dimer HAA. Then, the rhamnosyltransferase RhlB adds a rhamnose to the HAA using dTDP-rhamnose as substrate. If one more rhamnose is added by the rhamnosyltransferase RhlC a di-rhamnolipid is produced [105]. RhlC is encoded by *rhlC*, which is located in another genomic region of *P. aeruginosa*.

The production of rhamnolipids with *P. aeruginosa* has a few drawbacks. Examples are, the advanced regulation of the rhamnolipid synthesis by quorum sensing [126], which complicates fermentation procedures. Further, the highest rhamnolipid production is achieved using plant oils as substrates [331], which increases the effort and costs in downstream processing. At last, the before mentioned pathogenicity is critical for the permission of production processes and the resulting products. Thus, rhamnolipid production with a non-pathogenic host where the production is independent from host intrinsic regulation using renewable hydrophilic substrates is much aimed for. Many studies report the heterologous production of rhamnolipids in different organisms, e.g., *E. coli*, *P. fluorescens*, and *P. putida* KT2440 [147–149]. The latter species is a well-established chassis in industrial biotechnology and is known for its versatile metabolism and high redox cofactor regeneration rate [155,160,162,186].

Apart from the general microbial fermentation challenges, biosurfactant fermentations potentially have extensive foaming and high purification costs as further challenges. Excessive foaming is especially a challenge in aerated and agitated biosurfactant cultivations using hydrophilic substrates. The foaming facilitates biomass and product loss from the liquid phase. Especially the product can be enriched over 20 times in the foam phase [139,332]. The loss of the producing microbe has a negative impact on volumetric production rate [138]. While foaming is a true challenge, several strategies to control or prevent foaming exist. Antifoaming agents (or antifoamers) can be used, with the potential drawbacks of lowering the oxygen transfer rate, increasing the cost as they are expensive, and complicate downstream processing due to emulsification [139,140]. Another alternative is the use of mechanical foam breakers, which destroy the foam by shear stress [333]. These elements have

an additional energy demand and need maintenance, thus increasing also process costs. Further alternatives exist, e.g., adsorption of cell-containing foam, bubble-free membrane reactors, and foam fractionation [139,141,142]. However, all control or prevention methods have their limits, and usually add complexity to the fermentation. Hence, the development of alternative strategies to simplify the process, e.g., by controlling foaming, is a crucial step in process design and should be addressed in an early stage. Avoiding, e.g., antifoamers, reduces elaborative downstream processing and hence costs.

In times of massive production of greenhouse gases, the usage of alternative feedstocks is highly recommended. The overall goal is to establish a circular bioeconomy in which also lignocellulosic biomass and side- and waste streams are used as energy and carbon source. Several studies deal with the production of rhamnolipids from alternative resources, e.g., butane, agro-industrial waste, glycerol, and organic acids derived from bio-oil [102,150,152,334]. In the 1990s, ethanol was already considered as an alternative carbon source for rhamnolipid production [133]. Bioethanol is an interesting substrate because it can be produced from biomass constituents like sugars or sugar polymers (e.g., starch) [10]. But also attempts to use lignocellulosic sugars for bioethanol were made in the last decades, and various cellulosic ethanol plants are in operation [21,24,335]. Further, syngas fermentations can be used to produce ethanol from greenhouse gases [336]. A syngas-based industrial-scale ethanol production facility using *Clostridium autoethanogenum* as biocatalyst was brought into operation in 2018 [26]. Hence, CO and CO₂ can directly be used as substrates for sustainable ethanol production. The produced ethanol can be used as biofuel, as platform chemical (e.g., ethylene or ethylene glycol synthesis) or as a carbon source for the production of valuable products as proposed here for the synthesis of rhamnolipids.

In this work, we report heterologous rhamnolipid production with engineered *P. putida* KT2440 from ethanol. We used ALE to improve growth on and tolerance towards ethanol and identified the basis for adaptation by genome re-sequencing. An evolved mutant was characterized regarding growth and then engineered for mono-rhamnolipid production. Due to the improved growth parameters of the strain in the presence of ethanol, we were able to develop a unique rhamnolipid production process where ethanol takes over two functions – being the carbon source and the defoaming agent.

3.2.3 Results

3.2.3.1 Evolutionary engineering improves growth of *P. putida* KT2440 on ethanol

In order to establish a rhamnolipid production process based on ethanol, the growth behavior of *P. putida* KT2440 was investigated in minimal medium with 0.96%, 2%, 4%, 6%,

and 8% (v/v) ethanol as sole carbon source. 0.96% (v/v) ethanol was included, since this equals 0.33 Cmol, which is the carbon content used in all further experiments in this study. Interestingly, the wild type was able to grow natively in medium containing up to 4% (v/v) ethanol (Figure 10B). However, cell clumping was observed, indicating cell stress. The wild type exhibits a long lag phase of about 70 hours at 4% (v/v) ethanol (Figure 10B), which is likely caused by the inhibitory effect of the high ethanol concentration. While even at 0.96% (v/v) cell clumping was observed, the lag phase was significantly reduced to six hours and a growth rate of $0.21 \pm 0.03 \text{ h}^{-1}$ was reached (Figure 10A). However, the growth rate showed a sharp drop after approximately 16 hours. In general, the results show that *P. putida* KT2440 is able to utilize ethanol, which was not surprising since an ethanol oxidation system and several alcohol and aldehyde dehydrogenases exist in *Pseudomonas* species [186,337].

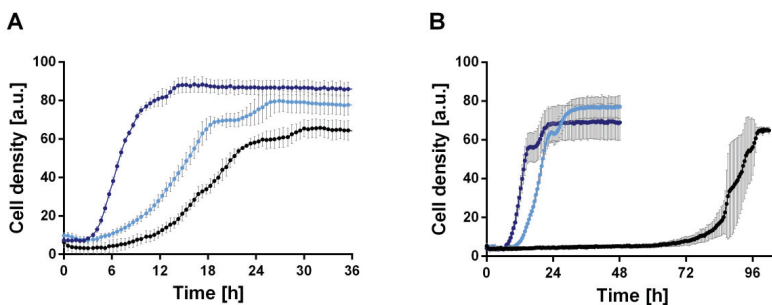


Figure 10: Comparison of growth of *P. putida* KT2440 (black) and two evolved strains, *P. putida* KT2440 E1 (light blue) and *P. putida* KT2440 E1.1 (dark blue), in M9 minimal medium containing ethanol. (A) Growth in medium containing 0.96% (v/v) ethanol and (B) growth in medium containing 4% (v/v) ethanol. Growth was detected using a Growth Profiler 960 in 96-well plates. Error bars indicate the standard deviation from the mean (n=3).

Because the cells clumped and had a moderate growth rate, we decided to improve these traits by ALE. ALE is a technique frequently used to improve features of microbes by adaptation to various environmental conditions, such as temperatures or non-preferred carbon sources. A first ALE experiment was performed using M9 minimal medium with 2 g L^{-1} glucose and 4% (v/v) ethanol to increase the solvent tolerance for 30 days corresponding to ~115 generations. Single colonies were obtained from the inhomogeneous culture, which were tested for growth (data not shown). The best-adapted colony (the strain with the highest growth rate and shortest lag phase after ALE) was designated *P. putida* KT2440 E1, which was used for a second ALE experiment in M9 minimal medium with 4% (v/v) ethanol for twelve days corresponding to ~37 generations. Again, single colony isolation, and growth rate determination was used; the best adapted strain was designated *P. putida* KT2440 E1.1. After

ALE, both strains showed reduced or no cell clumping in the presence of ethanol, which might correlate with ethanol tolerance. The growth of the two adapted strains was investigated in M9 minimal medium containing 0.96% or 4% (v/v) ethanol (Figure 10A,B). On 4% (v/v) ethanol, a significantly decreased lag phase of nine hours and six hours was observed for *P. putida* KT2440 E1 and *P. putida* KT2440 E1.1, respectively (Figure 10B). *P. putida* KT2440 E1 had a slightly reduced growth rate of $0.17 \pm 0.02 \text{ h}^{-1}$, while no drop of the growth rate when compared to the wild type in 0.96% (v/v) ethanol was observed. The growth rate of *P. putida* KT2440 E1.1 was increased to $0.51 \pm 0.03 \text{ h}^{-1}$.

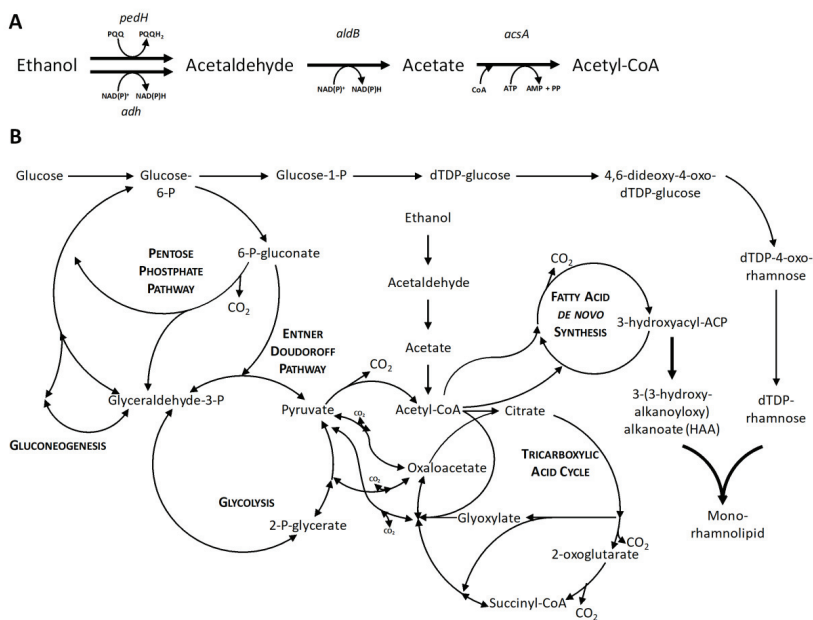


Figure 11: The metabolic network of *P. putida* KT2440. (A) Enzymatic steps of ethanol utilization. (B) Central carbon metabolism of *P. putida* KT2440 based on Sudarsan *et al.* [96] with the native ethanol utilization pathway and the heterologous mono-rhamnolipid production pathway. Bold arrows indicate the recombinant enzymatic steps towards mono-rhamnolipid production. *pedH*, quinoprotein ethanol dehydrogenase; *adh*, alcohol dehydrogenase; *aldB*, aldehyde dehydrogenase; *acsA*, acetyl-CoA synthetase; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; NAD⁺, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; PQQ, pyrroloquinoline quinone; PQQH₂, reduced pyrroloquinoline quinone.

Additionally, the formation of the intermediate acetate was examined. The intermediate acetaldehyde could not be measured due to its volatile character. The enzymatic steps and introduction in the central carbon metabolism are shown in Figure 11. Samples taken until 28 hours of the cultivation of *P. putida* KT2440 using 0.96% (v/v) ethanol could exclude the

presence of acetate. Hence, it is hypothesized that the conversion of acetaldehyde to acetate is the rate-limiting step and instead, an acetaldehyde accumulation took place, which caused growth inhibition (drop of the growth rate after 16 hours in Figure 10A) because aldehydes are highly toxic also at low levels. The evolved strains *P. putida* KT2440 E1 and E1.1 grew to higher cell densities, but they also showed higher acetate concentrations (3.1 ± 0.3 and 3.8 ± 0.1 g L⁻¹), respectively. Due to ALE, the overall concentrations of the intermediates were not growth-inhibiting anymore. As a consequence, the evolved strains were able to use ethanol and acetaldehyde more efficiently indicated by the higher biomass production. However, another imbalance was revealed after ALE, which resulted in a stronger acetate accumulation through a slow conversion of acetate to acetyl-CoA. Here, a higher growth rate on ethanol and a higher ethanol tolerance were achieved. The time period of the ALE process might be extended in order to select isolates with even more improved features, i.e., a higher growth rate and lower accumulation of intermediates. However, a short time period was chosen to minimize the appearance of random background mutations, simplifying the allocation of mutations to the observed phenotypes.

3.2.3.2 Rerouting of metabolism for carbon-efficient ethanol utilization caused by adaptation

To investigate the basis of the phenotypic changes after ALE, genome re-sequencing of both evolved strains and *P. putida* KT2440 as reference was performed. Furthermore, the metabolic network structure was examined by FBA. We could identify 60 mutations, including 36 single nucleotide polymorphisms (SNP) and 24 insertion-deletion polymorphisms (InDel) for the reference strain *P. putida* KT2440 from our strain collection compared to the genome sequence of *P. putida* KT2440 (AE015451.2; [184]). This phenomenon was observed before in other *P. putida* KT2440 genome re-sequencing studies [184,254]. Similar mutation patterns of two reference strains from different laboratories were observed, even though the reference strains share no common history [254]. Most of the mutations are located in non-coding regions, silent mutations, or errors due to low coverage and read quality. Seven additional SNPs and one InDel were identified for the evolved E1. *P. putida* KT2440 E1.1 carried three SNPs and six InDels in addition to E1. Four genomic regions, which probably have an influence on ethanol tolerance and utilization were identified (Figure 12). More specifically, all SNPs and InDels of strains E1 and E1.1 were considered except silent mutations, mutations in hypothetical proteins, mutations with low coverage and read quality, and mutations in non-coding regions (except one mutation in a probable promoter region). Three mutations were found in strain E1 and three additional mutations in strain E1.1 (Table A 1). Two physiological effects can be distinguished: enhanced ethanol tolerance and more efficient ethanol utilization (higher ethanol uptake rate).

The mutations, which might effectuate a higher ethanol tolerance, are present in both strains and were thus introduced in the first evolution round (Figure 12B,D). These include a missense mutation in *secE*, which encodes a protein translocase subunit, and a nonsense mutation in *fleQ*, which encodes a transcriptional regulator for flagellar and biofilm formation.

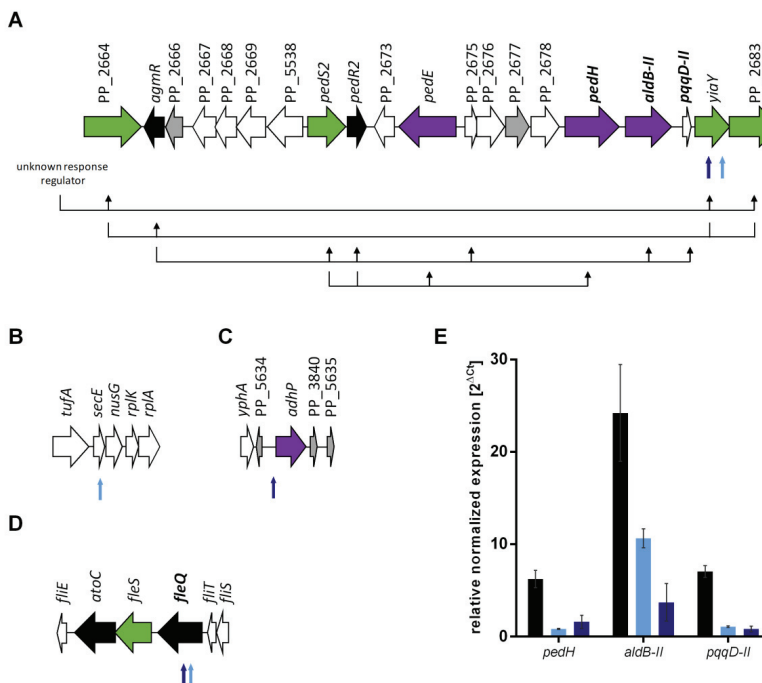


Figure 12: Genomic consequences of ALE on gene expression. The gene organization is based on the genome sequence AE015451.2 [184]. (A) Gene organization from PP_2664 to PP_2684 with a schematic hypothetical regulatory scheme below the genes based on regulation patterns in *P. aeruginosa* [338]. The black arrows display which genes have a positive regulatory influence on other genes. Further unidentified two-component systems genes are probably involved but not considered in this figure. The genes in bold font were investigated for gene expression levels. (B) Gene organization from *tufA* to *rplA* (PP_0440 to PP_0444). (C) Gene organization from *yphA* (PP_3837) to PP_5635. (D) Gene organization from *fleE* to *fliS* (PP_4370 to PP_4375). (E) Relative normalized expression of genes involved in ethanol metabolism of *P. putida* KT2440 (black bars), *P. putida* KT2440 E1 (light blue), and *P. putida* KT2440 E1.1 (dark blue) growing on 0.96% (v/v) ethanol. The $2^{\Delta CT}$ values were normalized to *rpoB* and error bars represent the maximum and minimum values of two independent measurements (n=2). Black arrows indicate genes coding for regulators, green arrows indicate genes coding for sensor kinases, purple arrows indicate genes involved in ethanol conversion, grey arrows indicate hypothetical proteins, and white arrows indicate genes with other functions. Smaller arrows below the gene organization show the identified mutations after ALE. The light blue arrows indicate the presence of the mutation in *P. putida* KT2440 E1 and the dark blue arrows indicate the presence of the mutation in *P. putida* KT2440 E1.1.

The presence of organic solvents leads to altered lipid compositions and leakage of the plasma membrane in microbes as reviewed by Heipieper *et al.* [339]. The mutation in *secE* was perhaps introduced to counterbalance this effect due to an altered translocation of

proteins across and insertion into the membrane. Furthermore, the nonsense mutation in *fleQ* likely causes the gene function to be negatively affected. In *P. putida* KT2440 E1.1, a frameshift was additionally introduced in *fleQ* to abolish the gene function. In recent studies, it was reported that *fleQ*-deficient *P. putida* strains showed a defect in swarming and reduced biofilm formation, which is caused by a lower expression of flagellar- and biofilm-related genes [340–342]. Further, a relation between the disruption of flagellar-related genes and solvent tolerance in *P. putida* was described [343,344]. To verify this hypothesis, the gene *fleQ* was deleted. This deletion mutant, the reference strain, and the evolved strain E1.1 were then cultivated in minimal medium containing glucose and increasing ethanol concentrations. *P. putida* KT2440 Δ *fleQ* and *P. putida* KT2440 E1.1 showed an identical growth behavior when concentrations from 1% to 3% (v/v) of ethanol were present (Figure 13). In contrast, the reference strain showed even in the presence of low ethanol concentrations a prolonged lag phase and decreased growth compared to the deletion mutant and the evolved strain (Figure 13). With the *fleQ* deletion we could show that i) the mutations in *fleQ* are likely to contribute to an increased ethanol tolerance and ii) the mutations indeed in all likelihood lead to a deactivation of regulation of flagellar and biofilm formation.

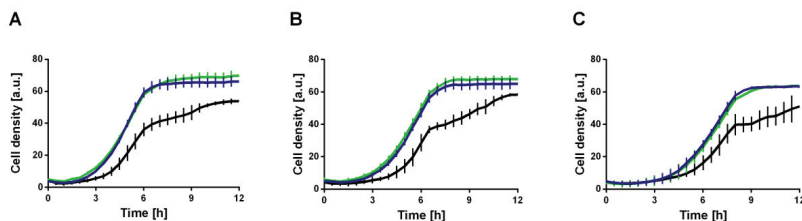


Figure 13: Ethanol tolerance of *P. putida* KT2440 and derivatives in M9 minimal medium containing different ethanol concentrations. *P. putida* KT2440 (black), *P. putida* KT2440 E1.1 (blue), and *P. putida* KT2440 Δ *fleQ* (green). (A) Minimal medium containing 10 g L⁻¹ glucose and 1% (v/v) ethanol. (B) Minimal medium containing 10 g L⁻¹ glucose and 2% (v/v) ethanol. (C) Minimal medium containing 10 g L⁻¹ glucose and 3% (v/v) ethanol. Growth was detected using a Growth Profiler 960 in 96-well plates. Error bars indicate the standard deviation from the mean (n=3).

Further mutations, which are likely assigned to ethanol utilization, were identified. Two mutations were found in a gene, of which the gene product is likely involved in the regulation of the ethanol oxidation system, which is encoded by genes localized in an operon (Figure 12A) [184,337]. A missense mutation was introduced in *yiaY* (PP_2682) and in the longer evolved strain an additional nonsense mutation was introduced upstream in the same gene (Table A 1). The gene *yiaY* is proposed to encode a Fe-containing alcohol dehydrogenase. A genome analysis using BLAST [305] showed that *yiaY* (PP_2682) and PP_2683 (a two-component system sensor histidine kinase/response regulator) of *P. putida*

KT2440 have sequence similarities of 84% to *ercA* (PA1991) and 72% to *ercS* (PA1992) of *P. aeruginosa* PAO1, respectively. The gene *ercA* forms an operon with *ercS* and the gene product is presumed to generate a signal that activates the sensor kinase ErcS. This has an impact on the regulator AgmR, which controls the transcription of genes encoding components for ethanol oxidation (Figure 12A). Consequently, the gene product of *ercA* is part of a complex regulatory system and controls the expression of the quinoprotein ethanol oxidation genes in *P. aeruginosa* [338,345]. Since the synteny of the ethanol oxidation system in *P. putida* and *P. aeruginosa* share similarities, we assumed that *yiaY* has a similar function in *P. putida* KT2440. To confirm this assumption, gene expression levels of the presumably regulated genes *pedH*, *aldB-II*, and *pqqD-II*, which are involved in ethanol oxidation (Figure 12A), in *P. putida* KT2440 and the two evolved strains E1 and E1.1 were determined via qRT-PCR. Interestingly, the transcript level of all three genes was highest for the reference strain (Figure 12E). *P. putida* KT2440 E1 had a lower transcript level of the regulated ethanol oxidation genes compared to the reference strain and the transcript levels of these genes of strain E1.1 were even lower. These findings indicate that *yiaY* is involved in the regulation of expression of ethanol oxidation genes since no other mutations were found in the surrounding kinases or regulators. Further, a low transcription of the genes involved in ethanol oxidation (*pedH*, *aldB-II*, and *pqqD-II*) could be observed in *P. putida* KT2440 E1.1, although a stop codon was introduced in *yiaY*. The stop codon was present in the second half of the gene and thus, there might be a residual activity. As mentioned before, the ethanol oxidation system is regulated by a complex regulatory system and other factors also might still regulate the transcription of the genes positively. However, it was observed that the transcript levels of the ethanol oxidation genes in the operon were more decreased the longer the strain was evolved by ALE. This fits the hypothesis, which was formulated in the beginning based on the growth of the strains. It was argued that the overall concentration of the intermediates was not growth-inhibiting anymore and that an adaptation to the intermediates happened. These changes were the basis for more efficient use of the substrate. The gradual reduction of transcript identified by qRT-PCR entailed a slower conversion of ethanol and thus, lower concentrations of the toxic intermediate acetaldehyde might be present. Accumulation of acetaldehyde was suggested as a reason for the sharp drop of the growth rate for the wild type, which was not present in both evolved strains. In strain E1, acetaldehyde accumulation might be reduced below a toxic level for the cells enabling the further conversion of acetaldehyde into acetate. However, acetate accumulation was observed, indicating a second bottleneck (slow conversion to acetyl-CoA). This could have led to the further decrease of transcript levels, which is probably caused by the missense mutation in

yiaY in strain E1.1. The mutation has a reductive effect on the expression of the quinoprotein ethanol oxidation gene, *pedH*, and *aldB-II* and possibly forces the strain to convert ethanol slower.

In *P. putida* KT2440 E1.1, the transcript levels of the genes for the ethanol oxidation enzymes, located in the operon (Figure 12E), were further decreased. However, other dehydrogenases may undertake the ethanol oxidation. One mutation in a gene, which is probably involved in ethanol degradation, occurred, causing that the encoded enzyme may undertake the conversion of ethanol to acetaldehyde. A SNP in the intergenic region before *adhP* (PP_3839), encoding an alcohol dehydrogenase, which is proposed to be located in the cytoplasm, was found (Figure 12C). The mutation upstream of *adhP* might alter the promoter activity upstream of *adhP*. Using the Softberry BPROM tool [346], different promoters were predicted for the reference strain and *P. putida* KT2440 E1.1, i.e., the -10 region of the probable promoter upstream of *adhP* is affected by the mutation (Figure A 2). Thus, the alteration caused by the mutation might have led to a significantly improved growth rate (Figure 10).

Although the mutations and resulting phenotypes, including the discovery of their underlying basis, were analyzed, the question why the quinoprotein dependent ethanol oxidation was downregulated and other dehydrogenases undertook their functions, are intriguing. To investigate this, FBA was used to predict fluxes and yields regarding biomass production using the genome-scale model *P. putida* KT2440 iJN1463 [185]. Two different scenarios, the PQQ-dependent and NAD^+ -dependent ethanol conversion, were tested. Maximal product yields of 37 mmol mol^{-1} and 41 mmol mol^{-1} were computed for the PQQ-dependent ethanol conversion and the NAD^+ -dependent ethanol conversion, respectively. To elucidate where the differences derive from, different fluxes were analyzed. In the reference strain *P. putida* KT2440 (PQQ-dependent), the electron pair is used in the respiratory chain for ATP synthesis and not for biomass production, thus a higher flux through the TCA cycle is needed to regenerate more NADH theoretically. With FBA, a higher respiration ($14.7 \text{ mmol g}_{\text{CDW}}^{-1} \text{ h}^{-1} \text{ O}_2$) and a higher flux through the TCA cycle, represented by the conversion of citrate to *cis*-aconitate ($4.87 \text{ mmol g}_{\text{CDW}}^{-1} \text{ h}^{-1}$), compared to the NAD^+ -dependent ethanol conversion was computed. This leads to a higher CO_2 production ($5.33 \text{ mmol g}_{\text{CDW}}^{-1} \text{ h}^{-1}$) using the PQQ-dependent ethanol conversion instead of $3.52 \text{ mmol g}_{\text{CDW}}^{-1} \text{ h}^{-1} \text{ CO}_2$ using the NAD^+ -dependent ethanol conversion. Thus, the changes obtained by ALE contributed to a more carbon-efficient route of ethanol degradation and led to an adaptation to the intermediates, which can explain the more efficient use of the substrate indicated by the higher biomass concentrations of the mutant strains E1 and E1.1.

3.2.3.3 Biosurfactant production is increased due to more efficient substrate utilization

The production of mono-rhamnolipids and HAAs (from now on referred to as biosurfactants) was consequently investigated with an efficient ethanol metabolizer (*P. putida* KT2440 E1.1) and compared to the wild type in 24-deep well plates (Figure 14). For the production, the biosurfactant synthesis module was integrated as a single copy into the genome of *P. putida* KT2440 E1.1. The biosurfactant producer was designated *P. putida* KT2440 E1.1_RL. For comparison, the wild type with the integrated biosurfactant synthesis module, *P. putida* KT2440 SK4, was used. The production was performed using 0.96% (v/v) ethanol or 10 g L⁻¹ glucose (both 0.33 Cmol L⁻¹) as the sole carbon source to compare the production capacities on ethanol and the conventionally used glucose. The cultivation was performed for 48 hours to ensure complete consumption of the substrates for both strains.

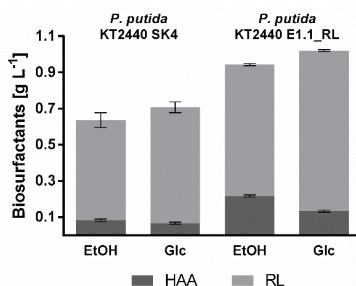


Figure 14: Proportion of different biosurfactants produced by *P. putida* KT2440 SK4 and *P. putida* KT2440 E1.1_RL in 24-deep well plates using M9 minimal medium. The minimal medium contained 0.96% (v/v) ethanol (EtOH) or 10 g L⁻¹ glucose (Glc) (both 0.33 Cmol L⁻¹). Shown are the product titers of HAAs (dark grey) and mono-rhamnolipids (RL; light grey). Error bars indicate the standard deviation from the mean (n=3).

The product to substrate yield for *P. putida* KT2440 SK4 was in a similar range for glucose and ethanol, while the product to biomass yield was significantly higher (1.6-fold) when ethanol was used (Table 8). Further, the distribution of the different congeners was similar and the proportion of HAAs was in a similar range for both substrates (13% and 9%), indicating that rhamnose provision is not significantly reduced when ethanol is used as a carbon source (Figure 14). The evolved strain E1.1 showed higher final biosurfactant titers, which is possibly caused by the higher biomass concentration. This also suggests that the evolved strain E1.1 uses the substrates more efficiently. Whereas the cause for the higher production on glucose remains unknown, in the case of ethanol, the rerouting of the metabolism is possibly the cause. As described before, the evolved strain downregulated the quinoprotein-dependent ethanol oxidation and instead likely used NAD⁺-dependent ethanol oxidation. With FBA not only for biomass production but also for rhamnolipid production, a

higher yield using NAD^+ as cofactor was predicted. The predicted maximal product yield using PQQ and NAD^+ as a cofactor is $0.63 \text{ mmol mmol}^{-1}$ and $0.71 \text{ mmol mmol}^{-1}$, respectively. Regarding the further evolved strain, E1.1 reached a similar product to substrate yield on ethanol compared to glucose but also reached a significantly higher product to biomass yield (Table 8). Further, the distribution of the different congeners was similar on both substrates, but a lower proportion of HAAs was produced on glucose (13%) compared to ethanol (23%) (Figure 14). This indicates a lack of activated rhamnose due to the conversion of ethanol to acetyl-CoA, which is then introduced in the TCA cycle or the fatty acid *de novo* synthesis (Figure 11B). To obtain dTDP-rhamnose, the strain has to undertake gluconeogenesis to synthesize glucose-6-phosphate, which is energy-intensive. Since this phenomenon was only observed for *P. putida* KT2440 E1.1_RL, a reduced relative metabolic flux in the direction of hexoses after ALE is assumed yielding in a higher share of HAAs.

Table 8: Cultivation characteristics of the recombinant biosurfactant producers. (CDW; cell dry weight, BS: biosurfactant (HAAs and mono-rhamnolipids), S: substrate)

Organism	Substrate	CDW ($\text{g}_{\text{CDW}} \text{L}^{-1}$)	Maximal titer ($\text{g}_{\text{BS}} \text{L}^{-1}$)	Product to substrate yield ($\text{Cmol}_{\text{BS}} \text{Cmol}^{-1}$)	Product to biomass yield ($\text{g}_{\text{BS}} \text{g}_{\text{CDW}}^{-1}$)
<i>P. putida</i> KT2440 SK4	Ethanol (7.6 g L^{-1})	1.11 ± 0.08	0.64 ± 0.03	0.10 ± 0.00	0.59 ± 0.07
<i>P. putida</i> KT2440 SK4	Glucose (10 g L^{-1})	1.94 ± 0.03	0.71 ± 0.03	0.11 ± 0.00	0.36 ± 0.01
<i>P. putida</i> KT2440 E1.1_RL	Ethanol (7.6 g L^{-1})	1.63 ± 0.05	0.94 ± 0.01	0.15 ± 0.00	0.58 ± 0.01
<i>P. putida</i> KT2440 E1.1_RL	Glucose (10 g L^{-1})	2.42 ± 0.05	1.02 ± 0.01	0.16 ± 0.00	0.42 ± 0.01

The product to biomass yield for each substrate is comparable for *P. putida* KT2440 SK4 and *P. putida* KT2440 E1.1_RL, with a higher product to biomass yield for ethanol. This points out that using ethanol leads to a higher production capacity per cell and that the stoichiometry of the ethanol degradation pathway is superior for biosurfactant production compared to glucose. Namely, ethanol is converted to acetyl-CoA directly being introduced in the fatty acid *de novo* synthesis without carbon loss, which is the precursor pathway for rhamnolipids (Figure 11B). Further, ethanol has a high degree of reduction, which means that more electrons are available and hence more energy is conserved in the substrate compared to glucose. This contributes to achieving a higher biomass or product yield. The theoretical product to substrate yield on ethanol is $0.93 \text{ Cmol Cmol}^{-1}$ for zero growth, of which 16% were

reached with *P. putida* KT2440 E1.1_RL. Further, the theoretical product to substrate yield on ethanol is 29% higher than on glucose.

3.2.3.4 The use of ethanol enables the development of an efficient biosurfactant production process

In order to increase the biosurfactant titer, a fed-batch fermentation procedure was developed. Biosurfactants tend to foam due to their amphiphilic structure. Thus, a fermentation process is challenging to control and often realized with additional equipment, chemicals, or process steps, which increases the costs and the effort in downstream processing. To keep the setup as simple as possible, an integrated foam recirculation with automated ethanol addition was developed.

The fermentations were conducted as biological duplicates with *P. putida* KT2440 E1.1_RL using ethanol as sole carbon source. In this setup, ethanol serves two functions. First, it is used as a carbon source for the production of biomass and biosurfactants. Second, it is used as a defoaming agent because the foam is destabilized when getting in contact with the ethanol. The collapsed foam with fresh carbon source was pumped back into the bioreactor, preventing thus loss of biomass and product while at the same time a fed-batch process is established.

The fermentation started with a batch phase (0.96% (v/v) ethanol), which ended after ethanol was consumed (9.5 hours). A biosurfactant titer of about 0.7 g L^{-1} was reached after 9 hours (Figure 15A). Further, a biomass concentration of around 3.5 g L^{-1} was reached (Figure 15A). The biosurfactant titer is 27% lower and the biomass concentration is twofold higher compared to the experiment in 24-deep well plates (Table 8). After the batch phase, the foam collection bottle was full of foam. To initiate the feed phase, 6 mL ethanol were added manually to collapse the foam. Subsequently, 3 mL ethanol were introduced automatically via the spraying nozzle by using a DO-based feeding script. The feed pulse was triggered when the DO signal increased to 40%. To account for the built-in inertia of the control loop, which is schematically demonstrated in Figure 16A, a break of five minutes was introduced after the feed trigger. During this pause, the DO signal dropped below 30%, causing the script to cease feeding. Once the DO signal reached again 40%, a new feed cycle was initialized, which can be seen in the oscillating DO signal (Figure 16B). In total, around 90 mL ethanol were fed. At the end of the fermentation a biosurfactant titer of about 5.3 g L^{-1} and biomass concentrations of about 22 g L^{-1} were reached. A growth rate of $0.14 \pm 0.00 \text{ h}^{-1}$ was reached in the feed phase, which marks a reduction by 60% compared to the batch phase. This was likely caused by carbon limitation due to the linear feeding profile (Figure 16B). With this setup, a product to substrate yield of $0.065 \pm 0.006 \text{ g}_{\text{BS}} \text{ g}_{\text{EtOH}}^{-1}$ was reached. The yield is 41% reduced

compared to the production experiment in 24-deep well plates, which can be explained by the high biomass production, which benefits from the high oxygen and nitrogen supply. Further, a space time yield of $0.23 \pm 0.01 \text{ g}_{\text{BS}} \text{ L}^{-1} \text{ h}^{-1}$ was achieved over the whole fermentation.

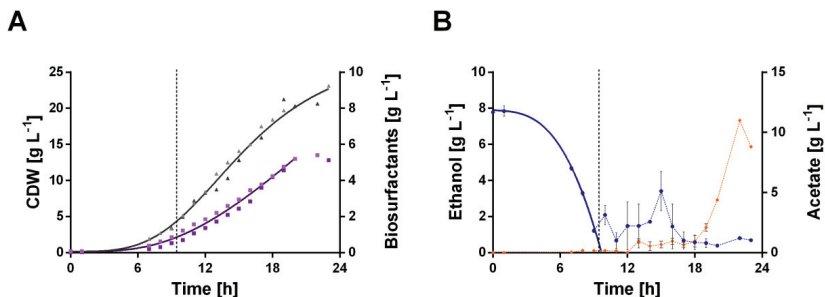


Figure 15: DO-triggered fed-batch fermentation for biosurfactant production with *P. putida* KT2440 E1.1_RL using ethanol as sole carbon source. The fermentation was performed as a duplicate. (A) CDW (▲, grey) and biosurfactant concentration (■, purple) and their respective fitted courses. The values are given as single representative values of two bioreactor cultivations. (B) Ethanol concentration (●, blue) and acetate concentration (◆, orange) and the respective fitted course for ethanol in the batch phase. The dashed lines indicate the switch from batch to the fed-batch phase. The values are given as mean values of two bioreactor cultivations and error bars represent the maximum and minimum values of two independent experiments. The data associated to this figure were taken from Tobias Karmainski's Master Thesis [347].

Again, acetate accumulation occurred after 18 hours of fermentation. At the end of the fermentation an acetate concentration of about 10 g L^{-1} was observed. This entails two issues: On the one hand, the carbon source was thus not completely available for biosurfactant production, which adulterates the yield. Since the accumulated acetate can theoretically be converted to biosurfactants, the actual yield is $0.072 \pm 0.007 \text{ g}_{\text{BS}} \text{ g}_{\text{EtOH}}^{-1}$. On the other hand, high acetate concentrations might cause growth inhibition. Contrary to the flask experiments of *P. putida* KT2440 E1.1, where approximately 3.8 g L^{-1} acetate accumulated, here no acetate was present after the batch phase. This might be caused by the introduction of additional metabolic demand for β -hydroxy fatty acids and thus acetyl-CoA (via biosurfactant production) and hence the increased conversion of acetate to acetyl-CoA.

This fermentation setup demonstrates the potential of using ethanol concomitantly as an efficient defoamer and carbon source. By automated ethanol addition, the supply of carbon is assured, resulting in an increase of biomass and biosurfactants throughout the whole fermentation. Additionally, no further equipment, such as mechanical foam breakers or antifoam, were necessary to control the foam.

Here, we show that the combination of strain and process design in an early stage resulted in the development of a novel fermentation setup. Strain engineering facilitated the use of ethanol, which subsequently could be used as foam-controlling agent and carbon source.

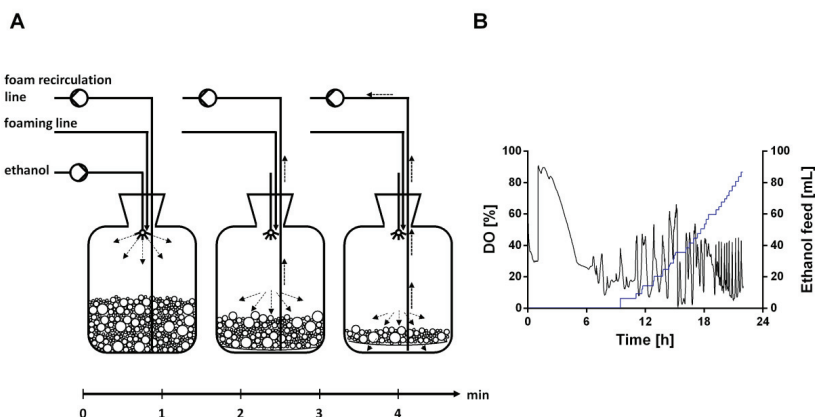


Figure 16: Ethanol feeding procedure. (A) Schematic overview of the ethanol feed in the foam collection bottle based on a DO-based feeding protocol. (B) DO signal (black), which is controlled by a DO-based feeding protocol, and ethanol feed volume (blue), exemplarily shown for one reactor. The data associated to this figure (B) were taken from Tobias Karmainski's Master Thesis [347].

3.2.3.5 Discussion

Taken together, the results show that a fed-batch rhamnolipid fermentation process can be developed without using conventional antifoam or mechanical foam breakers. To achieve this, we evolved *P. putida* KT2440 to an efficient ethanol metabolizer. The evolved strain was then characterized regarding growth and heterologous rhamnolipid production and was used to develop a unique rhamnolipid production process.

Considering the metabolic network configuration, ethanol is an ideal substrate for rhamnolipid production because it is converted to acetyl-CoA without carbon loss and feeds directly in the fatty acid *de novo* synthesis or the TCA cycle. Thereby, the formation of by-products derived from pyruvate can be circumvented and the yield may be increased. In addition, ethanol degradation delivers energy in form of NADH. This characteristic was already exploited for mevalonate production in *P. putida* KT2440 and rhamnolipid production in *P. aeruginosa* [133,348,349]. Moreover, bioethanol can be produced from alternative non-food substrates, e.g., lignocellulosic biomass [10,335]. Thus, the use of ethanol as a carbon source contributes to establishing a circular bioeconomy while also creating an advantage in terms of developing a long-lasting fermentation process. Ethanol has a defoaming property, which was described in a rhamnolipid production process using *P. aeruginosa* [350]. While the ethanol has no defoaming or antifoaming effect in the fermentation broth, foam in a foam collection bottle collapses if ethanol is sprayed on it. Therefore, a setup with foam collection

and recirculation was chosen (Figure 3). Sha *et al.* [350] used a similar setup but used colza oil as substrate and manually added ethanol to collapse the collected foam. While the addition of ethanol before 48 hours decreased the rhamnolipid and biomass production in their case, in our study, ethanol concomitantly served as carbon source and defoamer. The presence of ethanol to maintain growth and rhamnolipid production and collapsing of foam was ensured by a DO-based feeding script and hence occurred automatically. This setup theoretically allows the continuation of the fermentation beyond the batch phase for several days. Further, this study demonstrates the potential of combining strain and process development already in the early stages of process development. This integrated strain and process engineering approach was strongly emphasized by Kuhn *et al.* [351], while still many report consecutive optimization. Engineering a biosurfactant producer that is able to tolerate high ethanol concentrations and utilize ethanol as carbon source unlocked the usage of the carbon source also as defoaming agent. The general strategy that the carbon source acts as antifoam is prominent in conventional rhamnolipid production using plant oils as carbon source. However, ethanol features the striking advantage of being water-soluble, which significantly reduces subsequent downstream processing efforts.

Compared to previous reports about recombinant rhamnolipid production with *P. putida* KT2440, the here obtained results using *P. putida* KT2440 as host for heterologous rhamnolipid production are comparable or even superior. In this fed-batch fermentation, a product to substrate yield of $0.072 \text{ g}_{\text{BS}} \text{ g}_{\text{EtOH}}^{-1}$ and a space time yield of $0.23 \text{ g}_{\text{BS}} \text{ L}^{-1} \text{ h}^{-1}$ was reached. In a study with an integrated rhamnolipid adsorption system using glucose as carbon source, a yield of $0.05 \text{ g}_{\text{RL}} \text{ g}^{-1}$ and a space time yield of $0.073 \text{ g}_{\text{RL}} \text{ L}^{-1} \text{ h}^{-1}$ were reached [352]. Beuker *et al.* [353] reported a titer of 14.7 g L^{-1} , which corresponds to a yield of $0.09 \text{ g}_{\text{RL}} \text{ g}^{-1}$ and a space time yield of $0.2 \text{ g}_{\text{RL}} \text{ L h}^{-1}$ in a fed-batch fermentation using glucose and antifoam to control the foaming. With the native rhamnolipid producer *P. aeruginosa* a titer of $36.7 \text{ g}_{\text{RL}} \text{ L}^{-1}$ was achieved with a yield of $0.15 \text{ g}_{\text{RL}} \text{ g}^{-1}$ in a fermentation with foam separation using sunflower oil [29]. While in general, hydrophobic substrates are known to generate the best product yields [354], also other carbon sources were already investigated. A fed-batch cultivation of *P. aeruginosa* using a medium supplemented with yeast extract and ethanol resulted in $32 \text{ g}_{\text{RL}} \text{ L}^{-1}$ rhamnolipids, which corresponds to a yield of $0.58 \text{ g}_{\text{RL}} \text{ g}^{-1}$ [133]. Hence, further improvements are needed to reach yields and titers comparable to those of *P. aeruginosa* with the here described setup.

Improvements envisaged are: First, a higher proportion of HAAs was produced by the evolved *P. putida* KT2440 E1.1_RL strain on ethanol (Figure 14), which indicates a bottleneck in the availability of the precursor dTDP-rhamnose. This might be diminished by

the addition of hexoses as glucose, since glucose is directly used in the rhamnose pathway to yield dTDP-rhamnose [107]. Metabolic pathways for utilization of various substrates have natural stoichiometries leading to biosynthetic imbalance and suboptimal product yields [25]. Thus, using a mixture of substrates with introduction in several pathways may meet the requirements for optimal biosynthesis. However, some substrates are preferred and thus preventing the co-metabolization of less preferred substrates. This challenge was addressed by Park *et al.* with a synergistic substrate cofeeding approach, where a controlled limited feeding of glucose as dopant enabled a co-metabolization of acetate and glucose and enhanced product synthesis. Especially the use of least preferred carbon sources, such as CO₂ or acetate, can be facilitated with such an approach contributing to a sustainable bioeconomy [25]. Second, acetate accumulation in the fed-batch fermentation after 18 hours was observed (Figure 15B). A concentration of over 10 g L⁻¹ acetate likely caused growth inhibition. Recently, Arnold *et al.* [152] tested 10 g L⁻¹ acetate as the sole carbon source for rhamnolipid production with a recombinant *P. putida* KT2440 and also identified a growth inhibition. Further, an acetate accumulation during growth on ethanol was reported for *P. putida* KT2440 [348]. The authors hypothesized that the oxidation of ethanol to acetate is rapid and that the rate of acetate conversion to acetyl-CoA is limited and thus acetate accumulates. Yang *et al.* [348] produced mevalonate using ethanol and observed acetate accumulation for a strain carrying the empty plasmid, while no acetate was present when the strain produced mevalonate. This corresponds with our results from batch cultivations, promoting our hypothesis that there is an additional metabolic demand for acetyl-CoA when thereof derived products are synthesized.

In summary, we report rhamnolipid production with a recombinant *P. putida* KT2440 derivative using ethanol as an alternative carbon source and defoamer, increasing substantially the possibilities in the fermentation set-up.

Chapter 3.3

Genetic engineering of *P. putida* KT2440 for increased rhamnolipid production

Some of the deletion mutants and rhamnolipid producers were published in:

Tiso, T., Ihling, N., Kubicki, S., Biselli, A., Schonhoff, A., Bator, I., Thies, S., Karmainski, T., Kruth, S., Willenbrink, A.-L., Loeschke, A., Zapp, P., Jupke, A., Jaeger, K.-E., Büchs, J., and Blank, L. M. (2020) Integration of genetic and process engineering for optimized rhamnolipid production using *Pseudomonas putida*. *Frontiers in Bioengineering and Biotechnology*, 8, 1-24, <https://doi.org/10.3389/fbioe.2020.00976>

Blesken, C. C., Bator, I., Eberlein, C., Heipieper, H. J., Tiso, T., Blank, L. M. (2020) Genetic cell-surface modification for optimized foam fractionation. *Frontiers in Bioengineering and Biotechnology*, 8, 1-16, <https://doi.org/10.3389/fbioe.2020.572892>

Contributions:

Isabel Bator performed characterization experiments, prepared figures, and wrote this chapter. Strains and plasmids were constructed by Isabel Bator, Marie Dielentheis-Frenken, and Elena Recker. Cultivation of deletion strains was performed by Nina E. Sonntag with the help of Isabel Bator and coordination by Markus J. Müller and Nina Ihling. Overexpression experiments were performed by Marie Dielentheis-Frenken and Elena Recker. Till Tiso and Lars M. Blank conceived the study, advised on all experiments, discussed data, and reviewed this chapter.

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3.3 Genetic engineering of *P. putida* KT2440 for increased rhamnolipid production

3.3.1 Abstract

P. putida KT2440 gains more and more attention in industrial biotechnology as host for synthesis of secondary metabolites. Although the strain is known to produce only a few by-products, several regions on the chromosome encode proteins, whose metabolic products are metabolically demanding. In total, 17 dispensable regions containing 141 genes were determined, which could potentially have a negative impact on the strain's performance and rhamnolipid production by wasting resources. These regions were deleted with a homologous recombination-based method. After deletion, the strains were characterized in terms of growth performance, emerging phenotypes, and synthesis of mono-rhamnolipids as an example for production of heterologous secondary metabolites. While the growth of the mutants was not considerably impaired, they showed a decreased swimming ability and for some of the mutants an altered biofilm formation was observed. However, several mutants produced more rhamnolipids than the reference strain without deletions and the titer could be increased by about 70%, reaching a titer of 0.99 g L^{-1} . As accumulation of multiple beneficial deletions in one strain did not lead to a further increase, overexpression of genes encoding enzymes involved in precursor supply was performed. Using the combination of metabolic engineering and overexpression led to an increase of 94% in mono-rhamnolipid production, demonstrating the potential of genome engineering for improved product synthesis.

3.3.2 Introduction

Rhamnolipids are anionic glycolipid biosurfactants, which are classified as mono- or di-rhamnolipid depending on the number of rhamnose molecules. Around 60 different congeners are known and rhamnolipids are always produced as a mixture [92,330]. The opportunistic human pathogen *P. aeruginosa* is the most reported species for the microbial production of rhamnolipids. However, there are some disadvantages using this host for production. The regulation of the rhamnolipid synthesis genes is complex in *P. aeruginosa* [355]. Moreover, the highest titers are reached using hydrophobic substrates [331,354]. Further, the pathogenicity makes safe industrial production and the permission of the products difficult. All conditions increase the complexity, effort, and costs of the process.

The properties of biosurfactants and their more sustainable production make them promising alternatives to petrochemical surfactants. Several patents for the production and application of rhamnolipids exist [69], however their industrial-scale production is still in its infancy. Evonik is the first company producing rhamnolipids on industrial scale. Together

with Unilever, Evonik recently commercialized a household cleaning product [72]. It is expected that more industrial-scale plants will be commissioned in the future due to the high demand for “green” solutions.

The non-pathogenic *P. putida* KT2440 is a promising organism for the production of rhamnolipids in large scale. It is a robust host with a versatile metabolism, simple nutritional requirements, and high redox cofactor regeneration rates [155,160,162,186]. Further, *P. putida* KT2440 is only slightly growth inhibited at high rhamnolipid concentrations of up to 90 g L⁻¹ [147]. The rhamnolipid production by heterologous expression of the *rhlAB* operon from *P. aeruginosa* from renewable substrates is already established in this host [152,264,303]. In addition, high-titer rhamnolipid production using glucose was successfully implemented [353]. However, realization of an economically viable industrial production requires an efficient host that allows synthesis with high titers, yields, and rates.

P. putida KT2440 with integrated rhamnolipid genes was reported to produce 14% of the theoretical yield in minimal medium containing glucose [249]. Hence, there is still a lot of potential to improve rhamnolipid production. Since the beginning of the synthetic biology era, new gene arrangements can be constructed and existing gene arrangements can be altered. Many genetic tools for the engineering of *Pseudomonas* exist to build a chassis à la carte with improved features [164,225]. The genome of *P. putida* KT2440 is sequenced and well-annotated [184,185]. Moreover, a genome-scale model exists [185], which allows the understanding of the metabolic capabilities and genotype-phenotype relationships. This allows the establishment of a specific metabolic network in which the synthesis of valuable products from alternative substrates can be improved. By application of synthetic biology tools, a metabolic engineering strategy can be followed, where fluxes of pathways are altered in order to optimize production of a certain molecule and decrease by-product formation [356].

Strategies, such as improving heterologous gene expression by genome reduction or use of strong constitutive promoters for the expression of heterologous genes were already successfully applied in *Pseudomonas* [175,219,357]. Further, the overexpression of genes for increased product synthesis is a promising strategy, which was shown for phenylalanine production in pseudomonads [174]. These strategies could be applied to increase rhamnolipid titers and yields to be able to be competitive.

In this study, we deployed a combination of metabolic engineering and overexpression to engineer *Pseudomonas* strains for efficient rhamnolipid production. First, we deleted regions coding for metabolic costly cell compartments and molecules and evaluated the resulting phenotypes and the impact on rhamnolipid production. Second, we overexpressed genes

involved in precursor supply and deleted genes possibly altering the metabolic flux, whereby titers and yields are increased.

3.3.3 Results

3.3.3.1 Identification of metabolic demanding cellular components and molecules and their impact on the cell

Pseudomonads harbor many genomic regions/operons encoding proteins for, e.g., motility, biofilm formation, and by-product formation. These regions provide an additional metabolic burden for the cells. Hence, we looked for regions that are not necessary for bioprocesses or even negatively impact the effectivity in order to delete them. In total, 17 dispensable and metabolic costly regions were identified *in silico* and removed individually from the genome of *P. putida* KT2440 using an I-SceI-based knockout strategy [225]. The chosen regions are listed in Table 9. To determine the magnitude of the savings due to the removal, data from literature of bacteria, in particular *Pseudomonas*, was thoroughly researched.

Table 9: Genomic deletions carried out. The genetic segments are listed with a description, their genomic location according to AE015451.2 [184], their size, and the proportion of the total genome (EPS: exopolysaccharide, Lap: large adhesive protein). ^a The designations of the knockouts were defined based on the gene names or function. ^b The deletion of Δ flag is performed by sequential deletion of two genomic regions with a combined proportion of 1.01%.

Name ^a	Description	Genomic location	Size (bp)	Proportion of total genome (%)
Δ pvd	pyoverdines	4,768,854 - 4,793,839	24,986	0.40
Δ flag ^b	flagellar machinery - motion	4,917,602 - 4,936,589	18,988	1.01
		4,944,794 - 4,988,330	43,537	
Δ alg	alginate - biofilm	1,458,140 - 1,474,220	16,081	0.26
Δ bes	cellulose - biofilm	3,012,765 - 3,022,981	10,217	0.17
Δ pea	EPS A - biofilm	3,547,056 - 3,559,123	12,068	0.20
Δ peb	EPS B - biofilm	2,004,660 - 2,016,392	11,733	0.19
Δ lapA	LapA - biofilm associated	194,495 - 220,543	26,049	0.42
Δ lapF	LapF - biofilm associated	926,689 - 945,621	18,933	0.31
Δ pha	polyhydroxyalkanoates	5,699,523 - 5,705,802	6,280	0.10
Δ pili1	pili - motion	2,124,630 - 2,133,604	8,975	0.15
Δ pili2	pili - motion	2,690,661 - 2,697,071	6,411	0.10
Δ pili3	pili - motion	5,680,665 - 5,690,342	9,678	0.16
Δ pili4	pili - motion	5,801,595 - 5,805,208	3,614	0.06
Δ pili5	pili - motion	715,011 - 717,278	2,268	0.04
Δ curli	curli - motion	3,846,911 - 3,852,454	5,544	0.09
Δ pili6	pili - motion	2,259,413 - 2,262,148	2,736	0.04
Δ pili7	pili - motion	5,816,943 - 5,817,953	1,011	0.02

Pyoverdines are one of the few by-products produced by *P. putida*, which are detected after cultivation in standard M9 minimal medium [358]. Several pseudomonads produce pyoverdines in a different range. Concentrations of up to 457 mg L⁻¹ were reported for wild type *P. aeruginosa* [359]. Moreover, *Pseudomonas* strains are able to produce several exopolysaccharides (EPS), including alginate, cellulose, *putida* EPS a (Pea), and *putida* EPS b (Peb). Alginate production in bacteria, including *P. fluorescens*, was reported from different carbon sources reaching titers of 3 to 5 g L⁻¹ [360–362]. Cellulose concentrations of 1 g L⁻¹ and around 1.5 g L⁻¹ were reported for *Gluconacetobacter hansenii* and *P. aeruginosa*, respectively [363,364]. For Pea and Peb, no concentrations were found in the literature. As they are classified as EPS, we assume the production to be in a similar range as the other EPS. Especially in case of cellulose, they share the same precursor (glucose-1-phosphate) with rhamnolipids. Deleting cellulose synthesis may thus free up the precursor for rhamnolipid production. Another by-product sharing another precursor, i.e., the fatty acids derived from *de novo* synthesis, can be naturally produced by *P. putida*. This by-product is the storage polymer polyhydroxyalkanoate (PHA). *P. putida* KT2440 can produce 20 to 25 wt% PHA from different carbon sources, which amounts to up to 1 g L⁻¹ PHA [365,366]. However, much higher PHA contents were reported achievable through process optimization.

The EPS are not only by-products but also play a role in biofilm formation. Since this feature is needless in shaken laboratory cultivations, further genes contributing to biofilm formation were researched. Two proteins called large adhesive protein A and F (LapA and LapF), play a key role in biofilm formation and are the two largest proteins *P. putida* produces (888.2 and 615.7 kDa, respectively) [367]. LapA and LapF synthesis in the wild type has been demonstrated via SDS-polyacrylamide gels [367], but no quantified data (g L⁻¹ or %) are available. Since these proteins are quite large, one can imagine the metabolic burden caused by the synthesis of these two proteins.

Furthermore, *P. putida* harbors many genes coding for cell structures involved in motility. Pseudomonads are polar-flagellated and the flagellar machinery, their synthesis, and operation are encoded by a large number of genes (over 60) [226]. In the middle of the region, there is an area with flagellar-unrelated genes (PP_4345 – PP_4350), which was therefore left untouched during the deletion to prevent side effects and to be able to assign the potentially resulting phenotype to the removal of the flagellar genes. Hence, the flagellar deletion was performed by two sequential deletions inspired by Wynands *et al.* [227]. In *P. putida*, the ATP/ADP ratio is 30% higher and the NADPH/NADP⁺ ratio is 20% higher in non-flagellated *P. putida* cells [226]. Moreover, *P. putida* harbors segments for twitching motility and attachment to surfaces [368]. In total, nine regions were identified related to fimbriae/pili

formation, including type I and type IV pili and curli formation. These structures are smaller than the flagellum [369]. Type IV pili are assembled, extruded, and in complex type IV pili systems also disassembled by the action of ATPases [370,371]. Although it was shown that ATP is consumed, the impact of pili formation on the metabolism is not investigated in detail. Nevertheless, saving ATP due to the prevention of pili formation could increase the energy charge and hence positively influence the strain's physiological traits.

Taken together, for the synthesis of by-products and formation of large proteins or surface organelles a considerable amount of resources and energy is used. Thus, it is hypothesized that removing the genomic regions responsible for the synthesis and formation of the respective structures can free resources, which can be used for heterologous rhamnolipid production.

3.3.3.2 The removal of dispensable cell components and molecules leads to altered strain characteristics

After removing the 17 genomic regions, the influence on the growth performance and emergence of new phenotypes was determined. Therefore, all deletion mutants compared to the wild type were grown in M9 minimal medium containing 10 g L⁻¹ glucose in a μ RAMOS device. Against all expectations, as several studies demonstrated that reducing the metabolic load in *Pseudomonas* leads to better growth [227,357], the growth performance, i.e., the growth rate, was not improved for any of the deletion mutants. The growth rates are all in a similar range (0.49 – 0.58 h⁻¹) except for *P. putida* KT2440 Δ pili2, which is only 0.26 h⁻¹ (Table 10). The deleted fim2-region encodes type I pili. Other regions, such as fim1, also encode type I pili. Hence, it is not a general effect of the deletion of type I pili-encoding regions and probably a consequence of the deletion of this specific region. However, the reason for the decreased growth rate remains unknown. It could be speculated that during the knockout-procedure other mutations were introduced, leading to the observed phenotype. The similar growth of most of the mutants might be due to the relatively small reduction of the genome in each case (< 1%), which does not have a significant influence on the cell metabolism. Possibly, only multiple deletions would have an influence on the growth behavior due to altered fluxes.

This is likely also applicable to biomass production. No significant differences were observed for most of the mutants compared to the wild type (Table 10). *P. putida* KT2440 Δ flag was the only mutant, which showed a significantly increased biomass production (2.64 g L⁻¹). It was shown before that flagellum-deficient cells have an increased level of NADPH [226], which probably contributes to increased biomass formation as NADPH provides the reducing power needed for biosynthesis of all major cell components. Formate

addition to the medium of *P. putida* led to increased NADH formation, which also resulted in a higher biomass yield on glucose [372], supporting this assumption. Further, the biomass concentration was decreased twofold for *P. putida* KT2440 Δ pha (0.93 g L⁻¹). This probably indicates that all other mutants produced PHAs under the present conditions, reflected by the higher biomass concentration.

Table 10: Growth characteristics of *P. putida* KT2440 and the resulting deletion strains. The values are given as mean values with the standard deviation from the mean (n=4). The raw data (for determination of the growth rate) were produced by Nina E. Sonntag. (CDW: Cell dry weight)

Strain	Growth rate (h ⁻¹)	Biomass concentration (g _{cdw} L ⁻¹)
KT2440	0.56 ± 0.03	2.03 ± 0.11
KT2440 Δ pvd	0.53 ± 0.03	1.41 ± 0.05
KT2440 Δ flag	0.56 ± 0.03	2.64 ± 0.05
KT2440 Δ alg	0.58 ± 0.04	1.88 ± 0.19
KT2440 Δ bcs	0.54 ± 0.03	1.86 ± 0.10
KT2440 Δ pea	0.52 ± 0.03	1.91 ± 0.03
KT2440 Δ peb	0.55 ± 0.02	2.00 ± 0.05
KT2440 Δ lapA	0.55 ± 0.03	1.78 ± 0.07
KT2440 Δ lapF	0.55 ± 0.05	2.20 ± 0.13
KT2440 Δ pha	0.56 ± 0.04	0.93 ± 0.10
KT2440 Δ pili1	0.54 ± 0.02	1.88 ± 0.13
KT2440 Δ pili2	0.26 ± 0.04	1.57 ± 0.03
KT2440 Δ pili3	0.49 ± 0.03	1.72 ± 0.09
KT2440 Δ pili4	0.49 ± 0.03	1.61 ± 0.12
KT2440 Δ pili5	0.52 ± 0.05	1.94 ± 0.07
KT2440 Δ curl1	0.49 ± 0.03	2.00 ± 0.07
KT2440 Δ pili6	0.50 ± 0.03	1.33 ± 0.13
KT2440 Δ pili7	0.50 ± 0.04	2.05 ± 0.09

In addition to the growth performance, the swimming ability was investigated. All 17 deletion mutants and the wild type were grown in a preculture and then spotted in the middle of a swimming plate containing 0.3% agar. After growth for 48 hours, a picture of each plate was taken (Figure 17). While the wild type occupied almost the entire plate area through swimming, all mutants showed a reduced ability to swim and seven mutants completely lost their swimming ability. It was expected that the strain deficient in flagellar synthesis is not able to swim. Interestingly, half of the mutants deficient in pili/fimbriae formation (Δ pili2, Δ pili4, Δ pili5, Δ curl1), which are responsible for twitching motility, are also not able to swim anymore. As 0.3% agar was used in this experiment, the medium possibly was not viscous

enough for twitching. Twitching motility proceeds on surfaces of moderate viscosity, which is obtained by using 1% agar [368].

Further, the deletion of genes coding for EPS and LapF reduced the swimming ability. These components are usually involved in biofilm formation and contributed to the swimming ability here. It is reported that bacterial motility contributes to biofilm formation [373]. However, the phenomenon was observed vice versa and biofilm formation contributed to the swimming ability, which was decreased by deletion of these biofilm-forming components. Moreover, the swimming ability is decreased due to the lack of siderophores (Δ pvd), which was also observed in other bacteria [374]. Hence, the available iron in the cells seems to be crucial for the initiation of swimming. Overall, the swimming ability seems to be affected by more factors than only the structures for active movement (flagellum, pili).

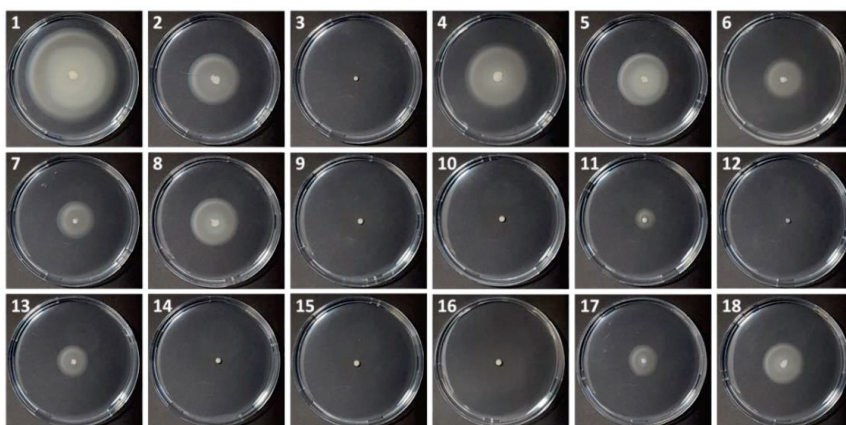


Figure 17: Swimming ability of *P. putida* KT2440 and 17 deletion mutants on M9 minimal medium plates containing 10 g L⁻¹ glucose and 0.3% (w/v) agar. 1: KT2440, 2: KT2440 Δ pvd, 3: KT2440 Δ flag, 4: KT2440 Δ alg, 5: KT2440 Δ bcs, 6: KT2440 Δ pea, 7: KT2440 Δ peb, 8: KT2440 Δ lapA, 9: KT2440 Δ lapF, 10: KT2440 Δ pha, 11: KT2440 Δ pili1, 12: KT2440 Δ pili2, 13: KT2440 Δ pili3, 14: KT2440 Δ pili4, 15: KT2440 Δ pili5, 16: KT2440 Δ curli, 17: KT2440 Δ pili6, 18: KT2440 Δ pili7. Pictures were taken after 48 h incubation at 30°C. One swimming plate of two biological replicates is exemplarily shown.

Furthermore, biofilm formation was investigated since several deletions are related to biofilm formation. Further, bacterial motility plays an important role in biofilm formation [375,376], especially in an early stage, and hence the impact on biofilm formation of the phenotypes observed before should be determined. For this, the wild type and all 17 deletion mutants were grown in a preculture and inoculated in fresh M9 minimal medium, which was transferred in a polyvinyl chloride well plate and incubated at 30°C. After growth for 13 hours, a picture of the well plate was taken and the biofilm was quantified (Figure 18A,B).

the main structural components of biofilms and decreased biofilm formation was observed in other mutants, which are impaired in EPS production in another study [380].

Thus, the deletion of individual regions involved in biofilm formation or motility only led to a few new phenotypes. This is likely due to the deletion of one respective region alone in the genome of *P. putida* and that the remaining biofilm-related regions and components could maintain the attachment to surfaces and the formation of a biofilm on the level of the wild type.

3.3.3.3 Rhamnolipid production is increased through minor genomic modifications

After discovering the effects on the growth performance, the impact of the deletions on rhamnolipid production was investigated. Therefore, the rhamnolipid cassette was introduced in the wild type and deletion mutants, which were designated *P. putida* KT2440 SK4, KT2440 Δ pvd_RL, KT2440 Δ flag_RL, KT2440 Δ alg_RL, KT2440 Δ bcs_RL, KT2440 Δ pea_RL, KT2440 Δ peb_RL, KT2440 Δ lapA_RL, KT2440 Δ lapF_RL, KT2440 Δ pha_RL, KT2440 Δ pili1_RL, KT2440 Δ pili2_RL, KT2440 Δ pili3_RL, KT2440 Δ pili4_RL, KT2440 Δ pili5_RL, KT2440 Δ curli_RL, KT2440 Δ pili6_RL, and KT2440 Δ pili7_RL.

The rhamnolipid production was performed in M9 minimal medium containing 10 g L⁻¹ glucose in 24-deep well plates and cultivation was stopped after 24 hours. Interestingly, the rhamnolipid production was improved for the majority of the strains (Figure 19), although the growth rates were not altered considerably. Twelve deletion mutants produced more rhamnolipids than the wild type with the rhamnolipid cassette (*P. putida* KT2440 SK4) and *P. putida* KT2440 Δ flag_RL showed the highest titer of 0.99 ± 0.05 g L⁻¹. While five deletions (Δ pvd, Δ flag, Δ alg, Δ bcs, and Δ pea) led to a strong improvement of rhamnolipid production of up to 66%, seven deletions (Δ lapA, Δ lapF, Δ pha, Δ pili1, Δ pili3, Δ pili4, and Δ pili5) only led to a slight improvement (7-24%). The remaining deletions showed no difference in production or even a slight decrease (Δ pili2) compared to the wild-type background. Moreover, all strains produced a minor fraction of HAAs. *P. putida* KT2440 SK4 produced 0.11 ± 0.01 g L⁻¹ HAAs, corresponding to 16% of the total biosurfactant amount (mono-rhamnolipids and HAAs). Most of the deletion strains produced HAAs in the same range (13-18%), whereas the best rhamnolipid producing strains (Δ pvd, Δ flag, Δ alg, Δ bcs) showed a decreased HAA formation with 10-11%. On the one hand, the presence of HAAs is due to the genetic context because the ratio of the transcript level for *rhIA* and *rhIB* is approximately 2:1 in a system based on genome integration at the *attTn7* site with an salicylate inducible promoter or the P_{ffg} promoter [240]. On the other hand, a bottleneck in dTDP-rhamnose supply is assumed, as the HAA proportion is reduced in the best producing

strains, which argues for an optimized metabolic network, supplying more of the rhamnose precursor.

Overall, the improvement of some strains is probably due to the savings of energy and carbon by deleting regions involved in the formation of the metabolic costly cellular components and molecules. If the titer was not increased, it is possible that a) the considered molecule or component has no major impact on the strain's performance or b) the cells do not synthesize them under the used conditions.

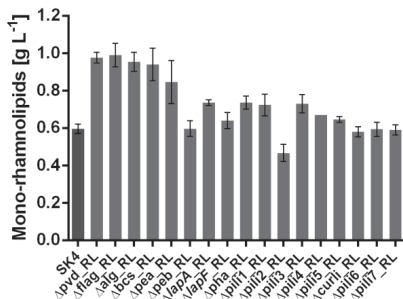


Figure 19: Rhamnolipid production of engineered rhamnolipid producers in 24-deep well plates using M9 minimal medium containing 10 g L⁻¹ glucose. The *P. putida* strains were cultivated in 24-deep well plates. Error bars indicate the standard deviation of the mean (n=3).

In addition to determining the impact of the deletions on rhamnolipid production, cultivation characteristics, such as substrate consumption, pH, and growth of the rhamnolipid producers, were investigated (Figure 20). The additional metabolic burden introduced by rhamnolipid synthesis did not alter the biomass production and all strains showed a similar growth behavior (Figure 20A). At about 14 hours, a peak of the OD₆₀₀ was observed for some of the mutants probably caused by morphology changes, when the cells entered the stationary phase. Interestingly, a sharp drop of the pH value was observed for four mutants (Δpvd, Δflag, Δalg, Δbcs), which correlates with the increased glucose consumption rate (Figure 20B,C). These four mutants consumed the complete glucose after 8 hours and showed a fast conversion of glucose to gluconate and 2-ketogluconate, which caused the pH decrease. At 8 hours, the 2-ketogluconate concentration was around 6 g L⁻¹ for the four mutants, while in comparison *P. putida* KT2440 SK4 produced only around 0.5 g L⁻¹ 2-ketogluconate. The glucose consumption rate was increased almost twofold to approximately 1.8 g L⁻¹. Together with the findings of the significantly increased rhamnolipid production and decreased HAA production of *P. putida* KT2440 Δpvd, KT2440 Δflag, KT2440 Δalg, and KT2440 Δbcs, this strongly indicates that freeing up resources leads to an alteration of the

metabolic network operation in the direction of central carbon metabolism intermediates, which are needed for precursor synthesis. The driven by demand concept examined by Tiso *et al.* [264] is thus likely promoted by the streamlining of the chassis.

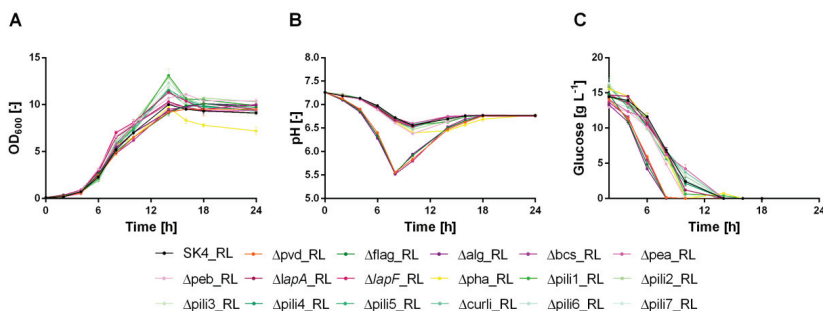


Figure 20: Cultivation characteristics of *P. putida* KT2440 derivatives in M9 minimal medium containing 10 g L⁻¹ glucose. The strains were cultivated in 500 mL shake flasks. OD₆₀₀ (A), pH values (B), and glucose concentrations (C) during 24 h cultivation are shown. The values are given as mean values and error bars represent the maximum and minimum values of two replicates (n=2).

As several single deletions led to improved rhamnolipid production, the question if the titer can be further increased by multiple deletions in one strain arose. In the course of generating the single-deletion mutants, also the first eight regions were sequentially deleted in *P. putida* KT2440 resulting in strain *P. putida* KT2440 GR18 ($\Delta pvd \Delta flag \Delta alg \Delta bcs \Delta pea \Delta peb \Delta lapA \Delta lapF$). The deletion of the eight regions resulted in a 2.95% reduced genome. However, accumulating all deletions in one strain could not be continued as the resulting strain showed severely impaired growth on agar plates making further cloning difficult. Hence, we decided to additionally construct a strain, only containing the knockouts, which led to improved rhamnolipid production. This strategy resulted in a strain designated *P. putida* KT2440 GR41 ($\Delta pvd \Delta flag \Delta alg \Delta bcs \Delta pea \Delta lapA \Delta lapF \Delta pha \Delta pili1 \Delta pili3 \Delta pili4 \Delta pili5$) with a 3.3% reduced genome. Both strains were equipped with the rhamnolipid production cassette (pSK02 [239]), resulting in *P. putida* KT2440 GR18_RL and *P. putida* KT2440 GR41_RL. Then, both rhamnolipid producers and the best-producing strain *P. putida* KT2440 $\Delta flag$ _RL were cultivated in M9 minimal medium containing 10 g L⁻¹ glucose in 24-deep well plates for 24 hours to determine if multiple deletions lead to a further increase in rhamnolipid production.

Unfortunately, the multiple deletion of costly metabolic products in one strain did not enhance the titer further compared to the single deletion of the flagellar machinery, which showed the biggest improvement (Figure 21). The rhamnolipid titers were slightly increased

due to a higher glucose concentration in the medium (12.4 g L^{-1}) compared to the previous result of *P. putida* KT2440 $\Delta\text{flag_RL}$. The final titer of *P. putida* KT2440 GR41_RL was even decreased compared to the others. Again, a reduced fraction of HAAs was produced with the multiple-deletion strains, which is consistent with the best single-deletion rhamnolipid producers. *P. putida* KT2440 $\Delta\text{flag_RL}$ and GR18_RL produced around 0.2 g L^{-1} HAAs, which corresponds to 11-12%. However, the HAA proportion produced by *P. putida* KT2440 GR41_RL amounted to only 6% of the total biosurfactant titer. The decreased rhamnolipid and HAA production of *P. putida* KT2440 GR41_RL is possibly due to product degradation, which has to be investigated in further studies.

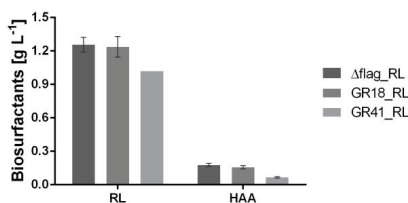


Figure 21: Rhamnolipid and HAA production by *P. putida* KT2440 $\Delta\text{flag_RL}$, GR18_RL, and GR41_RL in M9 minimal medium containing 10 g L^{-1} glucose. The cultivation was performed in 24-well plates and stopped after 24 h. Error bars indicate the standard deviation of the mean ($n=3$).

Although the accumulation of multiple deletions in one strain could not further increase the rhamnolipid titer, single deletions already reduced the metabolic load and increased the rhamnolipid titer (0.99 g L^{-1}). This corresponds to a product to substrate yield of $0.1 \text{ g}_{\text{RL}} \text{ g}_{\text{Glc}}^{-1}$, which equals $0.15 \text{ Cmol Cmol}^{-1}$. The theoretical yield per glucose is $0.72 \text{ Cmol Cmol}^{-1}$, of which 21% are reached. The reference strain *P. putida* KT2440 SK4 only reaches 13% of the theoretical yield.

Taken together, four deletions (Δpvd Δflag Δalg Δbcs) led to the highest improvement in rhamnolipid production. By sequential deletion of these regions and the subsequent analysis of rhamnolipid production, a further increase was expected due to freed-up resources. However, the GR18 mutant deficient in the regions *pvd*, *flag*, *alg*, and *bcs* produced rhamnolipids on the same level as the single deletion strains containing the rhamnolipid production module. Combined with the detection of additional HAAs in the supernatant, this strongly indicates a bottleneck in dTDP-rhamnose supply.

3.3.3.4 Overexpression of genes responsible for the synthesis of dTDP-rhamnose increases rhamnolipid titer

As the rhamnolipid titer could not be increased further with multiple deletions, a bottleneck regarding the precursor supply was investigated. For the production of rhamnolipids, dTDP-rhamnose is needed. The synthesis of this precursor involves a *pgm* and the gene products of the *rml*-operon [107,381]. Since it was observed that the reproducibility of rhamnolipid production depends on the used expression system, i.e., the promoter [249], the usually engaged rhamnolipid cassette pSK02 was replaced here. An expression system with a salicylate inducible promoter was used [249] instead to assign the results to the overexpression and not to the strong expression of the constitutive promoter and the rhamnolipids as a stress factor.

Three different *pgms* were used, which were cloned in the pSEVA441 vector: *xanA* from *P. putida* KT2440 (pWJ06; [242]), *algC* from *P. aeruginosa* PA01 (pLO03; [241]), and *algC* from *P. putida* KT2440 (pLO04; [241]). In addition, the overexpression of a *pgm* was performed in combination with the overexpression of the *rml*-operon and without. For that, a rhamnolipid cassette with the rhamnolipid genes and the complete *rml*-operon was used. The two cassettes pYTSK10K_0G7 (*rhlAB*) and pYTSK51K_0G7 (*rhlAB* + *rmlBDAC*) [240] were integrated into the *attTn7* site in *P. putida* KT2440 and *P. putida* KT2440 GR18. The respective strains were named *P. putida* KTsalmRL, *P. putida* GRsalmRL, *P. putida* KTsalmRL+Rha, and *P. putida* GRsalmRL+Rha and kindly provided by Sonja Kubicki (Heinrich Heine University, Düsseldorf, Germany). Then, the *pgm*-overexpression plasmids and the empty vector pSEVA441 were integrated in the respective strains. To determine the influence of the overexpression, all engineered strains compared to their control strains were cultivated in M9 minimal medium containing 10 g L⁻¹ glucose in 24-deep well plates (Figure 22). To induce the rhamnolipid production, salicylate to a concentration of 2 mM was supplemented after 4 hours. The cultivation was conducted for 48 hours because the plasmid-carrying strains grew slower. As the *rml*-operon was investigated in Sonja Kubicki's doctoral thesis, the focus is on the *pgm* here.

In general, the rhamnolipid production with the inducible promoter system is lower than with the usually used promoter (P_{rhl}). This is due to the stronger expression strength of the P_{rhl} promoter [249]. Further, all three overexpression plasmids led to a different increase of the rhamnolipid titer in the four different strain backgrounds. This reflects the strong metabolic burden caused by maintenance of the plasmid, which has a negative influence on the reproducibility here. Nevertheless, the titer was increased due to the use of any additional *pgm*. For example, the use of pLO04 led to an increase of 20% for *P. putida* KTsalmRL

(0.59 g L⁻¹) and the use of pLO03 led to an increase of 24% for *P. putida* KTsalmRL (0.75 g L⁻¹) compared to their respective origins. The additional overexpression of the *rml*-operon increased the titer further. In the case of *P. putida* KT2440 GR18, the titer was not significantly increased if a *pgm* was solely overexpressed. The initial strain *P. putida* GRsalmRL produced 0.64 g L⁻¹ rhamnolipids, while the highest rhamnolipid titer of 0.69 g L⁻¹ was reached by the respective strain carrying pLO04, which equals an increase of only 6%. If the *rml*-operon was overexpressed in addition, the titer was increased by 50% using pWJ06 as overexpression plasmid (0.97 g L⁻¹). If all strain engineering events are taken into account (genome reduction and overexpression of *pgm* and *rml*-operon), the titer was increased from 0.5 g L⁻¹ (*P. putida* KTsalmRL) to 0.97 g L⁻¹ (*P. putida* GRsalmRL+Rha + pWJ06). Hence, an increase of 94% was accomplished.

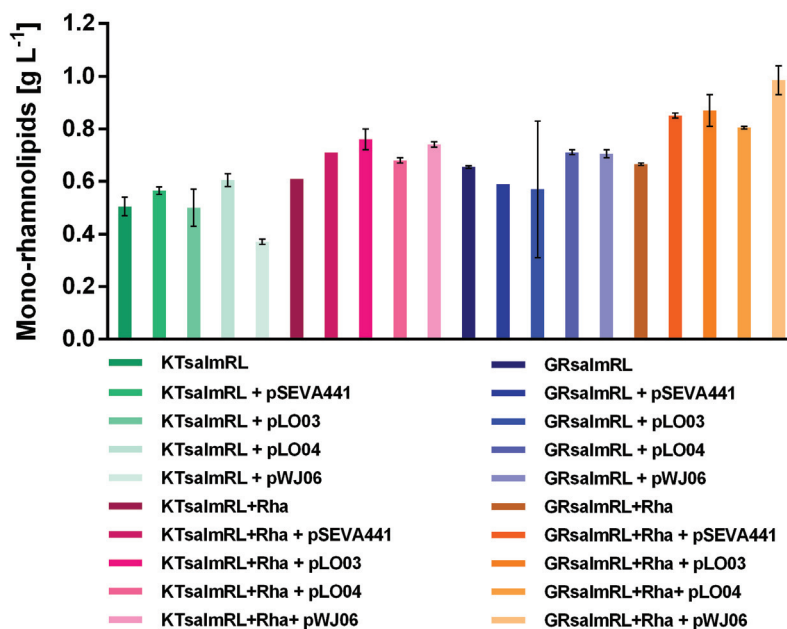


Figure 22: Impact of overexpression of genes involved in dTDP-rhamnose supply on rhamnolipid production by *P. putida* KT2440 derivatives. Cultivation was performed in M9 minimal medium containing 10 g L⁻¹ glucose in 24-deep well plates. The values are given as mean values and error bars represent the maximum and minimum values of two replicates (n=2). The figure was modified and adapted from Marie Dielentheis-Frenkens' Internship Report [257].

Moreover, a negative trend regarding HAA formation was observed (Figure A 3). The strains having an additional copy of the *rml*-operon integrated produced less HAAs than the

strains harboring only a *pgm*-overexpression plasmid. Also, the genome reduced mutants produced less HAAs than the strain with the wild-type background in general. This results in the smallest HAA production for the strains *P. putida* GRsalmRL+Rha carrying a *pgm*-overexpression plasmid, except pWJ06. Hence, the overexpression of the complete pathway (*pgm* + *rml*-operon) in a reduced background was most successful to direct the flux in the direction of the rhamnose precursor supply and consequently rhamnolipid synthesis.

Thus, the combination of metabolic engineering and overexpression of genes encoding enzymes involved in the precursor supply increased the rhamnolipid production. Furthermore, the assumed bottleneck in the precursor supply due to the stagnating rhamnolipid titer and HAA formation in the multiple-deletion strain was verified and eliminated by increasing the rhamnolipid titer and decreasing the HAA titer. However, the increase is still small and additional bottlenecks might be present.

3.3.3.5 Increasing β -hydroxy fatty acid supply for rhamnolipid synthesis

In addition to dTDP-rhamnose, β -hydroxy fatty acids are precursors for rhamnolipid synthesis (Figure 23). Hence, the flux should be directed to HAA synthesis and away from fatty acid *de novo* synthesis by metabolic engineering as it is suggested that RhIA competes with FabA for the fatty acids. As FabA is not essential for *P. aeruginosa* [109], a) the deletion of *fabA* and b) the overexpression of *fabG* was addressed. By performing these two modifications individually, an accumulation of *R*-3-hydroxyacyl-ACP is aimed for. This intermediate can then be used for rhamnolipid synthesis if the conversion to enoyl-ACP is slower than its use for the synthesis of HAAs.

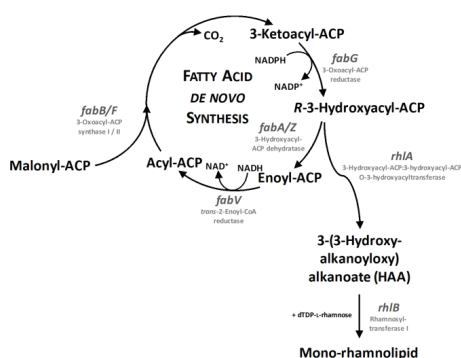


Figure 23: Overview of fatty acid *de novo* synthesis and rhamnolipid synthesis with the endogenous and heterologous enzymes. NAD(P)⁺, nicotinamide adenine dinucleotide (phosphate); NAD(P)H, reduced nicotinamide adenine dinucleotide (phosphate).

After successfully cloning pEMG-*fabA* for the deletion of *fabA* from the chromosome of *P. putida* KT2440 with the I-SceI-based method [225], the knockout procedure was conducted. The verification of the knockout was performed via colony PCR (Figure 24). A band at 1,905 bps was expected for the wild type and a band at 1,389 bps was expected for the *fabA* deletion mutant. For two colonies (8, 11), no band was observed, indicating that the DNA was not accessible probably due to failed lysis. Unfortunately, a band at 1,905 bps was observed for twelve of the 16 tested colonies and thus, the wild type was restored after recombination. For the other two colonies (9, 10), a band at 1,905 bps and the desired 1,389 bps was observed, indicating a mixed culture. To obtain a single isolate, which is deficient in *fabA*, the two colonies were grown in LB medium overnight and then plated on LB agar. Unfortunately, isolation resulted in the loss of the *fabA*-deletion and again only wild type bands were observed on the agarose gel after colony PCR (data not shown). These findings strongly indicate that FabA is essential in *P. putida* KT2440 and that no deletion mutant can be engineered.

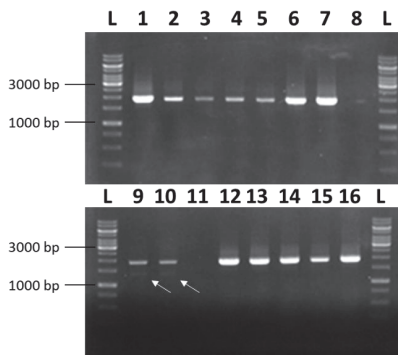


Figure 24: Agarose gel of colony PCR with 16 colonies after *fabA*-knockout procedure. L shows the lane with the GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, Waltham, MA, USA). Lane 1 to 16 show the amplified band by colony PCR of 16 different colonies obtained after the knockout procedure. The white arrows indicate the presence of a deletion mutant by showing the desired band.

As the first strategy failed, the overexpression of *fabG* was performed to increase the pool of *R*-3-hydroxyacyl-ACP, possibly resulting in a redirection of a higher fraction of this pool for rhamnolipid synthesis. For that, the plasmid pBT-*fabG* was transferred in *P. putida* KTsalmRL. To investigate the effect of the overexpression, the engineered strain and its control strain (*P. putida* KTsalmRL) were cultivated in M9 minimal medium containing 10 g L⁻¹ glucose in 24-deep well plates (Fig). To induce the rhamnolipid production, salicylate to a concentration of 2 mM was supplemented after 4 hours.

The strain carrying the overexpression plasmid *P. putida* KTsalmlRL + pBT-*fabG* produced 0.37 g L⁻¹ rhamnolipids and the control strain 0.46 g L⁻¹ rhamnolipids [250]. Hence, the rhamnolipid production was even decreased and the plasmid-carrying strain produced 20% less rhamnolipids. On the one hand, the maintenance of the plasmid is possibly the reason for the reduced production due to the metabolic burden, which should be elucidated in further research by using the empty plasmid as control. On the other hand, the fatty acid *de novo* synthesis is highly regulated and by overexpression of *fabG* the cells likely respond by automatically adjusting the fluxes through fatty acid *de novo* synthesis, resulting in a decreased supply of activated fatty acids for rhamnolipid synthesis.

3.3.3.6 Discussion

In this study, *P. putida* KT2440 was genetically modified to increase heterologous rhamnolipid production. On the one hand, the strain itself was modified by deletion of regions coding for metabolically demanding metabolic products. On the other hand, overexpression experiments were performed to increase precursor supply.

The streamlining of the genome is a promising strategy to create chassis, which is more often used due to the availability of many genomic tools [213]. The reduction of the genome can affect and improve several traits, such as plasmid stability, expression level of heterologous genes, or energy and carbon metabolism [357,382–384]. The targeted deletion of surface-related genes can also improve the accessibility of the cell envelope, which promotes the surface display of complex structures [385]. In several studies, the deletion of genes involved in the flagellar synthesis in pseudomonads was already addressed, as the synthesis and rotation of the flagellum costs resources and energy [226,227]. The deletion resulted in freeing up energy and showed a phenotype, which produces more biofilm than the wild type. This fits our data as we also observed a higher biofilm formation of *P. putida* KT2440 Δ flag. Although the biofilm formation should be prevented in aerated and stirred bioreactor systems, biofilms provide a protective environment and can be used in niche applications. Biofilms have increased resistance to antimicrobial agents or in general provided protective environments against stresses [386,387]. This characteristic is exploited to produce chemicals in biofilms [388,389]. For example, *P. taiwanensis* biofilms were used for (S)-styrene oxide production and *P. putida* biofilms even for rhamnolipid production [390,391]. Hence, *P. putida* KT2440 Δ flag could be used in such an approach.

While in two studies, an improved growth was observed for streamlined chassis [227,357], here no altered growth was observed for the deletion mutants, but a significantly increased rhamnolipid production. This phenomenon was observed before in a study, where 4.12% of the genome of *P. putida* KT2440 was deleted and green fluorescent protein (GFP)

expression and PHA production was increased, whereas the growth was comparable to the wild type [392]. Here, a rhamnolipid production increase of about 70% was achieved with four single knockout mutants (Δ pvd, Δ flag, Δ alg, and Δ bcs), resulting in roughly 1 g L⁻¹, which corresponds to 21% of the theoretical yield. This is the highest reported yield in M9 minimal medium from glucose in shake flask cultivations using recombinant *P. putida* KT2440. Further, the rhamnolipid production is growth independent and growth should be minimized in order to reach higher yields [147]. Thus, the use of the resources for increased rhamnolipid production and not growth is beneficial here.

The multiple deletions did not lead to a further increase contrary to previous studies [357,392], indicating a bottleneck and that the probable freed-up resources are not used. HAAs were detected in the supernatant, hinting to a bottleneck in dTDP-rhamnose supply. The fraction of HAAs produced amounts to 16% of the total biosurfactant titer for *P. putida* KT2440 SK4 and around 10% for *P. putida* KT2440 GR18_RL. Kubicki [240] reported also a reduced HAA fraction for the GR18-mutant using LB medium supplemented with glucose and an salicylate-inducible rhamnolipid expression system. Hence, it is likely that fluxes were redirected to dTDP-rhamnose supply through prevention of formation of by-products and cellular components, which is independent from the used medium and expression system. However, the reduction was substantial in the other system. This may indicate a better supply with nutrients in LB medium positively influencing rhamnolipid production, so that medium optimization of the minimal medium is another target for the future. This was already partially addressed in a study, where the M9 minimal medium was optimized for rhamnolipid production in small-scale reactors [261], however without the focus on reduction of HAA formation.

To further increase the rhamnolipid fraction, the overexpression of the *pgm* solely and in combination with the *rml*-operon was conducted and led to an increased rhamnolipid production. The overexpression was performed in a salicylate inducible rhamnolipid production system, which has a lower expression strength than the usually used rhamnolipid cassette with the constitutive P_{trg} promoter [219,249]. Hence, the rhamnolipid titer increase compared to the reference was almost 100%, however in the same range as the best producing strains with the usually used rhamnolipid cassette. The rhamnolipid fraction was further increased by overexpression of the *pgm* and *rml*-operon, matching the results in LB medium [240]. The *rml*-operon is considered to have the main impact on precursor availability here, which was also shown in another study [393]. The overexpression of the *pgm* was conducted via plasmid-based expression in this study and two other studies [240,393]. However, the reproducibility and consistency are not given in all studies. Thus, the cloning of the *pgm* and

rml-operon directly in the rhamnolipid production module for genomic integration to further increase the titer and decrease HAA formation was addressed by Kubicki [240] and should be combined with the genome-reduced mutant in future studies. The switch from a plasmid-based expression to chromosomal integration has advantages regarding the increase of gene stability, reproducibility, and reduction of the metabolic burden, which results in higher product yields [394,395]. The decrease of HAAs in the supernatant leads to a purer rhamnolipid fraction and thus reduces the effort for downstream processing for separation if a pure rhamnolipid fraction is needed for application.

As the rhamnolipid concentrations only marginally increased by overproduction of dTDP-rhamnose, the overexpression of *fabG* was conducted but did not result in an improved rhamnolipid production. The rate of fatty acid biosynthesis is tightly regulated and thus the overexpression of one gene would possibly not have an effect. The synthesis is regulated by negative feedback regulators, temperature, and transcriptional factors [396]. Thus, identifying and addressing a regulator in *P. putida* KT2440 would be a possible target. The overexpression of *fadR* coding for a fatty acid synthesis regulator in *E. coli* increased fatty acid production 7.5-fold, whereas the overexpression of single genes alone only led to a small increase [397]. In *P. aeruginosa*, DesT is a transcription factor, which represses the expression of the gene *fabB* [398], which provides the driving force for unsaturated fatty acid synthesis [397]. Deletion of a repressor with the same function in *P. putida* KT2440 possibly allows the increase of fatty acid synthesis and consequently rhamnolipid production.

In summary, we report the engineering of *P. putida* KT2440's genome and the consequent alterations in terms of their biofilm formation and swimming ability and the increased rhamnolipid production capacity. This study serves as a basis for further research because the multiple-deletion chassis potentially can be used in any study aiming at the production of secondary metabolites. In the specific case of rhamnolipid production, the genome-reduced chassis is only advantageous once the existing bottlenecks are eliminated.

Chapter 3.4

Valorization of lignocellulosic biomass for rhamnolipid production

Contributions:

Isabel Bator performed characterization experiments, prepared figures, and wrote this chapter. Strains and plasmids were constructed by Isabel Bator, Hans M. Böhne, and Jan Brinkmann. Jan Brinkmann performed cultivations and Tobias Karmainski performed fermentations. Till Tiso and Lars M. Blank conceived the study, advised on all experiments, discussed data, and reviewed this chapter.

3.4 Valorization of lignocellulosic biomass for rhamnolipid production

3.4.1 Abstract

The production of chemicals and fuels is mainly based on fossil resources. The reduced availability of these resources as well as the resulting pollution of the environment require alternative strategies to be developed. One approach is the employment of microorganisms for the production of platform molecules using renewable resources as substrates. Lignocellulosic biomass is one of the most abundant materials in nature and mainly inedible. Using also this fraction of plants would contribute to a sustainable bioeconomy and impede the food vs. fuel competition. Previously, we engineered *P. putida* KT2440 for heterologous rhamnolipid production from xylose, which is one of the main compounds in hemicellulose. However, some metabolic bottlenecks were identified, which inhibited efficient metabolization of the C₅ sugar. Using ALE, these bottlenecks could be overcome. Here, we addressed the use of xylose and in addition galacturonic acid, which is also present in hemicellulose and constitute a major part of pectin, as alternative substrates. The strains evolved for more efficient xylose metabolization were sequenced to reveal the nature of the bottlenecks and allow subsequent reverse engineering. Introducing one of the identified mutations resulted in restoring the phenotypic growth after ALE with a significantly reduced lag phase. Further, the metabolic burden caused by using expression plasmids was reduced by integration of the genes for the xylose utilization pathway into the genome. Moreover, expression of a xylonate dehydratase solely is efficient to enable growth on xylose. In the case of galacturonic acid, ALE was performed, which resulted in an improved growth rate of 0.43 h⁻¹. The efficient substrate metabolizers were engineered for heterologous rhamnolipid production and characterized regarding their growth and production performance in lab-scale bioreactors. This study serves as proof-of-concept that microbes can be applied for the sustainable production of bulk chemicals (here biosurfactants) based on renewable resources.

3.4.2 Introduction

Due to the ever-increasing energy demand and environmental pollution, the shift from a petroleum-based to a renewable biomass-based chemical industry is crucial. Renewable biomass, such as plant-based raw material, has the potential to substitute a large fraction of fossil resources as feedstock for industrial processes [399]. The renewable biomass can be microbially transformed into valuable products. To be competitive and to develop sustainable biotechnological production processes at larger scale, substrates have to be eco-friendly and cheap and optimally do not compete with food supply.

Lignocellulosic biomass, which can be found in plant cell walls and is gained from energy crops, e.g., switchgrass, and agricultural or wood residues, is a promising renewable substrate for the production of valuable products due to its abundant availability and low cost [21]. Lignocellulose is composed of cellulose, hemicellulose, pectin, and lignin, from which several sugars and organic acids can be derived, including arabinose, xylose, and galacturonic acid [12]. In recent years, much research has been conducted regarding the use of waste and side streams or lignocellulose for the production of biosurfactants. For example, lactose is the major constituent of whey, which is a by-product of the dairy industry, and was used for rhamnolipid production [131]. Further, rhamnolipid production using molasses was already reported. Molasses are by-products from sugar processing plants [134], still containing easily accessible sugars. But also lignocellulosic material was used for the production of, e.g., surfactin with *B. subtilis* [400], sophorolipids with *Cutaneotrichosporon mucoides* [401], and rhamnolipids with *P. aeruginosa* [402]. This shows that lignocellulose can be used as alternative cost-effective carbon source for biosurfactant production.

Despite the abundant availability of lignocellulose, the use of this feedstock is still sparse as many commonly used microbes in bioprocesses are not able to use the feedstock in its untreated form or even the monomers [17]. However, due to the progress in the field of synthetic biology this challenge can be addressed and microbes can genetically be modified to express genes or pathways for the utilization of this feedstock. One prominent example is the engineering of *S. cerevisiae* for the production of bioethanol from second generation feedstock [403].

The presence of pathways in several microbes was reported for the utilization of, e.g., arabinose, xylose, and galacturonic acid. Arabinose and xylose can be converted using the phosphorylative (isomerase) pathway, which was found in *E. coli* and *B. subtilis* [288,289,404,405]. Arabinose is sequentially converted to ribulose, ribulose-5-phosphate, and xylulose-5-phosphate. Xylose is directly converted to xylulose and xylulose-5-phosphate. The intermediate xylulose-5-phosphate is introduced in the pentose phosphate pathway. Furthermore, xylose and arabinose can be converted by oxidation. Xylose is converted to xylonate, 2-keto-3-deoxy-xylonate, α -ketoglutaric semialdehyde, and pyruvate and glycolaldehyde using the Dahms pathways or 2-oxoglutarate using the Weimberg pathway, which were found in *Pseudomonas* species [293,298]. For arabinose, the conversion via the pentonic acid to 2-oxoglutarate or pyruvate was also described for *Pseudomonas* species [293,314]. The resulting intermediates are introduced in the TCA cycle or glycolysis. Galacturonic acid can also be converted via an isomerase or oxidative pathway. In *E. coli*, galacturonic acid is sequentially converted to tagaturonate, altronate, 2-keto-3-deoxy-

gluconate, 2-keto-3-deoxy-6-phosphogluconate, and pyruvate and glyceraldehyde-3-phosphate [315]. The resulting intermediates are introduced in the glycolysis. Further, an oxidative pathway was described for *Pseudomonas* and *Agrobacterium tumefaciens* [316,406]. Galacturonic acid is converted to *meso*-galactarate, 5-keto-4-deoxy-glucarate, α -ketoglutaric semialdehyde, and 2-oxoglutarate, which is introduced in the TCA cycle [317]. The knowledge of these conversion pathways can be used for engineering microbes to efficiently utilize the substrates.

Since various *Pseudomonas* strains harbor genes, allowing the utilization of these three substrates, *P. putida* KT2440 seems to be a suitable microorganism for application in processes using these substrates as carbon and energy source. Over the last years, *P. putida* gained much attention in terms of recombinant biosynthesis of valuable products due to its native robustness against various sources of stress [155,166]. For example, *P. putida* was used to produce rhamnolipids, terpenoids, and amino acid-derived metabolites [166]. Furthermore, *P. putida* was already engineered to be able to use alternative raw materials, such as cellobiose, arabinose, xylose, and sucrose, to sustainably synthesize products in the near future [255,311,407].

In this study, we report heterologous rhamnolipid production with engineered *P. putida* KT2440 from xylose and galacturonic acid. Genome re-sequencing on previously evolved xylose metabolizing strains was applied to identify the genetic background for the adaptations. Further, the metabolic burden was reduced by replacing the plasmid carrying genes responsible for xylose metabolism with a genome-integrated cassette. ALE was used to improve growth on galacturonic acid. The evolved mutant was characterized regarding growth and subsequently modified to produce mono-rhamnolipids. The efficient xylose and galacturonic acid metabolizers were used for rhamnolipid production in a bioreactor.

3.4.3 Results

3.4.3.1 Deletion of the repressor PtxS eliminates xylonate import as bottleneck in efficient xylose catabolism

In Chapter 3.1.3.3, strains were engineered, which are able to grow on xylose. The strains harboring the respective plasmids showed a strong accumulation of xylonate and a resulting lag phase of 24 h and 34 h using the Weimberg and Dahms pathway, respectively. Through ALE the lag phase was reduced to approximately 3 hours, but the growth rate was decreased for the evolved Weimberg-strain (Figure 5). For further optimization (increasing the growth rate, while maintaining a short lag phase), reverse engineering is aimed for. Hence, to investigate the basis of the phenotypic changes, genome re-sequencing of both evolved strains

was performed. As already described in chapter 3.2.3.2, the wild type also harbors many mutations compared to the published sequence. Three additional SNPs and six InDels were identified for both evolved strains (*P. putida* KT2440 pWeim2 and *P. putida* KT2440ΔΔ pDahms2). Most of the mutations are located in non-coding regions, errors due to low coverage and read quality, or hypothetical proteins. Two genomic regions were identified, which probably have an impact on the lag phase (Figure 25). The first region includes the gene *gacS* (PP_1650), where six bps were deleted in *P. putida* KT2440 pWeim2 and one missense mutation was found in *P. putida* KT2440ΔΔ pDahms2 (Table A 2). Mutations in *gacS* were found in (evolved) *Pseudomonas* strains with improved growth and reduced lag phases [408,409]. However, this region was not investigated further so far, as the second region is believed to have a major impact on the lag phase due to the xylonate accumulation.

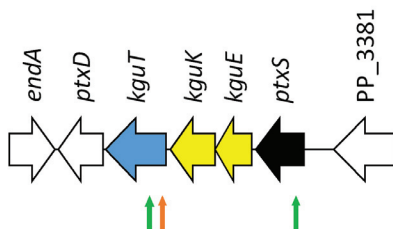


Figure 25: Overview of the mutated region after ALE on xylose. The gene organization is based on the genome sequence AE015451.2 [184]. Gene organization from *endA* (PP_3375) to PP_3381. A black arrow indicates a gene coding for a regulator/repressor, yellow arrows indicate genes involved in 2-ketogluconate conversion, a blue arrow indicates a transporter, and white arrows indicate genes with other functions. Smaller arrows below the gene organization show the identified mutations after ALE. The green arrows indicate the presence of the mutation in *P. putida* KT2440 pWeim2 and the orange arrow indicates the presence of the mutation in *P. putida* KT2440ΔΔ pDahms2.

The second region is originally involved in 2-ketogluconate conversion and transport. Interestingly, independently of the strain (Weimberg or Dahms-strain) a mutation in the same gene was found. Each of these is a missense mutation in *kguT*, encoding a putative 2-ketogluconate transporter. Further, insertion of an additional amino acid (histidine) was found for the Weimberg-strain in *ptxS*, encoding a 2-ketogluconate utilization repressor (Table A 2). As the glucose dehydrogenase Gcd was determined to be involved also in xylose conversion (3.1.3.2), the genes involved in 2-ketogluconate metabolism possibly have a side-activity for xylonate, which is an intermediate of the oxidative xylose pathways. To investigate if the mutations in *kguT* and *ptxS* influence xylose/xylonate metabolism, the mutations found in *P. putida* KT2440 pWeim2 were recreated. In detail, a point mutation (G → C) was introduced in *kguT* and *ptxS* was deleted from the chromosome via the I-SceI-based method [225]. Although an introduction of three bases does not lead to an interruption

of the open reading frame, the protein function might be negatively affected. Hence, we decided to delete the gene to imitate abolishing the protein function. The resulting strains were equipped with the plasmid for the Weimberg pathway and designated *P. putida* KT2440 $\Delta ptxS$ pWeim and *P. putida* KT2440 PP_3377_A117G pWeim. The effect of the changes was investigated by cultivation of the reference strains *P. putida* KT2440 pWeim and KT2440 pWeim2 and the engineered strains KT2440 $\Delta ptxS$ pWeim and KT2440 PP_3377_A117G pWeim in M9 minimal medium containing 10 g L^{-1} xylose in 24-deep well plates.

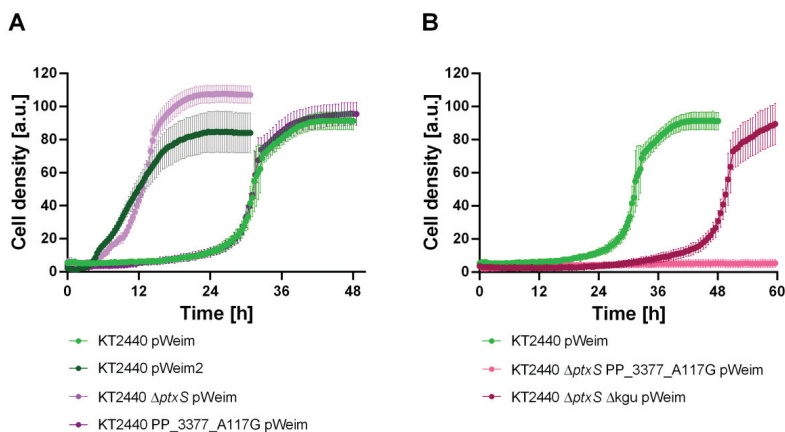


Figure 26: Comparison of growth of engineered *P. putida* KT2440 Weimberg-strains in M9 minimal medium containing 10 g L^{-1} xylose. Growth was detected using a Growth Profiler 960 in 24-deep well plates. Error bars indicate the standard deviation from the mean ($n=3$). The figure was modified and adapted from Jan Brinkmann's Master Thesis [251].

Unexpectedly, the strain *P. putida* KT2440 PP_3377_A117G pWeim harboring the point mutation in *kguT* showed no altered growth compared to the initial Weimberg pathway-harboring strain KT2440 pWeim (Figure 26A). Since in both evolved strains *P. putida* KT2440 pWeim2 and *P. putida* KT2440 $\Delta\Delta$ pDahms2 a mutation in *kguT* was observed, this mutation was considered as primary factor for the decreased lag phase. The deletion of *ptxS* on the other hand resulted in a significantly decreased lag phase comparable to the lag phase of the evolved strain *P. putida* KT2440 pWeim2 (Figure 26A). Further, the growth rate was not negatively affected as in the case of the ALE-strain and in a similar range as the growth rate of KT2440 pWeim (0.3 h^{-1}).

Additionally, the Dahms-plasmid was introduced in *P. putida* KT2440 $\Delta\Delta$ $\Delta ptxS$. The strain *P. putida* KT2440 $\Delta\Delta$ $\Delta ptxS$ pDahms showed the same phenomenon of the significantly reduced lag phase (data not shown). The expression of the *kgu* operon downstream of *ptxS*, which is usually involved in 2-ketogluconate metabolism and transport, is repressed by PtxS

and induced in the presence of glucose and its derived acids. However, *in vitro* assays showed that only in the presence of 2-ketogluconate, PtxS dissociates from the promoter region of the operon and that the RNA polymerase can then bind [189]. The described regulatory scheme and the described findings likely indicate that xylose and xylonate are not recognized by PtxS and that the expression of the genes of the operon is not induced. However, KguT is probably needed for the transport of xylonate from the periplasm into the cytoplasm. This might result in the extended lag phase of the initial strain. Once the repressor is not active anymore (*ptxS* deletion), the lag phase is reduced as the genes are expressed and xylonate can be transported into the cytoplasm.

To confirm this hypothesis and to exclude that PtxS is a positive regulator of the operon, which downregulates the expression of the operon by deletion of *ptxS*, the complete *kgu* operon (*ptxD*, *kguT*, *kguK*, and *kguE*) was deleted. This strain was equipped with the Weimberg plasmid and named *P. putida* KT2440 Δ *ptxS* Δ *kgu* pWeim. During growth, the lag phase was observed to be even further increased compared to *P. putida* KT2440 pWeim to approximately 42 hours (Figure 26B). Thus, the *kgu* operon is most likely involved in efficient xylose metabolization and KguT is required for transport of xylonate. Further, the double deletion strain is still able to use xylose for cell growth with an extended lag phase. This finding argues for another transporter contributing to the import of xylonate.

As two mutations were observed in *kguT* and *ptxS* in the evolved Weimberg strain, the effect of the double mutation was investigated. Therefore, the point mutation in *kguT* was introduced in *P. putida* KT2440 Δ *ptxS*. The resulting strain was equipped with the Weimberg plasmid and designated *P. putida* KT2440 Δ *ptxS* PP_3377_A117G pWeim. After cultivation, no growth was observed for over 60 hours (Figure 26B). Hence, the imitation of the genomic background after ALE by deletion of *ptxS* and mutation of *kguT* did not result in the expected phenotype with the reduced lag phase.

Taken together, the KguT transporter is needed for efficient transport of xylonate into the cytoplasm. However, the expression of the encoding gene *kguT* is repressed by the repressor PtxS. Xylonate can probably not bind to PtxS, which blocks the expression of *kguT*. By deletion of *ptxS* the release of PtxS from the DNA site is imitated, enabling the expression of *kguT* and thus the transport of xylonate. Moreover, reverse engineering can repeat the beneficial outcomes of an ALE experiment in a clean strain background.

3.4.3.2 Genomic integration of the xylose genes leads to a higher growth rate

As mentioned before, the chromosomal integration has advantages over a plasmid-based expression system, such as reproducibility, reduction of the metabolic burden, and avoiding the usage of antibiotics. The metabolic burden is mainly caused by the presence of multiple

plasmid copies, leading to a higher gene dosage [410,411]. To make use of these advantages, the integration of the genes coding for the Weimberg pathway into the genome was performed. For integration the mini-Tn7 delivery transposon vector (pBG) was used [216] to achieve a single integration at the *attTn7*-site. The vector contains the constitutive stacked P_{ig} promoter [219] and the genes PVLB_18550 to PVLB_18565 (pBG_PVLB_18550-18565) and was delivered to *P. putida* KT2440. The resulting strain was named *P. putida* KT2440 Tn7_PVLB_18550-18565 and tested for growth on xylose.

The genome-integrated strain showed a slightly reduced lag phase of 20 hours compared to the plasmid-harboring strain. Further, the growth rate was increased to $0.45 \pm 0.05 \text{ h}^{-1}$ (Figure 27). The reason for the drop of the cell density in the stationary phase is unknown but may be due to morphological changes. These results show that the xylose operon from *P. taiwanensis* is functionally expressed in *P. putida* KT2440, leading to improved growth compared to the plasmid-based expression. The higher growth rate is possibly due to the strong expression strength of P_{ig} and the reduction of the metabolic burden caused by plasmid maintenance.

Moreover, the question whether additional endogenous enzymes contribute to xylose utilization arose. In the beginning (3.1.3.2), a genome analysis was conducted and the xylose genes of *P. taiwanensis* were compared with the *P. putida* KT2440 genome. It was identified that PVLB_18565 is the only gene without any homolog in *P. putida* KT2440 and that there are endogenous genes in *P. putida* KT2440 catalyzing presumably the same reaction (Figure 4). To prove if only PVLB_18565 is needed to establish the Weimberg pathway, the mini-Tn7 vector pBG_PVLB_18565 was constructed and delivered to *P. putida* KT2440 $\Delta ptxS$ because this strain showed the shortest lag phase in previous experiments. The resulting strain was named *P. putida* KT2440 $\Delta ptxS$ Tn7_PVLB_18565 and was cultivated in medium containing xylose.

Growth of *P. putida* KT2440 $\Delta ptxS$ Tn7_PVLB_18565 was observed with a similar lag phase (4 h; Figure 27) compared to *P. putida* KT2440 $\Delta ptxS$ pWeim (Figure 26A). However, the expression system is different and the comparison has to be treated with caution. The growth rate was 0.27 h^{-1} and thus 40% decreased compared to the growth rate of *P. putida* KT2440 Tn7_PVLB_18550-18565. These results show that only one heterologous enzyme is needed to establish the Weimberg pathway in *P. putida* KT2440. The growth rate is possibly decreased using one gene instead of four genes due to the lower amount of the respective enzymes.

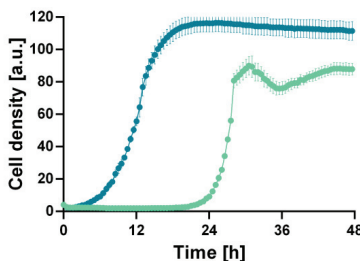


Figure 27: Growth of genome-integrated *P. putida* KT2440 strains using the Weimberg pathway. Cultivation of *P. putida* KT2440 Δ *ptxS* Tn7_PVLB_18565 (blue) and *P. putida* KT2440 Tn7_PVLB_18550-18565 (cyan blue) was performed in M9 minimal medium containing 10 g L⁻¹ xylose. Growth was detected using a Growth Profiler 960 in 24-deep well plates. Error bars indicate the standard deviation from the mean (n=3). The figure was modified and adapted from Jan Brinkmann's Master Thesis [251].

Overall, it was shown that the genomic integration of the xylose genes from *P. taiwanensis* VLB120 for establishing the Weimberg pathway was successful and that the genetic repertoire of *P. putida* enables the strain to grow on xylose by adding only one heterologous gene (PVLB_18565 from *P. taiwanensis* VLB120). As the resulting strain (*P. putida* KT2440 Δ *ptxS* Tn7_PVLB_18565) has a stable and improved phenotype without the drawback of plasmid instability, the strain can be used for further strain engineering for, e.g., the synthesis of valuable products based on xylose.

3.4.3.3 Alternative lignocellulosic biomass-derived substrates for production of rhamnolipids

Beside xylose, arabinose and galacturonic acid are interesting substrates for the sustainable production of valuable products as they are derived from lignocellulosic biomass. While arabinose cannot natively be used by *P. putida* [311], it is predicted that galacturonic acid can be used [184]. Performing a genome analysis using BLAST [305] revealed that *P. putida* KT2440 possesses genes, which might encode enzymes needed in galacturonic acid utilization. All genes required for utilization from *A. tumefaciens* were compared to the genome of *P. putida* KT2440. Homologous genes, which probably encode enzymes (with the EC numbers 4.2.1.41 and 4.2.1.42) for galacturonic acid degradation, are present with sequence identities of 65% and 66%, indicating that the oxidative pathway is used.

To be able to use arabinose, the plasmid pBT-*araBAD* with the genes of *E. coli* DH5 α was constructed. Unfortunately, after transformation of pBT-*araBAD* in *P. putida* KT2440, the cells were not growing in minimal medium containing arabinose. Therefore, the focus was on galacturonic acid.

To investigate the growth behavior of *P. putida* KT2440, the strain was grown in M9 minimal medium containing 10.8 g L⁻¹ galacturonic acid (0.33 Cmol L⁻¹). The strain showed already an efficient growth on galacturonic acid with a growth rate of 0.29 h⁻¹ (Figure 28B). However, a lag phase of 5 hours was detected.

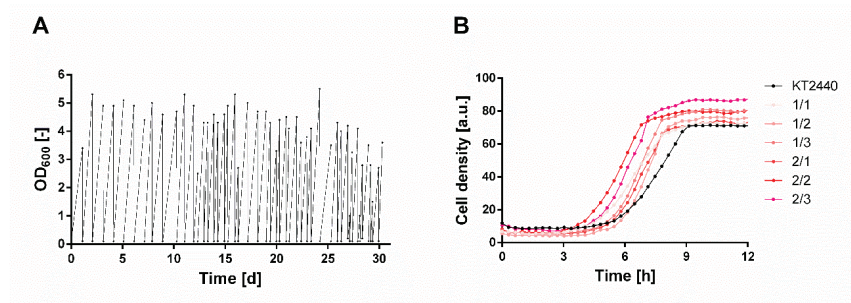


Figure 28: ALE and growth characteristics of isolates of *P. putida* KT2440 in modified M9 minimal medium containing 10.8 g L⁻¹ galacturonic acid. (A) ALE of *P. putida* KT2440 on galacturonic acid as sole carbon source. A single representative culture of two biological replicates is shown. (B) Comparison of growth for *P. putida* KT2440 (black) and three isolates from each replicative ALE cultures (red) on galacturonic acid, which are exemplarily shown. The best growing isolate is shown in pink. Growth was detected using a Growth Profiler 960 in 96-well plates.

ALE was applied to further improve the growth rate and reduce the lag phase. The strain was sequentially transferred to fresh minimal medium with galacturonic acid in two parallel cultures (Figure 28A). The growth performance of *P. putida* KT2440 was improved after 48 transfers, which corresponds to ca. 250 generations. The number of generations was calculated based on the starting OD₆₀₀ and the end OD₆₀₀ of every transfer. The ALE experiment was comparatively long (compared to the ALE on xylose and ethanol) due to the slow adaptation and minor improvement on galacturonic acid. Afterwards, several isolates were obtained from the inhomogeneous culture broth and up to twelve colonies were randomly selected and tested for growth on galacturonic acid for each culture (of which six are shown in Figure 28B). The evolved colonies showed a similar adaptation. The best isolate was designated *P. putida* KT2440 GA1. The evolved strain reached a growth rate of 0.43 h⁻¹, which indicates a 1.5-fold increase. Further, the lag phase was slightly reduced to 4 hours. To identify the genomic mutations, genome re-sequencing was performed but no obvious target, which could explain the improvement, was found.

Nevertheless, the application of ALE led to a significant improvement of the growth rate, expediting the use of galacturonic acid in industrial biotechnology.

In order to show the ability to use *P. putida* as a host for the synthesis of valuable products from galacturonic acid, the heterologous production of mono-rhamnolipids was

investigated. Therefore, the rhamnolipid synthesis module was integrated as a single copy into the genome of the efficient galacturonic acid metabolizer *P. putida* KT2440 GA1. The rhamnolipid producer was named *P. putida* KT2440 GA1_RL. The production was performed using 10.8 g L⁻¹ galacturonic acid (0.33 Cmol L⁻¹) as sole carbon source or 5 g L⁻¹ glucose and 5.4 g L⁻¹ galacturonic acid (both 0.165 Cmol L⁻¹) to compare the production capacities using one carbon source and mixed substrates. The cultivation was performed for 24 hours in comparison to the wild type carrying the rhamnolipid synthesis module *P. putida* KT2440 SK4.

Noticeably, the evolved strain had a higher growth rate and production rate compared to the wild type, leading to a faster consumption of galacturonic acid as sole carbon source. Whereas, the growth as well as the rhamnolipid production and substrate consumption was similar for both strains, when glucose and galacturonic acid are used simultaneously (Figure 29A-C). Specifically, *P. putida* KT2440 GA1_RL reached a biomass concentration of 1.51 ± 0.00 g L⁻¹ and produced 0.033 ± 0.008 g L⁻¹ h⁻¹ rhamnolipids in the first ten hours, meaning both approximately twofold higher than KT2440 SK4. This is probably caused by the ALE experiment, as the evolved strain has a higher growth rate and slightly reduced lag phase. The substrate consumption is also about twofold higher for *P. putida* KT2440 GA1_RL with 0.63 g L⁻¹ h⁻¹ in the first ten hours. After 24 hours, the rhamnolipid concentration of *P. putida* KT2440 GA1_RL is slightly reduced (0.61 g L⁻¹) compared to KT2440 SK4, which is probably due to the adaptation of the evolved strain towards galacturonic acid, resulting in a higher biomass production.

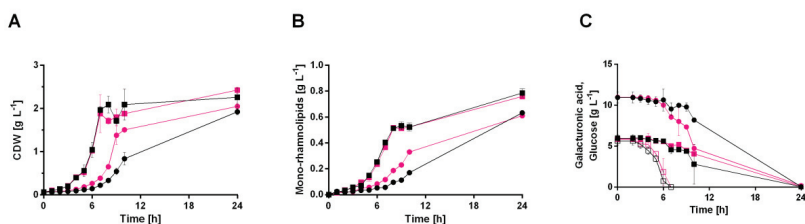


Figure 29: Mono-rhamnolipid production from galacturonic acid by engineered *P. putida* strains. Cultivation was performed in M9 minimal medium containing 10.8 g L⁻¹ galacturonic acid as sole carbon source (●) or a substrate mix of 5 g L⁻¹ glucose (□) and 5.4 g L⁻¹ galacturonic acid (■) in 500 mL shake flasks. Rhamnolipid producers *P. putida* KT2440 SK4 (black) and *P. putida* KT2440 GA1_RL (pink) were used for cultivation. Shown are the CDW (A), product titers of mono-rhamnolipids (B), and (C) consumption of the substrates. Error bars indicate the standard deviation from the mean (n=3). The figure was modified and adapted from Jan Brinkmann's Master Thesis [251].

In the case of the substrate mixture with glucose and galacturonic acid, no significant differences were observed. Glucose was the preferred carbon source and consumed in the first

seven hours by both strains (Figure 29C). Again after 24 hours, *P. putida* KT2440 GA1_RL reached a slightly higher final biomass titer ($2.43 \pm 0.06 \text{ g L}^{-1}$) compared to KT2440 SK4, which results in a slightly reduced rhamnolipid titer. Moreover, the rhamnolipid production rate was higher and the final rhamnolipid titer reached was almost 25% increased for both strains using two substrates. This is likely based on the introduction of the resulting metabolites in the central carbon metabolism. Glucose is directly converted into acetyl-CoA and dTDP-rhamnose, while galacturonic acid is presumably converted to 2-oxoglutarate, which is then converted to dTDP-rhamnose through gluconeogenesis. However, no increased HAA production was observed for the strains cultivated with galacturonic acid as sole carbon source compared to the mixed substrate use, indicating that dTDP-rhamnose supply is not a bottleneck and that glucose is better suited for rhamnolipid production due to the higher degree of reduction compared to galacturonic acid.

3.4.3.4 Production of rhamnolipids from renewable substrates in bioreactor scale

After it was shown that *P. putida* can produce rhamnolipids from renewable carbon sources, such as xylose (3.1.3.5) and galacturonic acid, up-scaling was performed in order to show the production efficiency in bioreactor scale. A fermentation setup with continuous foam recycling was used because production of biosurfactants in an aerated system leads to excessive foaming (Figure 3).

For rhamnolipid production from xylose, strain *P. putida* KT2440 $\Delta\Delta$ pDahms2_RL was used because the Dahms pathway is stoichiometrically favored over the Weimberg pathway for rhamnolipid production (3.1.3.1). The fermentation was carried out in modified M9 minimal medium containing 10 g L^{-1} xylose as duplicate over 26 hours. A final biomass titer of 2.44 g L^{-1} with a growth rate of 0.18 h^{-1} was achieved (Figure 30A). Between eight and 20 hours a linear growth was observed, indicating a limitation or bottleneck. As this behavior is also observed in shake flasks (Figure 5F), this behavior is assigned to the strain itself due to probable glycolaldehyde accumulation and not the bioreactor setup. The fermentation with xylose was significantly longer than the fermentation with galacturonic acid due to the slow growth rate (Figure 30A). After 20 hours, the strain stopped growing although xylose and the intermediate xylonate (not shown) were still present in the medium but remained producing rhamnolipids. The DO only decreased to 75% within 16 hours, hence the strain probably consumed less oxygen per hour due to the slow growth rate. As a consequence, the control cascade for the DO was not activated and the stirrer speed was constantly at 500 rpm during the whole cultivation (Figure 30B). The mono-rhamnolipid production showed a similar trend to the growth of *P. putida* KT2440 $\Delta\Delta$ pDahms2_RL (Figure 30B). Although 1 g L^{-1} xylonate was left at the end of the fermentation, a rhamnolipid titer of 0.65 g L^{-1} was reached. It is

mentioned, that the DO signal and the mono-rhamnolipid concentration of only one fermentation is presented, as a controller issue for the replicate appeared, which caused that the fermentation was controlled manually and that foaming out occurred, resulting in product loss.

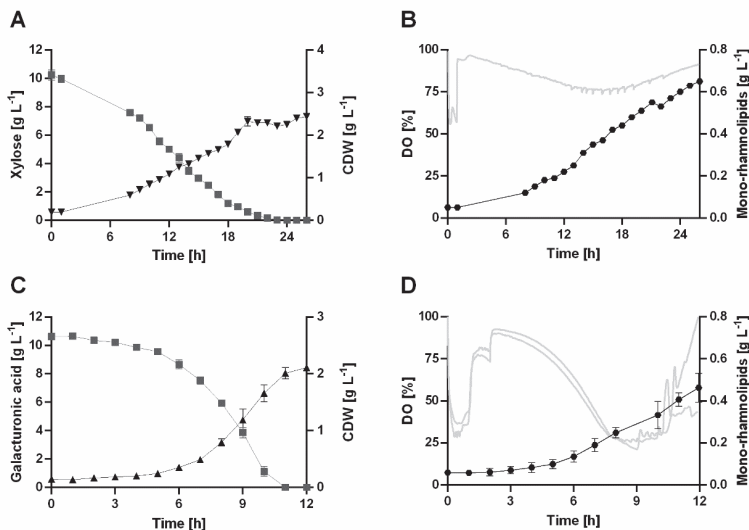


Figure 30: Batch fermentation for mono-rhamnolipid production from xylose or galacturonic acid with recombinant *P. putida* KT2440 strains. Fermentations were performed in a 1 L stirred tank reactor with foam recirculation in modified M9 medium containing 10 g L⁻¹ xylose (A, B) or 10.8 g L⁻¹ galacturonic acid (C, D). Shown are the CDW (▼), xylose or galacturonic acid concentration (■), mono-rhamnolipid concentration (●), and the DO (grey line). The values are given as mean values and error bars represent the maximum and minimum values of two replicates (n=2), except the DO and mono-rhamnolipid concentration using xylose (n=1). Each DO signal of the fermentation using galacturonic acid is shown. The figure was modified and adapted from Tobias Karmainski's Master Thesis [347].

For rhamnolipid production from galacturonic acid, the strain *P. putida* KT2440 GA1_RL was used and fermentation was performed as duplicate using 10.8 g L⁻¹ galacturonic acid. To be able to compare the results, a concentration of 0.33 Cmol L⁻¹ of the substrates was always used. The fermentation was carried out over twelve hours and a biomass concentration of 2.11 g L⁻¹ with a growth rate of 0.40 h⁻¹ was achieved (Figure 30C). Hence, the biomass concentration is lower compared to xylose (Table 11) but the growth rate is significantly higher, matching the obtained results in shake flasks. The DO signal decreased from 90% to 30% within 5.5 hours and after 7.5 hours the agitation speed increased automatically to 1,200 rpm to cope with the high oxygen demand (Figure 30D). Although the substrate was consumed after eleven hours, the strain still produced biomass and rhamnolipids. This is probably caused by the presence of intermediates, which unfortunately could not be measured

as the utilization pathway is not explored for *P. putida* KT2440. The rhamnolipid production had a more linear trend, but at the end of the fermentation a rhamnolipid titer of 0.46 g L^{-1} was reached.

These results indicate that both substrates are suitable to produce rhamnolipids. However, the rhamnolipid fermentations with the two substrates display different characteristics regarding the titer, yield, and space time yield. While a 40% higher maximal titer and a slightly increased product to substrate yield is reached with xylose as substrate, the space time yield is about 50% better for galacturonic acid (Table 11). Further, 17% and 20% of the theoretical product to substrate yields on xylose and galacturonic acid were reached, respectively. The theoretical product to substrate yield on xylose using the Dahms pathway is $0.60 \text{ Cmol Cmol}^{-1}$ and on galacturonic acid $0.36 \text{ Cmol Cmol}^{-1}$.

Table 11: Fermentation characteristics of the recombinant rhamnolipid producers. (CDW; cell dry weight, RL: mono-rhamnolipids, Yield_{ps}: product to substrate yield, STY: space time yield, GalA: galacturonic acid)

Organism	Substrate	CDW	Maximal titer	Yield _{ps}	STY
		(g _{CDW} L ⁻¹)	(g _{RL} L ⁻¹)	(Cmol _{RL} Cmol _s ⁻¹) (g _{RL} g _s ⁻¹)	(g _{RL} L ⁻¹ h ⁻¹)
<i>P. putida</i> KT2440 pDahms2_RL	Xylose	2.44	0.65	0.10	0.025
	(10 g L ⁻¹)			0.07	
<i>P. putida</i> KT2440 GA1_RL	GalA	2.11	0.46	0.07	0.038
	(10.8 g L ⁻¹)			0.04	

Overall, it was shown that the production of rhamnolipids is scalable and that it was possible to control the foam without the usage of antifoam agents or mechanical foam breakers using foam recycling. However, the foam tank was full of foam after the batch phase and further improvements, e.g., the collapsing of foam by a defoamer as presented in 3.2.3.4, are required to enable the conduction of a fed-batch fermentation.

3.4.3.5 Discussion

Taken together, these results demonstrate that xylose and galacturonic acid are suitable substrates for the production of rhamnolipids, also in larger scale. To achieve this, evolved strains, which feature a better substrate utilization, were engineered for heterologous rhamnolipid production. Further, targets were found to improve the growth on xylose (i.e., reducing the lag phase). Also, *P. putida* harbors endogenous genes catalyzing xylose conversion and foreign xylonate dehydratase is solely needed to enable growth on xylose.

Genome re-sequencing of xylose-evolved strains revealed a probable transporter problem, causing the long lag phase in the reference strains. Mutations in *kguT* (encoding 2-ketogluconate transporter) were found in the evolved Weimberg- and Dahms-strains and an

additional mutation in *ptxS* (encoding 2-ketogluconate utilization repressor) was found in the evolved Weimberg-strain. The conversion of glucose to gluconate/2-ketogluconate and xylose to xylonate is similar and happens in the periplasm. Glucose is converted to gluconate by glucose dehydrogenase (Gcd), which is converted to 2-ketogluconate by gluconate dehydrogenase (Gad). Gluconate can then be transported into the cytoplasm via GntT (gluconate transporter) and 2-ketogluconate via KguT [412], whereas flux studies revealed that most of the gluconate is converted to 2-ketogluconate [189]. As shown in 3.1.3.2, xylose is converted to xylonate by the action of Gcd. Thus, it is not surprising that the respective transporters (GntT and KguT) are probably involved in the transport into the cytoplasm. It is conceivable, that GntT undertakes the transport of xylose once *kguT* is deleted. The increased lag phase observed after deletion of the *kgu* operon possibly indicate that KguT has a higher affinity to xylonate than GntT (Figure 26).

Moreover, the growth rate was higher expressing all four Weimberg genes from *P. taiwanensis* than solely expressing the gene encoding the xylonate dehydratase (Figure 27). Recently, it was reported that product inhibition of the dehydrogenases and balancing of the dehydratase activities have a major impact on the Weimberg pathway performance [413]. The action of the xylonate dehydratase and aldehyde dehydrogenase were shown to be the rate-controlling steps [413,414]. Hence, the minimization of the expression of heterologous genes indeed could have led to the reduction of the growth rate because the additional aldehyde dehydrogenase was missing. This could potentially also explain the linear growth of the strain using the Dahms pathway due to an imbalance of the activities (Figure 5, Figure 30).

P. putida KT2440 was evolved on galacturonic acid and showed an improved growth rate and slightly decreased lag phase, but no (obvious) target could be identified after genome-resequencing, which probably improved the features. Beside mutations in genes coding for metabolic enzymes [415], which easily and clearly can be allocated to the improved phenotype, also transcriptomic re-organization can occur [416]. Hence, the expression level possibly could be altered due to adaptation.

Due to the improvements, xylose and galacturonic acid could be used to produce rhamnolipids in bioreactor scale. A product to substrate yield of 0.07 and 0.04 g_{RL} g_S⁻¹ for xylose and galacturonic acid was reached, respectively. Different studies report a product to substrate yield of 0.05 and 0.09 g_{RL} g⁻¹ for glucose using recombinant *P. putida* KT2440 [352,353]. The yields are in a similar range and these substrates could thus be used as alternative substrates for the production of rhamnolipids. Through the use of xylose and galacturonic acid, side or waste streams such as sugar beet pulp [417], wheat straw [418], or corn stover [419] could be implemented, contributing to a sustainable industry. However, the

theoretical yield on galacturonic acid is rather low ($0.36 \text{ Cmol Cmol}^{-1}$), which points to using substrate mixtures in a production process, e.g., in combination with glucose (as already presented, Figure 29) or other renewable carbon sources with theoretically high yields.

In summary, we report the efficient utilization of xylose and galacturonic acid for rhamnolipid production. Further, attempts were made to reduce the metabolic load by changing the expression system and re-engineer superior phenotypes. This study serves as a basis for the implementation of side and waste streams for sustainable rhamnolipid production.

Chapter 4

General discussion and outlook

Contributions:

This chapter was written by Isabel Bator and reviewed by Lars M. Blank.

4 General discussion and outlook

4.1 Microbial production of valuable products

The atmospheric concentration of greenhouse gases, such as CO₂, extremely increased due to human activities since the industrial revolution. Most of the emissions are due to burning fossil fuels, altered land use, and forestry [420]. To mitigate the resulting climate change, countries around the globe agreed in the Paris Agreement to limit the average global warming to 2°C above pre-industrial levels until 2100 by changing human activities [421]. A transition from a fossil-based to a bio-based industry would be required to reach this goal, which is a major challenge for our society. In this context, the production of chemicals from renewable substrates using microorganisms is promising [422].

A lot of research has been done in recent years to advance microbial production and develop economically viable processes. The microbial synthesis of many different products, such as aromatic compounds [423], organic acids [424], biopolymers [425], and biosurfactants [33], was reported. These products can be applied and used to potentially replace petroleum-derived products, for example, in the food and feed industry, therapeutics, cosmetics, and as precursors for bulk chemicals or fuels. For the synthesis, eukaryotic and prokaryotic organisms are used [36,173,426,427], each having different native advantages for the respective production process. However, most studies only deal with the demonstration of the synthesis in small scale (shake flasks) and many issues arise during scale-up [428]. Often, no established industrial processes exist because the scale-up is laborious and the processes are simply too expensive compared to petroleum-based production. The high process costs are mainly due to expensive feedstocks, the low performance of the microbes, and expensive downstream process operations [17,424,428,429].

Since surfactants can be found in many everyday products, they are an ideal product to strive for more sustainable production for the purpose of the environment. Biosurfactants were considered to replace chemical surfactants, such as carboxylates and sulphonates [430]. They can be divided into different classes, including glycolipids [33], one of the most studied biosurfactants. Rhamnolipids belong to the glycolipids, as they are comprised of a sugar moiety and a fatty acid moiety. The US Environmental Protection Agency already approved rhamnolipids as an environmentally friendly solution for the use in biofungicides [61].

Various bacteria were reported to be native rhamnolipid producers. However, most reported species belong to the genera *Pseudomonas* and *Burkholderia*, among which pathogenic species exist [112]. Rhamnolipid production by *P. aeruginosa* is studied in detail and high titers are reported [65]. However, the production of rhamnolipids with *P. aeruginosa*

is challenging in several ways. Rhamnolipid production is regulated by quorum sensing in this host, complicating the isolation of hyperproducing strains because the expression of rhamnolipid genes is induced at high cell densities by signal molecules [431]. Moreover, the highest titers are reached using plant oils as substrates [113,331]. Both aspects increase the effort and costs in fermentation and downstream processing. Besides, *P. aeruginosa* is an opportunistic pathogen, which complicates the permission of production processes due to the health risk [112]. Hence, to elude the challenges, the heterologous rhamnolipid production with a non-pathogenic host is a preferred alternative.

The microbial production of surfactants was addressed in this thesis. Specifically, this thesis focused on rhamnolipid production with the non-pathogenic bacterium *P. putida* KT2440. The use of renewable substrates for cost-reduced rhamnolipid production was one of the main aspects.

4.2 Up-scaling of biosurfactant production processes

In order to develop a viable production process, the knowledge and insights gained in small scale processes have to be transferred to large scale processes. Many parameters influence the productivity of a biotechnological process, such as pH, temperature, medium components, and oxygen supply [432]. Moreover, general challenges during scale-up have to be addressed, such as the duration of the production phase and the presence of inhibitory by-products [428]. Apart from the general microbial fermentation challenges, biosurfactant fermentations potentially have high purification costs and extensive foaming as further challenges. In the case of biosurfactants that are classified as heavy oils (e.g., sophorolipids), these challenges do not exist. In this case, a second product-containing phase is formed due to the low solubility and high concentrations, which is heavier than the fermentation broth and settles quickly after turning off the stirrer [433]. With an integrated sophorolipid production and gravity separation technique, the biosurfactants can directly be separated from the fermentation broth with recoveries of almost 90% [434]. In contrast, excessive foaming is observed in an aerated and agitated system for rhamnolipid production. The impact of foaming is described in detail in 3.2.2. The consequences require the development of alternative strategies to control the foaming.

As bioethanol is already available as renewable substrate and the main goal of this thesis was sustainable production, ethanol was used for rhamnolipid production. By exploiting the defoaming property of ethanol, a novel fermentation process was developed. The setup allowed the use of ethanol as carbon source and defoamer, enabling that foaming was controllable and rhamnolipids and HAAs were produced with a relatively high space time yield of $0.23 \text{ g L}^{-1} \text{ h}^{-1}$.

Various aspects can be considered to improve the process further. The carbon/nitrogen ratio has a major impact on production and carbon/nitrogen ratios of around 20 were reported to be best suited [135,136]. Here, nitrogen (NH_4OH) was supplied via automatic addition as base to control the pH, resulting in an excess of nitrogen. This probably led to increased growth and decreased production, thus striving for a decoupled feeding using a different base (e.g., KOH) and an optimized carbon and nitrogen feeding strategy. Moreover, the process could be divided into two phases, growth and production phase, by altering the medium composition after a certain biomass concentration was reached.

The loss of biomass is also a critical parameter, as rhamnolipid producers stick to the foam and remain in the foam tank until the collapsed foam is pumped back into the reactor, probably reducing the production efficiency. To circumvent this problem, either filter systems [435] or immobilized cell techniques [436] could be used to retain the cells in the fermentation broth. However, filter systems tend to block and backwashing has to be integrated.

Thus, the here presented results serve as a starting point. The setup could potentially be applied in combination with other strategies/methods to achieve sustainable and cost-effective production on an industrial scale.

4.3 *Pseudomonas putida* as production host

P. putida KT2440 is a suitable host for the application in biotechnological processes [155,166]. Its distinct tolerance to oxidative stress and other toxic compounds facilitates the use for redox-intensive reactions and processes, where conventionally used hosts would fail [163,191]. Further, the easy handling, fast growth, and versatile metabolism make the use promising. Not only the strain characteristics itself contribute to the adaptation of *P. putida* in industry, but also the availability of many tools for genetic engineering. This allows the modification of the strain towards the needs with almost no limits. Thereby, the strain is featured with new properties, enabling the use of novel substrates, such as cellobiose or arabinose [255,311], and synthesis of products of choice [437]. In this regard, the creation of tailor-made microorganisms was brought into play to maximize the performance of cells. To realize this, one approach is the streamlining of a chassis [438]. The reduction of the genome of pseudomonads resulted in an improved heterologous gene expression [357] and aromatics production [227]. The deletion of energy- and carbon-consuming molecules and structures was targeted in this thesis. Several deletions increased the rhamnolipid titer and yield, which shows the potential of this strategy.

Considering the use of *P. putida* KT2440 for rhamnolipid production, the strain is an ideal candidate. First, *P. putida* KT2440 can withstand high rhamnolipid concentrations of up

to 90 g L⁻¹, while other conventionally used hosts were significantly affected [147]. Second, the strain belongs to the family of the pseudomonadaceae, which also includes native rhamnolipid producers. Hence, the transferability is considered to be feasible. Specifically, pseudomonads harbor pathways for the synthesis of the precursors dTDP-rhamnose and fatty acids. As a result, only two additional genes, namely the rhamnolipid genes *rhlA* and *rhlB*, have to be introduced for mono-rhamnolipid production, keeping the metabolic burden and efforts for efficient expression small. However, effort for efficient gene expression should not be neglected. Various studies deal with rhamnolipid production using *P. putida* KT2440 by utilizing plasmids as expression system [147,150,152,352]. Plasmids can be lost during prolonged cultivation and have a negative impact on bacterial fitness [439]. Further, reproducibility can be impaired by varying plasmid copy numbers. Therefore in this thesis, the rhamnolipid genes were chromosomally integrated and under the control of a very strong, synthetic promoter [219], as very high expression is needed for increased production. Third, the expression of heterologous rhamnolipid genes results in production, which is uncoupled from growth [147]. This is advantageous as no regulatory issues arise compared to the use of the wild-type producer *P. aeruginosa* and resources can be optimally used for rhamnolipid production.

Overall, the application of *P. putida* KT2440 for rhamnolipid production is beneficial. By genetic engineering of the host, chassis for efficient rhamnolipid production from various renewable substrates can be designed, which was outlined in this thesis. Further, efficient production was achieved in minimal medium, preventing the use of costly complex medium.

4.4 Metabolic engineering for improved production

In order to establish efficient bioprocesses, the microbes have to perform close to the theoretical maximum regarding the titer, yield, and rate. To achieve this, microorganisms can be genetically engineered. One promising strategy is metabolic engineering, where fluxes are redirected in order to increase production rates and decrease by-product formation [356].

In the case of rhamnolipid production, the synthesis competes with other cellular pathways and compartments for the precursors. Fatty acids and dTDP-rhamnose are primarily needed for the formation of the membrane and LPS. Therefore, the increased production of rhamnolipids could impair essential cellular functions and the availability is thus a limiting factor. The increased synthesis of molecules needed for fatty acid metabolism might maintain cellular functions and lead to increased rhamnolipid production in addition. Overexpression of pyruvate dehydrogenase to yield more acetyl-CoA or acetyl-CoA carboxylase to yield more malonyl-CoA is thus a potential target.

The overexpression of one subunit of the pyruvate dehydrogenase resulted in a threefold increase of the HAA titer compared to the control strain carrying an empty plasmid in one study [440]. The overexpression of acetyl-CoA carboxylase in *E. coli* resulted in an increase in the intracellular level of malonyl-CoA and increased polyketide synthesis in *S. cerevisiae* [441,442]. Hence, the overexpression of these two targets could probably further increase the titer of the best rhamnolipid producer of this thesis. Another strategy could be the decrease of the flux through the TCA cycle, thereby increasing the malonyl-CoA pool and flux through fatty acid *de novo* synthesis indirectly. This strategy was demonstrated in *C. glutamicum* for polyphenol synthesis, where the native promoter of the pace-making enzyme citrate synthase was replaced by a weaker promoter, catalyzing the condensation reaction of acetyl-CoA and oxaloacetate to citrate [443].

Various hosts were engineered for heterologous production of rhamnolipids, however the concentration is relatively low compared to the rhamnolipid concentrations reached with the native rhamnolipid producer *P. aeruginosa* [149,444]. One study showed that precursor supply is a bottleneck and overproduction of dTDP-rhamnose resulted in an increased rhamnolipid titer in *E. coli* [445]. The improved supply with the precursor dTDP-rhamnose was also targeted in this thesis and higher rhamnolipid concentrations were measured.

However, the biggest improvement of rhamnolipid production was observed for the genome reduced chassis, where deletion of clusters for the synthesis of pyoverdines, flagellar, alginate, and cellulose was performed. The multiple deletions in one strain did not increase the titer further due to presumed bottlenecks in the precursor supply. Nevertheless, the engineered multi-deletion strains GR18 and GR41 could be used for other purposes. These strains feature a streamlined background with reduced by-product formation and could be applied as basis for the production of other valuable products. Further, they can be applied in approaches, where a reduced cell surface hydrophobicity is needed because several cell surface structures and proteins were deleted [446].

4.5 Utilization of alternative feedstocks

Implementation of biotechnological processes in the industry is challenging because the processes are not competitive with petroleum-based processes. The raw material costs have a major impact on the total costs. Hence, attempts are made to reduce costs by using cheap and renewable substrates. In this thesis, the use of lignocellulose-derived substrates (xylose and galacturonic acid) and ethanol for rhamnolipid production was investigated.

P. putida KT2440 has a versatile metabolism and can utilize various compounds, including glucose. Glucose is a monosaccharide that is preferred for microbial conversion because it is the most abundant sugar in nature and therefore cheap. It can be obtained by

processing sugarcane or crop. The metabolization is well studied in *P. putida* and high rhamnolipid titers can be achieved when using it [187,353]. However, the use in biotechnology competes with the food supply of humans. This fuels the food versus fuel debate and with a growing world population, the situation will get worse and food supply should be prioritized [9]. By utilization of further lignocellulosic fractions, the situation can be relaxed.

Hemicellulose is a promising candidate, as it contains several sugars, including pentoses and hexoses, such as xylose, arabinose, glucose, mannose, and galactose [12]. Xylose is the second abundant sugar and dependent on the source the xylose fraction can be around 25% [281]. The inclusion of alternative resources would thus bring more flexibility. *P. putida* KT2440 was already engineered for the utilization of xylose [255], arabinose [311], and galactose [447]. While these studies focused on implementing alternative pathways, the use of alternative feedstocks for the synthesis of valuable products was also demonstrated in this thesis.

The growth rate on galactose was significantly reduced compared to glucose, which was also the case for xylose [255,447]. However, in the study from Wang *et al.* [311], the growth rate on xylose and arabinose was comparable to glucose, which is surprising since other works with *P. putida* S12 also showed a decreased growth rate on xylose and arabinose compared to glucose [302]. The implementation of arabinose utilization was not successful in this thesis. Hence, two conclusions can be drawn, which strive for further research in the future. First, glucose is probably the preferred substrate for *P. putida* KT2440, when considering lignocellulose-derived substrates. As glucose can also be obtained from non-edible fractions, such as corn stover and wheat straw, the use of a mixture of glucose and alternative substrates is a suitable strategy. This would lead to a faster growth rate in the beginning and a higher production rate, as demonstrated in 3.4.3.2, where glucose and galacturonic acid were used simultaneously for rhamnolipid production, possibly resulting in a higher space time yield. Further, the major constituents of lignocellulose would be utilized for rhamnolipid production. Besides, dependent on the substrate and its degree of reduction, the product yield would be increased. This was also demonstrated for the simultaneous use of galacturonic acid and glucose in this thesis. The rhamnolipid titer increased and hence the yield when using both substrates compared to galacturonic acid as sole carbon source because the theoretical yield for galacturonic acid is rather low. This strategy was also applied in a study, where the highest theoretical poly- γ -glutamate yield was calculated for a substrate mixture of 30% glucose and 70% xylose [448]. Second, the phosphorylative pathways for utilization of pentoses seem to be unfavorable for *P. putida*, which was discussed in 3.1.4 in

detail. Hence, implementing an oxidative pathway for arabinose might result in efficient utilization, corresponding to the oxidative xylose pathways shown in 3.1.3.2. By using different utilization pathways, product yields can be theoretically optimized since the pathways have different stoichiometries, which was confirmed by producing pyocyanin and rhamnolipids with three different xylose pathways (3.1).

Thus, enabling *P. putida* to use these substrates will valorize waste streams. However, not only waste streams containing lignocellulosic biomass can be used, but also a by-product of biodiesel production and plastic waste. Glycerol is the main by-product of biodiesel production and produced in high amounts (1 kg per 9 kg biodiesel) [449], which have led to low prices. *P. putida* KT2440 is able to use glycerol natively, but a long lag phase with metabolically inactive cells is observed. The deletion of the GlpR regulator, which regulates the *glp* gene cluster, eliminated the long lag phase [450], thereby improving the space time yield for production processes. Crude glycerol contains impurities as fatty acids and methanol [451], which could impact the performance of the host. To be able to use this fraction, either the host has to be robust or crude glycerol has to be purified, which could increase the total costs. However, *P. putida* KT2440 is considered to be a suitable host due to the distinct stress tolerance. This was shown for *P. putida* S12, which performed better and produced more *p*-hydroxybenzoate on crude glycerol, whereas *E. coli* showed severe growth defects [452]. Production of valuable products, including rhamnolipids, from (crude) glycerol was already shown [150,175,453]. Hence, the utilization of crude glycerol for rhamnolipid production using *P. putida* KT2440 could be tested in the future.

One major issue of modern society is immense plastic pollution. To cope with this situation, microbes can be used to utilize the degradation products of plastic waste. This can be combined with the immediate conversion into value-added bioplastics, called bio(technological) upcycling [454,455]. Polyethylene terephthalate can be enzymatically degraded into terephthalic acid and ethylene glycol by the action of a PETase and MHETase [456]. These monomers can be utilized by *P. putida* GO16 and *P. putida* KT2440 (ethylene glycol) [457]. However, a long lag phase is observed for both strains on ethylene glycol, which can be reduced by ALE or deletion of the GclR regulator, which normally represses the glyoxylate carboligase pathway [254,454].

Overall, many alternative substrates are on hand that could be investigated for production of valuable products using *P. putida* KT2440. Nevertheless, the ideal substrate and composition of the carbon source have to be identified for each process and from an economic perspective, for each location (again price, price fluctuation, availability etc.), considering

stoichiometries, to reach the highest possible product yield and subsequently the highest margin.

4.6 Conclusion and outlook

In this thesis, the focus was on the production of rhamnolipids from renewable substrates with the non-pathogenic *P. putida* KT2440. Strains were successfully engineered to utilize xylose via three different bacterial pathways. Xylose is one of the most abundant sugars in nature and present in lignocellulose. Using the non-edible lignocellulosic biomass would contribute to a sustainable bioeconomy. Initially, only two pathways – Weimberg and Dahms – worked in our hands well and enabled efficient growth and consequently efficient production of secondary metabolites. However, we applied a further optimized strain using the Isomerase pathway and showed the production of secondary metabolites. The introduced xylose pathways have different stoichiometries, leading to different product yields for the same product. Hence, the various engineered strains can be used for different purposes to reach the highest product yield, contributing to process efficiency and cost reduction.

Moreover, galacturonic acid and ethanol were used as alternative sustainable carbon and energy sources for rhamnolipid production. The growth and rhamnolipid production rates were improved through laboratory evolution, demonstrating the potential of non-rational engineering strategies. In addition, a unique fed-batch process was developed using ethanol without the need for additional mechanical or chemical foam disruption, highlighting the freedom of developing new strategies to achieve scale-up.

Furthermore, *P. putida* KT2440 was genetically modified to reduce the metabolic load by deletion of energy- and carbon-wasting compounds and structures. Thereby, resources were freed up, which resulted in improved rhamnolipid production, enhancing the titer and yield, although no significant improvement of the growth performance was observed. By additional enhancement of the precursor supply, the yield was even further increased. Hence, the strategy of streamlined chassis and metabolic engineering was outlined and demonstrated to be a suitable strategy for improved process efficiency. These engineered strains can theoretically be applied for the establishment of other biotechnological processes, where *P. putida* KT2440 is used as production host, as the engineering targets are generic and not restricted to rhamnolipid synthesis.

The here presented results contribute to the sustainable production of valuable products. This is inevitable as climate change is progressing through the steady rise in using fossil resources. By combining the single disciplines of chassis engineering and the use of renewable substrates, the economic viability of biotechnological processes possibly can be further improved.

Appendix

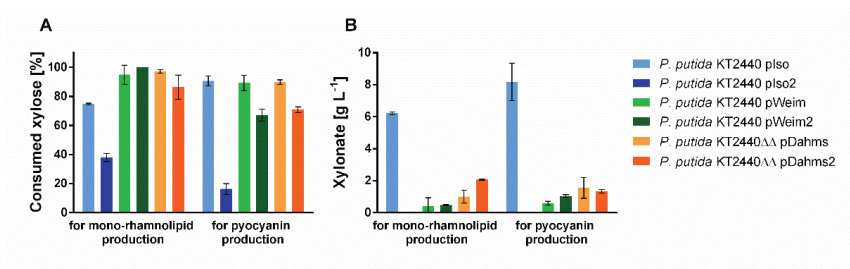


Figure A 1: Consumed xylose and formed xylonate by xylose utilizing *P. putida* strains during production of mono-rhamnolipids or pyocyanin. (A) Consumed xylose at the end of the cultivation and (B) formed xylonate, which was a leftover at the end of the cultivations of rhamnolipid and phenazine production strains. Error bars indicate standard deviation from the mean (n=3).

Table A 1: Identified mutations after genome re-sequencing of *P. putida* KT2440 E1 and *P. putida* KT2440 E1.1

Strain	Region	Position	Mutation	Outcome
<i>P. putida</i> KT2440 E1	PP_0441 (<i>secE</i>)	534,281	GAC (T) → GGC (A)	Missense mutation
<i>P. putida</i> KT2440 E1.1	PP_2682 (<i>viaY</i>)	3,071,601	CAG (Q) → TAG (Stop)	Nonsense mutation
<i>P. putida</i> KT2440 E1	PP_2682 (<i>viaY</i>)	3,072,054	CGT (A) → TGT (C)	Missense mutation
<i>P. putida</i> KT2440 E1.1	PP_4373 (<i>fleQ</i>)	4,964,766	A → ACGGCGCTTCG	Frameshift
<i>P. putida</i> KT2440 E1	PP_4373 (<i>fleQ</i>)	4,964,949	CAG (Q) → TAG (Stop)	Nonsense mutation
<i>P. putida</i> KT2440 E1.1	intergenic region upstream of PP_3839 (<i>adhP</i>)	4,362,872	C → T	Predicted -10 element affected

P. putida KT2440

> test sequence:

```
CCAGCCAGGCATACGCAGGAATTTTGTGGAAGCGCCACCTTGACCATGACCTGAAGCAGTTTTGCCTGGGCGGCAAGGT
CTATGCTATCCGACGATCACCAAGCTCACATCGGATAGACACGGAGGCTCTC
Length of sequence-      135
Threshold for promoters - 0.20
Number of predicted promoters -      1
Promoter Pos:      95 LDF-      2.28
-10 box at pos.      80 GTCTATGCT Score      52
-35 box at pos.      63 TTGCCT Score      56
```

P. putida KT2440 E1.1

> test sequence:

```
CCAGCCAGGCATACGCAGGAATTTTGTGGAAGCGCCACCTTGACCATGACCTGAAGCAGTTTTGCCTGGGCGGCAAGGT
CTATGCTATCCTGACGATCACCAAGCTCACATCGGATAGACACGGAGGCTCTC
Length of sequence-      135
Threshold for promoters - 0.20
Number of predicted promoters -      1
Promoter Pos:     100 LDF-      2.66
-10 box at pos.      80 TGCTATCCT Score      60
-35 box at pos.      63 TTGCCT Score      56
```

Figure A 2: Predicted promoter region upstream of *adhP* using the Softberry BPROM tool [346]. Shown is the alteration of the -10 element based on the introduced mutation (bold, red) in the evolved strain *P. putida* KT2440 E1.1 compared to the reference (*P. putida* KT2440).

Table A 2: Identified mutations after genome re-sequencing of *P. putida* KT2440 pWeim2 and *P. putida* KT2440ΔΔ pDahms2. The genes are located on the - strand, but the complementary bases are identified as mutations in the genome sequence (+ strand).

Strain	Region	Position	Mutation	Outcome
<i>P. putida</i> KT2440 pWeim2	PP_1650 (<i>gacS</i>)	1842970	TTGGCAG → T (+)	Deletion
<i>P. putida</i> KT2440ΔΔ pDahms2	PP_1650 (<i>gacS</i>)	1843829	GTC → GGC (+) GAC (D) → GCC (A) (-)	Missense mutation
<i>P. putida</i> KT2440 pWeim2	PP_3377 (<i>kguT</i>)	3822834	AGC → ACC (+) GCT (A) → GGT (G) (-)	Missense mutation
<i>P. putida</i> KT2440ΔΔ pDahms2	PP_3377 (<i>kguT</i>)	3823095	GGC → GAC (+) GCC (A) → GTC (V) (-)	Missense mutation
<i>P. putida</i> KT2440 pWeim2	PP_3380 (<i>ptxS</i>)	3825866	CTG → CTGGTG (+) CAG → CACCAG (+H) (-)	Insertion

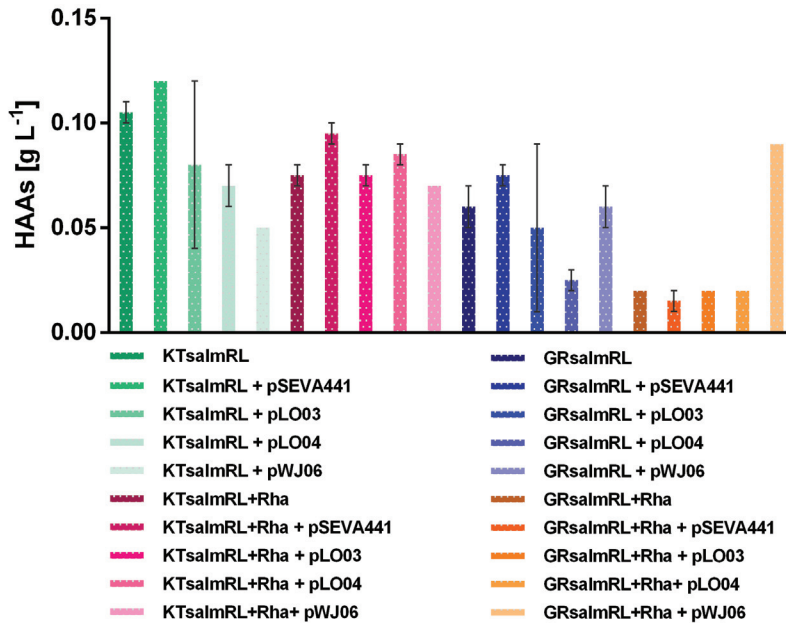


Figure A 3: Impact of overexpression of genes involved in dTDP-rhamnose supply on HAA production by *P. putida* KT2440 derivatives. Cultivation was performed in M9 minimal medium containing 10 g L⁻¹ glucose in 24-deep well plates. The values are given as mean values and error bars represent the maximum and minimum values of two replicates (n=2). The raw data associated to this figure were taken from Marie Dielentheis-Frenkens' Internship [257].

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Curriculum vitae

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Education

2017 - 2020	PhD student at the Institute of Applied Microbiology, RWTH Aachen University, Aachen, Germany
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Grants

2019	MSE (Molecular Science and Engineering) Grant to visit the Biochemical and Molecular Engineering XXI conference in Mont Tremblant, Quebec, Canada.
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Publications

Blesken, C. C., **Bator, I.**, Eberlein, C., Heipieper, H. J., Tiso, T., and Blank, L. M. (2020) Genetic cell-surface modification for optimized foam fractionation. *Frontiers in Bioengineering and Biotechnology*, 8, 1-16, <https://doi.org/10.3389/fbioe.2020.572892>

Kubicki, S., **Bator, I.**, Jankowski, S., Schipper, K., Tiso, T., Feldbrügge, M., Blank, L. M., Thies, S., and Jaeger, K.-E. (2020) A straightforward assay for screening and quantification of biosurfactants in microbial culture supernatants. *Frontiers in Bioengineering and Biotechnology*, 8, 1-11, <https://doi.org/10.3389/fbioe.2020.00958>

Tiso, T., Ihling, N., Kubicki, S., Biselli, A., Schonhoff, A., **Bator, I.**, Thies, S., Karmainski, T., Kruth, S., Willenbrink, A.-L., Loeschcke, A., Zapp, P., Jupke, A., Jaeger, K.-E., Büchs, J., and Blank, L. M. (2020) Integration of genetic and process engineering for optimized rhamnolipid production using *Pseudomonas putida*. *Frontiers in Bioengineering and Biotechnology*, 8, 1-24, <https://doi.org/10.3389/fbioe.2020.00976>

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Hosseinpour Tehrani, H., Becker, J., **Bator, I.**, Saur, K., Meyer, S., Rodrigues Lóia, A. C., Blank, L. M., and Wierckx, N. (2019) Integrated strain- and process design enable production of 220 g L⁻¹ itaconic acid with *Ustilago maydis*. *Biotechnology for Biofuels*, 12, 1-11, <https://doi.org/10.1186/s13068-019-1605-6>

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Bongartz, P., **Bator, I.**, Baitalow, K., Keller, R., Tiso, T., Blank, L. M., and Wessling, M. (2020) A scalable bubble-free membrane aerator for biosurfactant production. *Bioresource Technology* (in preparation)

Poster presentations

Bator, I., Karmainski, T., Tiso, T. and Blank, L. M. (February 17-19, 2020) Characterization of rhamnolipid production from renewable substrates with recombinant *Pseudomonas putida* strains in bioreactor lab-scale. BioProcessingDays 2020. Recklinghausen, Germany.

Kubicki, S., **Bator, I.**, Tiso, T., Thies, S., Blank, L. M. and Jaeger K.E. (November 18-19, 2019) Construction of recombinant cell factories for biosurfactant production. BioSC Symposium & Forum. Cologne, Germany.

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Bator, I., Böhne, H. M., Tiso, T., Blank, L. M. (October 24-26, 2018) Insertion of synthetic xylose-uptake pathways in *P. putida* for carbon efficient synthesis of secondary metabolites. 4th Applied Synthetic Biology in Europe. Toulouse, France.

Oral presentations

Bator, I. and Kubicki, S. (February 5, 2020) Construction of bacterial cell factories for heterologous rhamnolipid production. 7th BioSC Spotlight – Modular biotransformations in a circular bioeconomy. Aachen, Germany.

- 23 more contributions as co-author to conference presentations (poster and oral)