1 General introduction

1.1 The bio-economy is an attractive alternative to the petrochemical-based industry

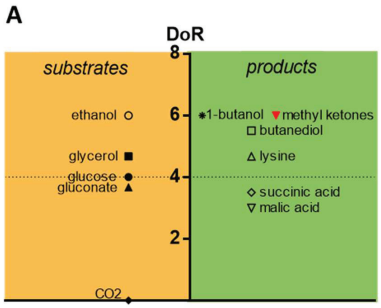
In the 20th century, the economy was dominated by the petrochemical industry. In recent years, an increased awareness rose that the world must deal with global challenges: limited resources, climate change, and ecosystem degradations. The awareness opens possibilities for biotechnology, which contributes to the change towards a more sustainable bio-based economy. The so-called bio-economy aims to convert renewable resources, waste streams, such as food waste or non-food biomass, into value-added products and bioenergy. One prominent biotechnological example is the production of amino acids, such as L-lysin with *C. glutamicum* (Leuchtenberger et al., 2005). An example from the bioenergy sector is the production of biofuel. The biochemical companies LanzaTech and Gevo, Inc. produce ethanol and isobutanol from corn-sugar with engineered yeast strain (Geleynse et al., 2018), respectively. In December 2019, the US airline Delta signed a contract with the biofuel-company Gevo Inc. to purchase 10 million gallons of sustainable aviation fuel (SAF) per year (Wolfsteller, 2019/12/17).

1.2 Challenges of the production of reduced compounds

Fuels are low-value-added products required at high quantities. The cost-effective production of bio-based products essentially challenges the petrochemical industry. Hence, any carbon substrate used requires a close to theoretical conversion to the product, including efficient re-routing of the carbon flow and reducing power from the substrate to the product.

The chosen microorganism acting as the biocatalyst converts the substrate (e.g., glucose) to the product, either reduced or oxidized relative to the substrate. It is very challenging for the biocatalyst if the target product is higher reduced than the substrate, additional reducing power is needed and can limit the maximum possible yield (Vickers et al., 2012) (Figure 1B). For a first estimation on can enumerate the degree of reduction (DoR) (Figure 1A), which describes the number of available electrons, and thereby the oxidative or reductive state

(Stephanopoulos et al., 1998). The nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) are redox cofactors, which play a vital role in metabolism, as they are ferrying reducing equivalents between redox reactions (Blacker and Duchen, 2016; Blank et al., 2010). The redox cofactors are involved in many biochemical reactions to sustain metabolism (Chen et al., 2014). The elementary role of cofactors emphasizes and underlines the difficulty to produce reduced products as additional reduced redox cofactors are needed.



B

$$C_aH_bO_cN_dS_eP_f$$

With C +4; H +1; O -2; N -3; S +6; P +5

$$DoR = \frac{4a + 1b - 2c - 3d + 6e + 5f}{a}$$

Figure 1. Degree of reduction of industrial attractive substrates and products (A) and the formula to calculate the degree of reduction (B)

The oxidized forms, NAD^+ and $NADP^+$, can receive electrons by adding a hydride ion to produce the reduced form NADH and NADPH. The cofactor $NADP^+$ functions mainly as an electron donor in anabolic pathways, whereas NAD^+ acts as an electron acceptor in catabolic pathways. NADH can fuel the electron transport chain and ultimately the oxidative phosphorylation of ADP to ATP through dehydrogenases, by that NADH is re-oxidized to NAD^+ (Sauer et al., 2004). The redox cofactors pool is tightly regulated and balanced in the cell (Blank et al., 2010) (Blacker and Duchen, 2016). *Pseudomonas* and other species, e.g. *E. coli,* possess pyridine nucleotide transhydrogenase, which catalyzes the reversible interconversion of NADH and NADPH (Sauer et al., 2004). Transhydrogenase activities fine-tune and balances redox cofactors, crucial for optimal biotransformation productivity (Zobel et al., 2017). For this reason, transhydrogenases are used as a metabolic engineering tool for redox cofactor manipulation (Sánchez et al., 2006). *Pseudomonas* species show high maximal NAD(P)H

regeneration rates under conditions where electrons, e.g. for transport processes are in demand. This phenomenon culminated in the driven-by-demand concept highlighting that the operation of the metabolic network depends strongly on the energetic requirements under a certain growth condition (Blank et al., 2008b) and hence that increased redox or energy demand should in principal lead to higher carbon substrate uptake (Tiso et al., 2016). The group of Peter R. Jensen from the DtU in Lyngby developed an ATPase that converts ATP to ADP without any proton transport, thereby generating strong energetic demand (Jensen et al., 1995; Koebmann et al., 2002). High carbon uptake is the consequence in a variety of organism and if properly aligned with ATP synthesis during product formation, results in high product formation (Friedrich and Scheide, 2000; Zahoor et al., 2020).

1.3 Strategy design to overcome redox limitation

Redox cofactors play a critical role in maintaining the intracellular redox homeostasis, which is necessary for robust growth and metabolism. A balanced NADH/NAD+ generally sustains the redox homeostasis and NADPH/$NADP^+$ ratio, fine-tuned by a complex reaction network (Liu et al., 2018). Redox cofactor-depending modifications or new pathways can introduce a redox imbalance, resulting in stress, decreased performance, or damage to the engineered production host (Chen et al., 2014). Engineering a production host for the production of especially a reduced product should consider this redox homeostasis. In recent years many cofactor strategies were developed to achieve an overall redox balance and a good production performance.

The ***deletion of cofactor-competitive pathways*** is one possibility to improve titer and yield. For example, NADH-dependent reactions involved in the production of side-products lactic acid, ethanol under anaerobic conditions are often targets for deletion (e.g., *adhE*, *ldhA*), which supported, for example, the production of propanediol, butyric acid, succinate, and methyl ketones in *Escherichia coli* (Berríos-Rivera et al., 2003; Lim et al., 2013; Park et al., 2012; Sánchez et al., 2005). The ***replacement of cofactor preferring enzymes*** by substituting a cofactor-dependent enzyme that prefers another cofactor is also established in metabolic engineering. For example, in *E. coli,* the NAD-dependent glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) was replaced against an NADP-dependent GAPDH from *Clostridium acetobutylicum* to increase the NADPH yield to drive the production of ε-caprolactone (Martínez et al., 2008). In another study, the fermentation of D-xylose to ethanol in *S. cerevisiae* was increased by supplementing the NADP(H)-dependent GAPD from *Kluyveromyces lactis* (Verho et al., 2003). Another example to mitigate the cofactor imbalance during methyl ketone production in *E. coli* was addressed with a heterologous expressed NADH-dependent FabG instead of an NADPH-dependent version (Goh et al., 2018). These studies show that the experimental implementation of such cofactor "swaps" is feasible and can promise increases in product yield. Alternatively, the ***modification of cofactor preferences*** of enzymes can be changed by site-directed mutagenesis from NADH to NADPH or vice versa. For example, the cofactor specificity of NADH-dependent 1,3-propandiol oxidoreductase was relaxed for both NADH and NADPH by introducing one mutation in *Klebsiella pneumoniae* (Ma et al., 2010). A constraint-based model for *E. coli* and *S. cerevisiae* was developed for optimal cofactor-specificity swaps to increase theoretical yield (King and Feist, 2014).

Another strategy for engineering redox homeostasis is the ***introduction of heterologous cofactor generation reactions***. One prominent example is the heterologous expression of NAD^+-dependent formate dehydrogenase (FDH, EC 1.2.1.2) of the yeast *Candida boidinii*. FDH catalyzes the practically irreversible oxidation of formate to CO_2 and the simultaneous reduction of NAD^+ to NADH. The NADH availability can be increased by supplementing the media or feeding formate (Berríos-Rivera et al., 2002). *Pseudomonas putida* KT2440 is endowed with a native NAD^+- dependent FDH. Co-feeding experiments with glucose and formate showed that *P. putida* KT2440 was able to drastically increase the NADH regeneration rate leading to an increased biomass yield on glucose. Additionally, it was observed that the increased NADH regeneration rate highly exceeded cellular needs in the absence of an NADH consuming redox biocatalytic reaction. Zobel et al. argued that this surplus of NADH formation could be beneficial for redox biocatalysis (Zobel et al., 2017). Another used cofactor-regeneration is the water-forming NADH-oxidase (NOX), which oxidized NADH to NAD^+ with concomitant reduction of oxygen (Riebel et al., 2002). NOX was expressed, for example, in *S. cerevisiae*, which resulted in a decreased NADH/NAD+ ratio, thus an increase in the formation of oxidized metabolites and allows regulation of by-products (Heux et al., 2006) (Kim and

Hahn, 2015; Vemuri et al., 2007). The ***reversible transformation of NAD(H) to NADP(H)*** is catalyzed by transhydrogenase to maintain redox homeostasis. There are two types of transhydrogenase: the energy-independent soluble pyridine nucleotide transhydrogenase (STH, UdhA) and the membrane-bound energy-dependent pyridine nucleotide transhydrogenase (PntAB) (Cao et al., 2011). The overexpression of *pntAB* improved, for example L-lysin production in *C. glutamicum* and 3-hydroxy propionic acid production in *E. coli* (Kabus et al., 2007; Rathnasingh et al., 2012). *P. putida* KT2440 encodes both the soluble and membrane-bound transhydrogenase. It was shown that these transhydrogenases act as a safety device of redox metabolism, as they drive the interconversion of redox cofactors whenever it is needed (Nikel et al., 2016b). Overall, this strategy is very attractive, as no other substrates are required.

1.4 Development of microbial cell factories applying metabolic engineering

Industrial biotechnology has the potential for various applications; however, the challenge is that a native organism's metabolism is not optimized to produce a target product. Consequently, the design and construction – the engineering - of a microbial production strain is necessary and is usually applied with tools of metabolic engineering and synthetic biology (Keasling, 2012). In 1973, Cohen, Boyer, and co-workers were pioneers and reported the first genetically engineered *Escherichia coli* (Cohen et al., 1973). The term metabolic engineering was developed ~20 years later and was described as "*Metabolic engineering is the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology*" by James E. Bailey in 1991. Metabolic engineering focuses on the rational deletion and overexpression of endogenous genes and introducing heterologous genes to produce the target product. This strategy can perturb the system and lead to changed metabolic fluxes, metabolite concentrations, and gene expression (Mijakovic et al., 2005). These problems need to be avoided as they can impact the production negatively.

Numerous tools are available to develop and incrementally improve a microbial cell factory. The DNA manipulation toolset comprises plasmids and chromosomal integration methods

(Keasling, 2012). One common strategy is the overexpression of gene(s) on plasmids (Martínez-García and de Lorenzo, 2017). The prerequisite of the successful use of plasmids is both the production host's stability and ability to carry large DNA fragments. As plasmids are easy to work with and allow a straightforward implementation, various vector toolsets are developed and available, e.g., the Standard European Vector Architecture (SEVA) collection for *Pseudomonas* (Martínez-García et al., 2015; Martínez-García et al., 2020; Silva-Rocha et al., 2013).

Several drawbacks of plasmid-based expression systems exist, such as the need for antibiotics for plasmid retention, as plasmids are non-essential DNA (Englaender et al., 2017), the cell metabolism can be affected as the plasmid DNA needs to be replicated and the genes to be expressed. These tasks can unbalance the cellular machinery, such as building blocks and DNA polymerases; this cellular challenge is named "metabolic burden" (Birnbaum and Bailey, 1991; Glick, 1995).

These disadvantages of vector systems can be overcome by integrating an expression cassette into the chosen host organism's chromosome. Genomic integration of expression cassettes can be achieved, e.g. by homologous recombination or by transposons (Martínez-García and de Lorenzo, 2017). The Tn5 transposon and derivatives thereof are used to integrate expression cassettes random into the chromosome. The here generated mutant library requires sequencing and screening, as the level of expression can vary depending on the location on the chromosome (de Lorenzo et al., 1990; Nikel and de Lorenzo, 2013; Sousa et al., 1997). In contrast to Tn5, the mini-Tn7 transposon inserts single-copy genes at the Tn7 attachment site, designated as *att*Tn7 (Lambertsen et al., 2004) (Choi and Schweizer, 2006). The *att*Tn7 site is located downstream of the highly conserved *glmS* (glutamine-fructose-6-phosphate aminotransferase). For the sequential use of transposons, the creation of a new landing pad for the transposase (Martínez-García and de Lorenzo, 2017) as well as the replacement of the antibiotic resistance marker (e.g., Flp-FRT system) (Hoang et al., 1998) needs to be considered. Whether plasmid-based or genomically-integrated expression is advantageous must be investigated individually.

Another metabolic engineering tool is exchanging the natural promoter of the heterologous gene with a promoter of defined and controllable expression strength (Keasling, 1999).

Different promoters, such as constitutive or inducible, are available. In a heterologous pathway, multiple promoters are needed: each gene can be placed under one promoter's control. Inducible promoters' expression strength, such as P_{BAD}, can be regulated by the inducer (Guzman et al., 1995).

Genome editing tools for replacing genes, eliminating branching, or developing a competitive pathway by deleting the corresponding genes are essential. Homologous recombination techniques are widely used (Martínez-García and de Lorenzo, 2011). For example, a scarless gene deletion method uses the double-strand break-induced by the endonuclease *I-SceI*, encoded on a plasmid. Other new techniques are explored and developed for *Pseudomonas*, such as CRISPR/Cas9 (Aparicio et al., 2018). This collection of metabolic engineering tools allows the genetic manipulation of the microbial cell factory to produce the product of interest. Such a newly developed cell factory's performance is typically evaluated by three key metrics: titer, rate, and yield (TRY). The cell factory's development follows an iterative cycle: i) design, ii) build, iii) test.

In recent years, the so-called "chassis"-strains (from French: chassis = frame; Latin capsa = container, box) were introduced, which evokes the basic frame of a car, to which several components can be added to the customer's need (de Lorenzo et al., 2021). In microbiology, a *chassis* is a well-characterized organism with a fully sequenced and well-annotated genome, an available genomic toolbox, a characterized metabolism including multi-omics. Finally, metabolic engineered enables easy plugging-in and -out of the desired pathway to produce the product of choice. The idea of such chassis strains is the fast construction of reliable production strains, with high resistance to harsh production conditions and ideally a product secretion system to enable easy downstream processing (Calero and Nikel, 2019).

1.5 Medium-chain methyl ketones C11-C17 as the target product of this study

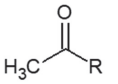
Methyl ketones (MK) are organic compounds with one methyl group as a ligand on the carbonyl carbon. The simplest methyl ketone is acetone (C_3H_6O). Here, in this study, we focus on the medium-chain methyl ketones in the range of C11 to C17.

In the last decades, the importance and interest of methyl ketones in the range of C11-15 strongly increased as they have a high potential to serve as a transportation fuel due to the

high cetane numbers and low freezing points in the range of diesel fuel (Goh et al., 2012b) (Table 1). ASTM's specification and requirements (formerly American Society for Testing and Materials, international standard organization) for biofuel require a minimum cetane value of 47, whereas the European specifications are more rigorous and require a cetane number of 51 (Hoekman et al., 2012). The methyl ketone with a chain of C11 matches both requirements (Table 1). Additionally, the fuel properties can be enhanced by chemical modification to dioxolanes resulting in a substantial increase of the cetane numbers to 80-90 (Harrison and Harvey, 2018). In comparison, a commercial diesel fuel such as No. 2 petroleum diesel has a cetane number of 40-45 (Hoekman et al., 2012).

The long carbon chain of the methyl ketones defines the hydrophobic properties and thus the insolubility in water. The hydrophobicity can be described with the log$P_{o/w}$, the logarithm of the partition coefficient *P* of a particular solvent between a 1:1 mixture of octanol and water (Heipieper et al., 2007; Weber and de Bont, 1996). For example, hydrophobic or better lipophilic compounds such as toluene have a log$P_{o/w}$ of 2.5 and are highly soluble in the cell membrane.

Table 1. Chemical structure and properties of the saturated methyl ketones of this study.

Structure methyl ketone congener	 R= C11, C13, C15		
Name	2-undecanone	2-tridecanone	2-pentadecanone
Molecular formula	$C_{11}H_{22}O$	$C_{13}H_{26}O$	$C_{15}H_{30}O$
Molecular Mass [g mol^{-1}]	170.29	198.35	226.4
Derived cetane number[a]	51.8	62.3	76.6
log*P*[b]	4.09	5.2	6.3

[a] The derived cetane number (DCN) calculated by Andrea König (AVT, RWTH Aachen University, DE) (Dahmen and Marquardt, 2015)

[b] Partition coefficient of a compound in a two-phase octanol/water system values are obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/)

The incorporation of lipophilic compounds into the membrane disturbs the lipid-lipid and lipid-water interaction, thereby destabilizing the membrane, which is often detrimental to microorganisms (Weber and de Bont, 1996). Solvents with a log$P_{o/w}$ value between log$P_{o/w}$ >4 are generally not toxic for microorganisms as they reach not a critical concentration in the membrane. Compounds with log$P_{o/w}$ >4 are often used in biotransformation reactions as a reservoir, the second phase for toxic compounds with lower log$P_{o/w}$ value (Inoue and Horikoshi, 1991; Vermuë et al., 1993). The methyl ketones in this study have a log$P_{o/w}$ value > 4 (Table 1).

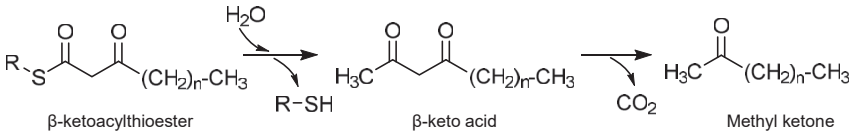
1.6 Applied strategies for the production of methyl ketones in different species

In 1858, the 2-undecanone ($C_{11}H_{22}O$) occurrence was first discovered by Greville Williams as a primary constituent of essential oil from *Ruta graveolens*, commonly known as rue (Williams, 1859). Since then, methyl ketones were confirmed as constituents of oils in other plants, such as cinnamon, coconut, and hops, mainly acting as insecticides (Forney and Markovetz, 1971) and as pheromones in plants (Antonious et al., 2003).

Different species of bacteria produce methyl ketones with various chain lengths. For example, *Pseudomonas methanica* and *Mycobacterium smegmatis* were found to produce short-chain methyl ketones (2-butanone, 2-pentanone, and 2-hexanone) from their corresponding alkanes, whereas *Mycobacterium rhodochrous* has been found to produce 2-undecanone from *n*-undecane (Leadbetter and Foster, 1959; Leadbetter and Foster, 1960; Lukins and Foster, 1963).

Methyl ketones are fatty acid-derived compounds, such as other biofuels and chemicals. Fatty acids are natural precursors of elementary building blocks of diverse membrane lipids, such as phospholipids and sphingolipids of all living organisms, and are vital components of the cell (López-Lara and Geiger, 2010). The essentiality of these metabolites (Janssen and Steinbuchel, 2014; López-Lara and Geiger, 2010) guarantees constitutive production. Successful examples show the possibility of rewiring microbial metabolism to synthesize fatty acid-derived products. For example, *Escherichia coli* has been engineered to produce long chain alkanes at a titer of 1.5 g/L (Cao et al., 2016).

Methyl ketones are biologically produced from β-keto acids, unstable intermediates that can spontaneously undergo decarboxylation under mild conditions (Kornberg et al., 1948)
Many metabolic engineering strategies have been developed to focus on producing unstable β-keto acids, e.g., trans-thioesterases or hydrolysis on β-ketoacyl thioesters (-ACP, -CoA).



R= ACP or CoA

Figure 2. Schematic reaction for the synthesis of methyl ketones. β-ketoacyl thioesters are hydrolyzed to unstable β-keto acids, which undergo a spontaneous decarboxylation.

In 2010, Yu et al. identified the thioesterases from wild tomato *Solanum habrochaites* (*Sh*Mks1/2), which hydrolyze 3-ketoacyl-acyl carrier (ACP) proteins to release β-keto acids (Yu et al., 2010). In 2012, Park et al. overexpressed the identified *Sh*Mks1/2 in *E. coli* to produce methyl ketones (Park et al., 2012). Yuzawa et al. engineered a modular type I polyketide synthases (PKS) in *Streptomyces albus* to produce short-chain methyl ketones (C5-C7)(Yuzawa et al., 2018) (Table 2). Zhu et al. focused on synthetic type I fatty acid synthase (FAS), a mega-enzyme, which faces an acyl carrier protein (ACP) domain. The enzyme *Sh*Mks2 was inserted into FAS to increase the cleavage activity to release β-keto acids in *S. cerevisiae* (Zhu et al., 2017).
Goh et al. compared the strategy of overexpressing the methyl ketone synthase *Sh*MKS1 and *Sh*MKS2 in *E. coli* with an overexpressed native acyl-CoA methyl ketone synthase FadM. FadM outperformed the two *Sh*MSK1/2 (Goh et al., 2012b). Additional to the overexpressed native thioesterase FadM, the codon-optimized acyl-CoA oxidase from *Micrococcus luteus* (Mlut_11700), an acyl-ACP thioesterase (TesA), and a bifunctional hydratase and dehydrogenase (FadB) were co-expressed. Furthermore, it was necessary to increase the fatty acid flux by overexpressing and balancing the fatty acid-responsive transcription factor (fadR) and fatty acyl-CoA synthetase (fadD). Host engineering strategies were necessary for *E. coli* to

increase the methyl ketone titer by deleting crucial acetate production pathway steps and the overexpression of the NADH-dependent β-ketoacyl-ACP reductase FabG from *Acholeplasma laidlawii* to mitigate the NADPH imbalance caused by the endogenous NADPH-dependent FabG. These metabolic engineering steps resulted in the so-far highest reported methyl ketone titer of 5.4 g L^{-1} (Goh et al., 2014; Goh et al., 2012b; Goh et al., 2018).

The fundamental strategy of the overexpression of FadM was very successful and was implemented in different species, such as *Yarrowia lipolytica* (Hanko et al., 2018) or *Ralstonia eutropha* (Müller et al., 2013). Yan et al. focused on a similar strategy and identified a FadM variant from *Providencia sneebia* that demonstrated higher activity on medium-chain substrates (C7-11), co-expressed with carbon-chain selective thioesterases (Yan et al., 2020).

Table 2. Overview of different metabolic engineering strategies, reported titer, yield and rate of methyl ketone production of different microorganisms (adapted and modified by (Yan et al., 2020)).

	Product	Titer / g L^{-1}	Yield /g g^{-1}	Rate /g L^{-1} h^{-1}	Cultivation strategies	Micro-organism	Metabolic engineering strategies	References
methyl ketone congener	C11 - C17	9.8	0.171 g g consumed glucose	0.132	bioreactor, fed batch	*P. taiwanensis*	Metabolic modeling prediction Δ*tesB*, Δ*pha*; deficient in β-oxidation (Δ*fadA*, Δ*fadE*, Δ*fadE2*, Δ*fadA2*); overexpression of *fadM*, '*tesA*, *fadB*, *Mlut_11700*	This study
	C11 - C15	5.4	~0.05 consumed glucose	0.05	bioreactor fed batch	*E. coli*	Overexpression of NADH-dependent FabG; deficient in β-oxidation (Δ*fadE*), Δ*poxB* Δ*ackA-pta*	(Goh et al., 2018)
	C13 - C23	0.31	0.024 consumed glucose	0.00155	bioreactor fed batch	*Y. lipolytica*	Δ*pot1*; overexpression of *fadM*, '*tesA*, *fadB*, *Mlut_11700*	(Hanko et al., 2018)
	C11 - C15	10			shake flask	*S. cerevisiae*		(Zhu et al., 2017)
	C9 - C13	0.5	0.017 g/g fed glucose	0.010	bioreactor, batch	*E. coli*	Overexpression of *Sh*MKS1/2, deletion of fermentative pathways (Δ*adhE* Δ*ldhA* Δ*poxB* Δ*pta*)	(Park et al., 2012)

	Product	Titer / g L^{-1}	Yield /g g^{-1}	Rate /g L^{-1} h^{-1}	Cultivation strategies	Micro-organism	Metabolic engineering strategies	References
Methyl ketone congener	C13 - C15	1.1	0.169 g/g	0.023	shake flask, batch	*P. putida*	deficient in β-oxidation (Δ*fadAB*), Δ*pha*; overexpression of *fadM*, '*tesA*, *fadB, Mlut_11700*,	(Dong et al., 2019)
	C13 - 15	0.18	-	0.0015	bioreactor, batch	*R. eutropha*	deficient in β-oxidation; overexpression of *fadM*, '*tesA*, *fadB*, *Mlut_11700*,	(Müller et al., 2013)
	C5 - C7	0.24	0.0028 g/g fed glucose	0.0011	bioreactor, batch	*S. albus*	Polyketide synthases (PKS)	(Yuzawa et al., 2018)
Methyl ketone	C7	4.4	0.035 g/g consumed glycerol	0.061	bioreactor, fed-batch	*E. coli*	*ΔfadAEIR;* overexpressing *fadD, Mlut_11700* and *FadM* variant of *Providencia sneebia*	(Yan et al., 2020)
	C9	3.0	0.028 consumed glucose	0.042	bioreactor, fed-batch	*E. coli*	*ΔfadAIR;* overexpression of ^{PS}*fadM,* thiolase *BktB* from *Ralstonia eutropha,* and C8-specific *Cuphea palustris FatB1* thioesterase	(Yan et al., 2020)
	C11	0.34	0.014 g/g consumed glycerol	0.007	shake flask, batch	*E. coli*	*ΔfadAEB ΔackApta,* overexpression of *fadB* of *P. putida, fadM* from *Providencia sneebia*	(Yan et al., 2020)

1.7 Selection of the production host: *Pseudomonas* as a suitable production platform

The top characteristics and requirements of a production host are genetic manageability, growth robustness, genetic stability, and the ability to accurately predict interactions between the heterologous pathway and the host metabolism (Beites and Mendes, 2015). With these characteristics in mind, the production host can be chosen from a list of available production strains, such as *Escherichia coli* (Pontrelli et al., 2018), *Bacillus subtilis* (Gu et al., 2018), *Streptomyces sp*. (Spasic et al., 2018), *Corynebacterium glutamicum* (Wendisch et al., 2016), and *Pseudomonas* putida (Nikel and de Lorenzo, 2018).

Pseudomonas, a Gram-negative, rod-shaped bacterium that belongs to the gamma-proteobacteria, has gained much interest in recent decades. *Pseudomonas*, a soil bacterium, is often continuously exposed to environmental contaminants in its habitat, along with all kinds of physicochemical stress and competing for microbial species (Copley, 2009; Nikel and de Lorenzo, 2018; Poblete-Castro et al., 2016). These conditions are one reason for the rapid growth, rapid adaptation due to the extremely versatile metabolism and low nutrient requirements (Nikel and de Lorenzo, 2018; Poblete-Castro et al., 2016). This versatile metabolism is advantageous in the bioremediation of aromatics-contaminated soils (Cao et al., 2009), as some *Pseudomonas* species can degrade aromatic compounds, such as toluene, xylenes, and benzoate (Jiménez et al., 2002). Other aromatic compounds, such as p-coumaric acid, ferulic acid, and vanillic acid, are found in lignin, which accounts for about 30% of the lignocellulosic biomass (Linger et al., 2014). Lignin itself is a complex aromatic polymer whose composition varies considerably depending on the starting material's source and how this starting material is processed (Beckham et al., 2016) (Elmore et al., 2020). *Pseudomonas* can metabolize the depolymerized lignin to produce value-added products (Borrero-de Acuna et al., 2020; Dong et al., 2019; Linger et al., 2014; Notonier et al., 2021).

Besides the utilization of aromatic compounds, *Pseudomonas* also prefers intermediates of the tricarboxylic acid cycle (TCA), such as succinate as a carbon source, as well as hexoses (glucose and fructose) (Poblete-Castro et al., 2012a). The glucose itself can either be phosphorylated in the cytoplasm to glucose-6P and further to 6P-gluconate, or glucose can be

oxidized to gluconate in the periplasm. Regardless of the glucose processing (either oxidation or phosphorylation), these pathways converge at the key intermediate 6-phosphogluconate, which is the starting point for three different routes: the Entner-Doudoroff-Pathway (ED), the incomplete glycolysis, and the pentose phosphate pathway (Figure 3) (Nikel and de Lorenzo, 2018). The glycolysis in *Pseudomonas* is incomplete as it lacks the 6-phosphofructo-1-kinase (Pfk), which catalyzes the ATP-dependent transformation of fructose-6-P (F6P) to fructose-1,6-bisphosphate. The lack of Pfk explains the intensive use of the ED pathway, in which the 6-phosphogluconate is converted into glyceraldehyde-3-phosphate and pyruvate (del Castillo et al., 2007a; Dos Santos et al., 2004; Nikel et al., 2015), and part of these trioses-phosphates are recycled back into hexoses-phosphate, using the incomplete glycolysis (Nikel et al., 2016a).

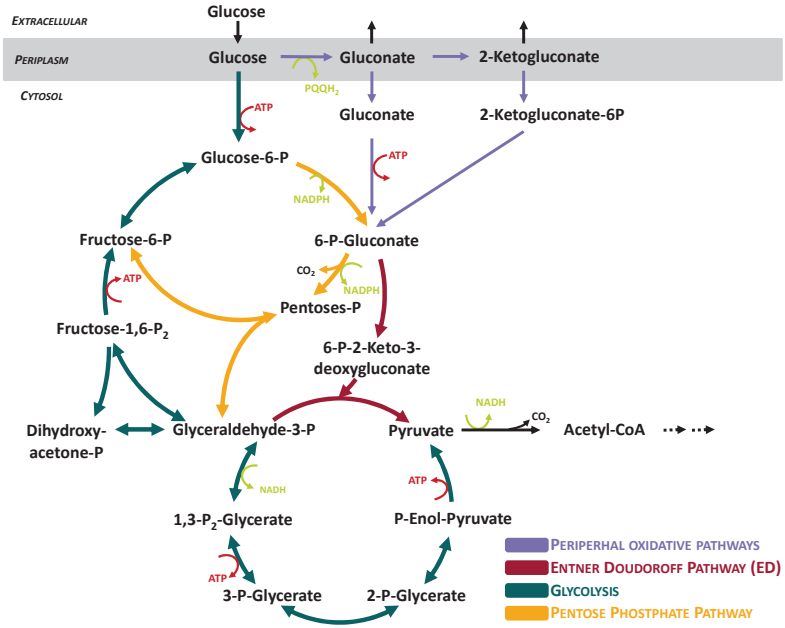


Figure 3. Schematic glucose catabolism organized in *Pseudomonas* composed of activities from different metabolic blocks, which are identified in colors.

The use of the ED pathway, the glycolysis, and pentose phosphate pathway plays a vital role in adjusting and regulating the redox status to sustain a high tolerance to oxidative stress (Kim and Park, 2014; Nikel et al., 2015; Nikel et al., 2021), this behavior is one part of the driven-by-demand phenotype. Another reaction to solvents: Under solvent stress, the solvent tolerant *Pseudomonas* responded with a drastically increased energy demand. This phenomenon was obtained with more than 2-fold carbon uptake and even an 8-fold increase in NAD(P)H regeneration rate compared to standard growth conditions (Blank et al., 2008b; Ebert et al., 2011; Rühl et al., 2009).

This behavior and requisite for implementing redox-expensive metabolic reactions hold great promise using *Pseudomonas* as a production organism (Akkaya et al., 2018; Nikel and de Lorenzo, 2018). *Pseudomonas* species were used as workhorses for biocatalytic production of bulk and fine chemicals, such as polyhydroxyalkanoates (PHA) polyesters (Borrero-de Acuña et al., 2014; Prieto et al., 2016), rhamnolipids (Wittgens et al., 2011) (Tiso et al., 2017), prodiginines (Klein et al., 2017), terpenoids (geranic acid) (Mi et al., 2014), and aromatics, such as anthranilate (Kuepper et al., 2015), L-phenylalanine (Molina-Santiago et al., 2016), phenazines (Schmitz et al., 2015), *para*-hydroxy benzoic acid (Yu et al., 2016), and phenol (Wierckx et al., 2005) (Wynands et al., 2018).

P. putida KT2440 is a typical workhorse but reaches its limits in producing interesting products like styrene or vinyl-phenol because these compounds are toxic for *P. putida* KT2440 due to the lack of solvent tolerance. Therefore, other *Pseudomonas* species are of emerging relevance, like *P. taiwanensis* VLB120. Several solvent tolerance mechanisms are observed in different *Pseudomonas* species, such as adaptive alteration of the membrane fatty acids and formation of vesicles loaded with toxic compounds. Efflux pumps, which pump excess solvent present in the cell to the outer medium, are considered the most efficient mechanism (Ramos et al., 2015). Efflux pumps are energy-dependent active efflux pumps, which belong to the resistance-nodulation-division (RND)-family. In *P. putida* DOT-T1E, three efflux pumps are present: TtgABC, TtgDEF, and TtgGHI. The expression is controlled by regulators of the efflux pumps (Ramos et al., 2002). *P. taiwanensis* VLB120 only harbors TtgABC and TtgGHI efflux pumps (Köhler et al., 2013) but possesses a high tolerance towards solvents as styrene, toluene, and octanol (Volmer et al., 2014).

In the presented thesis, *P. taiwanensis* VLB120 was chosen as the production host. It was isolated from forest soil in Stuttgart, Germany, as a styrene degrading organism. The available genome sequence, which consists of two replicons, a circular chromosome (6.6 Mbp) and a megaplasmid pSTY (361 kbp), allows targeted metabolic engineering (Köhler et al., 2013). In comparison, other industrial relevant Pseudomonads, such as *P. putida* DOT-T1E, *P. putida* KT2440, and *P. putida* S12, *P taiwanensis* VLB120, is nonpathogenic and able to assimilate xylose naturally (Köhler et al., 2015).

1.8 Scope of this thesis

This study aimed to demonstrate the high potential of *Pseudomonas taiwanensis* VLB120 to produce reduces compounds, focusing on methyl ketones with a chain length of C11 to C17 as a potential biofuel. Also, the NADH dehydrogenases type 1 and 2 were deeply characterized as potential redox targets.

Chapter 1 provides a general introduction to the production of reduced compounds and points out the challenges. Additionally, it highlights strategies to overcome and deal with redox limitations. Furthermore, chapter 1 introduces metabolic engineering strategies and pictures the state-of-the-art production of methyl ketones.

Chapter 2 describes all material and methods used in this study.

Chapter 3.1 provides an in-depth investigation of the three NADH dehydrogenases of the respiratory chain of *Pseudomonas taiwanensis* VLB120. The impact of the knockouts of corresponding genes was investigated on its physiology and metabolism and conferred to robust phenotypic behavior by a possible rerouting of metabolic fluxes.

The impact of deleted NADH dehydrogenase was investigated on the synthesis of the storage polymer PHA in Chapter 3.2. Additional, two potential redox-sink targets in the background of NADH-dehydrogenase knockout mutants were investigated to design a potential redox chassis for redox-dependent biocatalysis and were characterized by online-NADH measurement.

Chapter 3.3 describes the implementation of the heterologous methyl ketone pathway in *P. taiwanensis* VLB120. A model-driven metabolic engineering approach was successfully tested to increase further the methyl ketone production, which underlined the production host's potential.

In Chapter 3.4, the potential *Pseudomonas* redox chassis and the methyl ketone production was merged to study and compare the methyl ketone production with the best producer strain of Chapter 3.3.

Chapter 4 presents the results and achievements described in this thesis and are mutually discussed and placed in a larger context.