TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Chemie Biogener Rohstoffe

MICROBIAL OLEFIN PRODUCTION
–
TOWARDS A BIOREFINERY APPROACH

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Vollständiger Abdruck von der promotionsführenden Einrichtung
Campus Straubing
für
Biotechnologie und Nachhaltigkeit
der Technischen Universitat München
zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

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Die Dissertation wurde am 16.10.2019 bei der Technischen Universität München eingereicht und durch die promotionsführende Einrichtung Campus Straubing für Biotechnologie und Nachhaltigkeit am 10.02.2020 angenommen.

To All Loved Ones,
Especially To My Twins

Jannis and Kilian

The Family Circus

„YESTERDAY´S the past,

TOMORROW`S the future,

TODAY is a GIFT !

That`s why it´s called:
The PRESENT"

by Bil Keane

Zusammenfassung

In Zeiten des Klimawandels wird fieberhaft nach Alternativen zu fossilen Rohstoffen gesucht. Hierbei liegt ein Fokus auf nachwachsenden Rohstoffen. Die Hof-Bioraffinerie soll die gasförmigen Chemikalien Ethylen, Propylen und Isopren aus Silage bereitstellen. Hierzu sollen diese Chemikalien mittels Fermentation hergestellt werden. Die mikrobielle Bildung von Ethylen wurde bisher von drei Substraten beschrieben: (1) α-Ketoglutarat aus dem Zitratzyklus, (2) 1-Aminocyclopropan-1-Carbonsäure (ACC) aus dem Yang-Zyklus und (3) aus 2-keto-4-Methylthio Buttersäure (KMBA). Allen Stoffwechselwegen gemein ist eine suboptimale Stöchiometrie von Glukose ausgehend. Um wirtschaftliche Mengen bereitzustellen bedarf es der Erforschung neuer Stoffwechselwege zum Ethylen. Ziel der vorliegenden Arbeit ist die Suche nach stöchiometrisch optimaleren Wegen und enzymatischen Reaktionen. Hierzu wurde die lehrstuhlinterne Stammsammlung mittels headspace Gaschromatographie nach ethylenogenen und propylenogenen Mikroorganismen durchmustert. Im Anschluss wurde ein Teil der entdeckten Stämme weiter charakterisiert. Die Biosynthese des Isoprens ist verhältnismäßig gut verstanden, sodass diese Arbeit die Optimierung der Isoprenproduktion in *Cupriavidus necator* und die Etablierung der Isoprenproduktion in *Pichia pastoris* zum Ziel hatte.

Als Substrate für die Ethylenproduktion kamen 3-Phosphonooxy-Propansäure und β-Alanin zum Einsatz. Für Propylen 3-Phosphonooxy-Butansäure und 3-Sulfonooxy-Butansäure. Diese lassen sich aus nachwachsenden Rohstoffen herstellen und könnten von Decarboxylasen in Alkene umgewandlet werden. Die Stämme wurden drei Tage inkubiert, anschließend geerntet, gewaschen und zehnfach in Phosphatpuffer konzentriert. Nach dem Aufschluss der Zellen durch Hitze/Einfrier-Zyklen wurde das Zelllysat zusammen mit den Substraten in einem verschlossenen Gaschromatographie-Vial vier Stunden bei 30 °C inkubiert. Anschließend wurde die Ethylen- bzw. Propylenbildung analysiert. Die Ethylenogenen wurden weiter charakterisiert. Hierzu wurden Substrate (ACC, L-Methionin, α-Ketoglutarat, KMBA, S-Adenosylmethionin) der bekannten Stoffwechselwege in verschiedenen Kombinationen eingesetzt. Weiterhin wurde von drei Mikroorganismen das Genom sequenziert und eine Proteinaufreinigung durchgeführt. Für die Optimierung der Isoprenproduktion in *Cupriavidus necator* wurde zuerst ein Expressionsvektor mit (e)GFP als Reportergen identifiziert. Anschließend wurde die Isoprensynthase von *Pueraria montana* sowie das Mev-Operon, welche alle Gene für den MEP-Stoffelwechelweg enthält, in den Vektor pJeM1TcR und pMS137 kloniert. Für die Etablierung der Isoprenproduktion in *Pichia pastoris* X-33 wurde die Isoprensynthase aus *Pueraria montana* in das Integrationsplasmid pPICZa kloniert. Die Isoprenproduktion wurde mittels Gaschromatographie nach verschiedenen Inkubationszeiten und Medien nachgewiesen.

Proylenogene Mikroorganismen konnten nicht gefunden werden. Von 524 Stämmen zeigten 31 eine sehr geringe Ethylenproduktion. Die vier *Bacillus thurengiensis* Stämme SR-769, SR-778, SR-780, SR-792, *Lysinibacillus xylanilyticus* SR-86, *Bacillus cereus* SR-772, *Providencia* sp. SR-782 and *Bacillus luti* SR-783 produzieren Ethylen

über den KMBA-Weg, was ebenfalls auf *Lactobacillus brevis* SR-416 zutrifft. *Pichia fermentans* SR-265 zeigte hingegen ein Substratspektrum, welches zu erwarten ist, wenn Ethylen über den Yang-Zyklus gebildet wird. Die Genome von *Lysinibacillus xylanilyticus* SR-86, *Bacillus cereus* SR-772 sowie *Pichia fermentans* SR-265 wurden sequenziert. Dabei konnten nur in *Pichia fermentans* SR-265 homologe Proteine zur ethylenbildenden ACC Oxidase im Apfel (*Malus domestica*) gefunden werden. Diese zeigen die typische Triade aus His177, Arg179 und His234 für die Fe(II) Bindestelle. Die typische Bikarbonat-Bindestelle ist mit den Aminosäuren Thr157, Arg244 und Ser246 teilweise vorhanden. Weitere Versuche mit Aminosäuren und Metallen zeigten für die Proteinextrakte von Lysini, Lacto und Bacillus cereus, dass die Ethylenproduktion aus ACC durch die Anwesenheit von α-Ketoglutarat und Mn(II) gesteigert wird. Um Proteine zu identifizieren wurde eine Proteinaufreinigung mittels AEKTA vorgenommen. Das putative Ethylenbildene Enzym konnte aus Lysini über eine QXL- und QFF-Sepharose Anionentauscher gebunden und eluiert werden. Die Untersuchung der Fraktion brachte die 4-Oxalocrotonat Tautomerase hervor. Diese wurde in *Escherichia coli* BL21 kloniert und auf ihre Aktivität hin getestet. Es zeigte sich, dass sie nicht für die Ethylenbildung verantwortlich ist. Weitere Versuche zur Natur der Ethylenbildung über den Verdau der Proteine mittels Proteinase K zeigte, dass die Ethylenproduktion abnimmt. Kontrollversuche mit Bovines Serum Albumin (BSA) in Puffer nahm die Ethylenproduktion ebenfalls ab. Die Bildung von Ethylen aus KMBA wurde durch die Zugabe von EDTA, NAD(P)H und Fe(III) stimuliert. Die besten Vektoren für eine Genexpression in *Cupriavidus necator* waren pJeM1TcR und pMS137. Die Expression des Mev-Operons sowie der Isoprensynthase brachte jedoch keine Steigerung der Isoprenproduktion. Die Isoprenproduktion konnte in *Pichia pastoris* X-33 etabliert werden, war nach sechstägiger Inkubation jedoch sehr gering.

Die Ethylenproduktion in *Lysinibacillus xylanilyticus* SR-86, *Lactobacillus brevis* SR-416 und *Bacillus cereus* SR-772 aus ACC erwies sich als chemische Reaktion von ACC und α-Ketoglutarat, katalysiert durch Mn(II). Die Ethylenbildung aus KMBA ist in der Literatur für andere Mikroorganismen beschrieben und entspricht diesen. Eine Sequenz ist allerdings nicht publiziert. Die Aktivität der homologen Proteine in *Pichia fermentans* SR-265 kann mittels Expression überprüft werden. Um in der Hof-Bioraffinerie Ethylen produzieren zu können, muss weiterhin nach stöchiometrisch optimalen Stoffwechselwegen gesucht werden.

Basierend auf den Ergebnissen lässt sich ableiten, dass weiterhin nach effizienteren Stoffwechselwegen für die Produktion von Ethylen und Proyplen für die Hof-Bioraffinerie gesucht werden muss. Ebenfalls muss die Isoprenproduktion geeigneter Stämme für die Hof-Bioraffinerie verbessert werden.

Summary

In times of climate change the world is looking for alternative resources to move away from fossil resources. The farm-stead biorefinery aims to produce the gaseous chemicals ethylene, propylene and isoprene by fermentation from the renewable resource silage, in contrast to the recent production from fossil resources. A simple fermentation process would be desirable to provide the above-mentioned chemicals. This work has two major objectives: (1) find stoichiometrically favorable reactions to ethylene and propylene and their further characterization; (2) enhance the isoprene production in *Cupriavidus necator* and *Pichia pastoris* X-33. There are three pathways for the biosynthesis of ethylene known: the formation from α-keto-γ-methylthio-butyrate (KMBA), the ethylene-forming enzyme (EFE) reaction from α-ketoglutarate and the Yang-cycle in plants, in which ethylene is derived from 1-aminocyclopropane-1-carboxylic acid (ACC). From a biotechnological point of view, all pathways have a suboptimal stoichiometry. There is no pathway for the biological propylene production elucidated so far, but 3-hydroxyalkanoic acids converted by the mevalonate diphosphate decarboxylase are involved. Isoprene is biosynthesized via the mevalonate-independent and mevalonate-dependent pathway and optimization is in progress.

The screening for ethylenogenic and propylenogenic microorganisms in the department culture collection was examined by using gas chromatography. After three days of growth the culture was harvested, washed, concentrated ten-times and disrupted using heat/freeze cycles in phosphate buffer. Afterwards, the cell lysate was incubated with 3-phosphonooxy-propanoic and beta-alanine for ethylene production, and 3-phosphonooxy-butanoic acid and 3-sulfooxy-butanoic acid for propylene production in a sealed gas chromatography vial for four hours at 30 °C. Ethylene and propylene production were examined using headspace gas chromatography. The above-mentioned substrates can be produced from renewable resources and could be converted into alkenes by decarboxylation. Further characterization was done using molecular phylogenetic analysis, genomic sequencing and protein purification using AEKTA.

Enhancing isoprene production in *Cupriavidus necator* started with the identification of versatile expression vectors by using (e)GFP as reporter gene. The next step was to clone the isoprene synthase from *Pueraria montana* and a codon optimized Mev-operon into *Cupriavidus necator*. Establishing isoprene production in *P. pastoris* was achieved by cloning the isoprene synthase from *Pueraria montana* into the integrative vector pPICZa. Isoprene formation was detected using headspace gas chromatography after incubation of living cells in sealed gas chromatography vials on medium 1-6 days for *P. pastoris* and several hours for *Cupriavidus necator*.

A Screening of 524 strains for ethylenogenic and propylenogenic microorganisms was performed. In total, 31 strains were found to produce minuscule amounts of ethylene, but no propylene. Ten out of these strains were further characterized. Identification of the present ethylene biosynthesis pathway was elucidated with combinations of the substrates ACC, L-methionine, α-ketoglutarate, S-adenosyl methionine and ATP, key intermediates for the known pathways. Four *Bacillus thurengiensis* strains SR-769,

SR-778, SR-780, SR-792, *Lysinibacillus xylanilyticus* SR-86, *Bacillus cereus* SR-772, *Providencia* sp. SR-782 and *Bacillus luti* SR-783 were found to produce ethylene from KMBA, which is also true for *Lactobacillus brevis* SR-416. Additionally, the yeast *Pichia fermentans* SR-265 showed pattern in using the substrate in concordance with the Yang cycle. Interestingly, *Lysinibacillus xylanilyticus* SR-86, *Bacillus cereus* SR-772 and *Lactobacillus brevis* SR-416 showed production from ACC as well, but not the typical pattern of substrate usage for the Yang cycle. The genomes of *Lactobacillus brevis* SR-416, *Bacillus cereus* SR-772 were sequenced, but did not reveal known ethylene forming enzymes. The next step in order to identify ethylene forming enzymes was the purification by using AEKTA. The putative protein producing ethylene in *Lactobacillus brevis* SR-416 from ACC bound to QXL- and QFF sepharose in 40mM glycin NaOH buffer at pH9. In this fraction the 4-oxalocrotonate tautomerase was identified using mass spectrometry fingerprinting. Further tests with the protein extract showed that Mn(II) and α-ketoglutarate in combination with ACC stimulated the ethylene production of protein extracts from *L. xylanilyticus*, *B. cereus* and *L. brevis*. Addition of EDTA and proteinase K resulted in a decrease in ethylene production. A control experiment was done by supplementing the protein extract with bovine serum albumin (BSA), which resulted in a decrease as well. The genome of *Pichia fermentans* SR-265, which was also sequenced, revealed three sequences that showed homology to the ACC oxidase of *Malus domestica*. All three sequences show the typical facial triade, which is the Fe(II) binding site, comprising His177, Arg179 and His234. The bicarbonate binding site is partially present with Thr157, Arg244, Ser246. The ethylene formation from KMBA was stimulated by EDTA, NAD(P)H and Fe(III) in *Lactobacillus brevis* SR-416, *Lysinibacillus xylanilyticus* SR-86 and *Bacillus cereus* SR-772.

The best vectors for protein expression in *Cupriavidus necator* tested with (e)GFP are pJeM1TcR and pMS137. However, the expression of kISpS and the Mev-operon did not increase the isoprene production. *Pichia pastoris* X-33 showed very little isoprene production after incubating a culture for 6 days in methanol minimal medium, but no isoprene after one day. On the basis of the results of this research, it can be concluded that the ethylene formation from ACC in *Lysinibacillus xylanilyticus* SR-86, *Bacillus cereus* SR-772 and *Lactobacillus brevis* SR-416 are artifacts caused by Mn(II) from the culture medium. The ethylene production from KMBA seems to be as described in literature. More research needs to be done to identify enzymes involved in ethylene formation from KMBA, as no sequences are published. The expression of the putative ACC oxidases from *Pichia fermentans* SR-265 would provide further evidence for their identity. Metabolic engineering to enhance the isoprene production in *Cupriavidus necator* was unsuccessful, but little successful in *Pichia pastoris* X-33.

In conclusion, to establish a biorefinery, stoichiometrically efficient pathways are still need to be found or constructed. Furthermore, isoprene production of suitable strains needs to be optimized for the farm-stead biorefinery.

List of Publications

Michael E. Loscar, Christopher Huptas, Mareike Wenning, Volker Sieber, and Jochen Schmid (2016). Draft Genome Sequence of *Lysinibacillus xylanilyticus* SR-86.
In: Genome Announcement Vol. 4(6):e01317-16
DOI: 10.1128/genomeA.01317-16

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1 Introduction

Life on earth in the 21st century is challenging owing many problems humans are facing, such as over seven billion people on the planet, increasing CO_2 concentration in the atmosphere [143] and an expected shortage of petroleum [225]. One solution to the exhaustion of resources can be overcome by renewable resources concomitant with the sustainability mindset challenging our society. Feeding all seven billion people can only be achieved if farming of crops and energy plants are highly orchestrated [162]. As Sheikh Yamani, the Saudi Arabian minister of oil and mineral resources between 1962 to 1986, once proclaimed: "Stone age came to an end not because we had a lack of stones and the oil age will come to an end not because we have a lack of oil". It seems that biotechnology has the solutions for some of the problems in the 21st century [51, 111].

In the recent years, there has been significantly more research aiming to curb the usability of renewable resources. This research included the search for alternative sources for petroleum-based fine and bulk chemicals from renewable resources. On the one hand, new substrates, such as cellulose and lignocellulose are being tested [318]. On the other hand, new methods are being developed to produce bulk chemicals [48, 167, 325]. Looking at the economy over the last few thousand years we recognize that for the majority of time humans used biological resources instead of finite resources, e. g. petroleum [244].

One of the most important bulk chemicals amongst toluene, phenol and benzene is ethene (common name ethylene). It is used especially for the production of polymers, e. g. polyvinyl chloride (via vinyl chloride). At the moment, ethylene is mainly produced from petroleum by steam cracking, which is very energy intensive. The world wide production of ethylene in 2005 was $112.9 * 10^6$ t/yr, with $107 * 10^6$ t/yr in North America and $21.6 * 10^6$ t/yr in Western Europe [27, 136, 380].

The holy grail of the microbial ethylene production from renewable resources would be a process as simple and cost effective as the well-known ethanol production from *Saccharomyces cerevisiae* [206]. In the following sections, current production processes and biological formation of the short-chain olefins ethylene and propylene (see **Section 1.1**) as well as isoprene (see **Section 1.2**) are presented and the new concept of the “farm-stead biorefinery ” is introduced (see **Section 1.3**).

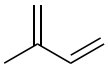
1.1 The Short-Chain Olefins Ethylene and Propylene

Olefins are defined by the International Union of Pure and Applied Chemistry (IUPAC) as:

> "*Acyclic and cyclic hydrocarbons having one or more carbon-carbon double bonds, apart from the formal ones in aromatic compounds. The class olefins subsumes alkenes and cycloalkenes and the corresponding polyenes.*" [230]

In this section, only the short-chain olefins ethylene and propylene (see **Tab. 1.1**) are considered. There is no strict rule for the term "short" in the literature, generally, medium-chain olefins start around C10. For oligomer molecules, the definition "A chain of low relative molecular mass" is found [230].

Table 1.1: *Names, identifiers and chemical structures of selected short-chain olefins.*

Common Name	Ethylene	Propylene	Isoprene
IUPAC Name	ethene	prop-1-ene	2-methyl-1,3-butadiene
CAS Number	74-85-1	115-07-1	78-79-5
PubChem CID	6325	8252	6557
Sum Formula	C_2H_4	C_3H_6	C_5H_8
Structure			
Other Names	NA	propene	isopentadiene, 2-Methylbutadiene
Molar Mass	28.05 g/mol	42.08 g/mol	68.12 g/mol
Boiling Point	-102.4 °C	-48.0 °C	34.0 °C
Melting Point	-169.0 °C	-185.0 °C	-145.9 °C
Flash Point	314.0 °C	-162.0 °C	-54.0 °C
Solubility in water (at 25 °C)	131 mg/L	200 mg/L	642 ppm

NA: not available. Data from PubChem [160]

Ethylene is the simplest olefin and the most produced platform chemical, contributing as feedstock for about 30% of petro-based chemicals [10]. Its annual production is $112.9{*}10^6$ t/yr in 2005 [380]. Other important platform short-chain olefins include propene (common name propylene) with a worldwide production volume of $63.2{*}10^6$ t in 2004 [10].

Ethylene and propylene today are mainly derived from fossil resources (petroleum and natural gas) by steam-cracking (see **Section 1.1.3.1**) [10, 26, 380].

Ethylene and propylene are known to occur as metabolites in various aerobic microorganisms (see **Sections 1.1.2** and **1.1.3.3**), which makes them targets for research on how to produce them from renewable resources by fermentation [12, 69, 101, 105, 204]. Naturally produced ethylene plays a role in microbial plant pathogenicity [342] and is a plant hormone [300].

Olefins are used to produce polymers, among other products. Ethylene is widely known for its use to form polyethylene (LDPE (low-density polyethylene), HPDE (high-density polyethylene)), which occurs in our every day life, e. g. in detergent bottles, water pipes and packaging material. The main products of ethylene include vinyl acetate, ethanol (by hydration) and styrene (polymerization with benzene and dehydrogenation) [10, 26, 380]. Propene is also used to produce polymers like polypropene, acrylnitrile, isopropanol and cumol [11, 26]. An overview of these compounds is given in the following sections.

1.1.1 Natural Pathways for the Biosynthesis of Ethylene

Today, there are three known mechanisms in which ethylene is produced (see **Fig. 1.1**). In plants, SAM is generated from L-Met (L-methionine) by SAM synthase and converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase [287]. This pathway was also found to be present in the slime mold *Dictyostelium mucoroides* [6] as well as the fungus *Penicillium cintrinum* [146].

Microorganisms are divided into two groups based on the used substrate [226]. The first group uses α-ketoglutarate and L-arginine to synthesize ethylene in a one-step reaction catalyzed by the enzyme “ethylene-forming enzyme” (EFE). The second group uses α-keto-γ-methylthio-butyrate (KMBA) which is thought to be derived from L-methionine via desamination by promiscuous transaminases. The stoichiometry of the known ethylene forming pathways is unfavorable for the biotechnological production (see **Tab. 1.2**). Suggestions have been made to produce acrylic acid by fermentation followed by an enzymatic decarboxylation to ethylene. However, the economic value of this reaction is doubtful, as acrylic acid is of higher value [299]. A detailed review of the three known pathways is given in the following.

Table 1.2: *Stoichiometry of ethylene production from glucose*

Pathway	Carbon Efficiency (g ethylene/g glucose)	Reference
ACC	0.12 g/g (optimized expression in *S. cerevisiae*)	[179]
Efe	0.19 g/g (optimized expression in *S. cerevisiae*)	[179]
KMBA	not available	
Chemical dehydration of EtOH	~0.31 g/g	[223, 298]
Theoretical yield limit	0.31 g/g	[3, 223, 298]

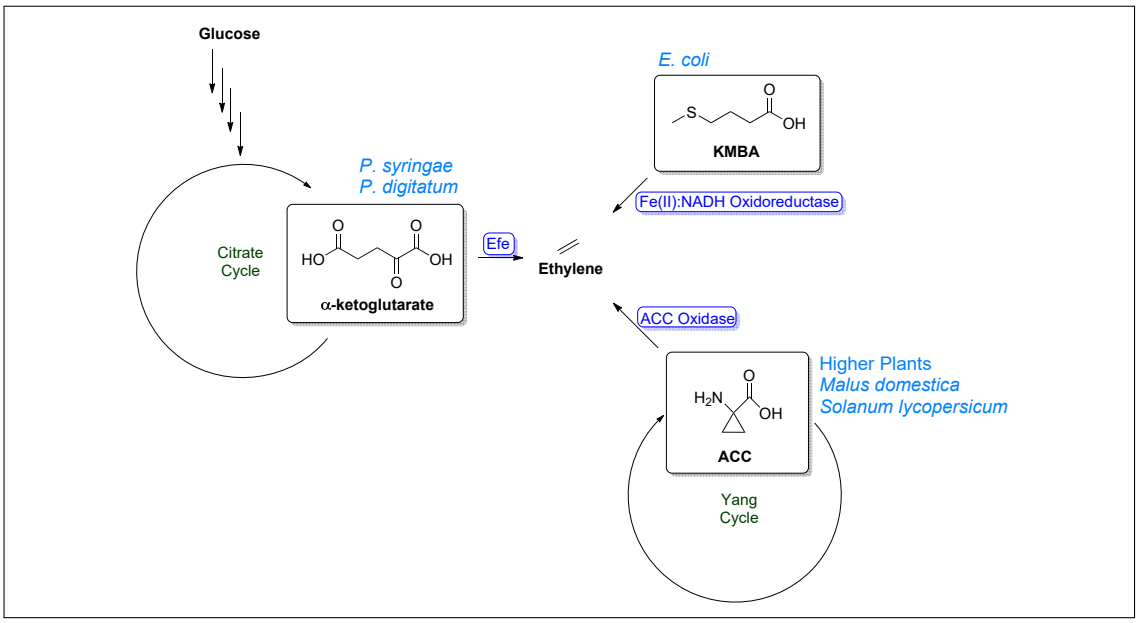


Figure 1.1: ***Overview of ethylene forming pathways.*** *Up to now, there are three known pathways in which ethylene is synthesized. In Penicillium digitatum and Pseudomonas syringae, ethylene is synthesized from its direct precursor, α-ketoglutarate, by Efe. In higher plants, ethylene is formed from 1-aminocyclopropane-1-carboxylic acid by the ACC oxidase. In a wide range of microorganisms including the E. coli strain SPO A and Bacillus sp., ethylene is derived from 2-keto-4-methylthiobutyrate (KMBA) by a Fe(III):NAD(P)H oxidoreductase. [100, 103, 138, 287].*

1.1.1.1 Ethylene Synthesis by Plants

1.1.1.1.1 Yang Cycle

In plants, ethylene is produced from L-methioine (L-Met) via 1-amino-cyclopropane-1-carboxylic acid (ACC). In this cycle, L-methionine and its sulfur atom are salvaged, which also gives the cycle the name methionine salvage pathway (see **Fig. 1.2**). It is named after its discoverer [300]. It might also be present in the slime mold *Dictyostelium mucoroides* [6] and the fungus *Penicillium cintrinum* [146].

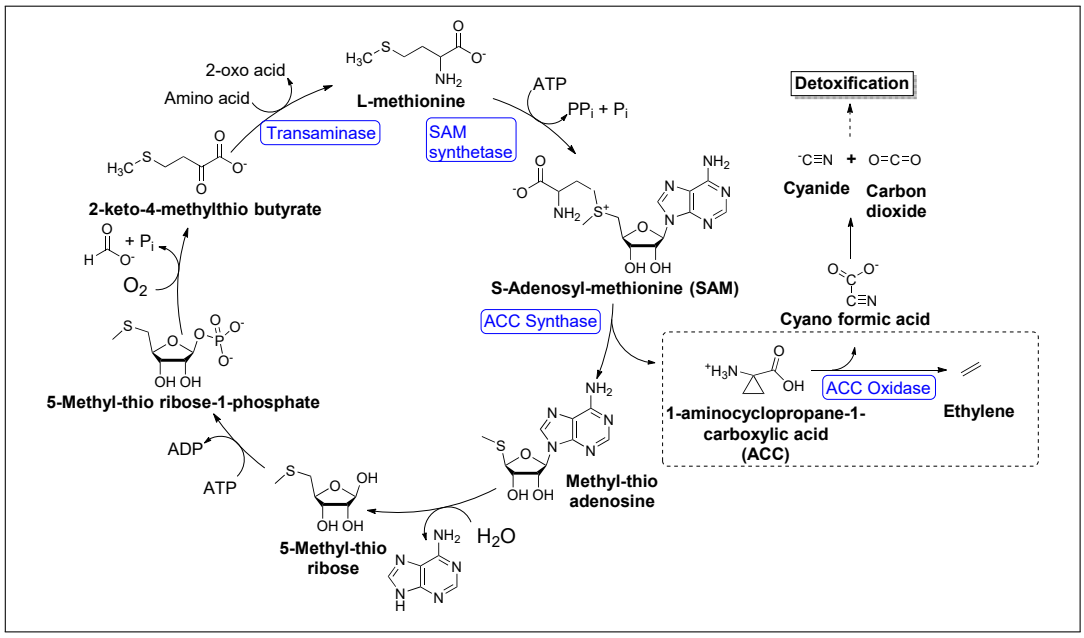


Figure 1.2: ***Ethylene formation via the Yang cycle.*** *This pathway is found in plants [300] and might be also present in some fungi [6, 146]. It is also known as the methionine salvage pathway. The key enzymes are the ACC synthase (EC EC 4.4.1.14) and the ACC oxidase (EC 1.14.17.4). For an easier presentation the steps from 5-methyl-thio ribose-1-phosphate to KMBA are not displayed in detail. For details please refer to the text. Figure according to [300].*

In the first step, S-adenosyl-methionine (SAM) is synthesized from L-methionine and ATP by SAM synthetase. In the next step, SAM is converted to ACC and methylthioadenosine (MTA) by ACC synthase (EC 4.4.1.14). ACC is then converted to ethylene and cyanoformic acid by ACC oxidase (ACCO). The latter product decays spontaneously into CO_2 and HCN, which is detoxified over β-cyanoalanine as asparagine and aspartic acid [220, 224, 300]. The second product of ACC synthase, MTA, is converted into methylthioribose (MTR) by MTA phosphorylase/nucleosidase [233, 273] and then into methylthioribose-1-phosphate (MTR-P) by MTR kinase [273]. MTR-P is then converted to methylthioribulose-1-phosphate by MTR-P isomerase [273]. Phosphate is then removed by dehydratase/enolase/phosphatase. Formic

acid is removed by acireductone dioxygenase to yield 2-keto-4-methylthiobutyrate [233, 273, 300]. To complete the cycle, it is then converted into L-methionine by a transaminase, which adds the amino group of an amino acid on KMBA to form L-methionine and a keto acid [273]. This way, plants do not have to synthesize L-methionine *de novo* for the ethylene synthesis, which is important in the fruits once detached from the mother plant [300].

From a biotechnological application point of view, one molecule ribose is converted to one molecule ethylene, only considering the carbon atoms (see reaction 1.1). If energy consumption is taken into account, the stoichiometry is less efficient.

$$\begin{aligned} &\mathrm{C_5H_{10}O_5} + \mathrm{NH_3} + 2\,\mathrm{O_2} + \mathit{Ascorbate} + \mathrm{NADH} + \mathrm{H^+} \longrightarrow \\ &\mathrm{C_2H_4} + \mathrm{CO_2} + \mathrm{HCN} + \mathrm{HCOOH} + 3\,\mathrm{H_2O} + \mathit{Ascorbate_{ox}} + \mathrm{NAD^+} \end{aligned} \tag{1.1}$$

ACC synthase is 5'-pyridoxalphosphate dependent and can be inhibited by typical PLP-dependent enzyme inhibitors, e. g. aminoethoxyvinlyglycine (50% inhibition at 2 μM), aminooxyacetate (50% inhibition at 6 μM) and L-α-aminooxy-propionic acid (50% inhibition at 3 μM) [8, 300]. Moreover, the ethylene formation is inhibited by α-aminoisobutyrate (AIB) [44], Co(II) and Ni(II) [180, 334, 368].

Ethylene synthesis is regulated by the transcription regulation of ACC synthase. Environmental factors, such as wounding, coldness, dryness and flooding of roots induce its synthesis [300]. The enzyme itself is inhibited by one of its products, methylthioadenosin [233]. ACC can also be irreversibly converted into 1-(malonylamino)cyclopropane-1-carboxylic acid which is stored in the vacuole and cannot be used to produce ethylene [130, 300].

1.1.1.1.2 Closeup: The ACC Oxidase

ACC oxidase (EC 1.14.17.4) catalyses the reaction from the unusual amino acid ACC to ethylene (HCN and CO_2) with the cofactors ascorbate and O_2 being essential (see **Fig. 1.2**). It is a member of 2-oxoglutarate-dependent dioxygenases (also 2OG oxygenases), a non-heme Fe(II) dependent enzyme family [35, 311].

Up to now, there has been only one 3D crystal structure published from *Petunia hybrida* (PDB number 1WA6) [375]. The main chain of one monomer consists of eleven α helices and 13 β helices. Eight of the β-helices form a distorted double-stranded β-helix (DSBH) which are common in all members of the 2OG oxygenases family. Residues for Fe(II) binding are His177, Asp179 and His234. This is the so-called 2-His-1-carboxylate facial triad (His-Arg/Glu-His) characteristic to the 2OG oxygenases [46]. The RXS motif is exclusively conserved in the structural subfamily of ACCO

[44, 126] and is comprised of Arg244, Ser246 together with Tyr162 and is responsible for interacting with the carboxylate of ACC. Various positively charged amino acids along with Lys158 and Arg299 are proposed as ascorbate and bicarbonate binding sites in α-helix 11 [77]. The mutation of the residues Arg244 and Ser246 decreases the activity [365] and the mutation of Lys158 completely destroys activity [183]. The mechanism of the ethylene formation from ACC is assumed to be a radical mechanism [55, 246], with a peroxy intermediate [258], but remains to be fully elucidated [46].

Ascorbic acid is stoichiometrically used during the reaction and converted to dehydroascorbic acid [78]. This is in contrast to most members of the 2OG oxygenases family (e. g. the isopenicillin N-synthase (IPNS)), which use α-ketoglutarate as a cofactor and convert it to succinate and CO_2 [66, 327].

Within the plant cell it is a monomer [78], but is has also been shown that it is active as a homodimer [375]. It also forms homotetramers when crystallized with the C-terminal part facing away from the active centre and interacting with an adjacent ACCO monomer [44, 365].

In apple, there are three ACC oxidases expressed in various tissues, which seem to be present mostly in the cytosol [62]. The three enzymes differ in their kinetics [35].

Thr157, Arg244 and Ser246 are believed to bind bicarbonate, an activator of ACCO [78, 215, 290, 364, 375].

The ACC oxidase is competitively inhibited by α-aminoisobutyric acid (AIB), a substrate analog to ACC [44, 217]. It has been shown that the ethylene formation was inhibited by 70% when 10 μM of Co(II) was present [334, 368].

1.1.1.2 Ethylene Biosynthesis from α-ketoglutarate

This pathway has been discovered and characterized in the microorganisms *Pseudomonas syringae* and *Penicillium digitatum* and others [82, 99, 103]. The ethylene-forming enzyme (EFE, 1.13.12.19), an oxidoreductase belonging to the mononuclear non-heme Fe(II)- and 2-oxoglutarate (2OG)-dependent oxygenase superfamily, catalyses the reaction from α-ketoglutarate (an intermediate in the citrate cycle) to ethylene. The Efe requires Fe(II), α-ketoglutarate, O_2 and L-arginine, with being highly specific for α-ketoglutarate and L-arginine (see **Fig. 1.3**).

The substrate ratio of the reaction is 3:1 α-ketoglutarate:arginine resulting in the product ratio 2:1:1 ethylene:succinate:L-Δ-1-pyrroline-5-carboxylate (or guanidine). The proposed dual-circuit mechanism sums up the two occurring reactions (EC 1.13.12.19 and EC 1.14.11.34) to 1:2 succinate:ethylene [103].

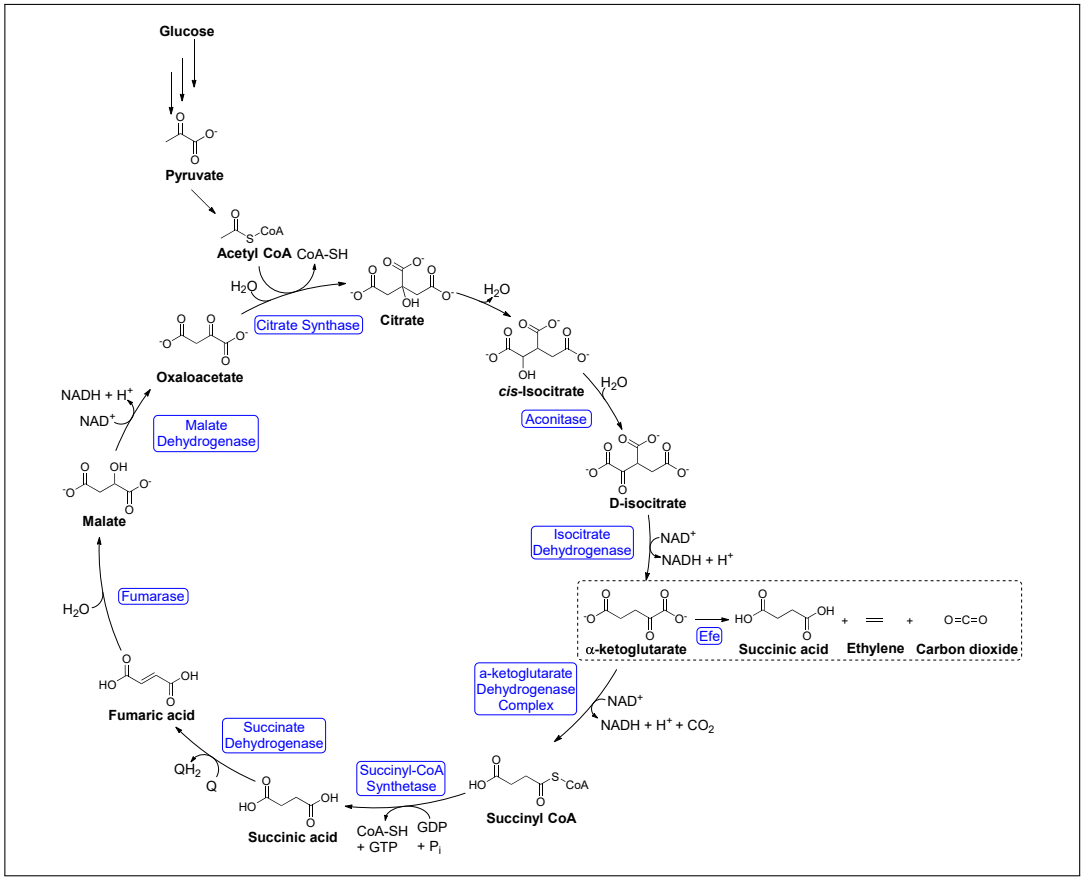


Figure 1.3: ***The ethylene formation from α-ketoglutarate (Efe pathway).*** *The biosynthesis pathway of ethylene found in microorganisms such as Pseudomonas syringae and Penicillium digitatum. The immediate precursor for ethylene is α-ketoglutarate which is produced by the citrate cycle. Essential cofactors are L-arginine, FeII and O_2. For details, refer to the text and Equations 1.2, 1.3, 1.4 and 1.5 on page 9 [56, 82, 103].*

The whole cascade is composed of three reactions, with reaction 1.2 being the ethylene forming reaction, and 1.3 and 1.4 being the succinate-forming and L-arginine-decomposing reactions. The sum of all reactions gives the complete reaction 1.5 of the ethylene-forming enzyme [103]. The whole catalysis works in a dual-circuit mechanisms, which works as follows. In the first and main reaction L-arginine remains bound as co-factor and two α-ketoglutarate molecules are converted into 6 CO_2 and two ethylene molecules. This reation occurs two times. Then, in the second reaction α-ketoglutarate and L-arginine are consumed into L-Δ-1-pyrroline-5-carboxylate, guanidine, CO_2 and succinate [82, 103].

$$2-\text{oxoglutarate} + \mathrm{O_2} \longrightarrow \mathrm{C_2H_4} + 3\,\mathrm{CO_2} + \mathrm{H_2O} \tag{1.2}$$

$$2-\text{oxoglutarate} + 0.5\,\mathrm{O_2} \longrightarrow \text{succinate} + \mathrm{CO_2} \tag{1.3}$$

$$\text{L}-\text{arginine} + 0.5\,\mathrm{O_2} \longrightarrow \mathrm{CH_5N_3} + \mathrm{C_5H_7NO_2} + \mathrm{H_2O} \tag{1.4}$$

$$3\,\mathrm{C_5H_6O_5} + 3\,\mathrm{CO_2} + \mathrm{C_6H_{14}N_4O_2} \longrightarrow$$
$$2\,\mathrm{C_2H_4} + 7\,\mathrm{CO_2} + \mathrm{C_4H_6O_4} + 3\,\mathrm{H_2O} + \mathrm{CH_5N_3} + \mathrm{C_5H_7NO_2} \tag{1.5}$$

Compared to the bacterial enzyme, there is not much information about the structure and mechanism of the fungal enzyme [126]. The Efe from *Pseudomonas syringae* pv. *phaseolicola* PK2 (PDB number 5V2U) is a monomeric enzyme [126]. It contains a double-stranded β-helix (DSBH) which is typical for Fe(II)/2OG-dependent oxygenases. The metal-binding site is the so-called 2-His-1-carboxylate facial triad (His-Arg/Glu-His), built by the amino acids H189, D191 and H268 [82, 227], which is typical for the enzyme family, and can also be found in ACC oxidase (see **Paragraph 1.1.1.1.1**). H268 and D277 might be involved in the α-ketoglutarate binding [126]. When L-arginine is bound, it introduces certain conformational changes to the active site. [211]

The ethylene production rate in crude enzyme preparation (precipitated with $(NH_4)_2SO_4$) increased 20-fold when $FeSO_4$ was added to a final concentration of 0.1 mM. The production rate decreased on adding $MgSO_4$, $CuSO_4$, $MnSO_4$ and was completely inhibited when $ZnSO_4$, $CoSO_4$, $NiSO_4$ were added at the same final concentration.
L-arginine is the only amino acid increasing the production rate drastically [99].

1.1.1.3 Ethylene Biosynthesis in Microorganisms from KMBA

There are several microorganisms known to produce ethylene from KMBA. The proposed enzyme is an NADH Fe(III):EDTA oxidoreductase. Up to date, there is no sequence available for this enzyme, although the enzyme could be purified over various steps [106]. It is assumed that the enzyme is a hydroxyl radical ($\mathrm{OH}\cdot$) forming enzyme.

Catalase suppresses the ethylene formation in various microorganisms, including *Citrobacter sp.*, *E. coli* and others, which indicates that the mechanism involves the formation of radicals [34]. This has been shown by the decrease of ethylene by adding catalase to the reaction. Electron scavenging reagents also lower the ethylene amount [14, 354].

It is suggested that any system generating hydroxyl radicals can produce ethylene from KMBA [106, 234]. This was shown by the generation of an ethylene forming aerobic system using milk xanthine oxidase and xanthine from methional. This system was suppressed by adding superoxide dismutase or catalase, indicating that both superoxide

anion radicals and hydrogen peroxide are necessary to produce ethylene in such a system [25]. The system was also tested for KMBA [76]. Horseradish peroxidase was also used to generate ethylene from methional and was inhibited by catalase [363].

It seems that the formation of KMBA from L-methionine is made by a promiscuous transaminase [54, 138]. KMBA is then split into ethylene and other compounds by hydroxyl radicals, either produced enzymatically, chemically [54], or photochemically [379]. The reaction is enhanced under ammonia limitation [202, 282]. The formation of KMBA is supposed to be a way to recover amino nitrogen from methionine [82].

A protein responsible for the ethylene formation from the microorganism *Cryptococcus albidus* was isolated, but no protein sequence was determined. The enzyme is NADH dependent, needs O_2 and Fe(III) (see **Fig. 1.4**), and is not a "truly" ethylene-forming enzyme [106], as the two known ethylene-forming enzymes are 2OG oxygenases [82]. The optimal pH for the chemical formation of ethylene from KMBA with flavin mononucleotide was 3.5-3.8 [34, 54]. Another protein partially purified from cauliflower produced ethylene from methional [203].

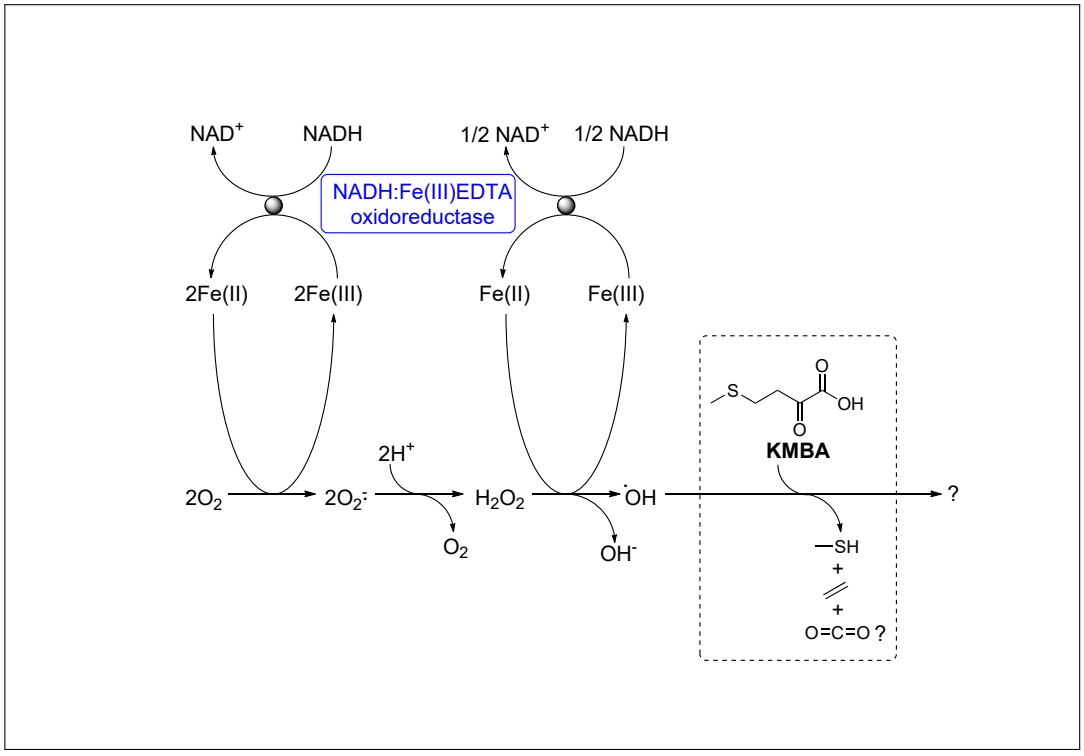


Figure 1.4: ***The ethylene formation from KMBA. Ethylene formation from KMBA.*** *The hydroxyl radical is the driving force, which means that any enzyme forming OH· can produce ethylene from KMBA. The enzyme is an NADH:Fe(III)EDTA oxidoreductase. Figure according to [106].*

1.1.1.4 Ethylene Biosynthesis in Soil

Ethylene has been found to be produced by various soil samples (forest, desert, loam) amended with various substrates, including L-met, ACC and KMBA [13, 14, 19, 199, 228]. The source is of biotic origin, as proven by autoclaved soil samples [14, 142, 229].

It was shown that various trace elements enhance ethylene biosynthesis in soil, with Co(II) being the most effective metal. In general, divalent ions are essential, as the activity drops by adding EDTA to the soils. The production of ethylene is also enhanced by various electron complexes including mannitol and hydroquinone. Ethylene formation seems to be a strictly biotic process, as amendment of autoclaved soils with ACC does not show significant ethylene production [158, 229]. The optimal pH for two soils tested in the above mentioned study was 7-7.5. The optimal temperature was between 30 °C and 35 °C. A *Bacillus* strain designated Bacillus ALK-7 has been isolated, which showed the production of ethylene from ACC [21]. Up to now, the mechanism is not clear and needs further elucidation.

Not only aerobic formation of ethylene was found. It was also shown to be formed from ACC in forest soils under anaerobic conditions with no information about the mechanism [142].

Interestingly, addition of EDTA inhibited the ethylene production from KMBA in soils [14], which is in contrast to the proposed enzyme mechanism by several studies [106, 138], which showed it to be increased (see **Section 1.1.1.3**). Considering the trace elements, Al(III) and Co(II) stimulated ethylene production, with the latter being the most effective. Fe(III) inhibited ethylene production.

Among the substrates KMBA, carbide (CaC_2), ACC and L-Met, ACC stimulated ethylene production the most. No ethylene was produced from ACC, KMBA and methionine from sterilized soils ($CaCl_2$ produced little amounts) [158, 228].

1.1.1.5 Ethylene Biosynthesis in Other Organisms

Ethylene synthesis was also shown in human lymphocytes during their response to pathogens [2] and is also found in rat liver microsomes from KMBA [92, 353, 355].

It has been shown that ethylene is also produced from forest soil samples in anaerobic conditions. The mechanism and the substrate are unknown until today [142]. It seems that the addition of L-Met has no effect on the formation of ethylene from soil samples under anaerobic conditions. However, addition of EDTA, ethionine and chlorogenic acid increased the anaerobic ethylene formation [303]. The anaerobic formation of ethylene might also arise from facultatively anaerobic microorganisms, such as *Pseudomonas* sp. [189].

Ethylene was also detected in sea sediments [358]. The mechanism of the production is not elucidated, but it has been shown that ethylene is formed from halogenated hydrocarbons (e. g. bromoethane sulfonate and 1,2-dichloroethane) in *Methanobacterium thermoautotrophicum* under anaerobic conditions [28]. Clear differentiation between abiotic [237] and biotic ethylene formation in sediments is not frugal. Unidentified anoxic pathways might also produce ethylene in sediments [358].

Ethylene was also found to be produced from organophosphates. In a screening, *E. coli* generated ethylene from ethylphosphonic acid, presumably by a radical based dephosphorylation mechanism [95].

An anaerobic version of the methionine salvage pathway was found to produce ethylene from L-methionine in the phototrophic bacteria *Rhodospirillum rubrum* and *Rhodopseudomonas palustris*. The direct precursor is believed to be 2-methylthioethanol [233].

1.1.2 Natural Pathways for the Biosynthesis of Propylene

The ability of short-chain olefin production is widely spread amongst microorganisms. In a screening aiming to identify propylene producers, the best identified yeasts were *Gliocladium roseum* IFO 7063 with 3.0 nL/mL culture/h and with 1.2 nL/mL culture/h *Schizosaccharomyces octosporus* IFO 0353 [98].

A screening for biodegradation of organophosphates by *E. coli* revealed that propylene was produced from the propylenophosphonic acid. The reaction mechanisms seem to be radical dephosphorylation based, as the same reactions were performed chemically with lead(IV) tetraacetate and electrochemical oxidation on a platinum anode [95].

Propylene was also detected in the fungus *Agaricus bisporus* [317] and under anaerobic conditions from methanogenic archaea in cow dung during a paper fermentation ranging from 0.13 to 0.21 ppm. *Penicillium digitatum* ATCC 10030 showed propylene production up to 1.2 ppm growing on potato glucose agar [71].

The pathway, involved enzymes and the mechanism remain unclear. However, the propylene production was shown to originate from 3-hydroxyalkanoic acids by the mevalonate diphosphate decarboxylase [72]. A cytochrome P450 from rabbit catalyzed the reaction from isobutyraldehyde to propylene. It is assumed that with the mono-oxygenase activity, O_2 is activated by NADPH as an electron source and the single oxygen atom is then transferred to the isobutyraldehyde, resulting in formic acid and propylene and water (from the other oxygen atom) [231, 257].

1.1.3 Current State Of Ethylene and Propylene Production

1.1.3.1 Chemical Production of Ethylene and Propylene

Pure ethylene and propylene do not occur naturally in fossil resources. Therefore, they need to be obtained by conversion of the crude naphtha. About 99% of ethylene is produced by steam cracking (also called pyrolysis) and catalytic cracking [374, 380].

Various feedstocks are used as starting material for ethylene and propylene: refinery gas, LPG, NGL, naphtha and gas oil [10, 380].

The most important products from steam cracking, are ethylene, propylene, butylenes and aromatic compounds, such as benzene, toluene and xylenes. These compounds exit the steam cracker as a mixture [26, 380].

In the process of steam cracking, a hydrocarbon stream is heated to 500-680 °C, depending on the feedstock. The stream is then heated to 750–875 °C with a strictly controlled temperature profile, a specific residence time and partial pressure. The hydrocarbons are cracked into ethylene, other olefins and di-olefins as the major products. These products interact and form other olefins, such as propylene. The products are cooled to 550–650 °C and separated by complex separation methods and chemical treatment steps. The typical yield for ethylene from steam cracked ethane is between 60–75%. [26, 380].

This technique is very energy demanding, as the cracking of hydrocarbons is highly endothermic [380]. In the US, about 67.5 $GJ/t_{ethylene}$ (and co-products) was used in 1994 [357]. The mechanism of olefin synthesis within a hydrocarbon stream is based on a radical mechanism. C-C bonds are cracked by homolysis, which produce more radicals from adjacent alkanes. Secondary hydrogen atoms from these alkanes are favorably abstracted to form alkenes.

1.1.3.2 Combined Biotechnological and Chemical Production of Ethylene and Propylene

This hybrid process has been described mainly for ethylene, but propylene can be derived from ethylene in this process. In this process, ethylene is produced from renewable resources (sugarcane) at an industrial scale. The Brazilian company Braskem produced 200,000 t ethylene in the year 2010 by the dehydration of ethanol. The ethanol was produced from sugarcane by fermentation of sucrose using a recombinant yeast strain and further processed to polyethylene [270, 325].

This process resembles the ethylene generation from petroleum in terms of using steam and high temperatures. It is long known [5] and the whole process is rather well

understood [90, 374] and rather simple due to the high selectivity of the catalysts used [380]. The bioethanol of 95% (w/w) purity is used as the raw material and dehydrated to ethylene using alumina-based catalysts (aluminum oxide or silicate/aluminum oxide catalysts [267]), molecular sieves (e. g. ZSM-5 type and Si-Al-phosphate (SAPO) type), temperatures of 300-500 °C and a pressure of 0.1–0.2 MPa [374] and is not affected by impurities of the ethanol [221]. A yield of 94–99% is achieved. Improvements are the focus of the research today, especially reactor design, as heating to at least 300 °C is necessary for a good yield. This process is believed to have a bright future [374].

Braskem also produces propylene, starting from ethanol-derived ethylene, followed by dimerization and metathesis [214, 298].

A second plant in Brazil produces 60,000 t/year (Solvay Indupa) and another facility is planned with a capacity of 190,000 t/year by Dow Chemical Company[90].

Advantages of the so-called “bioethylene process”, when compared to the petrochemical variant, are its better carbon footprint, market price stability and the relatively cheap nature of the process [286].

1.1.3.3 Biotechnological Production of Ethylene and Propylene

Recently, there has been intensive research to produce ethylene by an ethylene forming enzyme via α-ketoglutarate from microorganisms. These enzymes were cloned into appropriate production strains, such as *E. coli* [102], *Trichoderma viridae* [307], *Trichoderma reesei* [56] and *S. cerevisiae* [147, 245] (see **Tab. 1.3**). Some studies have also reported the enhancement of this enzyme by enzyme engineering techniques to obtain an increased production rate and overall yield. The biochemical details for the biosynthesis of ethylene via the α-ketoglutarate-dependent pathway is discussed in **Section 1.1.1**.

When reduction of CO_2 emissions and carbon footprint [205] is desired, it is possible to generate ethylene from *Synechocystis* sp. harboring the gene for the ethylene-forming enzyme (*efe*) [104, 122, 268, 320, 323, 371]. However, carbon footprint is only better when grown auxotrophically, but heterotrophic or mixotrophic growth releases CO_2 [82], which can be explained by the enzymatic reaction performed by the Efe protein, in which CO_2 is released (see **Section 1.1.1.2**).

Table 1.3: *Production rates or volumes of enhanced ethylene production in microorganisms*

Host	Volume/Rate of Ethylene Production	Substrate	Modifications	Reference
Saccharomyces cerevisiae	139 $\pm$ 10.1 $\mu g/g_{glucose}$	10 g/L glucose + 7.5 g glutamate	Expression of codon optimized *efe* from *Pseudomonas syringae pv. phaseolicola*	[147]
Saccharomyces cerevisiae	233 $\pm$ 0.8 $\mu g/g_{glucose}$	10 g/L glucose + 7.5 g glutamate	Expression of *efe* from *Pseudomonas syringae*	[148]
Saccharomyces cerevisiae	890$\pm$160 μg ethylene /g glucose	10 g/L glucose + either 5 g/L $(NH_4)_2SO_4$ or glutamate	Expression of *efe* from *Pseudomonas syringae*	[245]
Escherichia coli	10 μL ethylene/mg cell/h	LB medium	Expression of *efe* from *Pseudomonas syringae*	[141]
Trichoderma viride	1,059.7$\pm$119.99 nL/h/g dry wt	CI medium (contains 0.5% cellulose)	Expression of *efe* from *P.syringae pv. glycinea* ICMP2189	[307]
Trichoderma reesei	4,012 nL/h/L	wheat straw	Expression of *efe* from *P.syringae pv. glycinea* ICMP2189	[56]
Synechococcus PCC7942	10 nL/mL/h	CO_2 + light	Expression of *efe* from *Pseudomonas syringae*	[104]
Synechocystis sp PCC 6803	443 μL/L/h	CO_2 + light (220 μm photon/m^2/s^1)	Expression of codon optimized *efe* from *Pseudomonas syringae pv. phaseolicola*	[323]
Synechococcus sp. PCC 7942	33.4 nL/mL culture broth/OD_{730}/h	CO_2 + light ($1.39 * 10^4$ erg/cm^2/s)	Expression of *efe* from *Pseudomonas syringae*	[268]
Synechococcus elongatus PCC 7942	451 nl/mL/h/OD_{730}	CO_2 + light ($4.4 * 10^-4$ J/cm^2/s)	Expression of *efe* from *Pseudomonas syringae*	[306]
Synechocystis sp PCC 6803	7125 μg/L/h	CO_2 + light (50 mmol photons/m^2/s)	Expression of codon-optimized *efe* from *Pseudomonas syringae*	[320]
Synechocystis sp PCC 6803	$>$100 nl/ml/h	CO_2 + light (100 μE/m^2/s)	Expression of codon-optimized *efe* from *Pseudomonas syringae*	[122]

Selection of reported ethylene production improvements are taken from the review [82]. If various constructs and conditions were tested in the studies, the highest was taken to present in this table. NA: not available

Most of the studies so far used α-ketoglutarate and the ethylene-forming enzyme to generate ethylene. At the moment, these attempts are far from amounts useful for the chemical industry (for production rates and amounts, see **Tab. 1.3**). Ethylene production with engineered strains reaches from 6 $\mu g/g_{glucose}$/h ethylene with *S. cerevisiae*[1] [147] to 7125 μg/L/h with *Synechocystis* sp. PCC6803 [320].

So far, propylene has been biologically produced only on a laboratory scale. Propylene was produced from crotonic acid with an engineered 4-oxalocrotonate decarboxylase used in microorganisms up to 50 g/l/day [285].

A screening revealed that a P450 mono-oxygenases (in this case OleT) produced 1-alkenes using the corresponding C4-C22 acids as substrate. A cascade was designed using CamAB (a co-expressed electron transport system) as NADPH reducers and electron generators for the hydrogen peroxide production from oxygen by OleT, yielding in a continuous process to produce propylene from butanoic acid [74].

As mentioned above (see **Section 1.1.2**), a cytochrome P450 from rabbit produced propylene from isobutyraldehyde [231, 257]. It would be possible to generate isobutyraldehyde from glucose [298] and develop a process to form propylene [299]. A screening for biodegradation of organophosphates by *E. coli* revealed that propylene was produced from propylenophosphonic acid. The reaction mechanisms seem to be radical dephosphorylation based, as the same reactions were performed chemically with lead(IV) tetraacetate and electrochemical oxidation on a platinum anode [95].

In the screening, it was found that propylene was produced from 3-hydroxyalkanoic acids by the mevalonate diphosphate decarboxylase [72]. A process was developed in which 3-hydroxy-butyrate was generated from glucose and then converted into propylene by mevalonate diphosphate decarboxylase from *Picrophilus torridus* [208]. Another process is described using the same strategy. Here, 3-hydroxybutyrate is converted into propylene by the diphosphomevalonate decarboxylase from *Streptococcus salivarius* [9].

Several processes have been proposed by Harmsen, Paulien F. H., Hackmann, and Bos [124]: Propylene can be produced by the dimerization of ethylene into 1-butene, followed by a metathesis of the two to yield propylene. Fermentation of sugars can lead to propylene by the ABE fermentation to yield acetone, which is reduced to isopropanol. Another fermentation process converts sugars directly into isopropanol. This is dehydrated into propylene.

New theoretical pathways for the production of propylene are investigated using bioinformatic tools. It is possible to design the enzymes of these pathways using directed evolution and then implement them in microorganisms, such as *E. coli*. One such pathway starts from α-ketoglutarate to form S-adenosyl methionine (SAM), which is then converted to 2-methyl-2-ketoglutarate. From this compound, propylene is formed

[1] Calculated from 139 $\mu g/g_{glucose}$ divided by the fermentation duration of 24 h

using an engineered enzyme, resembling the pathway of ethylene production from α-ketoglutarate. Another pathway starts from pyruvate and leads over several steps and possible routes to 4-methyl-2-ketoglutarate, from which propylene is built. These pathways aim to come close to the theoretical stoichiometric maximum of 1.33 mol/mol (0.311 g/g) glucose in the overall reaction $3\,C_6H_{12}O_6 \longrightarrow 4\,C_3H_6 + 6\,CO_2 + 6\,H_2O$ [47].

The reactions of decarboxylases seem to be a buried treasure for the chemical industry. It would be interesting to find decarboxylases which use similar substrates to 3-hydroxyalkanoic acids and mevalonate diphosphate. This would maybe lead to a greater amount of decarboxylases being able to produce alkenes such as ethylene and propylene from substrates such as phosphono-oxy acids, which can be derived from glucose. With such pathways it would be possible to gain a productivity of about $2\,g_{alkene}$/L/h with a carbon balance of 0.66 [72, 165, 167, 204].

Global Bioenergies announced the production of propylene from glucose by direct fermentation on a laboratory scale [113, 124].

1.2 Isoprene

Isoprene is a C5 hydrocarbon and one of the simplest dienes first discovered in 1860 by C. G. Williams [352] (see **Tab. 1.1** for physico-chemical characteristics). It has two major significances: its role in the atmospheric sciences to contribute to the green house effect is being investigated with $600 * 10^6$ t/year emissions from trees [121, 278], and it is used to produce synthetic rubber and is the building block of natural rubber [11]. Furthermore, it is known across all three domains of life. Over 23,000 isoprenoids are made from its immediate precursor dimethylallyl pyrophosphate [265]. The current market volume is about 800,000 t per year [116].

Although isoprene is found as building block in a variety of compounds, such as terpenes and isoprenoids, isoprene itself is not released during production. The isoprene precursors isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are intermediates instead.

The biological role of isoprene in plants is to protect plants when exposed to stress, such as heat and reactive oxygen species, such as ozone [279]. Isoprene has been shown to protect the membrane lipid bilayer from degradation at 40 °C [277]. Similarly, the isoprene emission from *Bacillus subtilis* increases when H_2O_2, heat (48 °C) and 0.3% NaCl is applied to the culture [359].

1.2.1 Current Production Routes

Currently, isoprene is produced by steam cracking petroleum. Here, it is extracted from the naphtha originating C5 cracking fraction and by dehydration of C5 isoalkanes and C5-isoalkenes. Another way is the isoprene formation from synthetic reaction from smaller carbon units. These include the reaction of acetone with acetylene (10-40 °C, 20 bar, KOH in ammonia) to 2-methyl-3-butyn-2-ol with partial hydration (250-300 °C, 1 bar, Al_2O_3) to get isoprene. Another reaction is the dimerization of propene to isohexene (Ziegler catalyst), followed by the cleavage of a methane molecule (650-800 °C, catalytic amounts of HBr) among others [11]. It is used to produce *cis*-1,4-polyisoprene, natural rubber [11, 116, 159, 286]. In 1997, 0.85 t per year were produced worldwide, and the interest to it is growing [11]. Isoprene was listed in the Department of Energy's TOP 10 carbohydrates for biorefineries revised list (TOP 10 + 4) in 2010 [41].

There is no isoprene generated from renewable resources relevant to a world market, although global players like Goodyear are active in this field [348]. However, it should be noted that natural rubber (*cis*-1,4-polyisoprene) is produced solely from the tree *Hevea brasiliensis* with $12 * 10^6$ t in 2014 worldwide [26]. Calculations from 2015 showed that the price for bio-isoprene would be slightly higher than the actual market price for fossil isoprene. Thus, bio-isoprene could become competitive depending on the oil price [222, 299].

Isoprene in nature is used to build a huge number of valuable compounds, such as carotenoids, cholesterol and the pharmaceutical relevant artemisin (treatment for malaria) and taxol (treatment for cancer) [135]. Petrochemically produced isoprene is currently used together with 1,3-butadiene to produce synthetic rubber [11]. Future applications for isoprene, such as biofuel [263] and anti-knock agent [200] are discussed.

1.2.2 Biosynthesis of Isoprene

Pathways to generate isoprenoids are essential [261] and found in all domains of life, in bacteria, eukarya and archaea [164, 277]. However, isoprene is not built by all organisms, as the key enzyme is isoprene synthase (EC 4.2.3.27), which produces isoprene from dimethylallyl pyrophosphate. Isoprene formation in microorganisms was discovered about 20 years ago [174], whereas the discovery of isoprene was over 150 years ago [352]. To date, two pathways are known by which isoprene is produced: the mevalonate pathway (MVA pathway) and the 2*C*-methyl-D-erythritol-4-phosphate

pathway [2] (hereafter called MEP pathway). An overview of both pathways and their importance in the cell metabolic network is presented in **Fig. 1.5**.

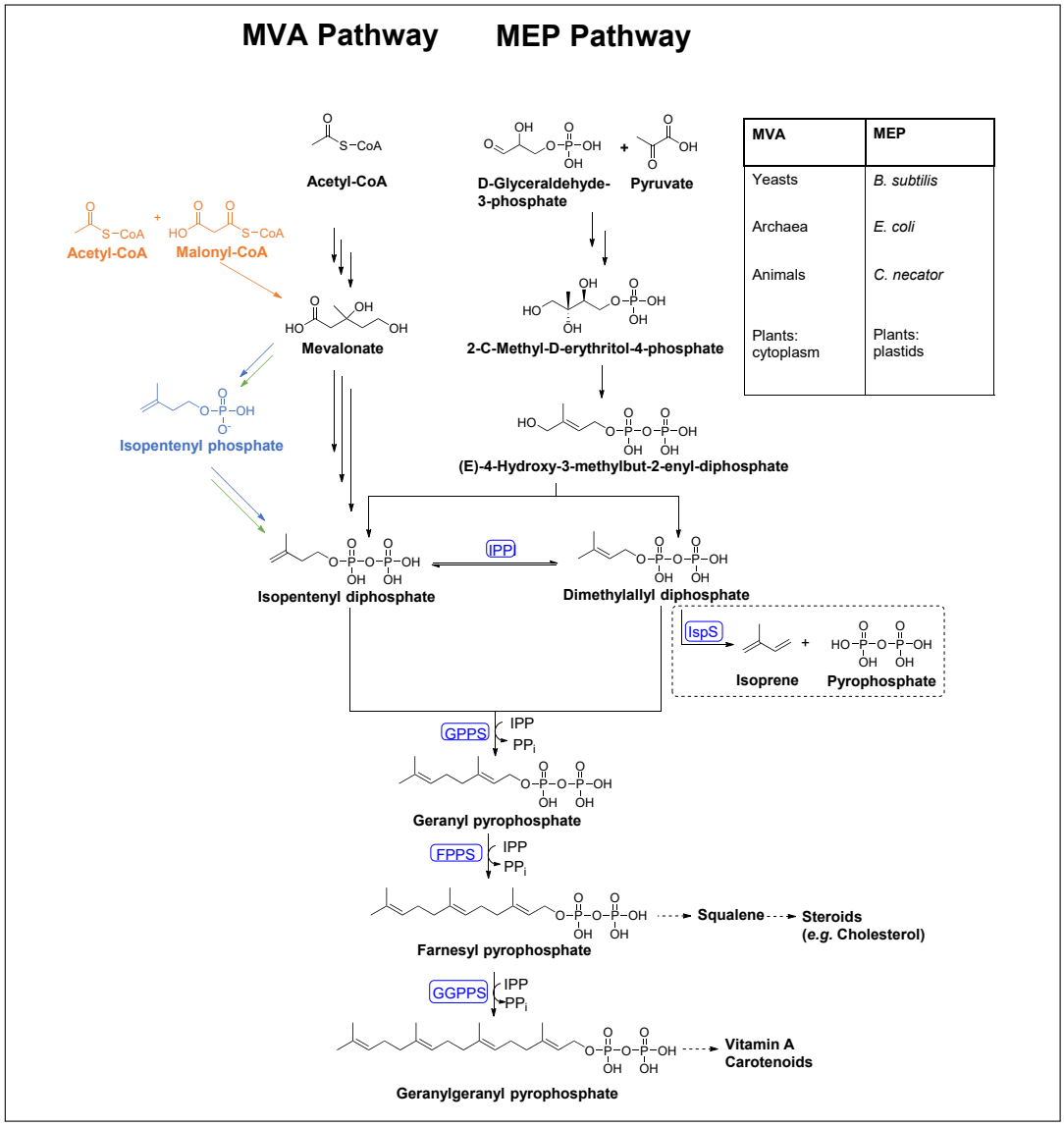


Figure 1.5: ***Isoprene biosynthesis pathways.*** *Up to now, two distinct pathways are known by which isoprene is synthesized. GPPS, geranyl pyrophosphate synthase; FPPS, farnesyl pyrophosphate synthase; GGPPS, geranylgeranyl pyrophosphate synthase [20, 329]. Black arrows: routes found in most organisms. Orange arrows: reaction in MVA pathway from Streptomyces sp. CL190 [175, 176, 235]. Blue and green arrows: alternative route proposed for Methanocaldococcus jannaschii [73, 118, 144, 164], Haloferax volcanii [322] and Thermoplasma acidophilum [17, 331–333]. Solid arrows indicate chemical reactions. Dashed arrows indicate pathway routes [232].*

[2] Also called non-mevalonate pathway, mevalonate indipendent pathway, 1-deoxy-D-xylulose-5-phosphate pathway (D(O)XP pathway) and GAP-Pyruvate pathway.

The MEP pathway exists exclusively in bacteria and in plastids of eukaryotes [193, 213], which were endosymbionts descending from prokaryotes [209, 275, 313], whereas the MVA pathway is predominant in eukarya and archaea. Today, it is thought that the isoprenoid pathway evolved independently in the bacterial and the archaeal lineage, with the last common ancestor (the cenancestor) having no isoprenoid synthesis [178, 193, 210], and thus being not cellular [163] or not enclosed by a membrane [210]. Some bacteria also have the MVA pathway like *Streptococcus pneumoniae*, *S. aureus* and *E. faecalis* [193, 262, 295, 335], which was first believed to be caused by horizontal gene transfer [39, 351], but recent phylogenetical analyses show that it was possibly present in the cenancestor [193].

The **MEP** pathway (see **Fig. 1.6**) is present in bacteria including Gram positive and negative ones, such as *B. subtilis* and *E. coli* and in plastids of plants.

This pathway starts from D-glyceraldehyde-3-phosphate, a central metabolite of glycolysis and formed in chloroplasts during the Calvin-Benson cycle [22] and pyruvate. From here, 1-deoxy-D-xylulose-5-phosphate is generated by 1-deoxyxylulose-5-phosphate synthase (DXS). This compound is converted to the pathway's namesake 2-C-methyl-D-erythritol-4-phosphate by 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR). Over three steps, 4-Hydroxy-3-methyl-but-2-enyl pyrophosphate is formed by 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase (HDS). It is then converted to isopentenyl diphosphate or dimethylallyl pyrophosphate by hydroxymethylbutenyl diphosphatereductase (HDR) [339], depending on whether the C2-atom (IPP) or the C4-atom (DMAPP) is protonated [340]. The ratio of IPP:DMAPP in plastids of tobacco BY-2 cells is 85:15 [315] and in *E. coli* 6:1 [260].

The **MVA** pathway (see **Fig. 1.7**) is predominantly present in eukarya, such as yeasts, animals, in the cytosol of plants and in the third domain of life: archaea. In the literature, it is sometimes divided into the upper MVA pathway, which produces Hydroxymethylglutaryl-CoA (HMG-CoA), and the lower MVA pathway, which produces IPP and DMAPP [361].

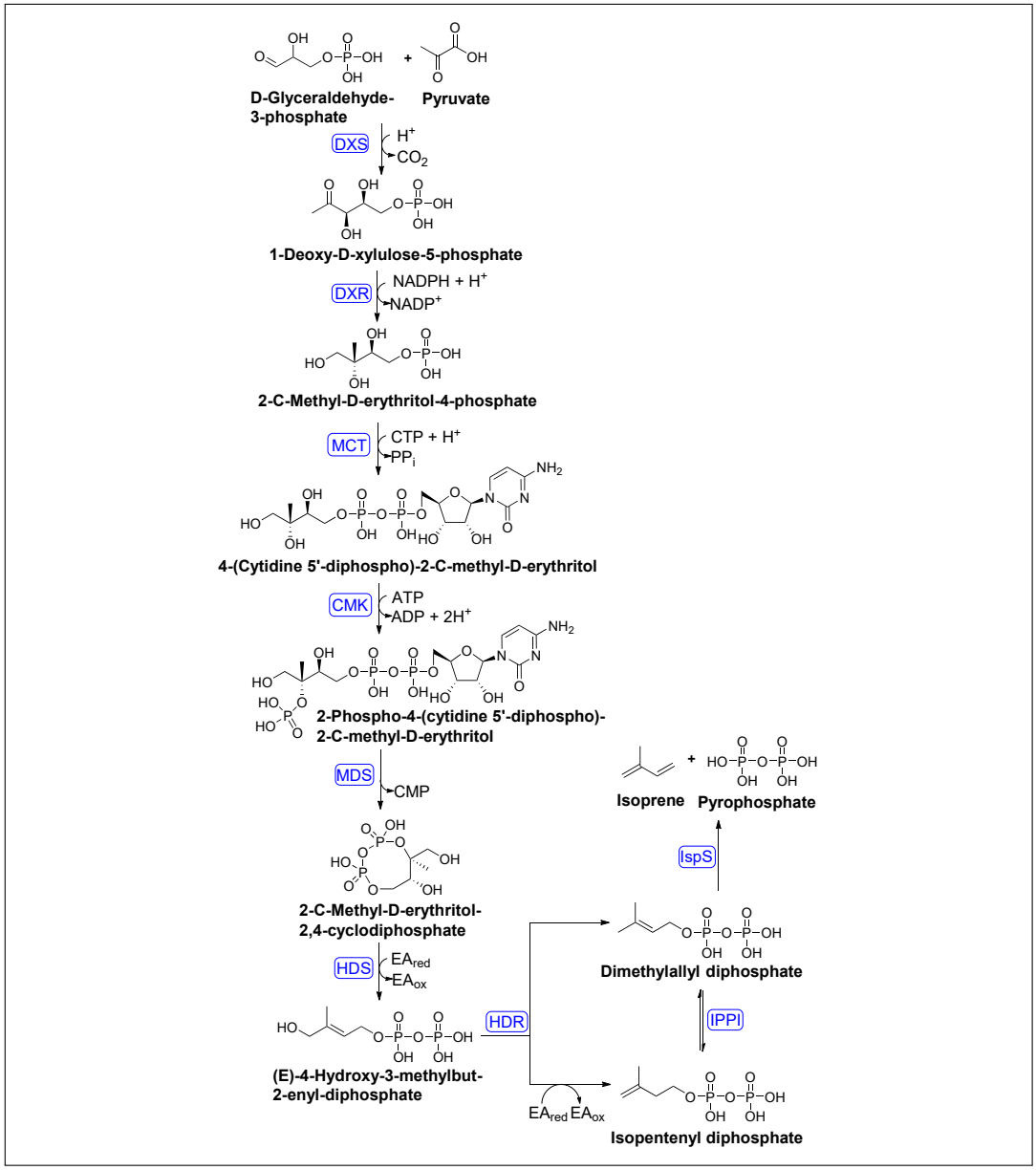


Figure 1.6: ***MEP-pathway for isoprene biosynthesis.*** *DXS, 1-deoxyxylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; MCT, 4-diphosphocytidyl-2-C-methyl-derythritol synthase; CMK, 4-diphosphocytidyl-2-C-methyl-derythritol kinase; MDS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase; HDR, hydroxymethylbutenyl diphosphatereductase; IPPI: isopentenyl diphosphate Δ-isomerase, IspS, isoprene synthase [20, 329].*

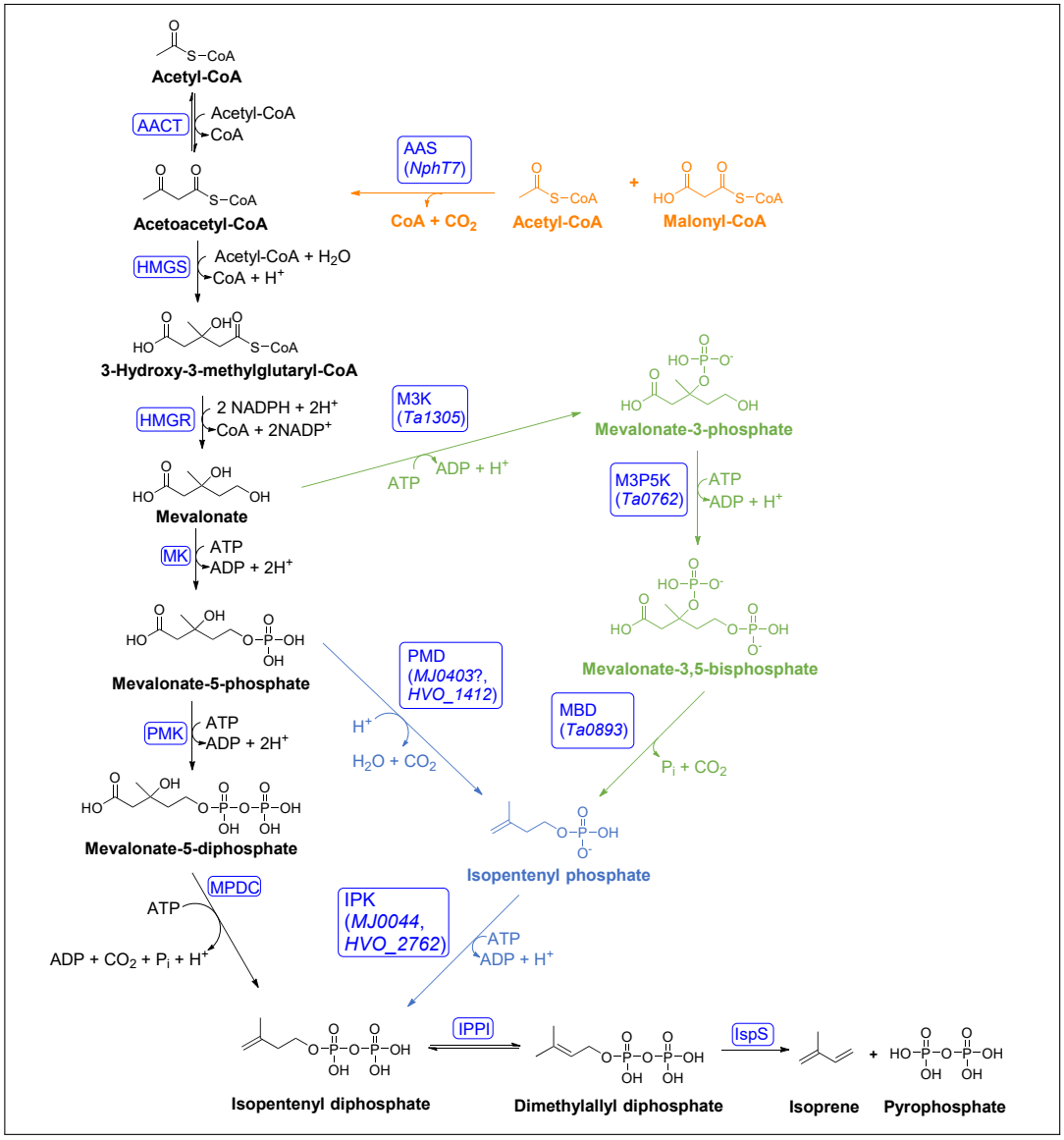


Figure 1.7: ***MVA-pathway for isoprene biosynthesis.*** *AACT, Acetyl-CoA C-acetyltransferase; HMGR, Hydroxymethylglutaryl-CoA reductase; HMGS, Hydroxymethylglutaryl-CoA synthase; MK, Mevalonate kinase; PMK, Phosphomevalonatedecarboxylase; MPDC, Diphosphomevalonatedecarboxylase; AAS, acetoacetyl-CoA synthase; IPK: Isopentenyl phosphate kinase, PMD: phosphomevalonate decarboxylase. M3K: mevalonate-3-kinase, M3P5K: mevalonate-3-phosphate-5-kinase, MBD: mevalonate-3,5-bisphosphate-decarboxylase. IPP, isopentenyl pyrophosphate; CMP, cytidine 5' monophosphate; CTP, cytidine 5' triphosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; PP_i, pyrophosphate; $NADP^+$, nicotinamide adenine dinucleotide phosphate reduced; NADPH, $NADP^+$, nicotinamide adenine dinucleotide phosphate oxidized; $EA_{red/ox}$, electron acceptor reduced/oxidized; CoA, coenzyme A; IPPI: isopentenyl diphosphate Δ-isomerase, IspS, isoprene synthase [20, 329]. Black arrow: standard pathway reactions. Blue arrow: alternative route proposed for Methanocaldococcus jannaschii [73, 118, 144, 164] and Haloferax volcanii [322]. Green arrow: MVA pathway proposed for Thermoplasma acidophilum [17, 331–333]. Orange arrows: reaction in MVA pathway from Streptomyces sp. CL190 [175, 176, 235].*

The pathway starts from acetyl-CoA, which is a central metabolite from various pathways, including glycolysis. Two molecules are combined by acetyl-coenzyme A C-acetyltransferase (AACT) to form acetoacetyl-CoA via a thioester-dependent Claisen condensation. From acetoacetyl-CoA, 3-hydroxy-3-methylglutaryl-CoA is built by HMG synthase (HMGS). From here, HMG reductase (HMGR) catalyzes the step to the eponymous compound, the mevalonate, by reducing HMG-CoA two times, each step requiring NAD(P)H. Mevalonate is then phosphorylated by mevalonate kinase (MK) and phospho-mevalonate kinase (PMK) and then converted by an ATP-coupled decarboxylation into one of the central compounds in isoprenoid metabolism: IPP. IPP is isomerized to DMAPP by IPP-Δ-isomerase (IPPI). From here, isoprenoids, such as carotenoids, chlorophylls and many more are built or isoprene is formed, if an isoprene synthase (IspS) is present [20, 329].

In some archaea, such as *Haloferax volcanii*, there is a modification to the MVA pathway, which branches from mevalonate-5-phosphate, generates isopentenyl phosphate and joins at isopentenyl diphosphate [73, 118, 144, 164, 322]. In *Thermoplasma acidophilum*, a third alternative to the classical MVA pathway was found, in which mevalonate-3-phosphate is formed and presumably also isopentenyl phosphate [17, 331–333] (see **Fig. 1.7**). Another modification of this pathway was found in *Streptomyces* sp. strain CL190, isolated from soil. Here, an acetoacetyl-CoA synthase, belonging to the thiolase superfamily, encoded within the MVA pathway gene cluster (gene: *nphT7*), is capable of condensing acetyl-CoA and malonyl-CoA to acetoacetyl-CoA and CoA [176, 235]. The pathway starting from acetoacetyl-CoA is the same as the classical pathway.

In the last step, IPP is isomerized to DMAPP, from which isoprene and isoprenoids are derived. There are two types of IPP isomerases (EC 5.3.3.2) [181], the IDI type II using flavin mononucleotide (FMN) as a cofactor, metal ions and NADPH, exclusively found in eubacteria (e. g. *B. subtilis*, *Streptomyces* sp. CL190) and archaea [24, 79, 132, 153, 193]. The IDI type I requires divalent cations (Mn(II) or Mg(II)) and is found, for example, in *E. coli* and two isoenzymes in humans [43, 64, 135] and insects [75]. The mechanism involves the formation of a carbocation [254, 301, 377].

Isoprene is then formed by isoprene synthase (IspS) by removing the pyrophosphate from dimethylallyl pyrophosphate. Another way for isopentenyl diphosphate and dimethylallyl pyrophosphate is to build isoprenoids. Within the class of isoprenoids, there are the divisions hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30) and tetraterpenes (C40) [232].

Isoprene synthases have only been identified [137, 219] and characterized [272] in plants. However, various microorganisms showed isoprene formation during growth [150, 174, 289], but no bacterial isoprene synthases have been identified so far [329].

1.2.3 Current State of Biotechnological Isoprene Production

There have been numerous strategies to produce isoprene from renewable resources. Two strategies are most predominantly found in the literature: firstly, engineering of *E. coli* to produce isoprene, as it is the most promising candidate [159, 329]. Secondly, the production of isoprene from CO_2 and light using photosynthetic microorganisms such as *Synechococcus elongatus* [110] and *Synechocystis* sp. [188]. Based on theoretical calculations (including economic evaluations, material efficiency, land use etc.), the photosynthetic production of isoprene from renewable resources by microalgae is more efficient than the bacterial production, mainly *E. coli*. However, with more cost optimization and further yield increases, *E. coli* would also be competitive to petroleum-based isoprene [212]. The maximum theoretical yield of isoprene from glucose is 0.30 g/g for the MEP pathway with the net reaction $C_6H_{12}O_6 + 2ATP + 3NADPH + NAD \longrightarrow IPP + CO_2 + 2\,ADP + 3\,NADP + NAD$ and 0.25 g/g for the MVA pathway assuming the net reaction $1.5C_6H_{12}O_6 + 2\,O_2 \longrightarrow C_5H_8 + 4\,CO_2 + 5\,H_2O$ [232, 299, 348]. However, the MEP pathway needs more energy in form of ATP and reducing equivalents [232].

To produce isoprene from light and CO_2, the isoprene synthase from *Pueraria montana* (kudzu) was heterologously expressed in *Synechocystis* sp. PCC 6803 as a proof-of-concept and produced about 50 µg/g cell dry weight/day isoprene [188]. Another study used *Synechococcus elongatus* PCC 7942 for isoprene production [110].

The effort to produce isoprene via fermentation was made possible by the discovery of the first isoprene synthase from *Populus alba* and functional expression in *E. coli* [219].

In order to produce isoprene at an industrial scale, two strategies are pursued: the yields are increased (see **Tab. 1.4**), and the purity is increased by decreasing fermentation by-products. This is achieved by interrupting the pathways leading from acetyl-CoA to a variety of metabolic intermediates [159].

Isoprene has been produced at 60 g/L and been purified from the fermenter off-gas to produce rubber and tires by Goodyear and Genencor [91, 348].

Scarce attempts have been made to improve or introduce isoprene production in other organisms. Species included were *Yarrowia lipolytica* with 0.5-1.0 µg/L in the culture headspace and *Trichoderma reesei* with 0.5 µg/L [42, 329], with an engineered *S. cerevisiae* with an isoprene production of 16.1 µg/L [127, 329] being the best.

Table 1.4: *Titers of Isoprene Enhancement*

Host	Amount of Isoprene	Substrate	Modifications	Reference
E. coli AceCo	1823 mg/L	NA	*E. coli* MG1655 Δ*ackA-pta poxB ldhA dld adhE pps atoDA*, *Populus trichocarpa* isoprene synthase, exogenuos MVA pathway, Codon optimization, adjusted RBS strength	[159]
E. coli BL21 (DE3)	6.3 g/L after 40 h	20 g/L glucose	Upper MVA pathway from *Enterococcus faecalis*, MvaS-A110G, *ispS* from *Populus alba*	[361]
E. coli BL21 (DE3)	314 mg/L after 40 h	20 g/L glucose	*ispS* from *Populus nigra* codon optimized, overexpression of *B. subtilis* ATCC 168 *dxs* and *dxr*	[376]
E. coli	60 g/L	NA	Overexpression of *Populus alba ispS*, bacterial/yeast hybrid MVA pathway with added *Methanosarzina mazei mvk*, phospho-nogluconolactonase from pentose phosphate way	[348]
E. coli	320 mg/L culture	NA	Overexpression of *Pueraria montana ispS*, overexpression of native MEP pathway, overexpression of MVA pathway	[381]
E. coli	2.727 mg/g/h (per dry weight)	NA	Overexpression of native *dxs*, *dxr*, *idi* and *ispS* from *Populus alba*	[198]
E. coli BL21 (DE3)	532 mg/L	20 g/L glucose	Coexpression of MVA pathway from *Saccharomyces cerevisiae* (non-codon optimized) and *ispS* from *Populus alba* (codon optimized)	[362]

Continued on next page

Host	Amount of Isoprene	Substrate	Modifications	Reference
Bacillus subtilis DSM10	2.37±0.53 ng/mL/OD_{600}	LB medium	Overexpression of native *dxs* and *dxr*	[359]
Bacillus subtilis DSM402	1275 μg/L/OD_{600}	LB medium	Overexpression of the non-codon optimized *ispS* from *Pueraria montana* kudzu	[114]
Bacillus subtilis DSM402	1.2 mg/L (batch), 30 mg/L	NA	Overexpression of *P. montana ispS* (similar results with MVA overexpression)	[329]
Synechocystis sp. PCC6803	50 μg/g cell dry weight/day	CO_2 + light	*Pueraria montana* kudzu *ispS*, codon optimized	[188]
Synechococcus elongatus PCC 7942	1.26 g/L after 21 d	CO_2 + light (100 μM of photons m^{-2} s^{-1})	Overexpression of fusion protein *E. globus idi* - *P. alba ispS*, *dxs*, *ispG* from *Thermosynechococcus elongatus*	[110]
Pantoea citra	10 μg/L	NA	Truncated and for *E. coli* codon-optimized *P. montana ispS*, in various constructs	[49, 329]
Pantoea ananatis	63 μg/L	NA	*M. bracteata ispS*	[127, 329]
Corynebacterium glutamicum	24.2 μg/L	NA	*M. bracteata ispS*	[127, 329]
Enterobacter aerogenes	316 μg/L	NA	*M. bracteata ispS*	[127, 329]
Saccharomyces cerevisiae	500 μg/L culture (+ by-products)	2% galactose (YPG medium), anaerobic incubation	Overexpression of codon-optimized *ispS* from *Pueraria montana var.lobata* kudzu, multiple copies genome integrated in rDNA loci	[131]
Saccharomyces cerevisiae	37 mg/L	2% glucose	Overexpression of native aldehyde dehydrogenase, alcohol dehydrogenase and *ispS* from *Populus alba*	[197]
Yarrowia lipolytica	0.5-1.0 μg/L	NA	NA	[42, 329]
Trichoderma reesei	0.5 μg/L	NA	NA	[42, 329]

NA: not available. Isoprene production yields were taken from the reviews [329], [119] and include own literature search.

There are also attempts to produce isoprene via non-natural occurring pathways. Bredow and colleagues are generating isoprene by a novel constructed pathway from 3-methyl-2-buten-1-ol over two enzymatic steps to isoprene [42, 329]. Another novel artificial way to produce isoprene is from mevalonate over 3-methy-3-buten-1-ol by $OleT_{JE}$ and by an oleate hydratase [360]. Isoprene is also produced by adding two vinyl groups to either of the precursors isobutyryl-CoA, 3-methyl-2-oxopentanoate, or 4-methyl-2-oxopentanoate [38].

Isoprene has also been produced in a cell-free enzyme cascade comprising 12 enzymes from pyruvate and phosphoenolpyruvate [166] and by using five enzymes from the mevalonate pathway, starting with the mevalonate kinase to form isoprene from mevalonate [57]. Advantages in using cell-free systems are that toxic products, by-products and intermediates do not affect titers and the enzyme amounts can be balanced more thoroughly in contrast to *in vivo* overexpression [329].

1.3 Biorefinery: Production of Chemicals from Biomass

1.3.1 Biorefinery

The processes taking place in a biorefinery have been defined by the "International Energy Agency (IEA) Bioenergy Task 42" as

> "*Biorefining is the sustainable processing of biomass into a spectrum of marketable products and energy.*" [58]

The American National Renewable Energy Laboratory (NREL) defines biorefineries as

> "*A biorenifery is a facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass. The biorefinery concept is analogous to today's petroleum refineries, which produce multiple fuels and products from petroleum. Industrial biorefineries have been identified as the most promising route to the creation of a new domestic biobased industry.*" [152]

To match this concept and to be profitable in the context of a bioeconomy, the biorefinery needs to produce several products from various feedstocks and produce

as little waste as possible [244]. The petroleum refinery uses petroleum, liquefied petroleum gas (LPG) and natural gas liquids (NGL) as feedstocks [11], whereas in a biorefinery carbohydrates, such as lignin and cellulose, proteins, lipids (fatty acids and oil) and starch are used [244] (see **Fig. 1.8**). Characteristic for refineries is the platform technology based on intermediates: specific chemicals produced from different feedstocks are processed to various products [244].

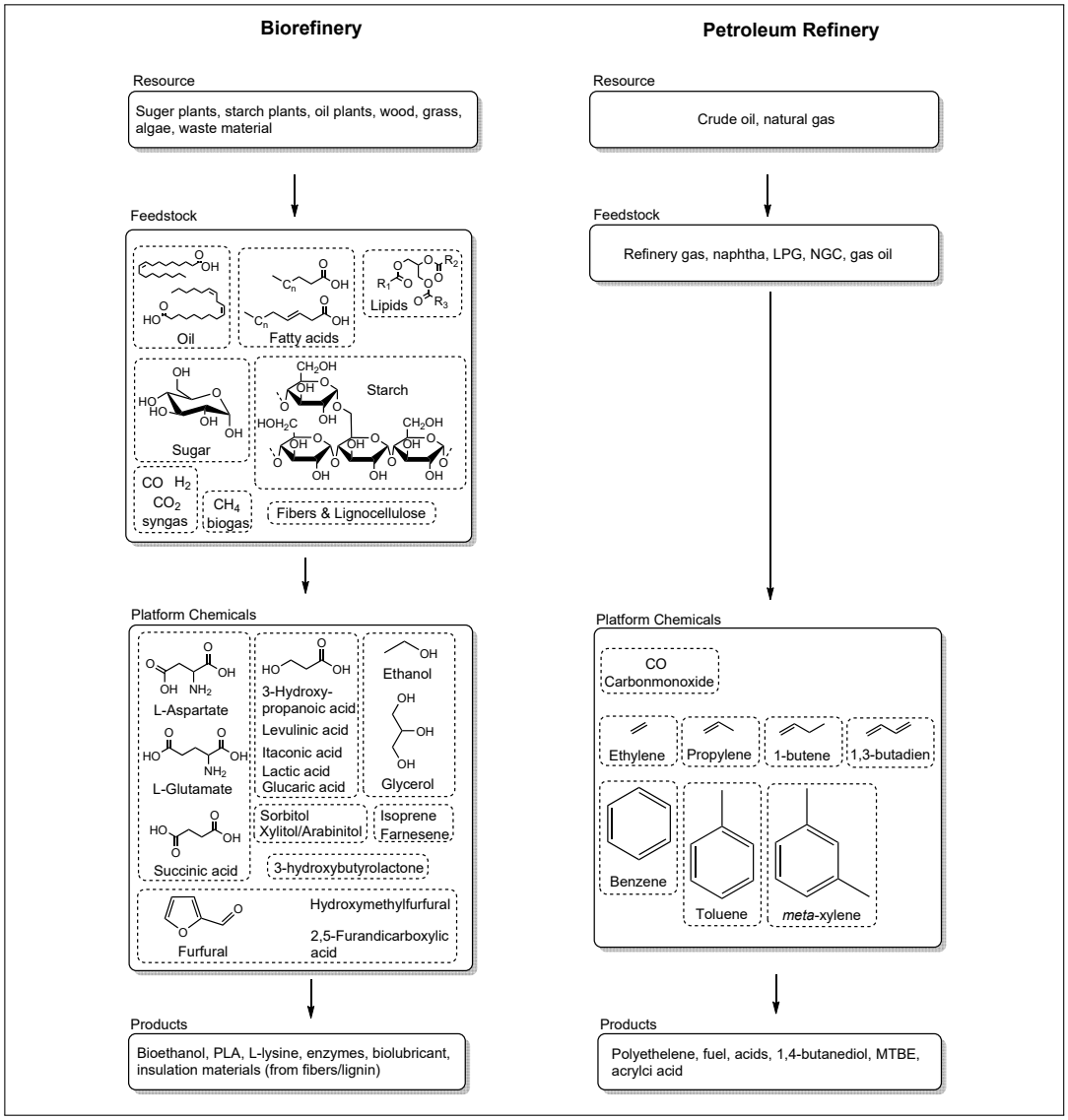


Figure 1.8: ***Feedstock and platform chemicals of biorefineries and petroleum refineries.*** *Comparison of feedstocks, platform chemicals and products of biorefineries and petroleum refineries. Based on [10, 26, 60, 244, 347, 380].*

Based on the technological status, there are three types of biorefineries, which are categorized as generation I, II and III. First generation biorefineries have a single

substrate, a fixed process and a single product. The second generation is characterized by a single substrate, various processes and diverse products. A generation III biorefinery is the most advanced biorefinery with all the traits of generation II, but with the option to use multiple substrates. Generation III-biorefineries are not operational at the moment, but are presumed to be functional in 2030 in Europe [86, 316], which is promoted by the European government [87].

The processing of biomass in biorefineries includes thermochemical, biochemical, chemical and mechanical treatment, among others. For example, lignocellusosic residues can be gasified to yield syngas (H_2 and CO), which can be processed to alcohols and Fischer-Tropsch biofuels [58]. Nowadays, syngas is also used in the industry from combusting fossil resources, i.a. brown coal and natural gas [10]. There already exist biorefineries in which bio-ethanol and polylactic acid i. a. are produced from extruded maize by fermentation [244]. A combination of chemical and enzymatic catalysts would also be a promising concept, combining the best of both worlds [324].

Another type of biorefinery is the “green biorefinery” which uses green plants as substrate. During the processing of the biomass, the cell juice (cytoplasm) is separated from the lignocellulose containing cell wall and treated differently in the following processes [152].

The idea of the biorefinery is to provide new chemicals and platform chemicals to the chemical industry, which they process today. This way, the change of resources is as smooth as possible. A good example is the case study made by Gevo, Toray and Coca Cola to produce 100% bio-based PET from bio-based isobutanol [60]. It is necessary to keep in mind the huge potential of newly discovered materials and chemicals that nature already synthesizes in a bioeconomical sense [316].

It is necessary to efficiently couple the glucose-degrading process with the formation of desired chemicals in order to get a competitive biorefinery [48]. The farm-stead biorefinery (see **Section 1.3.2**) is one of the concepts to highly integrate the whole process, from harvesting to the end product.

1.3.2 Concept of the Farm-Stead Biorefinery

The farm-stead bio-refinery is a concept for a “green biorefinery” located either on farms or distributed as hubs in the area (see **Fig. 1.9**). The feedstocks will be biomass from renewable resources, in order to be as sustainable as possible. An early definition of renewable resources is given as

> “The total range of living organisms providing man with food, fibres, drugs, etc [...]” [345]

The feedstocks will be namely grass-waste and (hydrolyzed) grass silage [165]. Excess permanent grassland with multiple harvesting has an annual dry matter yield of circa 8 t/ha in Europe [201]. The availability of unused grass is thought to increase, as dairy cow husbandry and cattle breeding is expected to decrease in the EU [152, 169] and it must be preserved as grass land according to European law [88]. The substrate for processes in the farm-stead biorefinery will be silage, separated into its liquid portion silage press juice, and its solid portion, fibers. Silage has the advantage of being available throughout the year without quality loss and hence, is feasibly stored [80, 319]. It has to be coupled to a biogas power plant in order to operate profitably [165].

Over the years, the definition became more differentiated as a branch to primarily produce chemicals and non-food products from renewable resources was established. This issue rose public awareness in the past years as the "food vs. fuel" debate [308]. Biomass is abundantly available and thus represents a highly promising renewable resource. If managed appropriately, it is possible to avoid an overlap in both food and fuel supply [162]. As the climate is changing, a new consideration has to be made in terms of using renewable resources. These renewable resources should act as a sink for CO_2 and are favored over renewable resources producing more CO_2, such as photosynthesizing organisms. When renewable resources are used to produce energy (by thermal utilization or in case of biofuels) at the end of their life cycle, only the same amount of CO_2 is released as was bound to build it. In this respect, renewable resources are carbon emission neutral [316] and can play an important role in stopping the climate change.

The facility will be modularized, in which module 1 (see **Fig. 1.9**) is selected depending on the substrate. The second module is chosen according to the product of the first module and the desired platform chemicals. As main target, the conversion of silage press juice (derived from silage) by microbial fermentation to gaseous products such as ethylene, propylene and isoprene is prioritized [165].

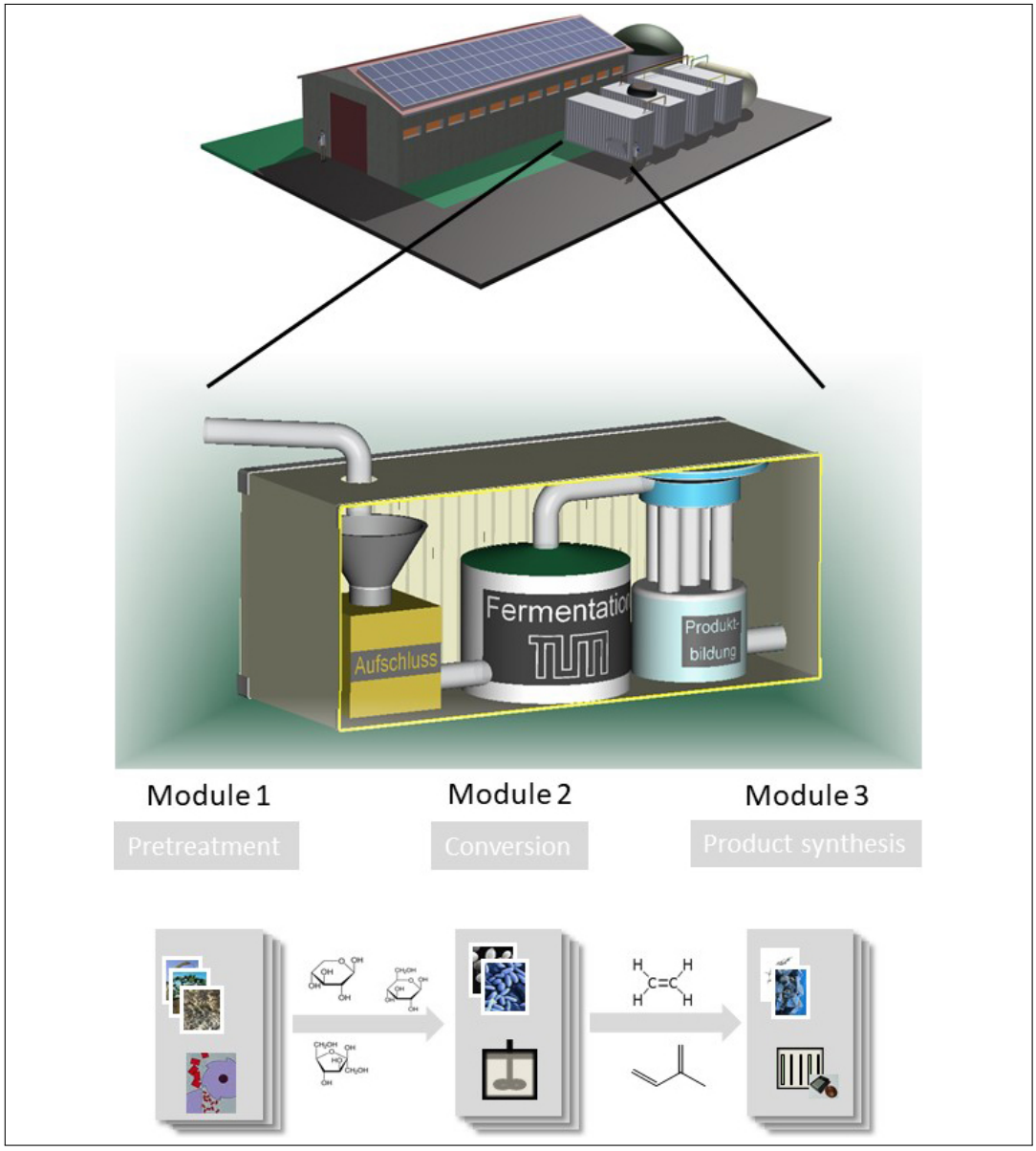


Figure 1.9: ***Concept of the farm-stead biorefinery.*** *The biorefinery is located on farms or as hubs in community areas. The conversion steps of the farm-stead biorefinery take place in the containers. Each container will be specialized for a specific substrate. The middle section shows one container from the inside. The substrate is pretreated in module 1 to sugars and organic acids. Then converted by microbial fermentation, chemical conversion or enzymes to platform chemicals in module 2. In module 3, products are made or platform chemicals are collected. The high adaptability of this approach is achieved by the exchangeable module. Figure taken from the Kompetenzzentrum für Nachwachsende Rohstoffe [165]. To be profitable, it is coupled with a biogas plant.*

Having the downstream process in mind, the separation of the products can be time, energy and chemicals consuming. Typical separation techniques include precipitation,

flotation, membrane separation, electrophoresis, chromatographic separation [59], distillation and separation with solvents. The main advantages of the “farm-stead biorefinery” are that purification of the product is easy as no separation from the fermentation broth is needed [204, 207, 336, 359]; and product inhibition does not occur, especially when the gas phase is continuously exchanged. Technologies to purify and separate desired products from the gas phase are available from the current production of olefins by steam cracking [380] and are being investigated intensively [29, 91].

Genencor and Goodyear developed a process in which *Escherichia coli* (*E. coli*) harbors a (synthetic) pathway for the production of isoprene. They were able to generate 60 g/L isoprene with high purity from the fermenter off-gas [91, 184, 207, 348]. With the selection of these products, the farm-stead biorefinery is following the so-called drop-in strategy, in which petro-based platform chemicals are provided from renewable resources [124]. Another advantage of the “farm-stead biorefinery” will be its short delivery ways for the substrates, reducing cost and transportation shortcomings.

The biggest challenge is to make this facility competitive with the petroleum based products in terms of production volume and price, as 1 t ethylene cost 1,103.66 $ in October 2017 [264]. Moreover, a competition with other alternative methods to steamcracking and feedsstocks for the generation of olefins from the chemical industry is challenging [7].

1.4 Aims of the Thesis

The farm-stead biorefinery concept integrates an easy way to convert renewable resources into valuable products, with a focus on bulk chemicals. In order to keep the process as simple as possible and cheap, the desired products – intermediates in the farm-stead biorefinery – should be easily separated from the fermentation broth. Hence, gaseous products such as ethylene, propylene and isoprene are preferred. An ideal process should come close to the theoretical stoichiometry from glucose of 0.31 g/g for ethylene [298] and 1.33 mol/mol for propylene [47], respectively.

The aim of this part is to find more and new enzymatic or microbial conversions to produce ethylene and propylene especially by decarboxylases, similar to the conversion of 3-hydroxyalkanoic acids to propylene by mevalonate diphosphate decarboxylase [72]. Accordingly, a screening (see **Fig. 1.10**) with 3-phosphonooxy propanoic acid, β-alanine, 3-sulfo-oxy butanoic acid and 3-phosphonooxy butanoic acid substrates was performed (see **Section 3.1**).

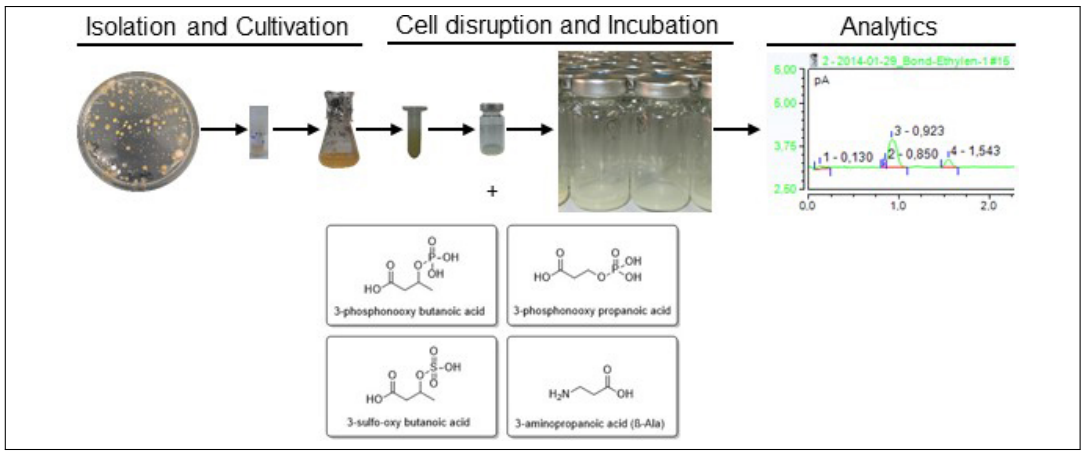


Figure 1.10: ***Screening scheme for short-chain olefin producers***

The second part aims to enhance the isoprene production. Isoprene is an omnipresent chemical in nature from which valuable products can be derived. As it is also easily separated from the fermentation broth, it is a suitable target product for the farm-stead biorefinery. Bio-isoprene needs to be produced with a better titer in order to compete with the chemical industry. The strains *Pichia pastoris* X-33 and *Cupriavidus necator* were chosen as hosts for genes to enhance isoprene production. *Pichia pastoris* X-33 is well known for genetic manipulation and expression of recombinant proteins [369] and *Cupriavidus necator* is on the verge to become as important for the future of generating valuable chemicals from CO_2 [123, 145, 255], as it grows chemolithoautotrophically with H_2 and CO_2 [274].

2 Material and Methods

2.1 Instrumentation

Autoclave	
Varioklav 135S	Thermo Fisher Scientific, Waltham, MA, USA
Centrifuges	
Galaxy MiniStar	VWR Interational GmbH, Darmstadt, Germany
HERAEUS FRESCO 21 Centrifuge	Thermo Fischer Scientific, Waltham, MA, USA
HERAEUS PICO 17 Centrifuge	Thermo Fischer Scientific, Waltham, MA, USA
ROTANTA 460R	Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany
Rotor F10-6x500y	Piramoon Technologies Inc., Santa Clara, CA, USA
Rotor F9-4x1000y	Piramoon Technologies Inc., Santa Clara, CA, USA
Rotor SH-3000	Thermo Fischer Scientific, Waltham, MA, USA
Rotor SS-34	Thermo Fischer Scientific, Waltham, MA, USA
SORVALL RC6+ Centrifuge	Thermo Fischer Scientific, Waltham, MA, USA
Thermo Shaker	
ThermoMixer C (1.5ml and 15ml adaptor)	Eppendorf AG, Hamburg, Germany
Tmix	Analytik Jena AG, Jena, Germany
Shaker	
HAT Minitron	Infors AG, Bottmingen/Basel, Switzerland
IKA R KS 4000 ic control	IKA Insturments,
MAXQ2000	Thermo Fischer Scientific, Waltham, MA, USA

MAXQTM Mini 4450	Thermo Fischer Scientific, Waltham, MA, USA
MTP shaker	Edmund Bühler GmbH, Hechingen, Germany
TH15	Edmund Bühler GmbH, Hechingen, Germany
TiMix 5 control	Edmund Bühler GmbH, Hechingen, Germany
Incubator	
30 °C room	Albert GmbH, Rain, Germany
Function Line B12	Thermo Fischer Scientific, Waltham, MA, USA
Heraeus B12	Thermo Fischer Scientific, Waltham, MA, USA
Heraeus kelvitron R kp	Thermo Fischer Scientific, Waltham, MA, USA
KBF 240 E5.1/C	Binder GmbH, Tuttlingen, Germany
Shakers for Incubators	
HT Minitron	Infors AG, Bottmingen/Basel, Switzerland
KS 4000 ic control	IKA-Werke GmbH & Co KG, Staufen, Germany
MaxQ 2000	Thermo Fischer Scientific, Waltham, MA, USA
Vortexer	
Vortex Genie 2	Scientific Industries Inc., Bohemia, NY, USA
VWR Vortexer	VWR Interational GmbH, Darmstadt, Germany
Microscope	
Axio Lab.A1	Carl Zeiss AG, Oberkochen, Germany
BA310	Motic Deutschland GmbH, Wetzlar, Germany
ZEISS Axiocam ERc 5s	Carl Zeiss AG, Oberkochen, Germany
pH meter	
FiveEasy™	Mettler Toledo Inc., Columbus, OH, USA

FiveGo™	Mettler Toledo Inc., Columbus, OH, USA
pH electrode InLab Expert Pro pH 0-14, 0-100 <U+2103>	Mettler Toledo Inc., Columbus, OH, USA
pH electrode InLab Micro Pro pH 0-14, 0-100 <U+2103>	Mettler Toledo Inc., Columbus, OH, USA
Balances	
440-47N	KERN & SOHN GmbH, Balingen, Germany
AW320	Shimadzu Corp., Kyōto, Japan
Pioneer PA214C	Ohaus Corp., Pine Brrok, NJ, USA
TB-215 D	Denver Instruments, Bohemia, NY, USA
TE1502S 2 digits	Satorius AG, Göttingen, Germany
TE6101 1 digit	Satorius AG, Göttingen, Germany
PCR Cycler	
C1000 TouchTM Thermal Cycler (equipped with CFX96TM Real-Time System)	Bio-Rad Laboratories GmbH, Munich, Germany
C1000TM Thermal Cycler	Bio-Rad Laboratories GmbH, Munich, Germany
MJ MiniTM Personal Thermal Cycler	Bio-Rad Laboratories GmbH, Munich, Germany
Photometer	
Multiskan Spectrum	Thermo Fischer Scientific, Waltham, MA, USA
Nanophotometer (equipped with lid5, lid 10 and lid 50)	IMPLEN GmbH, Munich, Germany
Ultrospec® cell density meter, 600 nm	Biochrom Ltd., Cambourne, United Kingdom
Varioskan	Thermo Electron Corporation, Waltham, MA, USA
Water bath	
CC1 Compatible Control	Peter Huber Kältemaschinenbau AG, Offenburg, Germany
Ministat 230 (IUL-accisiated)	Peter Huber Kältemaschinenbau AG, Offenburg, Germany

Steril hood

Herasafe™ KS (NSF)	Thermo Fischer Scientific, Waltham, MA, USA
MSC-ADVANTAGE	Thermo Fischer Scientific, Waltham, MA, USA

Gas chromatograph

Column: Rt-QS-Bond (length 15 m, 0.53 mm diameter, 20 µm particle size)	Restek GmbH, Bad Homburg vor der Höhe, Germany
RSH autosampler	Thermo Fischer Scientific, Waltham, MA, USA
Trace 1310	Thermo Fischer Scientific, Waltham, MA, USA

HPLC: UltiMate 3000 RS LC System

Auto sampler WPS 3000TRS	Thermo Fischer Scientific, Dionex, Idstein, Germany
Column compartment TCC 3000RS	Thermo Fischer Scientific, Dionex, Idstein, Germany
Column: Gravity C18	Macherey-Nagel GmbH & Co. KG, Düren, Germany
Degasser SRD 3400	Thermo Fischer Scientific, Dionex, Idstein, Germany
Diode array detector DAD 3000RS	Thermo Fischer Scientific, Dionex, Idstein, Germany
ESI-ion-trap unit	Thermo Fischer Scientific, Dionex, Idstein, Germany
Mass spectrometer HCT	Bruker Daltonik GmbH, Bremen, Germany
Pump module HPG 3400RS	Thermo Fischer Scientific, Dionex, Idstein, Germany

Gel Electrophoresis (DNA &Protein)

Gel caster with trays for MINI-SUB® Cell GT	Bio-Rad Laboratories GmbH, Munich, Germany
Gel iX Imager	Intas Science Imanging Instruments GmbH, Göttingen, Germany

Mini-PROTEAN® Tetra System	Bio-Rad Laboratories GmbH, Munich, Germany
MINI-SUB® Cell GT	Bio-Rad Laboratories GmbH, Munich, Germany
PowerPac™ Basic	Bio-Rad Laboratories GmbH, Munich, Germany
PowerPac™ HC	Bio-Rad Laboratories GmbH, Munich, Germany
Trans illuminator Dark Reader	Clare Chemical Research, Dolores, CO, USA
AEKTA™	
AEKTApurifier Box-900	General Electric Company, Boston, MA, USA
AEKTApurifier Frac-950	General Electric Company, Boston, MA, USA
AEKTApurifier INV-907	General Electric Company, Boston, MA, USA
AEKTApurifier M-925	General Electric Company, Boston, MA, USA
AEKTApurifier P-900	General Electric Company, Boston, MA, USA
AEKTApurifier P-960	General Electric Company, Boston, MA, USA
AEKTApurifier PV-908	General Electric Company, Boston, MA, USA
AEKTApurifier UPC-900	General Electric Company, Boston, MA, USA
HiTrap Desalting HiPrep 26/10 Desalting	General Electric Company, Boston, MA, USA
HiTrap™ DEAE FF 1 mL and 5 mL	General Electric Company, Boston, MA, USA
HiTrap™ HIC selection kit 11-0034-52 AG: Phenyl FF (high and low sub), butyl FF, butyl-S FF, Octyl FF	General Electric Company, Boston, MA, USA
HiTrap™ IEX selection kit 71-5017-51 AH: DEAE FF, CM FF, Q FF, SP FF, ANX FF	General Electric Company, Boston, MA, USA
HiTrap™ Q XL 1 mL and 5 mL	General Electric Company, Boston, MA, USA
Superdex 200 26/60 Desalting	General Electric Company, Boston, MA, USA
Pipettes	
Research Pro (8 x 1200 µL)	Eppendorf AG, Hamburg, Germany
Research Pro (12 x 300 µL)	Eppendorf AG, Hamburg, Germany
Transferpette S (10 µL)	BRAND GmbH + Co. KG, Wertheim, Germany

Transferpette S (100 µL)	BRAND GmbH + Co. KG, Wertheim, Germany
Transferpette S (1000 µL)	BRAND GmbH + Co. KG, Wertheim, Germany
Transferpette S (200 µL)	BRAND GmbH + Co. KG, Wertheim, Germany
Transferpette S (1 µL)	BRAND GmbH + Co. KG, Wertheim, Germany
Transferpette S (10000 µL)	BRAND GmbH + Co. KG, Wertheim, Germany
Transferpette S (2000 µL)	BRAND GmbH + Co. KG, Wertheim, Germany
Transferpette S (5000 µL)	BRAND GmbH + Co. KG, Wertheim, Germany
Transferpette S-12 (12 x 200 µL)	BRAND GmbH + Co. KG, Wertheim, Germany
Transferpette S-8 (8 x 100 µL)	BRAND GmbH + Co. KG, Wertheim, Germany
Transferpette S-8 (8 x 50 µL)	BRAND GmbH + Co. KG, Wertheim, Germany
Transferpette S-8 electronic (8 x 200 µL)	BRAND GmbH + Co. KG, Wertheim, Germany
Magnetic Heating Stirrer	
Heating stirrer plate MR Hei-Standard	Heidolph Instruments GmbH & Co.KG, Schwabach, Germany
M2	Ingenieurbüro CAT, M. Zipperer GmbH, Staufen, Germany
MR 3001 K	Heidolph Instruments GmbH & Co.KG, Schwabach, Germany
VMS-C7	VWR Interational GmbH, Darmstadt, Germany
Miscellaneous	
-20 °C freezer GNP 3056 Premium	Liebherr-International Deutschland GmbH, Biberach an der Riss, Germany

-20 °C freezer GTL 6105	Liebherr-International Deutschland GmbH, Biberach an der Riss, Germany
4 °C room	Albert GmbH, Rain, Germany
-80 °C freezer	GFL Gesellschaft für Labortechnik mbH, Burgwedel, Germany
-80 °C freezer Forma 906 -86 °C ULT	Thermo Fischer Scientific, Waltham, MA, USA
-80 °C freezer Thermo Forma 700 series	Thermo Fischer Scientific, Waltham, MA, USA
-80 °C freezer Thermo Forma 900 series	Thermo Fischer Scientific, Waltham, MA, USA
96-pin replicator	Boekel Scientific, Feasterville, PA, USA
Amicon ultra filtration unit 8050 und 8200 (and various filters)	Millipore GmbH, Schwabach, Germany
Blow torch gasprofi 1 SCS micro	WLD-Tec, Göttingen, Germany
Concentrator Savant SpeedVac Plus SC210A with Savant Refrigerated Vapor Trap RVT100	Thermo Fischer Scientific, Waltham, MA, USA
Conductivity meter Lab 970 with probe LF413T-ID	SI Analytics GmbH, Mainz, Germany
Constant Cell Disruption Systems	IUL Instruments GmbH, Königswinter, Germany
Electroporator MicroPulser™	Bio-Rad Laboratories GmbH, Munich, Germany
Elga Labwater PURELAB® R Classic	ELGA LabWater, Celle, Germany
Heating cabinet Function Line T12	Thermo Fischer Scientific, Waltham, MA, USA
Heating cabinet Heratherm OGS100	Thermo Fischer Scientific, Waltham, MA, USA
Heating cabinet U 27	Memmert GmbH + Co. KG, Schwabach, Germany
Ice Flaker AF80	Scotsman, Suffolk, United Kingdom
Lyophylle Alpha 2-4 Ldplus	Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany

Microwave MH 25 ED	ECG, Prag, Czech Republic
MSC-Advantage™ Class II Biological Safety Cabinte	Thermo Fischer Scientific, Waltham, MA, USA
Refrigerator KT 1440	Liebherr-International Deutschland GmbH, Biberach an der Riss, Germany
Rocking Platform	VWR Interational GmbH, Darmstadt, Germany
Sonicator UIS250L with “VialTweeter”	Hielscher Ultrasonics GmbH, Teltow, Germany
Stirrer plate KAMAG® Color Squid Magnetic Stirrer	IKA-Werke GmbH & Co KG, Staufen, Germany
Thoma Counting Chamber	VWR Interational GmbH, Darmstadt, Germany
Transilluminator	Thermo Fischer Scientific, Waltham, MA, USA
Ultra Turrax T18 basic	IKA-Werke GmbH & Co KG, Staufen, Germany
Vacuum pump PC 2004 VARIO	ACUUBRAND GMBH + CO KG, Wertheim, Germany
Vacuum pump RC 6	ACUUBRAND GMBH + CO KG, Wertheim, Germany
Water bath	Peter Huber Kältemaschinenbau AG, Offenburg, Germany
Water bath ED-33	JULABO Labortechnik GmbH, Seelbach, Germany

2.2 Glassware

All glassware used was made from borosilicate glass. A DURAN® laboratory bottle with DIN GL-45 thread in various sizes were purchased from the Duran Group GmbH, Wertheim, Germany.

2.3 Consumables

96 well plates, PS, F-bottom, clear, sterile	Greiner Bio-one International GmbH, Kremsmünster, Austria
96 well plates (deep well) 2.0 mL, V-bottom, sterile	Greiner Bio-one International GmbH, Kremsmünster, Austria
96 well plates, F bottom; 340 µL	Greiner Bio-one International GmbH, Kremsmünster, Austria
96 well plates, F96 Non-treated Black	Thermo Fisher Scientific, Waltham, MA, USA
96 well plates, V bottom; 1.1 mL	Harvard Apparatus, Holliston, MA, USA
96-well PCR plate, non-skirted, elevated wells, colorless, PP, DNase-free, RNase-free, DNA-free	BRAND GmbH + Co. KG, Wertheim, Germany
Axygen® 24 deep well plate with rectangular wells	Corning Inc., Corning, NY, USA
Axygen® breathable sealing film, sterile	Corning Inc., Corning, NY, USA
Axygen® PlateMax pierceable aluminium heat sealing film	Corning Inc., Corning, NY, USA
Cryo vials 1.8 mL CryoPure polypropylene	Sarstedt AG & Co. KG, Nümbrecht, Germany
Cuvettes, CryoPure; 1.8 mL; polypropylene, PMMA, semi-micro	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Cuvettes, PMMA, semi-micro, path length, 10 mm	VWR International GmbH, Darmstadt, Germany
Cuvettes, PS, semi-micro, path length 10 mm	BRAND GmbH + Co. KG, Wertheim, Germany
Cuvettes, PS, semi-micro, path length 10 mm	VWR International GmbH, Darmstadt, Germany
Filter membrane cellulose nitrate (CN); pore size: 0.45 µm; sterile	Sartorius Stedim Biotech GmbH, Göttingen, Germany
Filter membrane regenerated cellulose (RC); pore size: 0.2 µm; non-sterile	Sartorius Stedim Biotech GmbH, Göttingen, Germany

GC Vial crimp cap N20, magnetic	Machery-Nagel GmbH & Co. KG, Düren, Germany
GC Vial, Crimp neck N20, clear, flat bottom, rounded	Machery-Nagel GmbH & Co. KG, Düren, Germany
Gene Pulser Electroporation Cuvettes 0.2 mm	Bio-Rad Laboratories GmbH, Munich, Germany
Microreaction tubes 1.5 mL, 2.0 mL, PP	Sarstedt AG & Co. KG, Nümbrecht, Germany
Microreaction tubes 1.5 mL, 2.0 mL, PP, safe-lock	Sarstedt AG & Co. KG, Nümbrecht, Germany
Nitrile gloves	LLG Labware, Meckenheim, Germany
Pasteur pipettes, plastic	VWR International GmbH, Darmstadt, Germany
PCR cup strips, 200 µL, 8 cups	VWR International GmbH, Darmstadt, Germany
PCR domed cap strips, 8 caps	VWR International GmbH, Darmstadt, Germany
PCR tubes with attached flat caps	VWR International GmbH, Darmstadt, Germany
Rt-QS-Bond	Restek GmbH, Bad Homburg v. d. Höhe
Petri dish, 92x16 mm, PS, clear, sterile	Sarstedt AG & Co., Nümbrecht, Germany
Pipette tips (1 µL, 10 µL, 100 µL, 1000 µL, 5 mL, 10 mL)	BRAND GmbH + Co. KG, Wertheim, Germany
Pipette tips Research Pro 1250 µL	Eppendorf AG, Hambrug, Germany
Pipette tips, epT.I.P.S. Standard; uncolored; 1250 µL	VWR International GmbH, Darmstadt, Germany
Pipette tips, green; 1250 µL	Eppendorf AG, Hamburg, Germany
Pipette tips, uncolored; 10 mL	Zefa-Laborservice GmbH, Harthausen, Germany
Pipette tips, uncolored; 1000 µL	Sarstedt AG & Co., Nümbrecht, Germany

Pipette tips, uncolored; 20 µL	Sarstedt AG & Co., Nümbrecht, Germany
Pipette tips, uncolored; 200 µL	Sarstedt AG & Co., Nümbrecht, Germany
Pipette tips, uncolored; 5 mL	Zefa-Laborservice GmbH, Harthausen, Germany
Pipette tips, yellow; 300 µL	Eppendorf AG, Hamburg, Germany
Plastic disposable cuvette, 1.5 mL	BRAND GmbH + Co. KG, Wertheim, Germany
Plastic tubes 15 mL, 50 mL	Sarstedt AG & Co. KG, Nümbrecht, Germany
Plate mats, PCR-sealing mat for 96-well plates, TPE	BRAND GmbH + Co. KG, Wertheim, Germany
Plate mats, Whatman Capmats 96 wells, round, Silicone Rubber	GE Healthcare UK Ltd., Buckinghamshire, United Kingdom
Plate seals, Axygen breathable sealing film, sterile	VWR International GmbH, Darmstadt, Germany
Plate seals, Axygen sealing film, aluminium, non-sterile	VWR International GmbH, Darmstadt, Germany
Polystyrene 1.6 mL cuvettes	VWR International GmbH, Darmstadt, Germany
SCHOTT Laboratory glassware, various sizes	Duran Group GmbH, Wertheim, Germany
Sealing film parafilm M	VWR International GmbH, Darmstadt, Germany
Syringe 1 mL	Sarstedt AG & Co., Nümbrecht, Germany
Syringe 10 mL, LuerLock	Sarstedt AG & Co., Nümbrecht, Germany
Syringe 20 mL, LuerLock	Sarstedt AG & Co., Nümbrecht, Germany
Syringe 3 mL, LuerLock	Sarstedt AG & Co., Nümbrecht, Germany

Syringe 5 mL, LuerLock	Sarstedt AG & Co., Nümbrecht, Germany
Syringe 50 mL, LuerLock	Sarstedt AG & Co., Nümbrecht, Germany
Syringe filter 0.2 mm, cellulose acetate, sterile	Hartenstein GmbH, Würzburg, Germany
Syringe filters, cellulose acetate (CA), pore size: 0.2 μm, sterile	VWR International GmbH, Darmstadt, Germany
Syringe filters, cellulose acetate (CA), pore size: 0.45 μm	VWR International GmbH, Darmstadt, Germany
Syringe filters, cellulose acetate (CA), pore size: 0.45 μm, sterile	VWR International GmbH, Darmstadt, Germany
Syringe filters, cellulose mixed esters (MV); pore size: 0.2 μm	Machery-Nagel GmbH & Co. KG, Düren, Germany
Syringe filters, Polyvinyldifluoride (PVDF); pore size: 0.22 μm	Restek Corporation, Bellefonte, PA, USA
Syringe filters, regenerated cellulose (RC); pore size: 0.2 μm	Machery-Nagel GmbH & Co. KG, Düren, Germany
Syringe Infuject 50 mL	Sarstedt AG & Co., Nümbrecht, Germany
Test Tube, conical base, PP, red cap, 15 mL, 50 mL	Sarstedt AG & Co., Nümbrecht, Germany
Ultrafiltration-Membrane disc, PLBC, Ultracel, 3 kDa NMGG, 44.5 mm	Merck Millipore, Burlington, MA, USA
Whatman quantitative filter paper, 47 mm (type 40)	VWR International GmbH, Darmstadt, Germany
ZelluTrans Dialysis tubes 6.0 (cut-off 6-8 kDa)	Carl Roth GmbH, Karlsruhe, Germany

2.4 Software

AEKTA Unicorn 5.1	General Electric Company, Boston, MA, USA
Basic Local Alignment Search Tool (BLAST) [4, 50, 373]	National Center vor Biotechnology Information, Bethesda, MD, USA
ChemDraw Ultra 12.0	Perkin Elmer Informatics, Cambridge, MA, USA
Chromeleon 6.80 SR8 Build 2623 (156243)	Thermo Fisher Scientific, Waltham, MA, USA
Chromeleon 7.10 (P/N 43280000)	Thermo Fisher Scientific, Waltham, MA, USA
CloneManager 9	Scientific & Educational Software, Denver, CO, USA
Clustal Ω2 [283]	European Molecular Biology Laboratory (EMBL), Heidelberg, Germany
ClustalΩ [283]	EMBL-EBI, Cambridge, United Kingdom, https: //www.ebi.ac.uk/Tools/msa/clustalo/
Codon Usage Table Database [15]	hive.biochemistry.gwu.edu/review/ codon
EMBOSS [256]	EMBL-EBI, Cambridge, United Kingdom, https://www.ebi.ac.uk/Tools/emboss/
ezbiocloud [366]	ChunLab (http://www.chunlab.com/eng), https://www.ezbiocloud.net/
FastQC 0.11.4	Babraham Bioinformatics, Cambridge, United Kingdom, https://www.bioinformatics.babraham. ac.uk/projects/fastqc/
Gel Jet Imager	Intas Science Imaging Instruments GmbH, Göttingen, Germany

Jcat [120]	Andreas Grote, Institute for Microbiology, Technical University of Braunschweig, Braunschweig, Germany, http://www.jcat.de/
KEGG Database [154–156]	Kanehisa Laboratories, http://www.genome.jp/kegg/kegg1.html
Mascot [242]	Matrix Science Ltd, London, United Kingdom, http://www.matrixscience.com/search_form_select.html
Mass spectrometer control software HyStar 3.2.44.0	Bruker Corporation, Billerica, MA, USA
Mass spectrum analysis software DataAnalysis 4.0 SP 4 (Build 281)	Bruker Corporation, Billerica, MA, USA,
Mass spectrum quantification software QuantAnalysis 2.0 SP 4 (Build 281)	Bruker Corporation, Billerica, MA, USA
Microsoft Office	Microsoft Corporation, Redmond, WA, USA
National Center for Biotechnology Information (NCBI)	U.S. National Library of Medicine, Bethesda, MD, USA
NEB Interactive Tools	New England Biolabs GmbH, Frankfurt am Main, Germany, https://www.neb.com/tools-and-resources/interactive-tools
NGS QC Toolkit 2.2.3 [241]	https://omictools.com/ngs-qc-toolkit-tool
Protein Database (PDB) [30]	https://www.rcsb.org/
R version 3.2.2-3.5.1 (32-/64-bit) [251, 252]	R Core Team, https://www.R-project.org/,
Rapid annotations using subsystem technology (RAST) [18, 239]	National Microbial Pathogen Data Resource (NMPDR), Chicago, IL, USA, http://rast.nmpdr.org/
seqtk [186]	https://github.com/lh3/seqtk
SkanIt Software 2.4.3	Thermo Fisher Scientific, Waltham, MA, USA

SnapGene 2.3.2	GSL Biotech, LLC, Chicago, IL, USA
SPAdes 2.5.1 [23]	http://bioinf.spbau.ru/en/spades-2-5-1
Swissprot/UniProt [309]	The UniProt Consortium, https://www.uniprot.org/
Ubuntu 16.04.5 LTS on Windows Subsystem for Linux	Canonical Foundation, Ubuntu community, London, United Kingdom, https://www.ubuntu.com/
Windows 10	Microsoft Corporation, Redmond, WA, USA
Xcalibur	Thermo Fisher Scientific, Waltham, MA, USA

2.5 Chemicals and Biochemicals

In case of chemicals and complex media components the common trivial names under which they are marketed are given. The chemicals in **Section 2.5** are given in alphabetical order. If not noted otherwise, the highest possible purity was purchased.

1-aminocyclo-propane-carboxylic acid	Alfa Aesar, Kandel, Germany
1-aminocyclo-propane-carboxylic acid	Calbiochem, Merck AG, Darmstadt, Germany
1-Butanol	VWR International, Leuven, Belgium
2-Aminoisobutyric acid	Carl Roth GmbH, Karlsruhe, Germany
2-keto-4-methyl thiobutyrate	Sigma-Aldrich Chemie GmbH, Munich, Germany
3-(phosphonooxy)butanoic acid	Chair for Chemistry for Biogenic Resources, Straubing, Germany
3-(phosphonooxy)propanoic acid	Chair for Chemistry for Biogenic Resources, Straubing, Germany
3-(sulfo-oxy)butanoic acid	Chair for Chemistry for Biogenic Resources, Straubing, Germany
3-phosphonooxy butanoic acid	

5x Phusion HF Reaction Buffer	New England Biolabs GmbH, Frankfurt am Main, Germany
Acetic acid	Carl Roth GmbH, Karlsruhe, Germany
Acetonitrile	VWR International, Leuven, Belgium
Agar-agar	Carl Roth GmbH, Karlsruhe, Germany
Agarose	SERVA Electrophoresis GmbH, Heidelberg, Germany
Ammonia	Carl Roth GmbH, Karlsruhe, Germany
Ammonium carbonate	Carl Roth GmbH, Karlsruhe, Germany
Ammonium chloride	Carl Roth GmbH, Karlsruhe, Germany
Ammonium ferric citrate	Carl Roth GmbH, Karlsruhe, Germany
Ammonium sulphate	AppliChem GmbH, Darmstadt, Germany
β-alanine	Carl Roth GmbH, Karlsruhe, Germany
Beef extract	Carl Roth GmbH, Karlsruhe, Germany
Biotin	Carl Roth GmbH, Karlsruhe, Germany
β-mercaptoethanol	Merck KGaA, Darmstadt, Germany
Boric acid	Sigma-Aldrich Chemie GmbH, Munich, Germany
Bromphenol Blue	Carl Roth GmbH, Karlsruhe, Germany
C2-C6 Olefins: 100 ppm each of Ethylene, Proyplene, 1-Butene, 1-Pentene, 1-Hexene, in Nitrogen	Restek GmbH, Bad Homburg, Germany
Calcium chloride dihydrate	Carl Roth GmbH, Karlsruhe, Germany
Calibration buffer pH 10	VWR International, Leuven, Belgium
Calibration buffer pH 4	VWR International, Leuven, Belgium
Calibration buffer pH 7	VWR International, Leuven, Belgium
Chitin	Carl Roth GmbH, Karlsruhe, Germany
Cobalt chloride hexahydrate	Alfa Aesar GmbH + Co. KG, Karlsruhe, Germany
Copper sulphate pentahydrate	Fluka, Sigma-Aldrich Chemie GmbH, Munich, Germany
CutSmart 10x	New England Biolabs GmbH, Frankfurt am Main, Germany

Diammonium hydrogen citrate	Merck KGaA, Darmstadt, Germany
Dihydrogen	Westfalen AG, M"unster, Germany
Dimethyl sulfoxide	New England Biolabs GmbH, Frankfurt am Main, Germany
Dipotassium phosphate	Carl Roth GmbH, Karlsruhe, Germany
Disodium phosphate	Carl Roth GmbH, Karlsruhe, Germany
Dithiothreitol for molecular biology	VWR International, Leuven, Belgium
dNTPs mix (10 mM each)	Rapidozym Gesellschaft für Laborhandel und DNA Diagnostika mbH , Berlin, Germany
Ethanol	VWR International, Leuven, Belgium
Ethidium bromide	Carl Roth GmbH, Karlsruhe, Germany
ethyl acetate	Carl Roth GmbH, Karlsruhe, Germany
ethyl acetate	VWR International, Leuven, Belgium
Ethylenediaminetetraacetic acid (EDTA)	VWR International, Leuven, Belgium
Ferric sulphate heptahydrate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Formaldehyde	Carl Roth GmbH, Karlsruhe, Germany
Formic acid	AppliChem GmbH, Darmstadt, Germany
Glucose Monohydrat	SERVA Electrophoresis GmbH, Heidelberg, Germany
Glucose Monohydrat	Carl Roth GmbH, Karlsruhe, Germany
Glycerol	Carl Roth GmbH, Karlsruhe, Germany
Glycine	Carl Roth GmbH, Karlsruhe, Germany
Helium 5.0	Westfalen AG, Münster, Germany
Hydrochloric acid	Carl Roth GmbH, Karlsruhe, Germany
Isoprene	Sigma-Aldrich Chemie GmbH, Munich, Germany
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Carl Roth GmbH, Karlsruhe, Germany
L-(+)-Ascorbic acid	Carl Roth GmbH, Karlsruhe, Germany
Magnesium sulphate heptahydrate	Merck KGaA, Darmstadt, Germany

Malt extract	Carl Roth GmbH, Karlsruhe, Germany
Manganese sulfate tetrahydrate	Merck KGaA, Darmstadt, Germany
Mangenese chloride dihydrate	Merck KGaA, Darmstadt, Germany
Mangenese chloride tetrahydrate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Marine Bouillon	Carl Roth GmbH, Karlsruhe, Germany
Methanol	VWR International, Leuven, Belgium
MOPS (3-Morpholinopropane-1-sulfonic acid)	Carl Roth GmbH, Karlsruhe, Germany
MRS broth for microbiology	Carl Roth GmbH, Karlsruhe, Germany
Natriumacetat-3-hydrat	Carl Roth GmbH, Karlsruhe, Germany
Nitrogen	Linde AG, Munich, Germany
Plasmid-Saf^{TM} 10X reaction buffer and 25-mM ATP solution	Lucigen Corporation, Middleton, WI, USA
Potassium chloride	Carl Roth GmbH, Karlsruhe, Germany
Potassium dihydrogen phosphate	Carl Roth GmbH, Karlsruhe, Germany
Potassium hydroxide	Carl Roth GmbH, Karlsruhe, Germany
Saccharose	Carl Roth GmbH, Karlsruhe, Germany
S-Adenosyl methionine	New England Biolabs GmbH, Frankfurt am Main, Germany
SERVA DNA Stain G	SERVA Electrophoresis GmbH, Heidelberg, Germany
Sodium acetate x 3H2O	Carl Roth GmbH, Karlsruhe, Germany
Sodium acetate	Carl Roth GmbH, Karlsruhe, Germany
Sodium carbonate	Carl Roth GmbH, Karlsruhe, Germany
Sodium chloride	Carl Roth GmbH, Karlsruhe, Germany
Sodium dihydrogen phosphate	Carl Roth GmbH, Karlsruhe, Germany
Sodium dodecyl sulphate	SERVA Electrophoresis GmbH, Heidelberg, Germany
Sodium hydroxide	Carl Roth GmbH, Karlsruhe, Germany
Sodium molybdate	Carl Roth GmbH, Karlsruhe, Germany
Sodium nitrate	Merck KGaA, Darmstadt, Germany

Sodium tartrate dihydrate	Carl Roth GmbH, Karlsruhe, Germany
Sulphuric acid 96%	Carl Roth GmbH, Karlsruhe, Germany
Synthetic Air	Westfalen AG, Münster, Germany
Thiamine pyrophosphate (TPP)	Sigma-Aldrich Chemie GmbH, Munich, Germany
TRIS	Carl Roth GmbH, Karlsruhe, Germany
TRIS BASE	Carl Roth GmbH, Karlsruhe, Germany
TRIS hydrochloride	Carl Roth GmbH, Karlsruhe, Germany
Tryptone/Peptone	Carl Roth GmbH, Karlsruhe, Germany
Xylene cyanol	VWR International, Leuven, Belgium
Yeast extract for bacteriology	Carl Roth GmbH, Karlsruhe, Germany
Yeast Nitrogen Base (YNB)	VWR International, Leuven, Belgium
Zinc chloride	Merck KGaA, Darmstadt, Germany
Zinc sulphate heptahydrate	Merck KGaA, Darmstadt, Germany

2.6 Kits

Table 2.1: *Kits used in this work*

Kit	Manufacturer/Vendor
CloneJET PCR Cloning Kit	Thermo Fisher Scientific Inc., Waltham, MA, USA
NecleoSpin® Gel and PCR Clean-up	Machery-Nagel GmbH & Co.KG, Düren, Germany
GeneJET Plasmid Miniprep Kit	Thermo Fisher Scientific Inc., Waltham, MA, USA
DNeasy Blood & Tissue Kit	Qiagen GmbH, Hilden, Germany

2.7 Enzymes

All enzymes were stored according to the manufacturer.

Table 2.2: *Enzymes used in this work*

Enzyme	Concentration	Manufacturer/Vendor
2-log DNA ladder (0.1 - 10 kb)	NA	New England Biolabs GmbH, Frankfurt am Main, Germany
4-oxalocrotonate tautomerase from *Bacillus cereus* SR-772	NA	This work
4-oxalocrotonate tautomerase from *E. coli* DH5α	NA	This work
4-oxalocrotonate tautomerase from *Lactobacillus brevis* SR-416	NA	This work
4-oxalocrotonate tautomerase from *Lysinibacillus xylanilyticus* SR-86	NA	This work
ACCUzyme™	2500 U/mL	bioline, London, United Kingdom
BamHI-HF	20000 U/mL	New England Biolabs GmbH, Frankfurt am Main, Germany
BsaI-HF	20000 U/mL	New England Biolabs GmbH, Frankfurt am Main, Germany
EcoRI-HF	20000 U/mL	New England Biolabs GmbH, Frankfurt am Main, Germany
GoTaq® DNA Polymerase	5000 U/mL	VWR International, Radnor, PA, USA
KpnI-HF	20000 U/mL	New England Biolabs GmbH, Frankfurt am Main, Germany
LongLife™ Zymolyase®	20000 U/mL	GBiosciences, St. Louis, MO, USA
Lysozyme	$\geq$ 35000 FIP U/mg	Carl Roth GmbH, Karlsruhe, Germany
NEBuilder® HiFi DNA Assembly Master Mix	NA	New England Biolabs GmbH, Frankfurt am Main, Germany
OptiTherm DNA-Polymerase	500 U/ μL	Rapidozym Gesellschaft für Laborhandel und DNA Diagnostika mbH, Berlin, Germany
PciI	10000 U/mL	New England Biolabs GmbH, Frankfurt am Main, Germany
Phusion-HF® DNA Polymerase	2000 U/mL	New England Biolabs GmbH, Frankfurt am Main, Germany
Plasmid-Saf™ ATP-Dependent DNase	1000 units	Lucigen Corporation, Middleton, WI, USA

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Enzyme	Concentration	Manufacturer/Vendor
PmeI	10000 U/mL	New England Biolabs GmbH, Frankfurt am Main, Germany
Proteinase K	800 U/mL	New England Biolabs GmbH, Frankfurt am Main, Germany
RNAse A/T1	5000 U/mL	New England Biolabs GmbH, Frankfurt am Main, Germany
T4 DNA ligase	400000 U/mL	VWR International, Radnor, PA, USA
T7 DNA ligase	3000000 U/mL	New England Biolabs GmbH, Frankfurt am Main, Germany
Taq DNA Polymerase	5000 U/mL	VWR International, Radnor, PA, USA
Thermo Scientific™ PageRuler™ Unstained Protein Ladder	NA	Thermo Fisher Scientific, Waltham, MA, USA
Trypsin MS Grade (Pierce)	NA	Thermo Fisher Scientific, Waltham, MA, USA

NA: not available

2.8 Strains

In this section, purchased strains (see **Tab. 2.4**) are listed as well as strains for which extended experiments were carried out (see **Tab. 2.3**). A list of all strains being further used from the screening for ethylene and propylene production are listed in **Appendix A.2.2**.

Table 2.3: *Isolated strains used in this work. Strains were identified by 16S rDNA PCR (see **Sections 2.14.7** and **2.17.1** for the procedure)*

Strain designation	SR Strain collection number	Source
Bacillus sp.	SR-772	Environmental sample
Lysinibacillus xylanilyticus	SR-86	Environmental sample
Pichia fermentans	SR-265	Environmental sample
Lactobacillus brevis	SR-416	Environmental sample

Table 2.4: *Purchased strains used in this work*

Strain	Identifier	Genotype	Phenotype	Source
Cupriavidus necator H16	DSM-531	wild type	wild type	DSMZ
Cupriavidus necator H16	DSM-428	wild type	wild type	DSMZ
E. coli DH5α	NA	*F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* ϕ*80dlacZ*Δ*M15* Δ(*lacZYA-argF*)*U169, hsdR17*($r_K^- m_K^+$), λ^-	NA	Life Technologies, Darmstadt, Germany
E. coli XL-10 Gold	NA	*endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac Hte* Δ*(mcrA)183* Δ*(mcrCB-hsdSMR-mrr)173 tetR F'*[*proAB lacIqZ*Δ*M15 Tn10 (TetR Amy CmR)*]	NA	Stratagene (Agilent Technologies), Inc, Santa Clara, CA, USA
Pichia pastoris X-33	C180-00	wild type	Mut$^+$	Life Technologies, Darmstadt, Germany
E. coli BL21(DE3)	NA	F- *ompT gal lon* $hsdS_B(r_B^- m_B^-)$ λ(DE3)[*lacI lacUV5-T7p07 ind1 sam7 nin5*])$[malB^+]_{\text{K-12}}(\lambda^S)$	NA	Novagen/Merck, Darmstadt, Germany
E. coli S17.1	ATCC 47055, DSM-9079	*recA pro hsdR RP42Tc::MuKm::Tn7*	NA	American Type Culture Collection, Manassas, VA, USA

NA: not available

2.9 Oligonucleotides

All listed oligonucleotides (see **Tab. 2.5**) were synthesized by Biomers.net GmbH (Ulm, Germany) and the sequences are presented in 5'→3' direction.

Table 2.5: *Oligonucleotides used in this work*

Name	Sequence (5'→3')
27F	AGAGTTTGATCMTGGCTCAG
1525R	AAGGAGGTGWTCCARCC
5.8S-R	TCGATGAAGAACGCAGCG
ColPCR-pPICZB-f	ACTTTCATAATTGCGACTGG
ColPCR-pPICZB-r	GTGCCCAACTTGAACTGAGG
DH5a-4ot-f	ATATTACATATGCCGCACATCGACATTAAATG
DH5a-4ot-r	ATATCTCGAGTTAAGCATTCATGCTATAACCAGGTTTC
eGFP-MCS3-gg-f	ATATGGTCTCAagctATGACCATGATTACGCATCATCATC
eGFP-MCS3-gg-r	ATTAGGTCTCAGGATTTACTTGTACAGCTCGTCCATGC
eGFP-pMS137-f	ATAT ACATGT CGTAATGGAGATTCAT ATGACCATGAT TACGCATCATC
eGFP-pMS137-r	ATAT GGTACC TTACTTGTACAGCTCGTCCATG
gfp-pME6032-f	TATA GAATTC ATGAGTAAAGGAGAAGAACTTTTCACC
gfp-pME6032-r	ATCC GGTACC CTATTTGTATAGTTCATCCATGCC
klspS-MCS3-gg-f	ATATGGTCTCAAGCTATGCCATGGATTTGTGCTACGAG
klspS-MCS3-gg-r	ATTAGGTCTCAGGATTTACACGTACATTAGTTGATTGATTGG
klspS-pMS137-f	ATAT ACATGT CGTAATGGAGATTCAT ATGCCATGGA TTTGTGCTACGAGCTCTC
klspS-pMS137-r	ATAT GGTACC TTACACGTACATTAGTTGAT
klspS-pPICZB-EcoRI	ATAT GGTACC ATGCCATGGATTTGTGCTAC
klspS-pPICZB-KpnI	ATTA CCGCGG TTACACGTACATTAGTTGATTG
LR7	TACTACCACCAAGATCT
MCS3-gg-r	ATATGGTCTCAAGCTGTTTCCTGTGTGAAATTGTTATC
MCS3-mob-gg-f	ATTAGGTCTCACCGCGACCTTCGGGAG
MCS3-mob-gg-r	ATTAGGTCTCAGCGGGCCGTCTCTTGGG
MCS3-term-gg-f	ATATGGTCTCAGCGTTAATATTTTGTTAAAATTCGCG
Mev-MCS3-gg-f	ATATGGTCTCAAGCTATGACCCGTAAAGGCTATGGC
Mev-MCS3-gg-r	ATTAGGTCTCaGGATTTATTCGATGATTTCGATGCCGGTC
Mev-MCS3-mid-gg-f	GGTCTCACGTACCCCGATTGGCGTGTTTG
Mev-MCS3-mid-gg-r	GGTCTCATACGATACGCGGCGGCCAGAAC

Continued on next page

Name	Sequence (5' ⟶ 3')
Mev-pMS137-f	ATATACATGTCGTAATGGAGATTCATATGACCCGTAAAGGC TATGGCGAAAGC
Mev-pMS137-r	ATTAGGTACCGGCGCGCCCACGTGCCG
SR-416-4ot-f	ATATTACATATGCCAATCGTAAACATCGACTTAATTGC
SR-416-4ot-r	ATATTTATTTTTCGTCGCTCTTCAAAACGCC
SR-773-4ot-f	ATATTACATATGCCATACGTAACAGTGAAAATGCTAG
SR-773-4ot-r	ATATCTCGAGTTATTTGTCGCTTAAGCGTTTTCCGC
SR-86-4ot-f	ATATTACATATGCCATACGTAACAGTGAAAATGCTTG
SR-86-4ot-r	ATATCTCGAGTTATTTATCACTGTGGAGCACGCCTG
term-MCS3-gg-f	ATATGGTCTCAATCCGGCTGCTAACAAAGCC
term-MCS3-gg-r	ATTAGGTCTCAACGCCAAAAAACCCCTCAAGACCCG

2.10 Vectors

Table 2.6: *Vectors and plasmids used in this work*

Name	Size (bp)	Description	Source
pET-28a	5369	Kan^R ColE1 P_{lac} lacZ' *lacI*, protein expression vector with T7 expression system under the control of lac-operon	Novagen, Merck (Darmstadt, Germany)
pUC-gfp	3344	Amp^R, *gfp*	CBR
pME6032	9182	Tet^R, P_{tac}	CBR
pMS137	9310	Kan^R oriV *chnR* P_{chnB} *luc* 'chnE trfA	[292]
pPICZ B	3328	Zeocin® ^{R} P_{AOX1}	Thermo Fisher Scientific, Waltham, MA, USA
pET-24a	5310	Kan^R ColE1 P_{lac} lacZ' *lacI*, protein expression vector with T7 expression system under the control of lac-operon	Novagen, Merck (Darmstadt, Germany)
pKnock-Cm	1859	Chl^R, vector for genomic integration	[1]
pBBR1MCS-3	5228	Tet^R pBBR1 oriV P_{lac} lacZ'	[168]
pJeM1TcR	7504	Tet^R, *egfp* P_{rha} *rhaR rhaS*	[297]

Continued on next page

Name	Size (bp)	Description	Source
pGA1-Mev	9683	Amp^R, *mev*-operon, syntheitc	CBR
pBA2kIKmA2	6449	Kan^R Amp^R ColE1 lacZ' LacO PsbA2 ds/us	gift from Anastasios Melis (Addgene plasmid #39213), [188]
pET24a-SR-86-4-ot	5420	pET24a harboring the 4-Oxalocrotonate Tautomerase from *Lysinibacillus xylanilyticus* SR-86	This work
pET24a-SR-772-4-ot	5420	pET24a harboring the 4-Oxalocrotonate Tautomerase *Bacillus* sp. SR-772	This work
pET24a-SR-416-4-ot	5423	pET24a harboring the 4-Oxalocrotonate Tautomerase *L. brevis* SR-416	This work
pET24a-DH5α-4ot	5468	pET24a harboring the 4-Oxalocrotonate Tautomerase from *E. coli* DH5α CBR_S_76	This work
pMS137-eGFP	8331	Kan^R oriV *chnR* P_{chnB} *egfp* 'chnE trfA	This work
pMS137-kIspS	9282	Kan^R oriV *chnR* P_{chnB} *kisps* 'chnE trfA	This work
pMS137-Mev	14354	Kan^R oriV *chnR* P_{chnB} *mev* 'chnE trfA	This work
pBBR1MCS-3-eGFP	5725	Tet^R pBBR1 oriV P_{lac} lacZ' *egfp*	This work
pBBR1MCS-3-kIspS	6676	Tet^R pBBR1 oriV P_{lac} lacZ' *kIspS*	This work
pPICZB-kIspS	5026	Zeocin® ^{R} *kisps* P_{AOX1}	This work

NA: not available

2.11 Media

For all media, H_2O purified using the Elga^{TM} system was used. After preparation, media were autoclaved at 121 °C and 2 bar for 20 min.

For solid media, agar was added as indicated in the tables in this section. The agar was poured into petri dishes [243] under a sterile hood and was dried for several minutes. The agar plates were stored at 4 °C.

To 1 L of medium with the suffix “SE”, 1 mL of sterile filtered EPS trace solution (see **Tab. 2.25**) was added. Special media or modifications used for one specific method are described within the methods section.

2.11.1 Standard Media

Table 2.7: *Components for Y(E)PD(SE) medium*

Compound	Amount for 1 L
Yeast extract	10.0 g
Tryptone	20.0 g
Glucose	20.0 g
Agar	15.0 g
H_2O	1 L

Table 2.8: *Components for aMRS(SE) medium*

Compound	Amount for 1 L
MRS Bouillon	52.0 g
Agar	15.0 g
H_2O	1 L

The pH of the aMRS(SE) medium was adjusted to 5.0 using 37% HCl.

Table 2.9: *Components for LB medium*

Compound	Amount for 1 L
Yeast extract	5.0 g
Tryptone	10.0 g
NaCl	10.0 g
Agar	15.0 g
H_2O	1 L

Components according to [32] without glucose.

Table 2.10: *Components for LB low salt medium*

Compound	Amount for 1 L
Yeast extract	5.0 g
Tryptone	10.0 g
NaCl	5.0 g
Agar	15.0 g
H_2O	1 L

Table 2.11: *Components for ST1(SE) medium*

Compound	Amount for 1 L
Yeast extract	3.0 g
Tryptone	15.0 g
NaCl	6.0 g
Agar	15.0 g
H_2O	1 L

Table 2.12: *Components for ST1GSE medium*

Compound	Amount for 1 L
Yeast extract	3.0 g
Tryptone	15.0 g
NaCl	6.0 g
Glucose	5.0 g
EPS Trace elements solution	1 mL
Agar	15.0 g
H_2O	1 L

Table 2.13: *Components for DSMZ 1 medium*

Compound	Amount for 1 L
Tryptone	5.0 g
Meat extract	3.0 g
Agar	15.0 g
H_2O	ad 1 L

Table 2.14: *Components for NR medium*

Compound	Amount for 1 L
Yeast extract	10.0 g
Tryptone	10.0 g
Beef extract	5.0 g
Ammonium sulphate	5.0 g
Agar	15.0 g
H_2O	1 L

Table 2.15: *Components for SOC medium*

	Compound	amount for 1 L
	Yeast extract	5.0 g
	Tryptone	10.0 g
	NaCl	0.5 g
	Glucose (20%)	10 mL
	H_2O	1 L

Table 2.16: *Mineral salts medium*

Solution	Compound	Amount	Volume to combine for 1 L
Mineral salt components	$Na_2HPO_4 \cdot 12\,H_2O$	18 g	500 mL
	KH_2PO_4	3 g	
	NH_4Cl	1 g	
	$MgSO_4 \cdot 7\,H_2O$	0.4 g	
	$CaCl_2 \cdot 2\,H_2O$	0.04 g	
	Fe(III)NHCitrate	2.4 mg	
	H_2O	ad 1 L	
Agar component	Agar	16.5 g	400 mL
	H_2O	ad 1 L	
Carbon source			100 mL
EPS trace elements solution			1 mL

This medium is based on the DSMZ medium 81. However, it is slightly modified according to [294]. The carbon source was prepared in a 10x stock solution (w/v if solid, v/v if liquid). In addition, the orignal trace elements solution was replaced by the one described in **Tab. 2.25**.

Table 2.17: *EPS medium*

Solution	Compound	Amount	Volume to combine for 1 L
Solution 1	Pepton	1.50 g	800 mL
	$MgSO_4 \cdot 7\,H_2O$	1.34 g	
	MOPS	10.00 g	
	H_2O	800 mL	
Solution 2	$CaCl_2 \cdot 2\,H_2O$	0.06 g	160 mL
	Glucose	11.00 g	
	H_2O	160 mL	
Solution 3	KH_2PO_4	4.20 g	40 mL
	H_2O	100 mL	
EPS trace elements solution			1 mL
Vitamin solution			2 mL

2.11.2 Media for the Enrichment of Marine Microorganism

Table 2.18: *AMA medium*

Solution	Compound	Amount	Volume to combine for 1 L
Solution 1	Peptone	5.00 g	600 mL
	KH_2PO_4	0.30 g	
	KCl	0.60 g	
	$MgSO_4 \cdot 7\,H_2O$	2.40 g	
	$NaNO_3$	1.00 g	
	NH_4Cl	0.027 g	
	Agar	15.00 g	
	H_2O	600 mL	
Solution 2	Glucose	15.00 g	200 mL
	$CaCl_2 \cdot 2\,H_2O$	0.30 g	
	H_2O	200 mL	
Solution 3	KH_2PO_4	0.50 g	200 mL
	H_2O	200 mL	
EPS Trace Elements Solution			2 mL

The pH of the medium is at circa 6.5 after autoclaving and combining all the components. The components are autoclaved separately to prevent the salts from precipitating. After autoclaving, the solutions are cooled to at least 60 °C and then combined under a sterile hood.

Table 2.19: *Marine Bouillon*

	Compound	Amount for 1 L
	Marine Bouillon Powder	40.1 g
	Agar	15.0 g
	H_2O	1 L

2.11.3 Media for Transformation and Protein Expression in *Pichia pastoris* X-33

All media in this section are recommended by the *Pichia* expression kit manual (Thermo Fisher Scientific) [310].

Table 2.20: *Components for YPDS medium*

Compound	Amount for 1 L
Yeast extract	10.0 g
Tryptone	20.0 g
Glucose	20.0 g
Sorbitol	182.17 g
Agar	15.0 g
H_2O	1 L

Table 2.21: *Components for methanol minimal medium*

Solution	Compound	Amount	Volume to Combine for 1 L
10x YNB	Yeast nitrogen base (YNB)	134 g	100 mL
	Elga^{TM} H_2O	1 L	
500x Biotin	Biotin	20 mg	2 mL
	Elga^{TM} H_2O	100 mL	
Carbon Source (1%)	100% Methanol		10 mL
Elga^{TM} H_2O			888 mL

The 10x YNB solution and the 500x biotin solution were sterilized using a 0.2 µm cellulose acetate filter. The Elga^{TM} water and the 1 M phosphate buffer was autoclaved at 121 °C for 20 minutes, whereas the methanol was not treated for both minimal media in this section. If necessary, the buffer was adjusted to pH 6.0 using KOH.

Table 2.22: *Components for buffered methanol minimal medium*

Solution	Compound	amount	Volume to Combine for 1 L
10x YNB	Yeast nitrogen base (YNB)	134 g	100 mL
	Elga^{TM} H_2O	1 L	
500x Biotin	Biotin	20 mg	2 mL
	Elga^{TM} H_2O	100 mL	
Carbon Source (1%)	100% Methanol		10 mL
1 M potassium phosphate buffer, pH 6.0	K_2HPO_4	174.18 g	13.2 mL
	Elga^{TM} H_2O	1 L	
	KH_2PO_4	136.09 g	86.8 mL
	Elga^{TM} H_2O	1 L	
Elga^{TM} H_2O			700 mL

2.11.4 Media for the Isolation of *Streptomyces* sp.

For the isolation of *Streptomyces*, chitin agar plates were used, as a large portion of them are able to hydrolyze chitin [134, 190]. For enrichment after isolation GYM (also known as DSMZ Medium 65) plates were used. If the liquid form was needed, agar was left out.

Table 2.23: *Chitin agar and medium*

Compound	Amount for 1 L
Chitin	4.00 g
KH_2PO_4	0.30 g
K_2HPO_4	0.70 g
$MgSO_4 \cdot 7\,H_2O$	0.50 g
$FeSO_4 \cdot 7\,H_2O$	0.01 g
$ZnSO_4 \cdot 7\,H_2O$	0.01 g
$MnCl_2 \cdot 4\,H_2O$	0.01 g
Agar	20.00 g
H_2O	ad 1 L

Modiefied from [16].

Table 2.24: *GYM agar and medium (DSMZ Medium 65)*

Compound	Amount for 1 L
Glucose	4.00 g
Yeast extract	4.00 g
Malt extract	10.00 g
Agar	12.00 g
H_2O	ad 1 L

2.11.5 Media Supplements

Table 2.25: *Components for EPS trace elements solution*

Compound	Amount for 1 L
$MnCl_2 \cdot 4\,H_2O$	1.800 g
$FeSO_4 \cdot 7\,H_2O$	2.500 g
Boric acid	0.258 g
$CuSO_4 \cdot 7\,H_2O$	0.031 g
$ZnCl_2$	0.021 g
$CoCl_2 \cdot 6\,H_2O$	0.075 g
$NaMoO_4$	0.023 g
Sodiumtartrate $\cdot 2\,H_2O$	2.100 g

The EPS trace elements solution was stored at 4 °C and sterile filtered through a 0.2 µm cellulose acetate membrane filter.

The vitamin solution was purchased pre-mixed (for a list of components according to the manufacturer [284], see **Tab. 2.26**). The solution was sterile filtered through a 0.2 µm cellulose acetate membrane filter and stored in 2 mL aliquots at -20 °C.

Table 2.26: *Components for 100x vitamin solution*

Compound	Amount for 1 L
D-Biotin	0.02 g
Choline chloride	0.3 g
Folic acid	0.1 g
myo-Inositol	3.5 g
Niacinamide	0.1 g
p-Amino benzoic acid	0.1 g
D−Pentothenicacid · 0.5 Ca	0.025 g
Pyridoxal · HCl	0.1 g
Riboflavin	0.02 g
Thiamine · HCl	0.1 g
Vitamin B12	0.0005 g
KCl	0.2 g
KH_2PO_4	0.2 g
NaCl	8.0 g
Na_2HPO_4	1.15 g

2.11.6 Miscellaneous

Table 2.27: *Preparation of 60% glycerol for cryo stocks*

Compound	Amount for 100 mL
98% glycerol	60 mL
Elga^{TM} H_2O	40 mL

The 60% glycerol solution was autoclaved with the same parameters as the media.

Antibiotics were prepared by adding the appropriate amount into the desired solvent. The solutions were sterilized by filtering through a 0.2 µm cellulose acetate filter. All antibiotics were stored at -20 °C. Zeocin® was purchased ready to use (Thermo Fisher scientific, Munich, Germany). The end concentration in the medium was usally 1/1000th of the stock (1:1000 dilution), unless stated otherwise. In case of solid media, the antibiotics were added to the agar solution between 50–60 °C in order to prevent the antibiotics from being inactivated.

Table 2.28: *Antibiotic stock solutions*

Antibiotic	Concentration of stock solution	Solvent	Standard dilution in media
Ampicillin	100 mg/L	Elga^{TM} H_2O	1:1000
Carbenicillin	100 mg/L	Elga^{TM} H_2O	1:1000
Chloramphenicol	34 mg/L	96% ethanol	1:1000
Tetracyclin	2.5 mg/L	96% ethanol	1:200
Zeocin®			

2.12 Buffers

Buffers were stored at room temperature, if not stated otherwise. Buffers, which were only used in combination with a specific method, are given in the section describing this method. For example, the TFB I buffer was only used for preparing chemically competent cells and is henceforth found in **Paragraph 2.14.6.1.1**.

2.12.1 Buffers for Screening

Table 2.29: *Components for NaPi(SE) buffer*

Solution	Component	Amount	Volume to combine for 1 L
Solution 1	NaH_2PO_4 H_2O	6.00 g 250 mL	171.2 mL
Solution 2	Na_2HPO_4 H_2O	7.10 g 250 mL	78.8 mL
Solution 3	$MgSo_4 \cdot 7\,H_2O$ H_2O	3.08 g 50 mL	10 mL
Solution 4	TPP H_2O	46.10 mg 10 mL	1 mL
H_2O			740 mL
If SE version	EPS trace elements solution		1 mL

2.12.2 Buffers for Enzyme Assays and AEKTA™ Purification

Table 2.30: *Components 20 mM ammonium carbonate buffer*

Compound	Amount for 1 L
NH_4HCO_3	1.58 g
Elga™ H_2O	1 L

pH was adjusted to pH 9.0 using a 37% ammonia solution.

Table 2.31: *Components 40 mM glycin NaOH buffer*

Compound	Amount for 1 L
Glycine	3.0 g
H_2O	1 L

pH was adjusted to pH 9.0 using NaOH.

Table 2.32: *Components 50 mM MOPS buffer*

Compound	Amount for 1 L
MOPS	10.46 g
H_2O	1 L

pH was adjusted to pH 7.0 using NaOH.

Table 2.33: *Components for 50 mM phosphate buffer*

500 mM Stock	Compound	amount for 1 L
Component 1	Na_2HPO_4	71.00 g
	H_2O	1 L
Component 2	NaH_2PO_4	60.00 g
	H_2O	1 L

The two buffer components (see **Tab. 2.33**) were mixed in different volumes until the desired pH was reached (between 5.8 and 8.0). This solution was then diluted to the desired ionic strength, in this case, 50 mM.

2.12.3 SDS-PAGE Buffers

Table 2.34: *Composition of SDS-PAGE 10x running buffer stock solution*

Compound	Amount for 1 L
Tris	30.3 g
Glycine	144.1 g
SDS	10.0 g
Elga^{TM} H_2O	1 L

Table 2.35: *Composition of SDS-PAGE 4x upper and lower buffer stock solution*

Compound	Amount for 1 L upper buffer	amount for 1 L lower buffer
Tris	78.8 g	181.7 g
SDS	8.0 g	8.0 g
Elga^{TM} H_2O	ad 1 L	ad 1 L
pH adjusted with HCl	6.8	8.8

Table 2.36: *Composition of ammonium persulfate (APS) stock solution*

Compound	Amount for 1 L
Ammonium persulfate	12.114 g
Elga^{TM} H_2O	1 L

Additionally, a spatula tip of bromphenol blue was added to the upper buffer (see **Tab. 2.35**). The ammonium persulfate stock solution was aliquoted in 1 mL volumes and stored at -20 °C.

The SDS-PAGE buffer was used to mix 1 part of buffer with 4 parts of sample to load an SDS gel. The pH was adjusted to 6.8 using HCl and the buffer was stored in 1.5 mL aliquots at -20 °C. Opened aliquots were stored at 4 °C.

Table 2.37: *Composition of SDS-PAGE 5x sample buffer*

Compound	Amount for 100 mL
Glycerol	50 mL
β-mercaptoethanol	12.5 mL
SDS	7.5 g
Tris 1 M, pH 6.8	25 mL
Bromphenol blue	25 µg
Elga^{TM} H_2O	12.5 mL

2.12.4 Agarose Gel Buffers

Table 2.38: *Composition of 50x TAE buffer stock solution*

Compound	amount for 500 mL
Tris	242.3 g
EDTA	14.7 g
Glacial acetic acid	57.1 mL
Elga^{TM} H_2O	500 mL

The pH was adjusted to 8.0 using acetic acid. For the final usage, the stock solution was diluted at a factor of 1:50 with Elga^{TM} H_2O and was used as 1x TAE for gel electrophoresis (see **Section 2.14.5**).

Table 2.39: *Composition of the agarose gel 5x loading dye*

Compound	Amount for 75 mL
Tris	450 µg
EDTA	730 µg
Glycein	25 mL
Bromphenol blue	12.5 µg
Xylene cyanol	12.5 µg
Elga^{TM} H_2O	50 mL

This dye was used to load samples of DNA on agarose gels with 1 part dye and 4 parts of DNA solution. The pH was adjusted to 7.6 and stored in aliquots at -20 °C. Opened aliquots were stored at 4 °C.

2.12.5 Buffers for Working with *Pichia pastoris* X-33

Table 2.40: *Composition of 10x stock solution of phosphate buffer for the buffered minimal medium*

Compound	Amount for 1 L
K_2HPO_4	23 g
KH_2PO_4	118 g
Elga^{TM} H_2O	1 L

This buffer stock solution was used to prepare the buffered minimal medium (see **Tab. 2.40**). The pH was adjusted to 6.0 using 10 M KOH solution.

Table 2.41: *Composition of 5x stock solution of phosphate-buffered saline (PBS)*

Compound	Amount for 1 L
Na_2HPO_4	7.10 g
KH_2PO_4	1.22 g
NaCl	40.02 g
KCl	1.0 g
Elga^{TM} H_2O	1 L

This buffer was used to measure the OD_{600} of *P. pastoris* X-33 transformands and wild type in 96-well microtiter plates.

2.12.6 Buffers for HPLC Analysis

Table 2.42: *50% acetonitrile buffer*

Compound	Amount for 5 L
Acetonitrile	2.5 L
Elga^{TM} H_2O	1 L

Table 2.43: *0.1% formic acid in water*

Compound	Amount for 1 L
Formic acid	1 mL
Elga^{TM} H_2O	999 mL

Table 2.44: *0.1% formic acid in water*

Compound	Amount for 1 L
Formic acid	1 mL
Acetonitrile	999 mL

2.13 Microbiological Methods

2.13.1 Cell Growth and Cryo Stock

Cultures were usually shaken at 150 or 170 rpm on an orbital shaker. Cultures in 96-well deep well plates were grown at 1000 rpm and 24-well plates were grown at 800 rpm on an orbital shaker. The temperature depended on the organisms optimal growth temperature. Typically, *E. coli* was grown at 37 °C, environmental isolates and yeast strains at 30 °C. The time of the incubation is given in **Chapter 3**. Antibiotics were added to the medium when necessary.

Cells were stored in a cryo stock with a final concentration of 25% glycerol (v/v). Cryo stocks of 1 mL volume were prepared by adding 400 µL of 60% glycerol (v/v) (see **Tab. 2.27**) to 600 µL of cell culture in a 1.8 mL screw cap tube (Sarstedt AG & Co. KG) and stored at -80 °C.

Cryo stocks in 96-well micro titer plates (Greiner Bio-one International GmbH) were made by adding 65 µL of 60% glycerol to 135 µL overnight culture using a multi-channel pipette if indicated and stored at -80 °C.

2.13.2 Determination of Cell Density

2.13.2.1 Measuring the Cell Density at 600 nm

2.13.2.1.1 OD_{600} in Cuvettes

Depending on the cell density judged by the naked eye, there were two different approaches used. If the cell density seemed to be low, 1 mL of the culture was transferred into a 1.6 mL polystyrene cuvette (VWR International GmbH, Darmstadt, Germany) and the OD_{600} was measured at 600 nm using the Ultrospec® cell density meter (Biochrom Ltd., Cambourne, UK) or nano photometer P330 (Implen GmbH, Munich). If the cell density was greater than 1 for the first measurement or by judgement by the naked eye, it was diluted in medium accordingly or until the measured OD_{600} was smaller than 1. For all measurements, sterile medium was used as a blank. If cultures were diluted using 0.9% NaCl, sterile medium was diluted with the same factor and used as a blank.

2.13.2.1.2 OD_{600} in 96-Well Microtiter Plates

Cultures were diluted in 0.9% NaCl for bacteria and 1x PBS for *Pichia pastoris*. The OD_{600} was measured at 600 nm using the Varioskan (Thermo Fisher Scientific, Waltham, Maine, USA) or Multiskan Spectrum (Thermo Fisher Scientific, Waltham, Maine, USA) with their associated software. If the cell density was greater than 1 for the first measurement, it was diluted in medium accordingly, until the measured OD_{600} was smaller than 1. For all measurements, cultures the sterile medium was diluted in 0.9% NaCl for bacteria and 1x PBS for *Pichia pastoris* with the same factor and used as a blank.

2.13.2.2 Determining the Cell Number using the Thoma Counting Chamber

For the determination of the cell denisty after disruption, a Thoma Counting Chamber was used with 0.01 mm depth and an area of 0.0025 mM ^{2}.

In the chamber, precisely 5 μL were pipetted and counted using the 40x magnification objective and phase contrast on the microscope. If the cell count was to big to count, the cell suspension was diluted and counted again. The cell density is calculated differently, depending on the field counted (see **Fig. 2.1**). The cell density is calculated as

$$V_c = 0.0025mm^2 * 0.01mm = 0.00025mm^3 = 0.25 * 10^-6mL \tag{2.1}$$

$$\bar{x} = \frac{\sum countedcells}{a} \tag{2.2}$$

$$N(cell) = \frac{\bar{x}}{V_c} \tag{2.3}$$

with V_c being the volume of one c-field in the used Thoma counting chamber (see **Fig. 2.1**), $\bar{x}$ being the average of cells counted and N (cell) being the cell count in cells/mL. The factor a depends on the fields counted: if the c-fields are counted, then a is the amount of the c-fields counted. If the b-field is counted, $a = 16$.

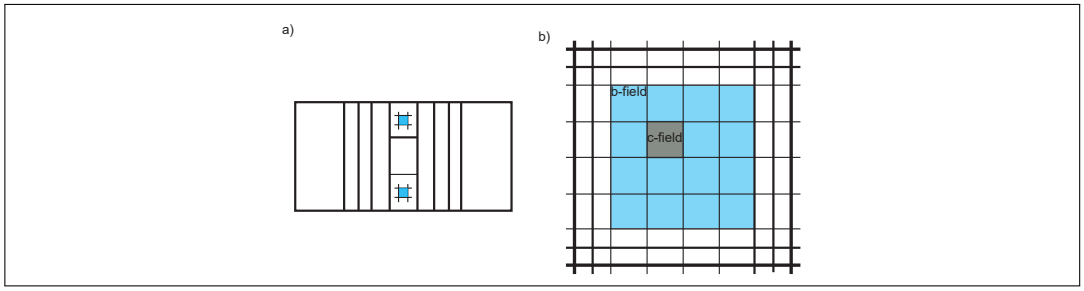

Figure 2.1: ***Thoma Counting Chamber.*** *Layout of a Thoma counting chamber. The chamber is devided into 2 b-fields which consist of 16 c-fields with an area of 0.0025 mm^2. a) Thoma chamber b) b-field in blue, c-field in brown. Naming and scheme according to [293, p. 332-333]*

2.13.3 Isolation of Microorganisms from Various Environments

Depending on the aggregate state of the environment, the sample was pretreated. If the sample was liquid, different dilutions of the sample were spread on different agar media plates. If the sample was solid, it was incubated in the same liquid media, as it was spread on agar plates afterwards for one hour at 30 °C. When possible, single colonies were transferred on new agar plates to reach purity of the environmental isolate. The standard technique of the streak method was applied until single colonies could be picked.

Isolates were stored as cryo cultures in 25% glycerol at -80 °C in micro titer plates and as 1 mL stock in 1.8 mL screw cap tubes.

A special protocol was used to isolate *Streptomyces* from the environmental samples. A suspension was made from soil samples by adding 0.2-0.5 g soil with 10 mL sterile Elga^{TM} H_2O with 0.001% Triton X100 (v/v) in a 25 mL Erlenmeyer flask shaking at 150 rpm for 30 minutes. After a short sedimentation of soil particles, 0.1 mL of the suspension was mixed with 9.9 mL phenol water (1 mL phenol in 99 mL Elga^{TM} H_2O (v/v) and was incubated for 15 minutes at room temperature. 100 µL were pipetted on chitin agar plates (see **Tab. 2.23**) and incubated at 30 °C until it was possible to pick colonies. Colonies were purified on GYM agar plates (see **Tab. 2.24**).

2.13.4 Antibiogram

In order to assess the antibiotics to which the strain *Cupriavidus necator* H16 was resistant, an antibiogram was performed in two different ways, as described in the following sections.

2.13.4.1 Antibiogram with Whatman™ Filter Paper

One hundred microliter of an overnight culture were spread on plate and autoclaved Whatman® filter paper of 0.5 cm in diameter was aseptically transferred on the plates using forceps. Five microlitre of the diluted antibiotic in question was added on the filter paper. Agar plates were then incubated at 30 °C over night with the lid facing up. Growth was examined the next day. If no growth occurred, the plates were incubated for up to 3 days. Negative controls of the H_2O, filter paper and antibiotics were made. As a positive control, 100 µL of the overnight culture was spread on plate and incubated. The zone of inhibition was estimated using a ruler on four sides of the filter paper (see **Fig. 2.2**).

2.13.4.2 Antibiogram with Gradient Plates

Gradient plates (see **Fig. 2.2**) were prepared with different antibiotics and varying concentrations according to the procedure described in [305]. First, 15 mL medium with agar was poured into a 50 mL falcon tube and the desired amount of antibiotics was added and mixed. This mixture was poured into petri dishes elevated on one side for approximately 2 mm. After the agar was solidified and dried, the plate was leveled and 15 mL of agar medium without antibiotics was added on top. Once solidified and dried, the plates were stored for up to one day, bottom facing up or used after storage of at least 1 hour, bottom up to let the antibiotics diffuse into the upper agar layer. The result is an agar plate with a concentration gradient, if incubated bottom up.

One hundred microliter of an overnight culture were spread on plate and incubated for up to 3 days or until growth occurred. If no growth on a gradient plate with a specific antibiotic occurred, a new gradient plate was made with a lower starting concentration of the antibiotic. As a positive control, 100 µL of the overnight culture was spread on plate and incubated alongside.

The minimal concentration of inhibition was estimated by measuring the distance from the edge of the petri dish marking the value "0% antibiotic" to the edge of the growth smear. This value was divided by the diameter of the petri dish multiplied with the concentration of 100% in µg/mL.

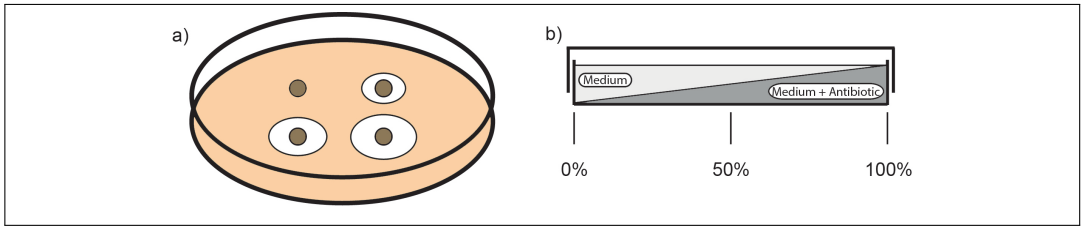

Figure 2.2: ***Gradient Plate.*** *Antibiogram using a) antibiotics soaked filter paper and b) gradient plate as it is used for an antibiogram. Layout inspired by [305].*

2.13.5 Screening for Ethylene and Propylene Producers

The number of isolates to be tested was inoculated as an overnight culture in a 96 deep well plate using the appropriate medium from a cryo culture in 96-well micro titer plate. The plate was shaken at 1000 rpm at 30 °C. One percent of the pre-culture was used to inoculate the main culture in a 25 mL shaking flask with 10 mL. The medium was the same as used for the pre-culture. The main culture was incubated at 150 rpm and 30 °C for three days. Afterwards, the culture was harvested at 4600 *xg* for 20 minutes at room temperature and resuspended in 1/10 volume (e. g. 1 mL) NaPi-SE-TPP buffer (see **Tab. 2.29**) supplemented with 1% trace solution stock (see **Tab. 2.25**). The cells were washed once in the 1 mL sodium phosphate buffer and then disrupted by a three times iteration of 1 minute liquid nitrogen and 10 minutes 60 °C water bath. Afterwards, 720 µL of the whole cell lysate was pipetted in a 10 mL GC vial and 80 µL of pooled substrates stock solution with a concentration each of 100 mM was added. The GC vials were sealed and incubated for four hours in the dark at 30 °C, 150 rpm. The substrates were 3-(phosphonooxy) butanoic acid and 3-(sulfo-oxy) butanoic acid for propylene, and 3-(phosphonooxy) propanoic acid and β-alanine for ethylene. Positive hits were re-screened with the same substrate solution.

2.13.6 Applying Substrate Combinations to Various Isolates

All isolates were grown in individual pre-cultures overnight at 30 °C at 150 rpm in 10 mL medium in 25 mL Erlenmeyer flasks. The main culture was inoculated with 1% of the volume in 500 mL baffled flasks with 120-150 mL medium. After incubation of three days, the cultures were harvested, washed once in 1/10 of the original culture volume in NaPi buffer and resuspended in NaPi-SE-TPP buffer to yield the same OD_{600}. Cells were disrupted using the IUL system operated at 2.4 kbar pressure and cooled to 5 °C

with six iterations for the total sample volume. The obtained total cell lysate was used for the ethylene assay. To 720 µL of total cell lysate 80 µL of substrate combinations were added in 10 mL GC vials and sealed. The samples were incubated for 4h at 30 °C at 150 rpm and then put on the GC autosampler and analyzed using the method for screening (see **Section 2.15.1**).

2.14 DNA Methods

2.14.1 Purification of Genomic DNA

2.14.1.1 Isolation of gDNA using the DNeasy Blood & Tissue Kit

All strains regardless of identity where inoculated from the cryo stock. An overnight culture was grown at the organisms-preferred growth temperature and medium. If the culture was not turbid enough as inspected with the naked eye, growth time was extended until it was turbid enough. At least 1 mL of the culture was harvested in a 2 mL microreaction tube. The specific centrifuging parameters for each cell type is given in the paragraphs below.

The supernatant was discarded. If required, the cell pellet was stored at -20 °C until the purification was proceeded.

All centrifugation steps were carried out at room temperature.

2.14.1.1.1 Isolation of gDNA from Yeasts

The cell pellet was resuspended in 100 µL 1M sorbitol, 100mM EDTA and 1% β-mercapto-ethanol buffer per 1 mL culture. The mixture was homogenized by vigorous vortexing for 1 min. Afterwards, the homogenate was incubated for 5 minutes on ice. In order to digest the cell wall, 10 µL of Zymolayse (VWR International, Radnor, USA) was added per 100 µL for 30–60 minutes at 37 °C. The spheroblasts were pelleted at 3000 *xg* at room temperature for 10 minutes. These were resuspended in 180 µL Buffer ATL. Twenty microliters of proteinase K were added, vortexed briefly and incubated for 15 minutes at 56 °C. Afterwards, 15 µL RNase T1/A was added and incubated for 30 minutes at 37 °C. After vortexing for ca. 15 seconds, 200 µL Buffer AL was added, vortexed and 200 µL 96% ethanol was added and again mixed by vortexing. This solution was added into a provided mini spin column and centrifuged at 6000 *xg* for 1 minute. The column was placed into a new collection tube, 500 µL Buffer AW1 was added and centrifuged at 6000 *xg* for 1 minute. It was again placed in a new collection tube, 500 µL buffer AW2 was added and centrifuged for 3 minutes at 20000 *xg*. It was placed in a 1.5

mL microreaction tube, and 100 µL buffer AE was added and incubated for 1 minute at room temperature before it was centrifuged at 6000 *xg* for 1 minute. In order to increase the overall amount of DNA, the last step was repeated and the DNA was eluted in a fresh microreaction tube.

2.14.1.1.2 Isolation of gDNA from Gram Positive Bacteria

The cell pellet was resuspended in 180 µL enzymatic lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM Na-EDTA, 1.2% Triton X-100, lysozyme 20 mg/ mL) and incubated for at least 30 minutes at 37 °C. Afterwards, 25 µL proteinase K and 200 µL buffer AL (without ethanol) were added and incubated at 56 °C for 30 minutes. Fifteen microliter of RNase T1/A was added and incubated for 30 minutes at 37 °C. Two hundred microliter 96–100% ethanol was added and mixed by vortexing. The solution of the previous step was transferred into a provided column and centrifuged for 1 minute at 6000 *xg*. The column was placed in a new collection tube, 500 µL buffer AW1 was added and centrifuged for 1 minute at 6000 *xg*. The column was again placed in a new collection tube and buffer AW2 was added and centrifuged for 3 minutes at 20000 *xg*. The DNA was eluted from the column by placing it in a 1.5 mL microreaction tube, adding 100 µL buffer AE and incubating for 1 minute at room temperature, before it was centrifuged at 6000 *xg* for 1 minute. The overall yield of DNA was increased by eluting once more in a new microreaction tube with the same parameters.

2.14.1.1.3 Isolation of gDNA from Gram Negative Bacteria

The cell pellet was resuspended in 180 µL buffer ATL and 20 µL proteinase K was added and incubated at 56 °C for 30 minutes. Fifteen microliter of RNase T1/A was added and incubated for 2 hours at 37 °C. Two hundred microliter buffer AL was added and mixed by vortexing. Two hundred microliter 96–100% ethanol was added and mixed by vortexing. The solution of the previous step was transferred into a provided column and centrifuged for 1 minute at 6000 *xg*. The column was placed in a new collection tube, 500 µL buffer AW1 was added and centrifuged for 1 minute at 6000 *xg*. The column was again placed in a new collection tube and buffer AW2 was added and centrifuged for 3 minute at 20000 *xg*. The DNA was eluted from the column by placing it in a 1.5 mL microreaction tube, adding 10 µL buffer AE and incubating 1 minute at room temperature before it was centrifuged at 6000 *xg* for 1 minute. The overall yield of DNA was increased by eluting once more in a new microreaction tube with the same parameters.

2.14.2 Determining DNA Concentration

The concentration of DNA was measured using the NanoPhotometer P330 (Implen GmbH, Munich, Germany) according to the manufactures instructions.

2.14.3 Butanol Precipitation of DNA

In order to concentrate DNA solutions, -20 °C 100% butanol with at least 10 times the volume of the sample (minimum 1 mL) was added. The solution was mixed by using a vortexer until no phase separation was noticeable and incubated for 10 minutes at -20 °C. The mixture was then centrifuged with 21100 *xg* at 4 °C for 10 minutes. The supernatant was discarded and the pellet was resuspended in 70% ice-cold ethanol and centrifuged with the same parameters. The supernatant was discarded and the pellet was dyed at room temperature or at 60 °C until the pellet was almost completely dry. The pellet was then resuspended in Elga water or buffer with the desired volume.

2.14.4 PCR for Cloning

The first step before performing the PCR was to design the primers. Usually, the primer pairs were designed to have a melting temperature of 60 °C with no more difference than 5 °C. The length of binding nucleotides withing the template was at least 20 bases ending with a C or G at the 3'-end. Adjacent to the 5'-end of the template, the restriction side was added with additional bases (usually 4 bases) to enhance the ion enzymes' efficiency. For cloning into expression vectors, prevention of frame shifts was given special consideration. The design of primers and *in silico* cloning was performed using CloneManager 9 or SnapGene 2.3.2. The typical master mix of PCR reactions for various polymerases is given in **Tab. 2.45** and for the ACCUzyme® master mix in **Tab. 2.46**.

For more than one sample, the PCR mix was upscaled excluding the differing parts. The differing parts were put in PCR tubes and the master mix was added in each tube.

For templates with complex structure, up to 3% DMSO from a 100% DMSO Stock was added (replaced the same volume of water).

Tab. 2.47 gives an overview of the optimal conditions according to the manufacturer for all polymerases and master mixes used.

Typical PCR-programs for the used polymerases used are given in **Tab. 2.48**.

The lid was heated to 105 °C.

Table 2.45: *Standard PCR mix for one reaction for different polymerases*

Compound	NEB Taq		NEB Phusion®		
Total volume	10 µL	40 µL	10 µL	40 µL	50 µL
Elga™ H_2O	6.70 µL	29.8 µL	5.85 µL	26.40 µL	31.00 µL
dNTPs (10mM each)	0.20 µL	0.80 µL	0.10 µL	0.40 µL	1.00 µL
Primer fw (10pmol/ µL)	0.50 µL	2.00 µL	0.50 µL	2.00 µL	2.50 µL
Primer rv (10pmol/ µL)	0.50 µL	2.00 µL	0.50 µL	2.00 µL	2.50 µL
Buffer*	1.00 µL	4.00 µL	2.00 µL	8.00 µL	10.00 µL
Polymerase	0.10 µL	0.40 µL	0.05 µL	0.20 µL	0.50 µL
Template	50 ng plasmid DNA, 100 ng gDNA				

* The buffer for Taq was 10x, for Phusion was 5x concentrated.

Table 2.46: *PCR master mix for one reaction of 50 µL using ACCUzyme™ PCR master mix*

Compound	Amount for 10 µL
H_2O	20.0 µL
Primer fw (10pmol/ µL)	2.0 µL
Primer rv (10pmol/ µL)	2.0 µL
Mastermix 2x	25 µL
Template	1.0 µL

Table 2.47: *Various parameters for PCR for all polymerases used*

Parameter	NEB Taq	NEB Phusion®	ACCUzyme™
Time for 1 kb	1 minute/kb	30 s/kb	1.5-2 minute/kb
Elongation Temperature	68 °C	72 °C	72 °C
Concentration of gDNA	1 ng-1 µg	50-250 ng	5-500 ng
Concentration of plasmid DNA	1 ng	1 pg-10 ng	50 pg-10 ng

Table 2.48: *PCR cycler programs for all polymerases used for cloning*

Step	NEB Taq	NEB Phusion®	ACCUzyme™
Initial denaturation	95 °C for 30 s	95 °C for 30 s	95 °C for 30 s
30 Cycles	95 °C for 30 s 45-68 °C for 30 s 68 °C for 1 minute/kb	95 °C for 30 s 45-72 °C for 30 s 72 °C for 30 s/kb	95 °C for 30 s 55-60 °C for 30 s 72 °C for 2 minutes/kb
Final extention	68 °C for 5 minutes	72 °C for 5 minutes	72 °C for 5 minutes
Hold	16 °C for ∞	16 °C for ∞	16 °C for ∞

2.14.4.1 Golden Gate Cloning

Golden Gate Cloning (for details see [84, 85, 346]) was used to clone inserts into vectors where no appropriate restriction sites were available. To do so, at first, the insertion site in the vector was chosen. Then primers were designed in a way that the restriction enzyme cutting was BsaI, which cuts outside of its recognition site. This allows to generate four nucleotide long 5'-overhangs with a selectable random sequence. All fragments used for the Golden Gate assembly were produced by PCR, including the vector backbone (see **Section 2.14.4**). The fragments were purified by a 1% agarose gel (see **Section 2.14.6.6**). The next step was to assembly the fragments as shown in **Tab. 2.49**. After the assembly was complete, the remaining linear PCR products were removed by adding 1.5 µL plasmidsafe enzyme, 1.5 µL plasmidsaf buffer, 1.5 µL ATP (10 mM) and 0.5 µL Elga^{TM} H_2O to the Golden Gate assembly reaction. The reaction was put in a thermo cycler with the programme shown in **Tab. 2.50**. This reaction was then completely used for transformation (see **Section 2.14.6.10**) or stored at -20 °C until transformation.

Table 2.49: *Reaction mix for Golden Gate assembly*

Compound	Amount for 10 µL
BsaI-HF	0.75 µL
T7 DNA ligase	0.25 µL
10x Cut Smart Buffer	1.00 µL
ATP (10 mM)	1.00 µL
DTT (10 mM)	1.00 µL
Vector backbone	50-100 ng
Insert(s)	equimolar to vector backbone
H_2O	ad 10 µL

Table 2.50: *Thermocycler program for the Golden Gate assembly*

Step	Value
30 Cycles	37 °C for 5 minutes 20 °C for 5 minutes
Enzyme inactivation	80 °C for 20 minutes
Hold	12 °C for ∞

2.14.4.2 NEB HiFi DNA Assembly

This method is a version of the Gibson assembly. The procedure was performed according to the manufacturers manual[1]. The primers were designed using the NEBuilder online tool[2]. The overlap of the 5'-end of the primers were set to 25 nucleotides and the guidelines for 2-3 fragments was used. All fragments used were cleaned up using the methods described in (see **Sections 2.14.6.5** and **2.14.6.6**), in order to prevent wrong assemblies. The vector to insert molar ratio was 1:2, the total amount of fragments was circa 0.2 pmol (50-100 ng of vector). Ten microlitres of the "NEBuilder HiFi DNA Assembly Master Mix" was added and the reaction volume was adjusted to 20 µL using Elga^{TM} water. The tube was transferred in a PCR cycler and incubated for up to 60 minutes at 50 °C. After the reaction was completed, 2 µL were used to transform *E. coli* DH5α cells (see **Section 2.14.6.10**) or was stored at -20 °C until further use.

Usually, no positive or negative control was performed, as clones formed on plate were screened for the correct insert (see **Paragraph 2.14.6.11.1**). Details are given in **Chapter 3**.

2.14.5 Detection of DNA Fragments on Agarose Gels

For the detection of plasmids, PCR fragments and genomic DNA a 1% agorse gel was prepared. In 250 mL, 1x TAE buffer 2.5 g of agarose (Serva Electrophoresis GmbH, Heidelberg) was added and heated in a microwave until it was completely dissolved. Afterwards, the agarose was stored at 60 °C. Two methods for staining DNA and thus preparing the agarose gels were used.

2.14.5.1 Agarose Gel Staining Using Ethidium Bromide

Gels were poured into the mold and the ridge was put in the gel. The agarose was given the chance to solidify. Afterwards, it was transferred to the electrophoretic chamber with 1x TAE buffer. Samples mixed with 5x loading dye were loaded on the gel as indicated in the results section. In general, gels were run with 110 V as constant values (PowerPac Basic, Bio-Rad Laboratories GmbH, Munich, Germany). Short gels were run for 25 minutes, long gels for 50 minutes. Afterwards, the gel was dyed for 10 minutes in staining solution consisting of 250 mL 1x TAE and 5 drops of a 10 g/L ethidium bromide

[1] https://www.neb.com/products/e2621-nebuilder-hifi-dna-assembly-master-mix, last access: 2017-08-24

[2] http://nebuilder.neb.com/, last access: 2017-08-24

solution for 10 minutes. After destaining for 10 minutes in 1x TAE, an image of the gel was taken (Gel iX Imager, Intas-Science-Imaging instruments GmbH, Göttingen, Germany) using the accompanying software Intas GDS with optimal adjusted parameters.

2.14.5.2 Agarose Gel Staining Using SERVA DNA Stain G

The second method of staining DNA on agarose gels was using SERVA DNA Stain G. Before the gel solidified, one drop was added to 50 mL 1% agarose (2 drops to 100 mL 1% agarose) from a working solution, which was a dilution of the purchased solution of 1:20000. The DNA was visualized using blue light (450 nm) or UV light (300 nm). Values for running the gel and taking the image were the same as mentioned above.

2.14.6 Cloning

2.14.6.1 Preparation of Chemically Competent *E. coli* Cells

In the following, the methods of how competent cells were made are presented. All of these methods were used. The main difference in the result between the methods is the efficiency of the competent cells.The protocol using rubidium chloride (see **Paragraph 2.14.6.1.1**) generates the most efficient cells.

The given methods were used for all *E. coli* strains.

2.14.6.1.1 Rubidium Chloride Method

An overnight culture of typically 10 mL LB was inoculated from a cryo stock and incubated at 37 °C at 150 rpm. After growth, the OD_{600} was measured and 1 L of LB medium + 20 mM $MgSO_4$ (typically 20 mL 1 M $MgSO_4$ to 1 L of LB medium, prepared as 12.3 g $MgSO_4 \cdot 7\,H_2O$ in 50 mL Elga^{TM} H_2O) was inoculated to OD_{600} = 0.1. The culture was incubated at 37 °C at 150 rpm until an OD_{600} of 0.6 was reached. The culture was rested on ice for 15 minutes and then harvested by centrifuging at 4000 rpm for 10 minutes at 4 °C. The pellet was then resuspended in 100 mL TFBI buffer and divided equally in 50 mL falcon tubes. After centrifugation with the same parameters, the pellets were resuspended in 25 mL TFBI buffer each. After centrifugation the pellets were resuspended in 2.5 mL TFBII buffer each. Aliquots of 100 µL were frozen by immersing in liquid nitrogen and stored at -80 °C. The pH of buffer TFB I was adjusted to 5.8 using 50% acetic acid. This buffer was prepared fresh on the day of the experiment.

The pH of buffer TFB II was adjusted to 6.5 with KOH. This buffer was stored at 4 °C. Both buffers were sterilized by filtering the buffer through a 2 µm filter.

Table 2.51: *Composition of TFB I buffer*

Compound	Amount for 1 L
Potassium acetate	2.94 g
RbCl	120.1 g
$CaCl_2 \cdot 2\,H_2O$	1.48 g
$MnCl_2 \cdot 4\,H_2O$	9.9 g
Glycerol	150 mL
Elga^{TM} H_2O	ad 1 L

Table 2.52: *Composition of TFB II buffer*

Compound	Amount for 1 L
MOPS	2.1 g
RbCl	1.2 g
$CaCl_2 \cdot 2\,H_2O$	11.02 g
Glycerol	150 mL
Elga^{TM} H_2O	ad 1 L

2.14.6.1.2 Preparation of Chemically Competent *E. coli* Cells According to [140]

A pre-culture was prepared from the cryo stock in 10 mL SOB medium and grown at 37 °C at 150 rpm. Two hundred fifty microliters of SOB medium in a 2 L flask were inoculated to an OD_{600} of 0.1 and grown at 200 to 250 rpm and 18 °C until an OD_{600} of 0.6 was reached. The culture was chilled on ice for 10 minutes and centrifuged at 2500 *xg* for 10 minutes at 4 °C. The pellet was resuspended in 80 mL ice-cold TB and incubated for 10 minutes on ice and centrifuged as previously depicted. The pellet was then resuspended in 20 mL ice-cold TB buffer and DMSO was added to a final concentration of 7% with gentle mixing. The cells were chilled on ice for 10 minutes. The cell suspension was then aliquoted in 500 µL portions in 1.5 mL microreaction tubes and frozen quickly in liquid nitrogen and stored at -80 °C until used.

The pH of the TB buffer was adjusted to 6.7 using KOH and sterilized by filtering the buffer through a 2 µm filter.

Table 2.53: *Composition of TB buffer*

Compound	Amount for 1 L
HEPES	10 mM
$CaCl_2$	15 mM
KCl	250 mM
$MnCl_2$	55 mM
Elga^{TM} H_2O	ad 1 L

2.14.6.2 Preparation of Electro Competent *E. coli* Cells

This method was used for all strains of *E. coli* used in this thesis. A single colony was used to inoculate a 5 mL pre-culture in LB-low medium (see **Tab. 2.10**) and incubated overnight at 37 °C at150 rpm. The 100 mL main culture was inoculated to OD_{600} = 0.1 and grown to an OD_{600} of 0.5–0.6 at 37 °C at 150 rpm. Afterwards, the main culture was chilled on ice for 15 minutes and then transferred to two 50 mL Falcon tubes. The culture was then harvested with 3500 *xg* for 15 minutes at 4 °C. The pellet was resuspended in 50 mL autoclaved Elga^{TM} H_2O by gentle inverting of the tubes. Cells were centrifuged with the above mentioned parameters and resuspended in 50 mL 10% (v/v) glycerol. Cells were centrifuged again (as mentioned above) and resuspended in 300 µL 10% (v/v) glycerol and adjusted to an OD_{600} of 50 with 10% glycerol. The competent cells were frozen in liquid nitrogen as 70 µL aliquots in 1.5 mL reaction tubes and stored at -80 °C.

2.14.6.3 Preparation of Electro Competent *Cupriavidus necator* DSM428 and DSM531 Cells

Electrocompetent cells of *Cupriavidus necator* were prepared according to [240]. An overnight culture in NR medium (see **Tab. 2.14**) inoculated from the cryo stock was used to inoculate the main culture at an OD_{600} of 0.1 and grown until an OD_{600} of 0.6–1.0 at 30 °C at 150 rpm. The culture was then harvested at 3000 *xg* for 10 min at 4 °C. Afterwards, it was washed three times in 50 mL ice-cold deionized water with the aforementioned centrifugation parameters. In the final step, the cells were resuspended

in 400µl of 10% (v/v) sterile glycerol. Aliquots were prepared with a volume of 50µl, frozen quickly by immersing the aliquots in liquid nitrogen and stored at -70 °C.

2.14.6.4 Preparation of Electro Competent *Pichia pastoris* X33 Cells

An overnight pre-culture was inoculated in 5 mL YPD from the cryo stock and incubated at 30 °C in a 25 mL Erlenmeyer flask. The main culture of 500 mL YPD in a 2 L flask was inoculated from the pre-culture to an OD_{600} of 0.1 to 0.5 and grown at 30 °C overnight to a final cell density of OD_{600} 1.3 to 1.5. Cells were harvested by centrifugation at 1500 *xg* for 5 minutes at 4 °C, which are the centrifugation parameters for the following steps as well. The pellet was resuspended in 500 mL ice-cold water and centrifuged and afterwards resuspended in 250 mL ice-cold water and centrifuged. The pellet was then resuspended in 20 mL ice-cold 1M sorbitol, centrifuged and resuspended in 1 mL ice-cold sorbitol. The resuspended cells were stored on ice until transformation on the same day (cells were stored for the long term).

2.14.6.5 Purification of Plasmids

Plasmids were purified according to the manufactures' protocol with the GeneJET Plasmid Miniprep Kit K0503 (Thermo Fisher Scientific Inc., Waltham, Maine, USA). In general, a 10 mL liquid culture of *E. coli* strains with the desired plasmid and the appropriate antibiotics was inoculated from cryo stock and incubated at 37 °C at 150 rpm overnight. The pellet was resuspended in 250 µL of the Resuspension Solution and transferred to a 2 mL micro-reaction tube. Thereafter, 250 µL of Lysis Solution was added and the solution was mixed by gently inverting the tube 6 times. Before centrifuging the solution for 10 minutes at 21100 *xg*, 350 µL of Neutralization Solution was added and gently inverted 6 times. The supernatant was then transferred to a provided column. After centrifugation for 1 minute at 12000 *xg*, 500 µL of Wash Solution was added to the column and centrifuged for 1 minute at 12000 *xg*. This step was repeated once. The plasmid DNA was eluted into a fresh micro-reaction tube by adding two times 25 µL 75 °C heated Elution buffer, sequentially. DNA was stored at -20 °C. Concentration was determined as described in **Section 2.14.2** and, if necessary, purity was checked on a 1% agarose gel (see **Section 2.14.5**).

If more than 30 µL of plasmid preparation was desired, the procedure was applied to more than one column.

2.14.6.6 Purification of DNA Fragments

PCR and DNA restriction products were purified using the NucleoSpin Gel and PCR clean-up Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). All steps were conducted as suggested by the manufacturer.

2.14.6.6.1 Purification of PCR Product

To clean up PCR, 1 part of PCR was mixed with 2 parts of buffer NTI (v/v). The sample-buffer mix was transferred to a provided column and centrifuged for 30 s at 11000 *xg*. If the volume exceeded the column volume, the remaining sample was added sequentially to the same column. The sample was washed two times by adding 700 µL Wash Buffer and centrifuging for 1 minute at 11000 *xg*. The column was dried by centrifuging at 11000 *xg* for 1 minute. To elute the DNA, two times 15 µL of 70 °C heated NE buffer was added to the column, incubated for 1 minute at room temperature and centrifuged for 1 minute. DNA was stored at -20 °C. Concentration was determined (see **Section 2.14.2**) and, if necessary, purity was checked on a 1% agarose gel (see **Section 2.14.5**).

2.14.6.6.2 Extraction of DNA Fragments from Agarose Gels

After the gel run was finished, the gel was dyed by the ethidium bromide method or the SERVA G Stain method (see **Section 2.14.5**). If stained with SERVA Stain G, the gel was transferred onto a transilluminator (Thermo Fischer Scientific, Waltham, MA, USA) and the bands were cut from the gel. If the gel was stained using the ethidium bromide method, the DNA bands were visualized using the Gel iX Imager and cut using a scalpel from the gel. The gel slice was weighted and double the amount of milligram in microliters of NTI buffer was added to the slice (w/v). It was heated and vortexed at 50 °C until the agarose was completely dissolved. The solution was transferred to a column. From here, the steps were similar to the procedure described above.

2.14.6.7 Restriction Digest of DNA Fragments and Plasmids

All enzymes and buffers were provided by New England Biolabs (see **Tab. 2.2**). Restriction digests were usually performed in a double-digest way. The buffer giving the enzymes' best performance was chosen via the NEB online tool "double digest finder"[3] or using a table specifying the activity of restriction enzymes in various buffers provided by NEB. Enzyme and buffer combinations were chosen in a way that the activity of a

[3] https://www.neb.com/tools-and-resources/interactive-tools/double-digest-finder

given enzyme was never below 25%, preferably 50%. High fidelity enzyme versions were used if available. The reaction mixture (see **Tab. 2.54**) was incubated at 37 °C for 1 hour (1.5–2 hours if the activity of at least one enzyme was 25%) and stopped by heating to 65 °C for 20 minutes. If enzymes which could not be inactivated by heat were involved, no inactivation occured. If necessary, the restriction digestion was stored at -20 °C. If the desired amount of DNA could not be achieved, either less was accepted or the DNA was concentrated as described in **Section 2.14.3**.

Table 2.54: *Typical master mix for restriction digestion of DNA fragments*

Compound	Volume
Restriction enzyme 1	1 µL
Restriction enzyme 2	1 µL
DNA fragment	100 µg
Buffer	4 µL
Elga^{TM} H_2O	ad 40 µL

The complete mixture was put on an agarose gel and run with standard parameters (see **Section 2.14.5**). Afterwards, the desired fragment was extracted from the gel and purified (see **Section 2.14.6.6**). The DNA concentration was measured (see **Section 2.14.2**) and if necessary stored at -20 °C until further use.

2.14.6.8 Ligation of Vectors and Fragments

Ligations were performed either at room temperature for 60 minutes or at 4 °C (refrigerator) or 16 °C (PCR cycler) overnight.

Table 2.55: *Composition of the ligation master mix of DNA fragments*

Compound	Volume
T4 DNA ligase	1 µL
T4 DNA ligase 10x buffer	1 µL
Digested plasmid	100 µg
Digested insert	2–3x plasmid concentration
Elga^{TM} H_2O	ad 10 µL

The volume of the insert V_I was calculated using

$$V_I = \frac{\frac{m_P * L_I}{L_P} * \left(\frac{n}{1}\right)}{c_I} \tag{2.4}$$

with m_P being the mass of the plasmid in ng, m_I being the mass of the insert in ng and L_I being the length of the insert and L_P being the length of the plasmid in kb, n being the desired excess of insert and c_I being the concentration of the insert. The concentration of the plasmid and, therefore, the volume was preset by defining its amount (typically 100 ng).

Practically, the amount of insert was limited by the vector concentration and the volume of the ligation mixture of 10 µL. Rather than matching all parameters to the figure, a balance of the highest possible plasmid amount (not less than 50 ng) and excess of insert (not less than 2x) was chosen to meet the best performance in ligation and transformation. Calculations were usually performed using an Excel template.

The ligation mixture was used immediately for transformation (see **Section 2.14.6.10**) or stored at -20 °C.

2.14.6.9 Ligation of Fragments and pJET1.2/blunt

PCR products for cloning into pJET1.2/blunt (Thermo Fisher Scientific, Munich, Germany) were always generated using NEB Phusion polymerase, as it generates blunt ends. The PCR product was purified as depicted in **Section 2.14.6.6**. The cloning was conducted according to the manufacturer's recommendations[4]. Briefly, 10 µL 2X reaction buffer, 1 µL of pJET1.2/blunt vector, 1 µL T4 DNA ligase was mixed with 0.15 pmol ends of the PCR product to be cloned into the vector. Elga™ water was added to a final volume of 20 µL and then incubated at room temperature for 5 minutes (30 minutes for fragments $>$ 3 kb). For the transformation (see **Section 2.14.6.10**) of *E. coli* DH5α, 1-2 µL of the reaction was used and clones bearing the plasmid were selected on LB agar plates with ampicillin or carbenicillin overnight at 30 °C.

2.14.6.10 Transformation

2.14.6.10.1 Electroporation of *E. coli*

Electro competent cells (see **Section 2.14.6.2**) were used for the electroporation of *E. coli* for all strains used in this work. The competent cells were first thawed gently on ice. Various amounts of plasmids were added to 50 or 100 µL of electrocompetent

[4] https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0012707_CloneJET_PCR_Cloning_20rxn_UG.pdf

cells and chilled for 5 minutes on ice. The mixture was then transferred to a 0.2 mm electroporation cuvette and treated with the programme specified for *E. coli*. The cells were immediately added to 1 mL of SOC medium and incubated at 37 °C at 150 rpm for 1 to 2 hr. Cells were then spread on a plate containing the appropriate antibiotics for selection and incubated overnight at 37 °C.

2.14.6.10.2 Chemical Transformation of *E. coli*

The competent cells were thawed on ice before use. Various amounts of plasmids or ligation mixture were added to 100 µL chemical competent cells and incubated on ice for 30 minutes. After a heat shock step of 42 °C for 90s the cells were transferred to 1 mL SOC medium and incubated for 1 to 2 hours at 37 °C at 150 rpm. Cell were then spread on plate containing the appropriate antibiotics for selection and incubated overnight at 37 °C.

In case of chemical competent cells prepared according to the rubidium chloride method (see **Paragraph 2.14.6.1.1**), the heat shock was 42 °C for 45 s.

2.14.6.10.3 Electroporation of *Pichia pastoris* X33

To 80 µL freshly prepared cells 5 to 10 µg in 5 to 10 µL of linearized pPICZ DNA were added. The mixture was transferred in an ice-cold 0.2 mm electroporation cuvette and incubated on ice for 5 minutes. The cuvette was then placed into the MicroPulser™ (Bio-Rad Laboratories GmbH, Munich, Germany) and set to the program "Pic", which applied one pulse of 2.0 kV for 5 ms. Immediately after the pulse, 1 mL ice-cold 1M sorbitol was added and the cells were transferred into a 15 mL Falcon tube and incubated at 30 °C for 2 hours without shaking. The cells were then spread on YPDS-plates (see **Tab. 2.20**) with 100 µg/mL zeocin in portions of 50 to 200 µL and incubated at 30 °C for 1 to 3 days. For each construct, 10 to 20 colonies were picked and purified on fresh YPDS + 100 µg/mL zeocin and subjected to colony PCR.

2.14.6.10.4 Electroporation of *Cupriavidus necator* DSM428 and DSM531

For the electroporation of *C. necator* according to [240], a 2 mm cuvette was pre-cooled on ice. A 50 µL aliquot of competent cells was transferred to the cuvette and mixed with 100 to 300 ng DNA and incubated for 5 minutes on ice. The cuvette was then placed in the MicroPulser™ and set to “EC2”, which applied a pulse of 2.5 kV in one pulse for 5 ms. After the cells were treated with the pulse, 1 mL of 30 °C warm NR medium was immediately added. The cells were incubated for 2 hours at 30 °C with shaking at 150 rpm. After incubation, 100 µL of the cell suspension was spread on NR or LB

plates containing the appropriate concentration of antibiotics. The remaining cells were centrifuged for 1 minute at 21100 *xg*, the supernatant was poured off and the pellet was resuspended in the remaining medium and spread onto a similar plate. The plates were incubated for 48 hours at 30 °C. Growing colonies were subjected to colony PCR. In a minor modification to this method, only the cell pellet was spread on plate.

2.14.6.10.5 Mating of *Cupriavidus necator* with *E. coli* S17-1

After the desired plasmid has been brought into *E. coli* S17-1 via the chemical transformation protocol (see **Paragraph 2.14.6.10.2**), *E. coli* bearing the desired plasmid and the wild type strain of *C. necator* were grown overnight with appropriate antibiotics (*E. coli*) at 150 rpm in 10 mL LB at 37 °C or 30 °C, respectively. A portion of 1 mL of both cultures was harvested by centrifugation at room temperature for 1 minute at 21100 *xg* and washed at least once in fresh LB medium using the same centrifugation parameters. Both pellets were resuspended in 50 µL LB medium and combined. This mixture was spotted in the middle of an LB plate and incubated with the lid facing upwards overnight at 30 °C. Afterwards, a loopful of the cell mixture was resuspended in medium or 0.9% NaCl and spread on a mineral medium (see **Tab. 2.16**) plate containing the proper selective antibiotic for the plasmid. After incubation overnight at 30 °C, colonies were picked and purified by streaking on a new plate. The purified colonies were used for colony PCR.

2.14.6.11 Identification of Correct Clones

2.14.6.11.1 Colony PCR

Colonies from a transformation plate were picked and dissolved 20 µL LB medium (without antibiotics). From this solution, 1 µL was used as a template for the colony PCR and, if positive, it was used to inoculate and overnight culture for cryo stock and plasmid preparation. The typical master mix of colony PCR reactions for various polymerases is given in **Tab. 2.56** and the program is given in **Tab. 2.57**. The lid was heated to 105 °C.

One to two positive clones were inoculated in a 5–10 mL LB medium overnight culture and incubated at 37 °C at 150–170 rpm. The cultures were harvested and plasmids were purified (see **Section 2.14.6.5**) and send for sequencing (see **Section 2.14.8**).

2.14.6.11.2 Identify Correct *Pichia pastoris* X-33 Clones Containing kIspS

Colonies were transferred two times on fresh YPDS agar plates with 100 µg/mL Zeocin™ and on YPD with 100 µg/mL Zeocin™. Several clones were then screened for the integrated kIspS. Hence, a colony PCR was performed as described in

Table 2.56: *Standard PCR master mix for one reaction of 10 µL for different polymerases*

Compound	NEB Taq	GoTaq®
H_2O	6.70 µL	6.35 µL
dNTPs (10mM each)	0.20 µL	0.20 µL
Primer fw (10pmol/ µL)	0.50 µL	0.20 µL
Primer rv (10pmol/ µL)	0.50 µL	0.20 µL
Buffer	1.00 µL (10x)	2.00 µL (5x)
Polymerase	0.10 µL	0.05 µL
Template	1 µL cell suspension	

Table 2.57: *PCR cycler programs for colony PCR*

Step	NEB Taq	GoTaq®
Initial denaturation	95 °C for 30 s	
30 Cycles	95 °C for 30 s	
	45-68 °C for 30 s	45-72 °C for 30 s
	68 °C for 1 minute/kb	72 °C for 1 minute/kb
Final extention	68 °C for 5 minutes	72 °C for 5 minutes
Hold	16 °C for ∞	

Paragraph 2.14.6.11.1, except that the cells were resuspended in 20 µL 20 mM NaOH, incubated at 95 °C for 10 minutes, cell debris spinned down. The supernatant was used as the template.

2.14.6.11.3 Restriction Digestion of Purified Plasmid

Another way to confirm the successful integration of genes into vectors was the digestion of the purified plasmid, especially when the colony PCR did not work. Colonies were randomly picked from the plate after the transformation (see **Section 2.14.6.10**) and inoculated in a 5-10 mL overnight culture in LB medium containing the selection marker. The culture was harvested and the plasmid was purified as described in **Section 2.14.6.5**. Then the plasmid was digested using appropriate restriction enzymes as described in **Section 2.14.6.7**. Modification of this method includes a shorter

incubation at 37 °C for 30 minutes and a smaller reaction volume of 10 µL. The restriction digestion was then loaded onto a 1% agarose gel as described in **Section 2.14.5** and the fragment pattern was examined. The expected bands were generated using CloneManager 9 or SnapGene 2.3.2. Among positive clones, one to two were chosen for sequencing (see **Section 2.14.8**) and the correctness of the sequence was confirmed.

2.14.6.12 Cloning of *gfp* and *egfp* into Expression Vectors for *Cupriavidus necator*

The *gfp* for pME6032 was amplified from CBR_P_15 using the primers gfp-pME6032-f and gfp-pME6032-r and cloned using EcoRI-HF and KpnI-HF. *gfp* was amplified using Phusion-HF (NEB), as described in **Section 2.14.4**. To clone *egfp* from pJeM1TcR into pMS137, it was amplified using Taq DNA Polymerase (NEB) using the primers eGFP-pMS137-f and eGFP-pMS137-r, at an annealing temperature of 60 °C and elongation time of 1 minute among standard parameters (see **Section 2.14.4**). The PCR fragment was then cloned into pMS137 by PciI (NEB) and KpnI-HF (NEB). Positive clones were identified using colony PCR (see **Paragraph 2.14.6.11.1**) and sequenced (see **Section 2.14.8**). *C. necator* was transformed with the plasmids (see **Paragraphs 2.14.6.10.4** and **2.14.6.10.5**). All successful constructs are listed in **Tab. 2.6**.

egfp from pJeM1TcR was cloned into pBBR1MCS-3 using Golden Gate cloning (see **Section 2.14.4.1**). Hence, four fragments are amplified using PCR (see **Tab. A.35**). In order to destroy the BsaI site, the vector had to be split and amplified as two fragments using the primer pairs MCS3-mob-gg-f, MCS3-gg-r and MCS3-term-gg-f, MCS3-mob-gg-r. The T7 terminator from pET28a was amplified using primers term-MCS3-gg-f, term-MCS3-gg-r and *egfp* was amplified using eGFP-MCS3-gg-f, eGFP-MCS3-gg-r.

The fragments were then combined using the Golden Gate technique (see **Section 2.14.4.1**). Positive clones were identified using colony PCR (see **Paragraph 2.14.6.11.1**) and sequenced (see **Section 2.14.8**).

C. necator was transformed with the constructs as described in **Paragraph 2.14.6.10.4**.

2.14.7 PCR for Amplification of 16S and Fungal rDNA Gene Sequences

Strains were identified using 16S or 5.8S rDNA and LSU gene sequence. Depending on the media used and partially on microscopic data, the strains were applied to either amplification of the 16S or the 5.8S rDNA and LSU gene.

2.14.7.1 Amplification of the 16S rDNA Gene

As a template for the PCR, 10 µL of an overnight culture were diluted in 90 µL Elga^{TM}-H_2O. From this 1:10 dilution 10 µL were added to 10 µL of the PCR mastermix comprising 3.40 µL H_2O, 0.40 µL 10mM dNTP mix, 4.00 µL 5x buffer, 0.20 µL Taq polymerase (Rapidozyme, Berlin) and 1.00 µL of each primer. The Primers used were 27f and 1525r [177] (see **Tab. 2.5**). The PCR reaction tubes were placed in a PCR cycler (Bio-Rad Laboratories GmbH, Munich, Germany) and the program **Tab. 2.58** was started with the lid heated to 105 °C.

Table 2.58: *PCR cycler program for the amplification of 16S rDNA*

Step	Values
Initial denaturation and cell lysis	95 °C for 10 minutes
30 cycles	95 °C for 30 s 56 °C for 30 s 72 °C for 1:30 minute
Final extension	72 °C for 5 minutes
Hold	16 °C for ∞

The water used to prepare the dilutions and the PCR mastermix was used as a negative control. The success of the PCR was evaluated by applying 2 µL of PCR product mixed with one drop of 5x loading dye to a 1% agarose gel as described in **Section 2.14.5**. Positive PCRs were send to sequencing (see **Section 2.14.8**). Negative PCRs were performed again without changed parameters or with a different dilution of the template.

2.14.7.2 Amplification of Fungal rDNA Fragments

As a template for the PCR, 10 µL of an overnight culture were diluted in 90 µL Elga^{TM}-H_2O. From this 1:10 dilution, 10 µL were added to 10 µL of the PCR mastermix comprising 3.40 µL H_2O, 0.40 µL 10mM dNTP mix, 4.00 µL 5x buffer, 0.20 µL Taq polymerase (Rapidozyme, Berlin) and 1.00 µL of each primer. Primers used were 5.8S-R and LR7 (see **Tab. 2.5**), which amplify parts of the 5.8S and 24S rRNA genes [330]. The PCR reaction tubes were placed in a PCR cycler (Bio-Rad Laboratories GmbH, Munich) and the program **Tab. 2.59** was started with the lid heated to 105 °C.

Table 2.59: *PCR cycler program for the amplification of 5.8S rDNA and LSU gene*

Step	Values
Initial denaturation and cell lysis	95 °C for 10 minutes
35 cycles	95 °C for 30 s 50 °C for 30 s 72 °C for 2 minute
Final extension	72 °C for 5 minutes
Hold	16 °C for ∞

Positive PCRs were sent to sequencing (see **Section 2.14.8**). Negative PCRs were performed again without changed parameters or with a different dilution of the template.

2.14.8 Sequencing of 16S, Fungal rDNA and Cloned Fragments

All DNA fragments and genes were sequenced using Sanger sequencing by either GATC Biotech AG or Eurofins Genomics. Both provide a reading length of circa 1 kb with Sanger sequencing. If fragments with more than 1.5 kb were sequenced, more primers were used to cover the complete sequence. The DNA from the 16S rDNA PCR or 5.8 rDNA and 24S gene PCR (see **Section 2.14.7**) was checked for success on a 1% agarose gel and purified (see **Section 2.14.6.6**). The concentration was measured (see **Section 2.14.2**).

For sequencing with Eurofins Genomics as “Value Reads”, 15 µL of the purified DNA fragment with a concentration between 50–75 ng/ µL and 2 µL of 10 pmol/ µL primer were mixed and shipped. Briefly, for sequencing with GATC Biotech AG as “LIGHTrun^{TM} ”, 15 µL of purified DNA fragments with a concentration of 10 ng/ µL and

2 µL of 10 pmol/µL primer were mixed and shipped. After the sequencing was done, the sequences were checked for overall quality by visually examining the AB1 trace files. If the quality of the sequence was too bad (e. g. no curves, sequencing stopped early, etc.), the sequencing was performed again. Otherwise, the sequences were processed as described in **Section 2.17.1**.

2.14.9 Sequencing of Genomic DNA

The genomic DNA was prepared from an overnight culture as described in **Section 2.14.1**. The samples were sent to Freising with a concentration of at least 75 ng/µL in 55 µL minimum. The details regarding the computational part are given in [195] and in **Section 2.17.2**.

2.15 Analytics

2.15.1 Gas Chromatographic Measurement of Ethylene, Propylene and Isoprene

The gas chromatographic method for the measurement of ethylene and isoprene was as follows. The oven was set to an initial temperature of 40 °C and was heated to 150 °C with a rate of 10 °C/min. This temperature was held for 4 min. The injector was operated at 200 °C and constant pressure mode with 20 kPa. The split ratio was set to 1:10. The detector used was an FID operated at 200 °C. The makeup gas flow rate was set to 15.0 mL/min, the air was 350.0 mL/min and the hydrogen was set to 35.0 mL/min. The agitator of the autosampler was set to 40 °C. The head space syringe (2.5 mL volume) was heated to 40 °C as well. One milliliter of the sample was injected into the inlet after 1 minute of incubation into the agitator with 10s shaking and 10s pause in order to get more ethylene into the head space. The filling and injection speed was 50 mL/min. Post syringe flush was 30s with N_2. The column applied was a Rt-QS-Bond from Restek GmbH.

2.15.2 Gas Chromatographic Measurement of Ethylene

A second method was used in order to increase the sampling frequency for the ethylene production of various substrate combinations of four organisms and protein

fractions from AEKTA™ (see **Section 2.16.6**). Detector and injector setting were the same as in the method mentioned above.

2.15.3 Generation of Standards for Gas Chromatography

2.15.3.1 Ethylene Calibration Curve

A 100 ppm mixture of ethylene amongst other olefins pre-mixed (Restek GmbH, Bad Homburg vor der Höhe, Germany) was used to generate an ethylene calibration. For each point, three headspace GC vials were sealed and flushed with the standard for 1 minute. As blank, air was used. Various volumes of gas samples were retrieved: 200 µL, 400 µL, 600 µL, 800 µL and 1 mL. The concentration of 100 ppm ethylene (40 °C, normal atmospheric pressure) in g/ mL was calculated as

$$\beta_i = \frac{0.1 * M * p * X_j}{R * T} = \frac{0.1 * 28.054 g/mol * 1000 mbar * 100 ppm}{8.314472 J/kmol K * 313.15 K} \tag{2.5}$$

$$= 109.15 mg/m^3 = 109.15 * 10^{-6} g/L = 109.15\,\mu M \tag{2.6}$$

with M being the molar mass (g/mol), p pressure (mbar), R being gas constant (8.314472 J/kmolK), T temperature (K), X_j the concentration in ppm and β_i being the concentration in mg/m^3.

A calibration curve (see **Appendix A.1**) could be prepared and the equation

$$y = 0.0079 * x - 1.35, \text{with } R^2 = 0.977 \tag{2.7}$$

was derived.

2.15.3.2 Isoprene Calibration Curve

For the calibration curve, ≥99% isoprene (Sigma-Aldrich Chemie GmbH, Munich) was diluted in Elga™ H_2O or ethyl acetate in 2 mL reaction tubes. **Tab. 2.60** gives the steps and the ratio of the isoprene and the solvent. The first step was to generate a 100 mM solution by adding 100 µL of pure isoprene to 9.9 mL of solvent. From this solution, a 5 mM stock solution was made by adding 0.5 mL of the 100 mM solution to 9.5 mL of solvent. All following dilutions were made from this solution, which was generated in triplicates. The final dilution step of 1:10 was achieved by pipetting 200 µL of the dilution

series in 1800 µL solvent in 10 mL gc headspace vials. The solvent without isoprene served as blank. All steps were carried out in triplicates except for the measurement of the gc headspace vial. The calibration was done with the gc method for measuring isoprene, ethylene and propylene (see **Section 2.15.1**).

The molarity of the isoprene solution was calculated as follows:

$$c = \frac{680g/l}{0.68g/cm^3} = 9.98l/mol \rightarrow 0.10mol/l \tag{2.8}$$

The calibration curve of the isoprene in ethyl acetate is shown in **Fig. A.2**. The curve equation and regression coefficient was calculated using R, with the concentration being on the y-axis and the area (pA*min) being on the x-axis. The curve parameters are

$$y = 0.0036 * x + 0.0027; R^2 = 0.9997 \tag{2.9}$$

Table 2.60: *Dilution series for the calibration curve of isoprene*

Final concentration	0 µM	0.5 µM	2.0 µM	7.8 µM	31.3 µM	0.125 mM	0.5 mM
Solvent	1.0 mL	0.75 mL	0.75 mL	0.75 mL	0.75 mL	0.75 mL	0 mL
Volume added from previous tube	0 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	0.25 mL	1 mL
Solvent in gc vial	1.8 mL added to all						
Dilution solution	200 µL added to all						

The dilution series was prepared from right to left. The 5 mM solution was the previous solution for the most right tube.

2.16 Protein Biochemical Methods

2.16.1 Protein Extract

For preparation of protein extract for protein purification and further experiments, a 10 mL overnight culture from the cryo stock was prepared. From this culture, the main culture was inoculated with 1% and incubated at the appropriate temperature overnight (unless stated otherwise) with 150–170 rpm.

The cells were harvested using a Sorvall RC6+ centrifuge for 20 minutes, 4 °C, 4580 *xg*. The pellet was resuspended in 1/10 volume of the culture of buffer and cells were centrifuged in a Sorvall RC6+ at 4580 *xg* or Haereus Rotanta 460R at 4600 *xg* for

20 minutes at 4 °C. The cells were then resuspended in buffer calculated to a specific OD_{600} and then thoroughly resuspended using the Ultra Turrax T18 basic for 1 minute at level 5.

The cells were then disrupted using an IUL at 2.4 kbar, 5 °C 6 times. The cell debris was removed by centrifuging the cell lysate using a Sorvall RC6+ at 4 °C and 20,000 *xg* for 30 minutes to yield the protein extract. The protein extract was stored on ice or at -20 °C for storage longer couple in the range of days.

2.16.2 Measuring the Expression of *gfp* and *egfp*

Cultivated cells were diluted 1:10 with 0.9% NaCl to measure the OD_{600} and fluorescence. The fluorescence was determined using fluorescence plates (Greiner Bio-one International GmbH). The fluorescence was measured using the Varioskan and the accompanying software SkanIt Software 2.4.3 (Thermo Fisher Scientific Inc.). The plates were shaken for 10 s at 600 rpm with a diameter of 1 mm. The fluorescence was determined with an excitation wavelength of 485 nm with an excitation bandwidth of 12 nm and an emission wavelength of 520 nm. The dynamic range was set to "AutoRange" and the measurement time to 100 ms.

The OD_{600} was determined using a 96 well micro titer plate (Greiner Bio-one International GmbH). The culture was diluted 1:10 with 0.9% NaCl. The plate was shaken for 5 s at 600 rpm with a diameter of 1 mm. The OD_{600} was then determined at 600 nm with a bandwidth of 5 nm and a measurement time of 100 ms.

Fluorescence is given as arbitrary unit and is normalized according to the OD_{600} by dividing the fluorescence by the OD_{600} .

2.16.3 SDS-PAGE

2.16.3.1 Gel Preparation and Run

The gels were prepared using a preparation chamber (Bio-Rad Laboratories, Munich, Germany). The chambers were assembled according to the manufacturers' instructions. As the preparation chambers suffered from being leaky, 1.5% agarose was applied to every critical spot. The separation gel was prepared first and poured into the chamber. It was covered with Elga^{TM} water in order to prevent air bubbles. After the gel polymerized, the water was poured off and the collection gel was prepared and pipetted on top of the separation gel. Before the gel was polymerized, the sample comp (15 or

10 poured) was put into the collection gel. If necessary, the gels were stored up to one week at 4 °C wrapped in wet paper towels in a plastic bag.

Table 2.61: *SDSD-PAGE: 12% separation gel*

Compound	Amount for 1 gel
bis-acrylamide	2.00 mL
H_2O	1.645 mL
4x Lower buffer (containing SDS)	1.25 mL
10% APS	50.0 µL
TEMED	5 µL

Table 2.62: *SDSD-PAGE: 15% separation gel*

Compound	Amount for 1 gel
bis-acrylamide	2.50 mL
H_2O	1.145 mL
4x Lower buffer (containing SDS)	1.25 mL
10% APS	50.0 µL
TEMED	5 µL

Table 2.63: *SDSD-PAGE: collection gel*

Compound	Amount for 1 gel
bis-acrylamide	0.415 mL
H_2O	1.385 mL
4x Upper buffer (containing SDS)	0.625 mL
10% APS	25 µL
TEMED	2.5 µL

The gels were assembled with the running chamber filled with the running buffer and usually 40 µL protein sample was mixed with 10 µL 5x loading buffer (see **Tab. 2.37**) and heated for 10–15 minutes at 95 °C in a thermo block or thermo cycler. The samples were then filled in the pockets of the SDS gel. One gel run with 35mA constantly usually until the bromphenol blue front of the loading buffer exited the gel. The gel was then stained according to the following protocols in this section.

As protein standard, the PageRuler™ unstained protein ladder (Thermo Fisher Scientific Inc., Waltham, Maine, USA) was used to estimated the protein size.

2.16.3.2 Visualization of Protein Bands Using Standard Coomassie Staining

Two distinct methods were used. The first is as follows: after the gel was extracted from the chamber, it was directly stained with the staining solution for 15 minutes while shaking. The staining solution was removed and the gel was washed three times by adding fresh ddH_2O, heating in a microwave for 2:30 minutes with a paper towel on top. The gel was then destained in fresh ddH_2O for at least 1 hour with shaking or as long as the blue background was removed adequately. The staining solution was prepared by adding 2 mg Coomassie Brilliant Blue G-250 and 2 mg Coomassie Brilliant Blue R-250 to a mixture of 400 mL water, 500 mL ethanol and 10% acetic acid. The solution was stirred for circa 2 hours and stored at room temperature. The solution was re-used several times.

In the second method according to [182], the gel was washed in distilled water, heated in the microwave for 60 s and submersed in aqueous Coomassie Brilliant Blue (CBB) solution and heated in the microwave for 30 s. Afterwards, it was shaken for 5 minutes and submersed in a water/ethanol mixture and heated in the microwave for 30 s. To clean away all the remaining stain, the gel was shaken for 5 minutes in the water/ethanol mixture. The gel was then analyzed and documented using the Gel iX Imager (Intas-Science-Imaging Instruments GmbH, Göttingen, Germany) under white ligth. The Coomassie Brilliant dye solution was prepared by dissolving 60–80 mg of CBB R-250 in double deionized water by stirring at room temperature for 2–4 hours. In the last step, 3 mL concentrated HCl was added and stirred for a few more minutes. The solution was stored at room temperature and was re-used several times.

2.16.3.3 Visualization of Protein Bands Using Sensitive Coomassie Staining

All steps (according to [81]) were carried out on a vertical shaker at room temperature. After the gel was separated from the chamber, it was washed 3x in Elga^{TM} water. The gel was subjected to the fixation solution for 30 minutes. Afterwards, it was stained using the colloidal coomassie staining solution overnight. The background was destained using destaining solution for 10 to 60 minutes. In the final step, the gel was washed in Elga^{TM} water for 10 minutes. A photo of the gel was taken by using the Gel iX Imager (Intas-Science-Imaging Instruments GmbH, Göttingen, Germany) under white light.

The staining solution was prepared as follows: the aluminum sulphate was first dissolved in Elga^{TM} water. Then the ethanol was added, homogenized using a magnet stirrer, and the coomassie G-250 dye was added to the solution. When the solution was

completely dissolved, the 85% phosphoric acid was added, which gave the solution its characteristic green color. The solution was filled up to the desired volume with Elga^{TM} water. The solution was prepared fresh for every use.

Table 2.64: *Sensitive coomassie staining: fixation solution*

Compound	Amount for 1 L
H_2O	680 mL
100% Ethanol	300 mL
85% Phosphoric acid	20 mL

Table 2.65: *Sensitive coomassie staining: staining solution*

Compound	Amount for 1 L
H_2O	876.5 mL
Aluminium sulphate	50 g
100% Ethanol	100 mL
85% Phosphoric acid	23.5 mL
Coomassie G-250	0.2 g

Table 2.66: *Sensitive coomassie staining: destaining solution*

Compound	Amount for 1 L
H_2O	880 mL
100% Ethanol	100 mL
85% Phosphoric acid	20 mL

2.16.3.4 Visualization of Protein Bands Using Silver Staining

This protocol is based on a laboratory course protocol found online at the University of Graz[5]. The steps done at 4 °C in the protocol were carried out at room temperature. After the gel was extracted from the chamber, it was shaken in the fixation solution for 30 minutes with the exchange of solution after 20 minutes. All shaking steps were carried out on vertical shaker at room temperature. The gel was washed two times in wash solution. Afterwards, it was washed for 30 minutes in water with changing the water after 10 minutes. The gel was then shaken in a sensitizer solution for 1 minute. Then it was washed 2 times in water for 30s each. Afterwards, it was stained in the silver solution

[5]http://lipotox.uni-graz.at/protocols/Ag_Faerbung_ger.pdf, last access: 24.08.2017

for 20 minutes while shaking. The silver solution was completely removed and the gel was washed in water 3x for circa 15 s each. The gel was then incubated in developer solution with shaking for a maximum of 3 minutes. The staining process was stopped by pouring off the developer and washing the gel with water. In the final step, the staining was stabilized by shaking the gel in 5% acetic acid for 5 minutes. The gel was then transferred to 1% acetic acid. A photo of the gel was taken by using Gel iX Imager (Intas-Science-Imaging Instruments GmbH, Göttingen, Germany under white light).

If necessary, the gel was stored at 4 °C for several days.

Table 2.67: *Silver staining solutions: fixation solution*

Compound	Amount for 1 L
H_2O	500 mL
100% Ethanol	400 mL
Acetic acid	100 mL

Table 2.68: *Silver staining solutions: washing solution*

Compound	Amount for 1 L
H_2O	700 mL
100% Ethanol	300 mL

Table 2.69: *Silver staining solutions: sensitizer solution*

Compound	Amount for 1 L
H_2O	1 L
$Na_2S_2O_3$	0.5 g

Table 2.70: *Silver staining solutions: silver solution*

Compound	Amount for 1 L
H_2O	1 L
$AgNO_3$	16.99 g
37% Formaldehyd	0.54 mL

Table 2.71: *Silver staining solutions: developing solution*

Compound	Amount for 1 L
H_2O	1 L
$Na_2CO_3 \cdot H_2O$	20 g
37% Formaldehyd	1.08 mL

2.16.4 Measurement of Protein Concentration with the Bradford Reagent

The protein concentration was determined using Roit-Quant (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) according to the instructions for protein determination in micro titer plates [52]. Firstly, 10 mg/ml BSA in water stock solution was prepared. Starting with this solution, different concentrations ranging from 20 µg/ml to 160 µg/ml were prepared. The working solution of the bradford solution was prepared by mixing 2 parts of the 5x Roti-Quant solution and 5.5 Elga water. If necessary, the protein solutions were diluted. For the assay, 50 µL of sample or standard was mixed with 200 µL of the bradford wokring solution and incubated for 5 minutes. The concentration was determined at a wave length of 595 nm using varioskan or multiskan photometer. For each buffer, a blank was determined. To calculate the concentration, the absorption was plotted against the concentration of the standard and the equation and the coefficient of determination was determined. If not stated otherwise, one sample was measured in triplicates.

2.16.5 Ammonium Sulphate Precipitation

In order to separate proteins in solutions according to their hydrophobicity, ammonium sulphate was applied. The ammonium sulphate was first pestled and then added to the solution at 4 °C under constant stirring. As there are more local effects when ammonium sulphate is used in its powder form rather than in a solution, the salt was added slowly: more salt was added as soon as the previous amount dissolved completely.

After stirring for two hours at 4 °C and 100–300 rpm (depending on the stirrer and size of the stirring bar), the solution was transferred to 2 mL microreaction tubes and centrifuged at 21100 *xg* for 30 minutes at 4 °C. The supernatant was separated from the pellet and the pellet was resuspended in the buffer of the protein sample and the same volume. The solution was transferred into dialysis tubes (ZelluTrans Dialysis tubes 6.0, Carl Roth GmbH, Karlsruhe, Germany), which were soaked in Elga™ water for at least 15 minutes. The tubes were then put in at least 2 L of the the desired buffer. The procedure was carried out at 4 °C with stirring at 100-300 rpm (depending on stirrer

and size of stirring bar). Typically, the first dialysis step was carried out overnight. The following day, the buffer was exchanged two times with each step being about 3 hours. If possible, the total factor of dialysis was 1000x. Details concerning the exact numbers are given in the results section (see **Chapter 3**).

2.16.6 AEKTA^{TM}

All columns and the AEKTA^{TM} system were stored at room temperature in 20% ethanol. Before every change of buffer, the pumps were purged with it, controlled by the Unicorn software. Before use, the column was purged with water 10x of its volume. Following this, the column was purged with 10x its volume of elution buffer and flushed using binding buffer until the conductivity was in the range of the binding buffer. After this step, the system was ready to be used.

2.16.6.1 Purification Using Various Physico-Chemical Properties of Enyzmes

Usually, the whole run was performed automatically by using the Unicorn software, if not stated otherwise. Desalting and separation according to molecular weight (also called gel filtration chromatography) was performed operating the software in manual mode.

After the run was completed, the column was flushed with the elution buffer 10x its volume, the buffer was echanged with water by purging it with 10x volume and finally purged 10x with 20% ethanol and stored at room temperature.

2.16.6.2 Desalting of Protein Extracts

Protein extracts were desalted using a HiPrep^{TM} 26/10 Desalting column (GE). General operation parameters were the same as mentioned above. The column was equilibrated with the desired buffer with five column volumes. The sample pump was equilibrated with the protein extract buffer. The sample was injected on the column at 5–7 mL/min in a maximum total volume of up to 15 mL. Subsequently, the sample was eluted with up to 10 mL/min and either used directly for assays or stored at -20 °C.

2.16.7 Assay for Ethylene from AEKTA™ Fractions and Protein Extracts

If not noted otherwise, 450 µL of protein extract or fraction was combined with 50 µL substrate solution and incubated as described above. The gas chromatographic method used is described under **Section 2.15.2**.

The specific activity was calculated as ethylene production as pA*min divided by the protein concentration as µg/mL (measured with Bradford reagent, see **Section 2.16.4**).

Total activity was calculated as the product of the volume as mL and the ethylene production as pA*min.

2.16.8 Mass Finger Printing of Peptides

2.16.8.1 SDS-PAGE and Sample Preparation

AEKTA™ fractions were used as a sample, generated as described under **Section 2.16.6**.

Before an ordinary SDS-PAGE (see **Section 2.16.3**), the samples were reduced by mixing 80 µL sample and 10 µL 100 mm DTT in Elga™ H_2O, freshly prepared . The sample's pH was between 7.5–8.5 and adjusted using 50 mM ammonium carbonate buffer (see **Tab. 2.30**) if necessary. The solution was incubated for 15 minutes at 60 °C. After the solution was cooled to room temperature, 10 µL 600 mM iodoacetamide, freshly prepared, was added and incubated for 15 minutes at room temperature. The sample was mixed with 5x SDS sample buffer (see **Tab. 2.37**) and heated for 15 minutes at 95 °C. A SDS-PAGE was performed afterwards, as described in **Section 2.16.3**.

After the SDS-PAGE bands were cut out by using the OMX-S® device[6]. The "picker" was put in the "peptide-sampler" and centrifuged for 2 minutes at 13000 *xg* with the "reactor" facing down. To the gel pieces in the "reactor" 20 µL Pierce™ trypsin (Thermo Fisher Scientific Inc., Waltham, Maine, USA) working solution – trypsin stock 1:10 diluted in 40 mM Glycin NaOH pH 9 – was added, shortly spinned, and incubated at 50 °C for 45 minutes at 500 rpm in a thermo block ThermoMixer C (Eppendorf AG, Hamburg, Germany). The OMX-S® device was then centrifuged for 3 minutes at 1000 *xg* and further processed for sequencing (see the next section) or stored at -20 °C until further use.

[6] OMX Proteome X Solutions does no longer exist and the material can no longer be purchased. The lot used in this work was produced in 2012.

2.16.8.2 Liquid Chromatography-Mass Spectrometry and Database Query

The samples generated from the previous step were mixed 1:1 with 50% acetonitrile (v/v) and 10 µL were directly used for mass fingerprinting. The following paragraphs give more information about the HPLC method and the parameters for the database Mascot ([242]). The HPLC system (Ultimate 3000RS, Dionex) is comprised of a degasser SRD 3400, the pump module HPG 3400RS, the auto sampler WPS 3000TRS, the column compartment TCC 3000RS, the diode array detector DAD 3000RS and an ESI-ion-trap unit (HCT, Bruker). The software used to control the HPLC and analyze the data were Bruker Hystar, QuantAnalysis and Dionex Chromelion. Buffers are described in **Section 2.12.6**.

Ten microliter of the sample were injected splitlessly onto a Gravity C18 column (100 mm length, 2 mm i. d., 1.8 µm particle size, Macherey-Nagel) using the autosampler. The flow was set to 0.2 µL/min with the column oven at 40 °C. A gradient program was performed using the mobile phase C (0.1 % formic acid in water) and the mobile phase D (0.1 % formic acid in acetonitrile) as follows: the gradient started with 10 % D for 4 minutes and then increased to 75 % D over 45 minutes. The gradient was decreased to the initial parameters of 10 % over 1 minute and equilibrated for 5 minutes. The autosampler was set to a temperature of 10 °C. The run was terminated after 60 minutes.

The HPLC column was splitlessly injected into the mass spectrometer with the ion-trap set to positive and operated in the ultra scan mode (26000 *m/z/s*) from 300–1500 m/z. The ICC smart target was set to 200000 with a minimum accumulation time of 150 ms and five averages. The ion source parameters were 3 kV capillary voltage, nebulizer at 40 psi, dry gas at 9 L/min at 365 °C. Auto-MS-mode with the smart target mass 15.00–3000.00 m/z was used and a MS/MS fragmentation amplitude of 0.5 V was used.

The acquired mass spectrometry data were exported using the Bruker Hystar software (see **Tab. 2.72**).

Table 2.72: *Export parameters for MS data from the Bruker Hystar software*

Category	Parameter	Setting
Compound detection (AutoMS(n))	Intensity threshold	250000, neg. 10000
	Maximum number of compounds	1000
	Retention time window [min]	0
	Fragments qualified by	amino acids
Mass list	Apex	on
	Peak width (FWHM [m/z])	0.1
	S/N threshold	1
	Relative intensity threshold (base peak)	0.1%
	Absolute intensity threshold	100
	Use peak finder to calculate peak position	on
Charge deconvolution	Peptide/small molecules	on
	MS Full Scan	on
	MS(n)	on
	Maximum charge	auto
	Allow precursor deconvolution from fragment spectra	on

The exported MS data were uploaded to the Mascot MS/MS Ions Search server and searched against the databases “NCBIprot (AA)” and “Swissprot (AA)”. The taxonomy was restricted to *Firmicutes* (Gram-positive bacteria) and the enzyme was set to trypsin. Up to one miss cleavage was allowed and the variable modifications were set to acetyl (N-term), oxidation (HW) and oxidation (M). The data format was set to “Mascot generic” and the instrument to “ESI-TRAP”. All other parameters were used with their default values. The significant hits were then investigated using further online databases (see **Section 2.4** for a list of databases used in this thesis).

2.16.9 Concentration of Protein Solutions

The standard method used to concentrate AEKTA™ fractions was by freeze-drying. The samples were collected in 50 mL Falcon tubes and put into a freeze-dryer (Alpha 2-4 Ldplus). The samples were dried until the desired volume remained at -40 °C at 20 mbar, usually overnight

With fractions from the AEKTA™ runs, filtration with the 3 kDa filter (Merck Millipore) was tested (Amicon® stirred cell) as described by the manufacturer [83].

2.17 Bioinformatical Methods

2.17.1 16S and Fungal rDNA Sequence Generation

Before applying the sequences to further analysis, the sequences were checked for overall quality by visually examining the AB1 trace files. If the quality of the sequence was too bad (e. g. no curves, sequencing stopped early, etc.), the sequencing was performed again. The AB1 trace files were first converted into the fastq file format using the EMBOSS tool seqret [256]. Sequences were then trimmed according to quality using the tool seqtk and converted into the fasta file format [186]. If the filename indicated a reverse sequence, the reverse complement was generated using the EMBOSS tool revseq [256]. Afterwards, fasta sequences were renamed according to the filename and the sequence length was determined for both, raw and trimmed sequences, using R 3.2.3 for 64bit Linux [251]. The process was automated using SHELL scripts. The scripts ran on Ubuntu 16.04.5 LTS on Windows Subsystem for Linux on a Windows 10 machine.

There were two separately treated cases for DNA sequences. For rDNA gene fragments with only successful forward or reverse sequencing, the files were analyzed using a SHELL script and the sequences were trimmed with a threshold of $p = 0.05$. For fragments with successful forward and reverse sequencing, another SHELL script was used with a threshold for trimming of $p = 0.01$ for a good merging. It should be noted that before merging the sequences, the length of the combined forward and reverse run was checked to sum up to at least 1308 bp to ensure overlapping of at least 10 bp. If no overlapping occurred, the sequences were treated individually.

The sequences were then assigned to a species using the ezbiocloud service[7] [366] in case of 16S rDNA (identification of bacteria). In case of fungal rDNA, NCBI Nucleotide BLAST[8] was used [50, 373]. The best hit was taken.

[7] https://www.ezbiocloud.net
[8] https://blast.ncbi.nlm.nih.gov/Blast.cgi

2.17.2 Draft Genome Generation

In brief, the data were generated using the Illumina MiSeq system. The Read data were trimmed and quality filtered using the NGS QC Toolkit 2.2.3. The high-quality read pairs were analyzed using FastQC 0.11.4 and then assembled with SPAdes 2.5.1. The annotation was performed by uploading the assembly onto the RAST server[9] [18, 239]. This method is described in [195].

2.17.3 BLAST

Homologous sequences were searched using the NCBI BLAST Tool 2.2.30+[10] using blastp in order to find protein sequences using a protein sequence as query. In order to find protein sequences in genomes, tblastn was used using a protein sequence query. For a refined search, PSI-BLAST was used with four iterations. In each iteration, only the annotated sequences were used. All other parameters were set as standard and the web tools were used.

2.17.4 Multiple Sequence Alignment

Multiple sequence alignment was performed using the ClustalΩ[11] [283] web tool with standard parameters.

2.17.5 Codon Usage

The codon usage of *Cupriavidus necator* was retrieved from the "Codon Usage Table Database"[12] [15]. The sequences of the genes to be compared (see **Appendix A.3**) were first translated into RNA by substituting thymidine into uracil. Next, the number of codons was calculated as follows:

$$N_{\text{codons}} = \frac{\text{gene length in bp}}{3} \tag{2.10}$$

[9] http://rast.nmpdr.org/
[10] https://blast.ncbi.nlm.nih.gov
[11] https://www.ebi.ac.uk/Tools/msa/clustalo
[12] hive.biochemistry.gwu.edu/review/codon

Afterwards, the codons were extracted of the genes and their frequency was counted. The percentage of the codons was calculated as follows:

$$Frequency_{codons} = \frac{\text{Number of codons in gene}}{\text{total codon number}} * 100, \text{ codon usage in genes} \quad (2.11)$$

(2.12)

$$Frequency_{codons} = \frac{\text{Frequnecy in 1000 codons}}{1000} * 100, \text{ global codon usage} \quad (2.13)$$

This process was done using R version 3.2.2-3.5.1 (32-/64-bit) [251, 252]. The CAI value was derived from Jcat[13] [120] for the organisms *Ralstonia eutropha* JMP134.

[13] http://www.jcat.de/

3 Results

3.1 Screening for Ethylene and Propylene Producers

3.1.1 Isolation of Microorganisms

The microorganisms used in this work were isolated (method described in **Section 2.13.3**) from different environmental habitats and sampling sites. The samples were inoculated in several growth media (AMA, DSMZ medium 1, GYM, LB, marine broth, ST1, YEPD) and grown on agar plates to generate individual colonies. These colonies were then purified and preserved at -80 °C (cryo cultures). **Tab. 3.1** shows a list of the number of microorganisms (total: 524) obtained from various sampling sites. Media usage is summarized in the appendix (see **Tab. A.1**).

Table 3.1: *Habitats and sampling sites of environmental isolates (524 strains)*

Sampling site	Number of isolates derived
Streptomyces from Soil, Straubing	8
Air-exposed agar plates	53
Banana field, India	30
Cham-Katzberg, Silage from Heinrich Hausladen	85
Coconut field, India	25
DSMZ-strains	4
Fresh Water, Allachbach, Straubing	10
Juice of water melon, Straubing	9
Marine sample (Seawater, beach)	21
Orange peel, Straubing	8
Organic waste, Straubing	144
Plant cut infusion, Straubing	55
Purchased watermelon, Straubing	6
Rice field, India	39
Soil, Straubing	10
Water from salted lake, Romania	17

3.1.2 Detection of Ethylene and Propylene Producers

The 524 microorganisms, isolated and cultivated as mentioned above, were subjected to a screening protocol. In brief, the isolated microorganisms were inoculated in a 10 mL culture from a 1 mL overnight pre-culture and grown for three days in appropriate media at 30 °C. Cells were then harvested, washed in 10 mL NaPi once, resuspended in 1 mL NaPiSE-TPP and disrupted using three cycles of liquid nitrogen and a 60 °C waterbath. The entire cell lysate was incubated with substrates (pooled, 10 mM each) for 4 h in sealed 10 mL GC headspace vials at 30 °C and 150 rpm (for details, see **Section 2.13.5**). For ethylene production, 3-phosphonooxy propanoic acid and β-alanine were added. For propylene production, 3-sulfo-oxy butanoic acid and 3-phosphonooxy butanoic acid were added.

Among the 524 strains tested, 31 produced ethylene (see **Tab. 3.2**) and none produced propylene. In order to reproduce the results, 18 of the positive hits were incubated with the pooled substrates again. Positive hits from the replicate were further used to elucidate substrates for the ethylene production. All ethylene producers of the first round (screening) were identified using 16S/fungal rDNA sequencing as described in **Section 2.14.8**. Fifteen strains belonged to the genus *Bacillus*, two belonged to the genus *Pseudomonas* and *Stenetrophomonas*, one to *Proteus*, *Buttiauxella*, *Staphylococcus*, *Providencia* and *Staphylococcus*, six to *Pichia* and one belonged to the family *Enterobacteriaceae*. Further information about the sequencing data is presented in **Appendix A.2.2**.

Table 3.2: *Ethylene production (pA*min) from screening for ethylene producers with the substrates 3-phosphonooxy propanoic acid and β-alanine. All values represent one single measurement. Substrates for known pathways: L-Met, ATP, α-KG (10 mM each), *: additionally 10 mM ACC. Details about the sequencing results are presented in the* ***Appendix A.2.2****.*

Strain	Screening	Replication	Substrates for known pathways	Identity (16S/fungal rDNA)
SR-12	0.005	0	0.016*	*Pseudomonas vranovensis*
SR-16	0.011	0.01	NA	*Stenotrophomonas maltophilia*
SR-19	0.004	0	NA	*Buttiauxella gaviniae*
SR-24	0.002	0	0.022*	*Pseudomonas alkylphenolica*
SR-4	0.004	0	NA	*Stenotrophomonas lactitubi*
SR-26	0.004	0	0.029	*Bacillus luti*
SR-27	0.003	0	0.013	*Bacillus luti*
SR-73	0.003	NA	0.03	*Bacillus toyonensis*
SR-76	0.004	0.006	0.036	*Bacillus cereus*

Continued on next page

Strain	Screening	Replication	Substrates for known pathways	Identity (16S/fungal rDNA)
SR-768	0.003	0	0.11	*Bacillus* sp.
SR-790	0.003	0	0.07	*Proteus alimentorum*
SR-792	0.004	0.005	0.063	*Bacillus thuringiensis*
SR-798	0.005	NA	0*	*Staphylococcus epidermidis*
SR-932	0.004	NA	NA	*Bacillus cereus*
SR-955	0.005	NA	NA	*Bacillus* sp.
SR-228	0.004	0	NA	*Enterobacteriaceae*
SR-234	0.004	NA	NA	*Paenibacillus chitinolyticus*
SR-256	0.003	NA	0*	*Pichia fermentans*
SR-261	0.016	NA	0.007	*Pichia fermentans*
SR-263	0.009	NA	0.01	*Pichia fermentans*
SR-265	0.017	NA	0.019	*Pichia fermentans*
SR-266	0.039	NA	0.011	*Pichia fermentans*
SR-268	0.017	NA	0.01	*Pichia fermentans*
SR-423	0.005	NA	0*	*Bacillus megaterium*
SR-430	0.005	NA	0*	*Bacillus megaterium*
SR-782	0.004	0.004	0.114	*Providencia* sp.
SR-974	0.003	0	NA	*Bacillus cereus*
SR-70	0.004	0.004	0.023	*Bacillus cereus*
SR-71	0.004	0.004	0.012	*Bacillus* sp.
SR-763	0.04	0.003	0.009	*Bacillus cereus*
SR-780	0.004	0.004	0.078	*Bacillus thuringiensis*

NA: Not available.

In order to increase the phylogenetic diversity for further experiments, 39 strains showing an ethylene peak below the integration limit of the Chromeleon software were chosen to be incubated with L-Met, α-ketoglutarate, ATP and ACC pooled to a concentration of 10 mM each (see **Tab. 3.3**). Eleven out of fourteen *Bacillus* strains and one out of three *Lysinibacillus* strains showed ethylene production above the integration limit. One *Klebsiella*, one out of two *Aeromonas* species and three *Myroides* species showed ethylene production. No ethylene was produced by the *Staphylococcus* species. Additionally, one *Lactobacillus* was found to produce ethylene. Ten strains remained unidentified.

Table 3.3: *Ethylene production (pA*min) from additional screening for ethylene producers from substrates for known pathways (ATP, α-KG, L-Met, ACC 10 mM each). All values represent one single measurement. *: additionally 10 mM ACC added. Details about the sequencing results are given in the* ***Appendix A.2.2****.*

Strain	L-Met, ATP, α-KG (10 mM each)	Identity (16S/18S rDNA)
SR-58	0	*Bacillus subtilis subsp. inaquosorum*
SR-59	0	*Bacillus licheniformis*
SR-78	0.038	*Bacillus thuringiensis*
SR-85	0.086	*Bacillus luti*
SR-25	0.023	*Bacillus mycoides*
SR-30	0.004*	*Lysinibacillus sphaericus*
SR-36	0.019	*Bacillus nitratireducens*
SR-41	0	*Lysinibacillus cresolivorans*
SR-44	0.028	*Bacillus cereus*
SR-45	0.041	*Bacillus luti*
SR-772	0.104	*Bacillus cereus*
SR-778	0.115*	*Bacillus thuringiensis*
SR-779	0.08	NA
SR-783	0.14	*Bacillus luti*
SR-784	0.053	*Klebsiella pneumoniae subsp. ozaenae*
SR-786	0	*Enterobacteriaceae*
SR-788	0.043	*Aeromonas aquatica*
SR-86	0.448*	*Lysinibacillus xylanilyticus*
SR-794	0	*Aeromonas aquatica*
SR-795	0	*Staphylococcus capitis subsp. capitis*
SR-796	0	*Bacillus* sp.
SR-801	0.024	*Bacillus* sp.
SR-88	0.111*	*Myroides* sp.
SR-89	0.039	*Myroides* sp.
SR-90	0.041	*Myroides* sp.
SR-92	0	*Aeromonas media*
SR-769	0.108	*Bacillus thuringiensis*
SR-412	0*	NA
SR-413	0*	NA
SR-414	0*	NA
SR-415	0*	NA

Continued on next page

Strain	L-Met, ATP, α-KG (10 mM each)	Identity (16S/18S rDNA)
SR-416	0.004*	*Lactobacullus brevis*
SR-418	0*	NA
SR-419	0*	NA
SR-420	0*	NA
SR-421	0*	NA
SR-422	0*	NA

NA: Not available.

For further characterization, the strains:
Lysinibacillus xylanilyticus SR-86, *Pichia fermentans* SR-265, *Bacillus cereus* SR-772, *Lactobacillus brevis* SR-416, *Providencia* sp. SR-782, *Bacillus luti* SR-783 and the *Bacillus thuringiensis* strains SR-792, SR-769, SR-778, SR-780 were selected.

3.2 Characterization of Ethylene Producers

3.2.1 Substrate Combinations

3.2.1.1 Identification of Precursors for Ethylene Formation from Whole Cell Lysates

In order to determine the pathway by which ethylene is produced, various concentrations of substrates were added to whole cell lysates (prepared as described in **Section 2.13.6**) of various isolates and incubated in sealed 10 mL GC headspace vials for 16 h at 150 rpm at 30 °C in a total volume of 800 µL (**Fig. 3.1** and **3.2**).

The bacterial population tested included six *Bacillus* strains, one *Providencia*, and one *Lysinibacillus* species. There was a great variability in the amount of ethylene produced among the microorganisms and replicates tested, which is evident from the large standard error. In all cases, for almost all the microorganisms, the ethylene formation decreased with increasing ATP concentrations. Additionally, all microorganisms produced ethylene from ACC with the maximum amount being produced from L-methionine and α-ketoglutarate or ACC. A minuscule amount of ethylene was produced when using α-ketoglutarate as the substrate. The maximum amount of ethylene was produced from ACC as seen in *Bacillus luti* SR-783 from L-methionine and α-ketoglutarate from *Bacillus cereus* SR-772 for 10 mM L-Met and

20 mM α-ketoglutarate. The majority of the isolates showed decreased ethylene production with increased ATP concentrations from L-Methionine.

Interestingly, there was a high variability in the ethylene production from the *Bacillus thuringiensis* strains (see **Fig. 3.1**). All strains produced ethylene from ACC, but SR-778 and SR-792 showed little to no ethylene production from L-Methionine and α-ketoglutarate, in contrast to SR-780 and SR-769.

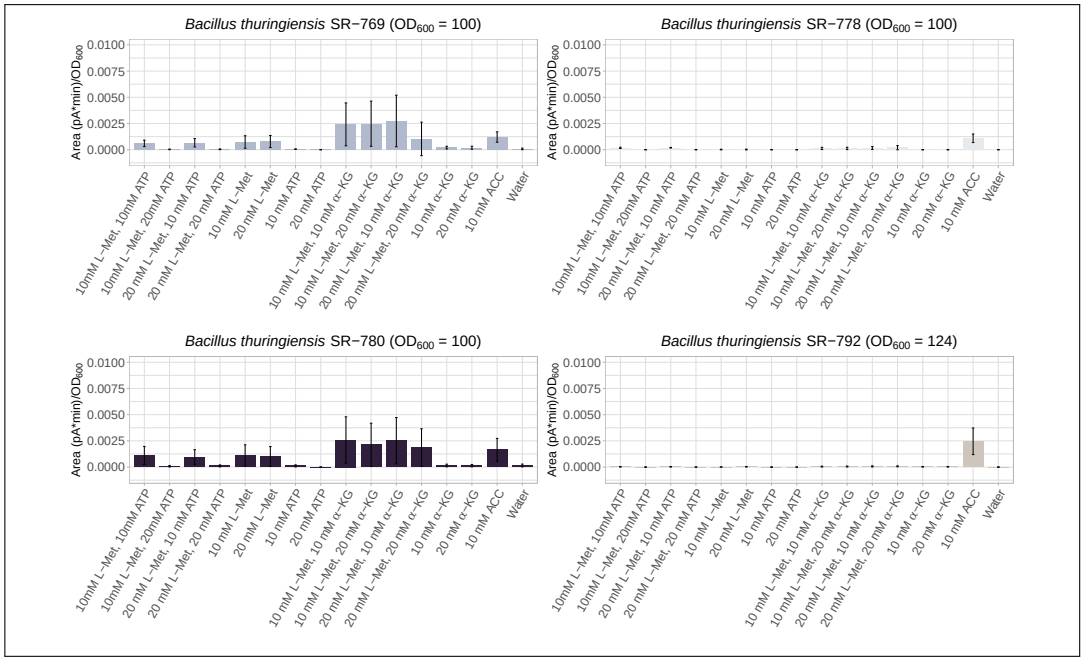

Figure 3.1: ***Ethylene production from various substrate combinations applied to four Bacillus thuringiensis strains from the screening.*** *The values are normalized by dividing the area by the OD_{600} of the concentrated culture. Three-day cell cultures were harvested and concentrated to a specific OD_{600} . 720 µL whole cell lysate and 80 µL substrates were incubated in sealed 10 mL GC headspace vials for 16 h at 30 °C. Error bars indicate standard error (n=3). The ethylene concentrations are given in table* ***Tab. A.5*** *and the OD_{600} values in table A.6. GC data have to be divided by the OD_{600} to generate the data used for this figure.*

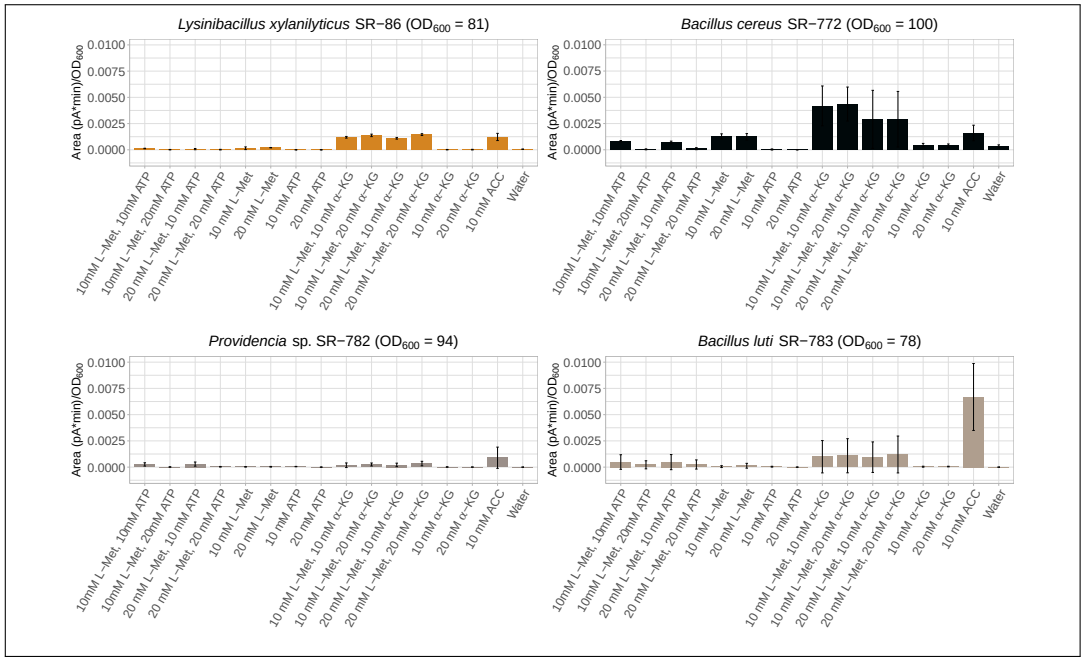

Figure 3.2: ***Ethylene production from various substrate combinations applied to four strains from the screening.*** *The values are normalized by dividing the area by the OD_{600} of the concentrated culture. Three-day cell cultures were harvested and concentrated to a specific OD_{600}. 720 µL whole cell lysate and 80 µL substrates were incubated in sealed 10 mL GC headspace vials for 16 h at 30 °C. Error bars indicate standard error (n=3). The ethylene concentrations are given in table A.5 and the OD_{600} values in table A.6. GC data have to be divided by the OD_{600} to generate the data used for this figure.*

In the following, the strains *Bacillus cereus* SR-772 and *Lysinibacillus xylanilyticus* SR-86 were further investigated. In order to increase the phylogenetic diversity, the strains *Lactobacillus brevis* SR-416 and *Pichia fermentans* SR-265 were also analyzed.

3.2.1.2 Further Characterization of Four Environmental Isolates According to Ethylene Production from Substrate Combinations

In order to further characterize the pathway by which ethylene is produced, two organisms from **Section 3.2.1.1** were used in addition to two organisms belonging to a genus other than *Bacillus* from **Section 3.1.2**, to add more phylogenetic diversity.

All strains were grown at 30 °C at 150–170 rpm in a 1 L baffled Erlenmeyer flask with 250 mL liquid culture. *Lysinibacillus xylanilyticus* SR-86 and *Bacillus cereus* SR-772 were grown in ST1GSE, *Lactobacillus brevis* SR-416 in aMRSSE and *Pichia fermentans* SR-265 was grown in YPDSE. Cells were washed once in the NaPi buffer subsequent to harvesting and resuspended to OD_{600} = 100 (*Lactobacillus brevis* SR-416 OD_{600} = 40). The protein extract was made as described in **Section 2.13.6**.

To 450 µL of protein extract 50 µL of substrates were added in 10 mL GC headspace vials and sealed. Ethylene amount was assessed after incubation for 16 h at 150–170 rpm at 30 °C (see **Section 2.15.2**).

All organisms showed ethylene production from ACC (see **Fig. 3.3**), with *Lactobacillus brevis* SR-416 showing the highest at both concentrations.

Lysinibacillus xylanilyticus SR-86 showed little ethylene production when L-Methionine was added along with ATP. Less ATP resulted in slightly higher ethylene amounts. The addition of ACC resulted in the highest ethylene production from all Yang cycle associated substrates.

Bacillus cereus SR-772 showed a similar pattern when L-Methionine and ATP were added to the reaction mixture. *Lactobacillus brevis* SR-416 showed only ethylene production from ACC, and none when treating the reaction mixture with Yang cycle intermediates, with the exception of 1.6 mM SAM. *Pichia fermentans* SR-265 showed ethylene production when ACC was added. The ethylene production increased with increasing ATP concentrations, showing a contrary effect to the aforementioned organisms.

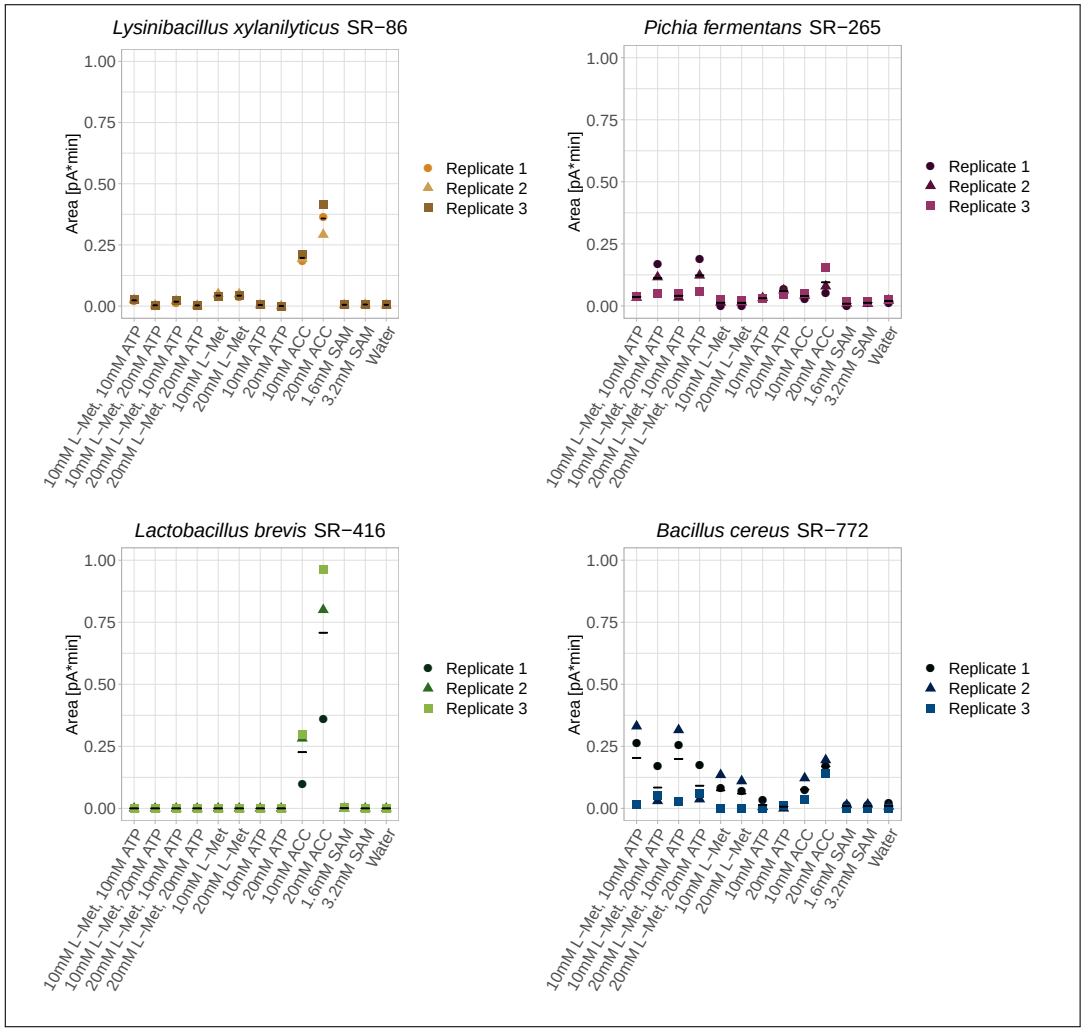

Figure 3.3: ***Ethylene production from substrate combinations identifying the ACC pathway for Bacillus cereus SR-772, Lysinibacillus xylanilyticus SR-86, Lactobacillus brevis SR-416 and Pichia fermentans SR-265.*** *Protein extract was incubated for 16 h at 30 °C at 150–170 rpm in sealed 10 mL GC headspace vials. Strains were grown at 30 °C over three days and resuspended to OD_{600} = 100 for Lysinibacillus xylanilyticus SR-86, Bacillus cereus SR-772 and Pichia fermentans SR-265; Lactobacillus brevis SR-416 was resuspended at OD_{600} = 40 as its growth was not as dense as the grwoth of the other strains. The values are given in* ***Tab. A.7****.*

All organisms showed ethylene production when KMBA was added to the reaction in the presence of the cell lysate, with *Lysinibacillus xylanilyticus* SR-86 producing the highest amount of ethylene (see **Fig. 3.4**). It is to be noted that there seems to be a natural fluctuation of the ethylene production, which can be deduced from the large standard error. Moreover, *Lysinibacillus xylanilyticus* SR-86 and *Bacillus cereus* SR-772, both showed an increased amount of ethylene production, when increased amount of L-Methionine was added to the reaction mixture.

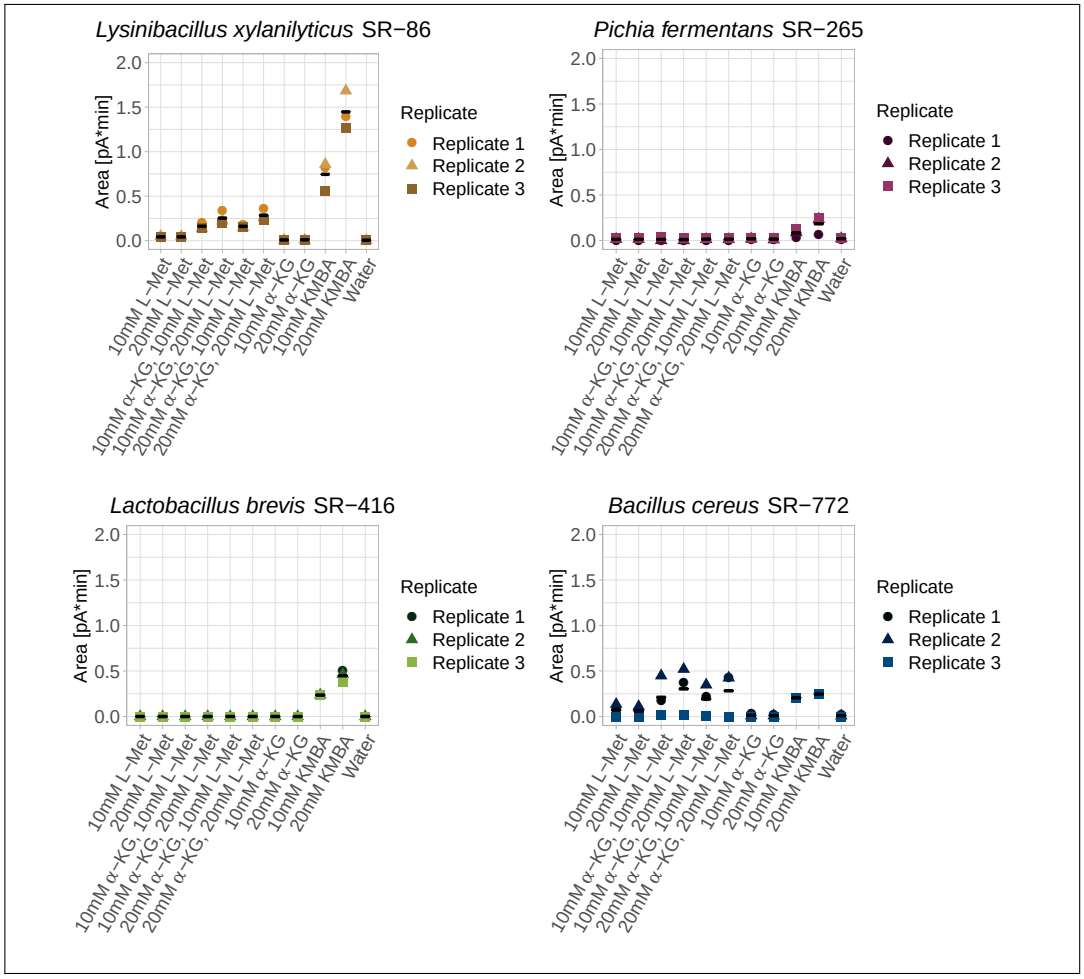

Figure 3.4: ***Ethylene production from substrate combinations identifying the KMBA and Efe pathway for Bacillus cereus SR-772, Lysinibacillus xylanilyticus SR-86, Lactobacillus brevis SR-416 and Pichia fermentans SR-265.*** *Protein extract was incubated for 16 h at 30 °C at 150–170 rpm. Strains were grown at 30 °C over three days and resuspended to OD_{600} = 100 for Lysinibacillus xylanilyticus SR-86, Bacillus cereus SR-772 and Pichia fermentans SR-265; Lactobacillus brevis SR-416 was resuspended at OD_{600} = 40 as it did not grow as dense as the other strains. The values are given in* ***Tab. A.8****.*

3.2.1.3 Effect of Metals and Co-Substrates on Ethylene Production

The effect of various metals, organic acids and electron complexes on ethylene production in *Lysinibacillus xylanilyticus* SR-86, *Bacillus cereus* SR-772 and *Lactobacillus brevis* SR-416 was investigated.

All protein extracts from overnight cultures were prepared in 50 mM MOPS buffer at pH 7 (see **Section 2.16.1**). *Lactobacillus brevis* SR-416 was cultivated in aMRSSE and the Erlenmeyer flask was sealed with parafilm. The other two microorganisms were cultivated in ST1SE without parafilm. The reaction was setup by mixing 450 µL protein extract and 25 µL ACC (concentration in protein extract of 10 mM) and 25 µL 10 mM co-substrate or 0.1 mM metals/electron complexes in 10 mL GC headspace vials.The samples were incubated at 30 °C, 170 rpm for 16 h. As the control, 10 mM ACC without additional substrates was used.

The ethylene production from 10 mM ACC with cobalt(II) in *Bacillus cereus* SR-772 was about 23-fold higher compared to 10 mM ACC alone (control). When EDTA was added, no ethylene production could be determined. For *Lactobacillus brevis* SR-416 the ethylene produced from ACC when cobalt(II) was added was circa 2.4-fold when compared to the control with only ACC added. The addition of EDTA resulted in an activity loss. A similar result was seen for *Lysinibacillus xylanilyticus* SR-86 with a 15.6-fold increase from when cobalt(II) was added (see **Fig. 3.5**). The other combinations did not show any significant changes.

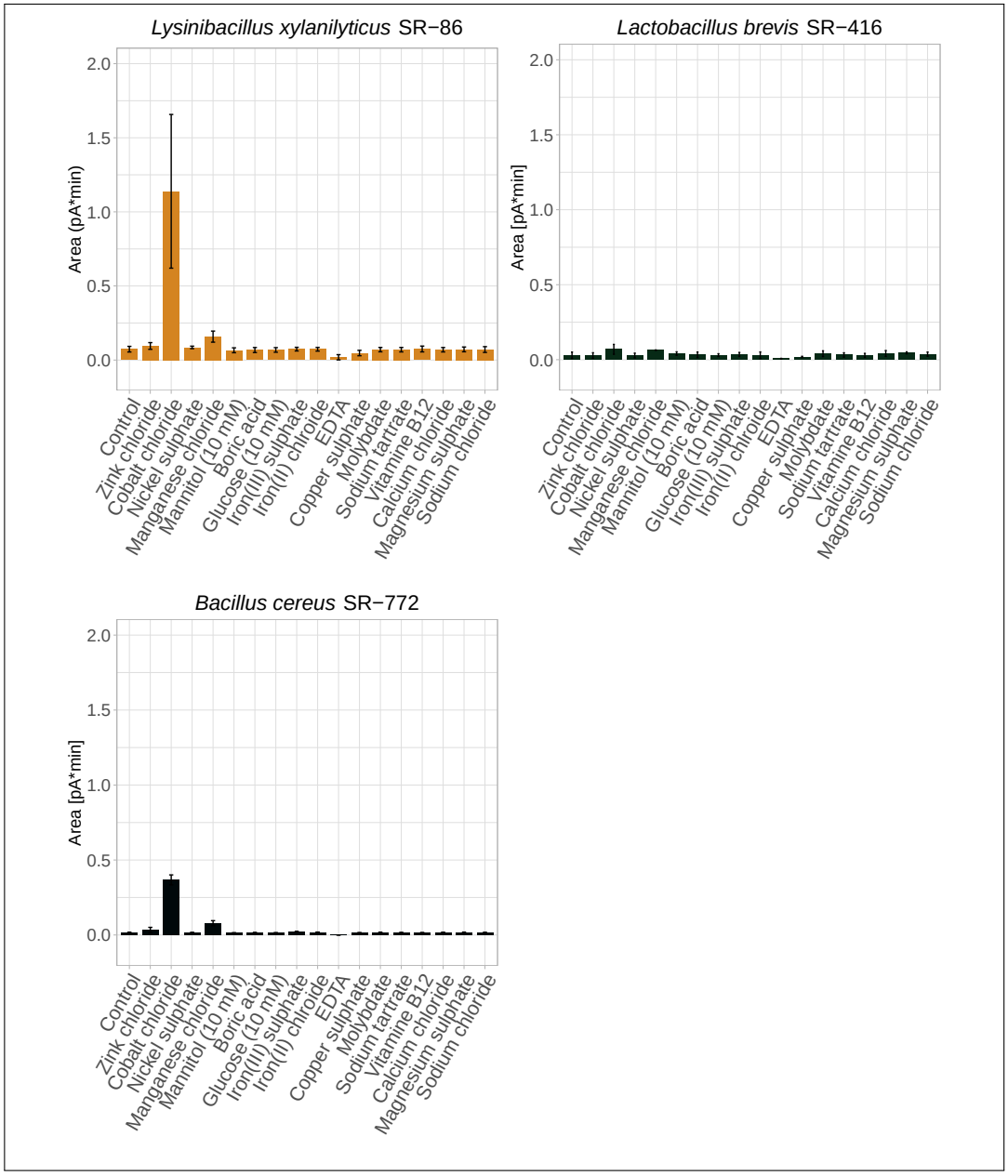

Figure 3.5: ***Effect of added metals and electron complexes on ethylene production from ACC in the protein extract of Bacillus cereus SR-772, Lactobacillus brevis SR-416 and Lysinibacillus xylanilyticus SR-86.*** *Control: 10 mM ACC. Triplicates of overnight grown culture were used to prepare the protein extract. 25 µL ACC (concentration in protein extract of 10 mM) and 25 µL 10 mM co-substrate or 0.1 mM metals/electron complexes were used. Sealed 10 mL GC headspace vials were incubated to 16 h at 30 °C, 170 rpm. Ethylene was analyzed using gas chromatography. Error bars indicate standard error (n=3). The values are given in* ***Tab. A.9****.*

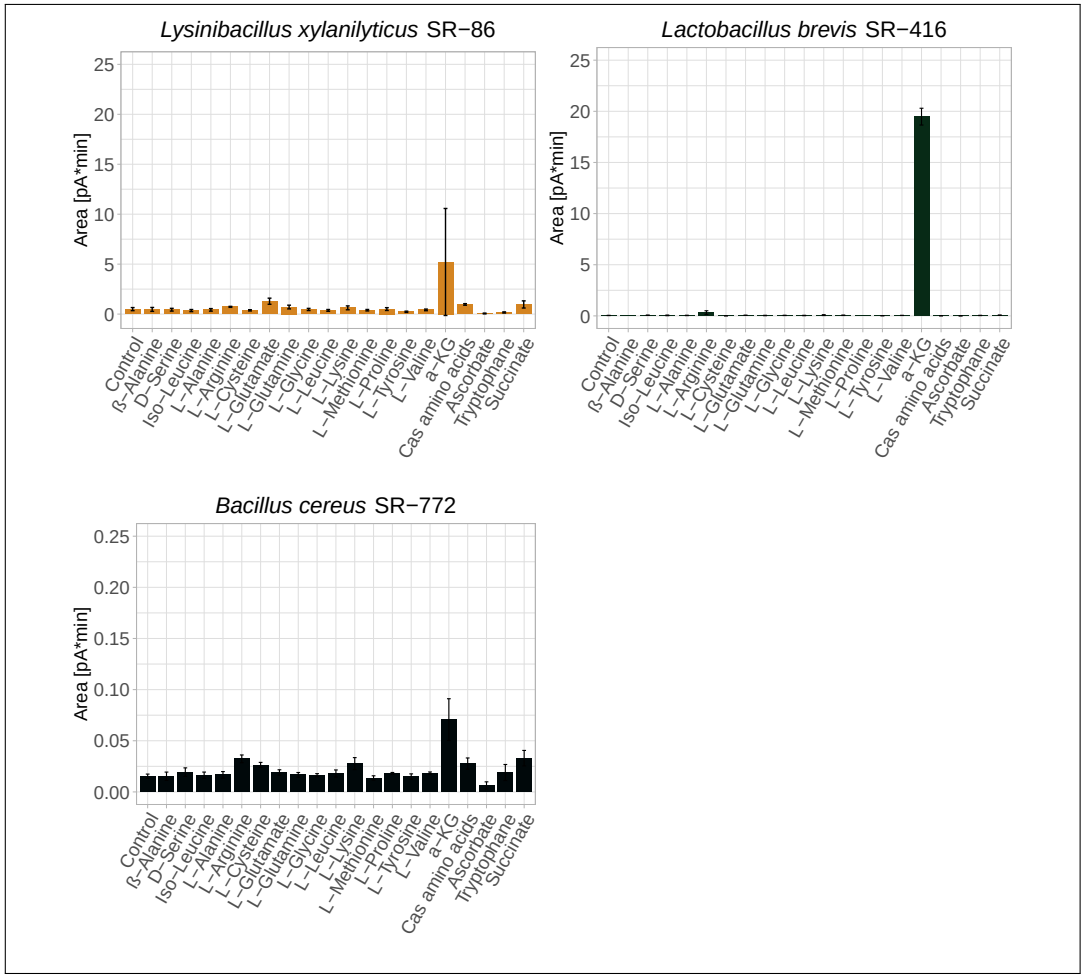

Figure 3.6: ***Effect of added amino acids and organic acids on ethylene production from ACC in the protein extract of Bacillus cereus SR-772, Lactobacillus brevis SR-416 and Lysinibacillus xylanilyticus SR-86.*** *Control: 10 mM ACC. Note that the scale for Bacillus cereus SR-772 is 100 times smaller, as no difference would be seen if the scale of the other strains would be applied. 25 µL ACC (concentration in protein extract of 10 mM) and 25 µL 10 mM co-substrate or 0.1 mM metals/electron complexes were used. The final volume was 500 µL. Sealed 10 mL GC headspace vials were incubated to 16 h at 30 °C, 170 rpm. Ethylene was analyzed using gas chromatography. Error bars indicate standard error (n=3). The values are given in* ***Tab. A.10****.*

In regard to the amino acids and organic acids, all three tested strains show the highest response when α-ketoglutarate was added to the protein extract and ACC (see **Fig. 3.6**). *Lysinibacillus xylanilyticus* SR-86 showed a 10-fold increase of ethylene production, *Bacillus cereus* SR-772 showed a 4.7-fold increase and *Lactobacillus brevis* SR-416 showed the highest increase of 488-fold. In all organisms, ascorbic acid showed an inhibitory effect on the ethylene production. L-tryptophane, L-tyrosine, L-leucine and iso-leucine showed inhibited production in *Lysinibacillus xylanilyticus* SR-86, while L-tyrosine, L-proline, L-leucine, L-gluatime, L-cysteine, L-arginine and beta-alanin inhibited *Lactobacillus brevis* SR-416.

The effect of other α-keto acids on ethylene production was examined for *Lysinibacillus xylanilyticus* SR-86 (see **Fig. 3.7**). α-ketoglutarate had the maximum effect on ethylene production of up to 5-fold when compared to ACC alone. 2-aminoisobutyric acid (AIB) had the least effect with a 1.6-fold increase. However, no ethylene was detected when the protein extract was incubated with α-keto acids without ACC.

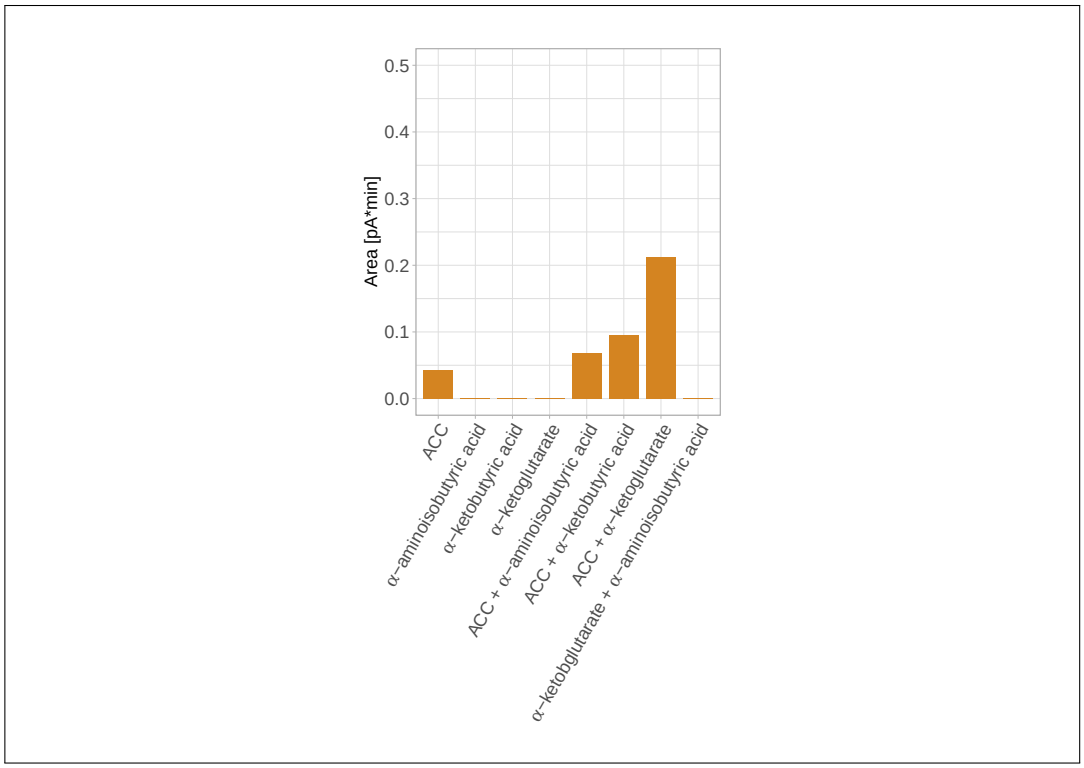

Figure 3.7: ***Effect of added α-keto acids on ethylene production from ACC in the protein extract of Lysinibacillus xylanilyticus SR-86 (n=1).*** *α-keto acid were added to a final concentration of 10 mM to the protein extract. The final volume was 500 µL consisting of 450 µL protein extract, 25 µL ACC and 25 µL α-keto acid both 10 mM final concentration. Sealed 10 mL GC headspace vials were incubated to 16 h at 30 °C, 170 rpm. The amount of ethylene was analyzed using gas chromatography (n=1). The values are given in* ***Tab. A.11****.*

In order to understand the ethylene forming system better, radical scavengers and proteinase K were added to the reaction (see **Fig. 3.8**). The protein extract for proteinase K was heated to 37 °C for 10 minutes in a thermo shaker. There was no loss or gain in activity when the protein extract was heated to 37 °C without proteinase K compared to the non-heated protein extract in all organisms. Treatment with proteinase K had an effect on the ethylene formation in all organisms. The strongest effect was seen for *Lysinibacillus xylanilyticus* SR-86 with less than 48% ethylene being produced and *Lactobacillus brevis* SR-416 with 47% less ethylene formed. The effect in *Bacillus cereus* SR-772 was less with about one third ethylene formed.

Argon had a minimal effect on *Lactobacillus brevis* SR-416, with a decrease in ethylene production by 15%. However, for *Bacillus cereus* SR-772 and *Lysinibacillus xylanilyticus* SR-86, the ethylene production decreased by 46% and 85%, respectively. The addition of catalase affected ethylene production the most. In *Lysinibacillus xylanilyticus* SR-86 a decrease of 56%, while in *Lactobacillus brevis* SR-416, a decrease of 39% was seen. In *Bacillus cereus* SR-772, there was an increase by 25% when catalase was added.

When the protein extract was heated to 95 °C for 10 minutes, a 82% drop of the ethylene amount in *Lysinibacillus xylanilyticus* SR-86 and 19% in *Lactobacillus brevis* SR-416 was observed. In *Bacillus cereus* SR-772, the ethylene amount increased by 88%.

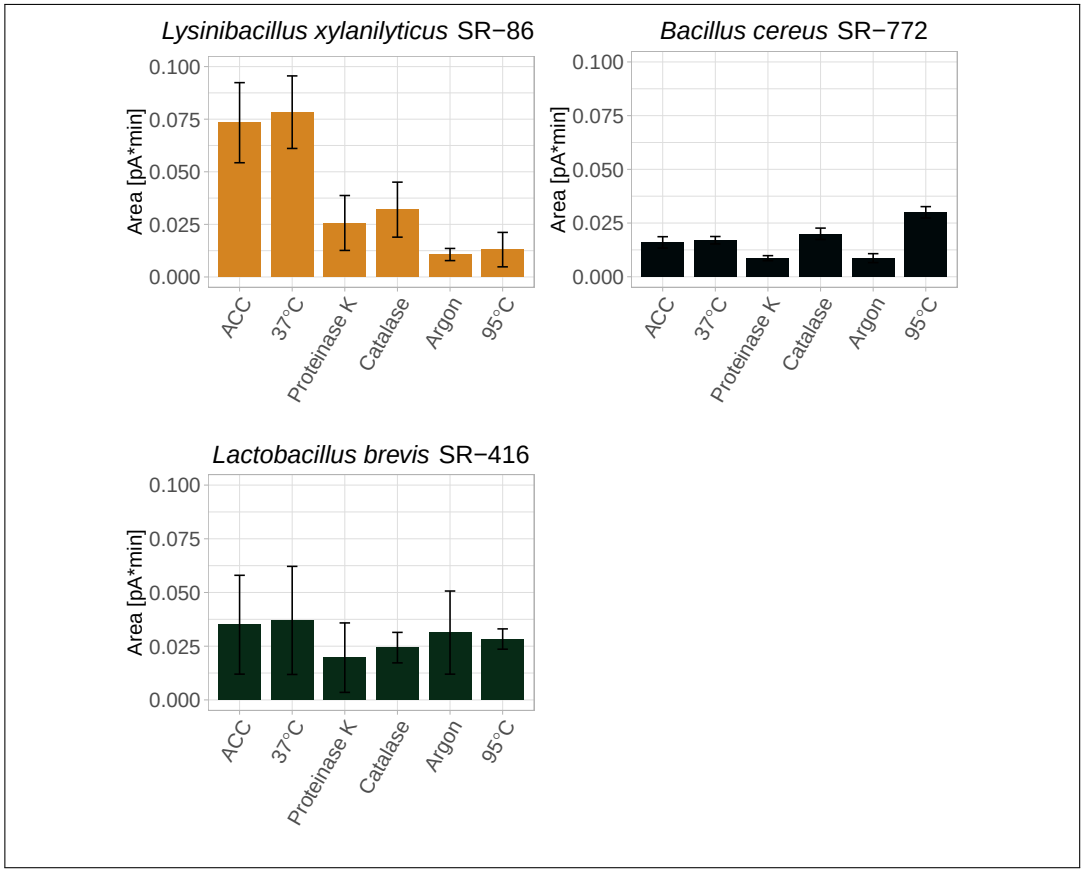

Figure 3.8: ***Effect of radical scavangers, heat and proteinase K on ethylene production from ACC in the protein extract of Lysinibacillus xylanilyticus SR-86, Lactobacillus brevis SR-416 and Bacillus cereus SR-772.*** *ACC was added to a final concentration of 10 mM to the protein extract. The final volume was 500 µL consisting of 450 µL protein extract, 50 µL ACC at 10 mM final concentration. The protein extract was heated to 37° C for 10 minutes before pipetting it in the GC vials fo the 37° C sample. The protein extract was heated to 95° C for 10 minutes. The protein extract for all other samples was not pretreated. Sealed 10 mL GC headspace vials were flushed with Argon for 10 minutes. 1 µL of proteinase K was added and 5 µL of catalase (1000 units in 50 mM MOPS at pH 70). Sealed 10 mL GC headspace vials were incubated to 16 h at 30° C, 170 rpm. The amount of ethylene was analyzed using gas chromatography (n=1). The values are given in* ***Tab. A.12****.*

In order to investigate the possibility that the pathway by which ethylene is produced could be via the efe pathway, the combination of L-arginine, α-ketoglutarate and ACC was tested (see **Fig. 3.9**).

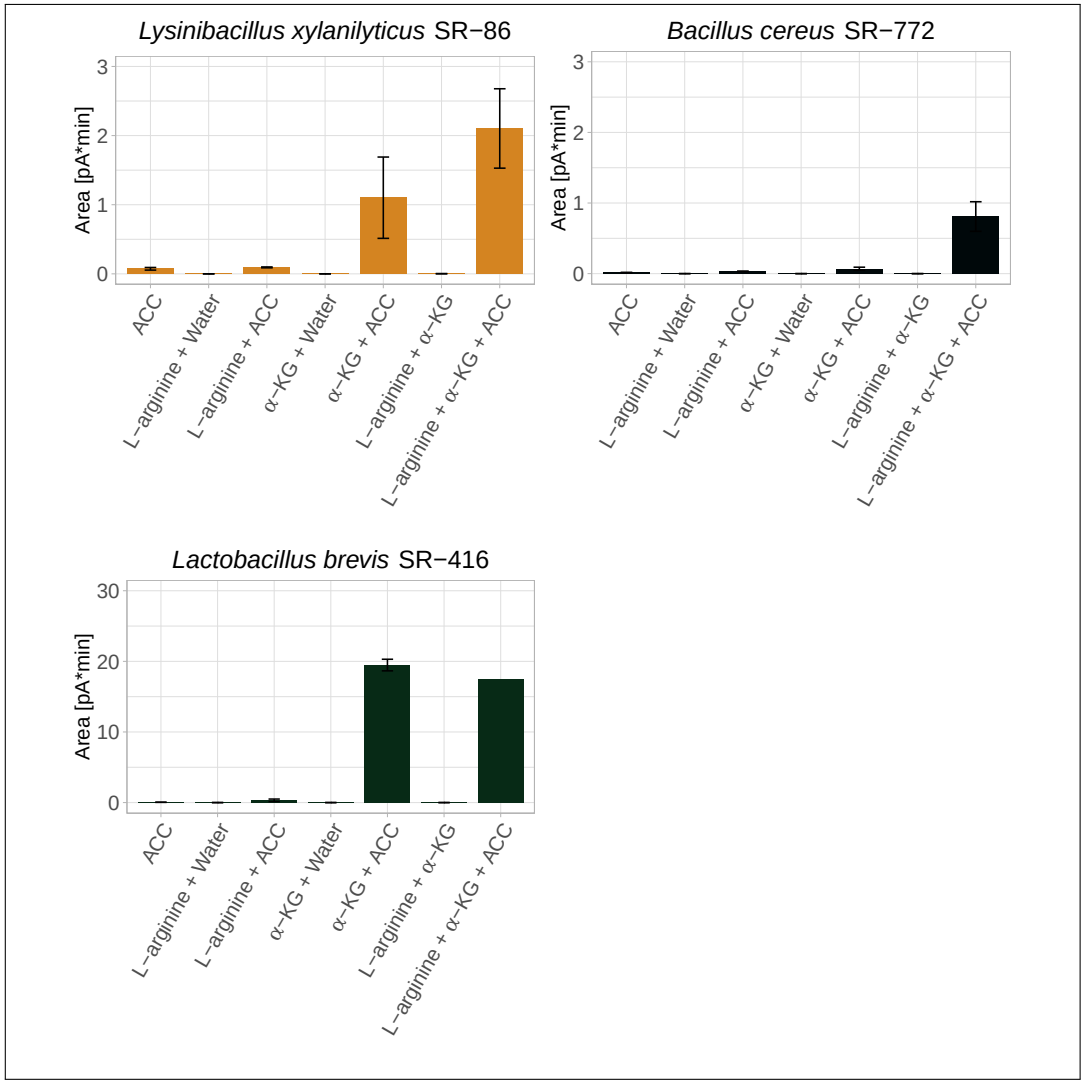

Figure 3.9: ***Substrates for the efe pathway in Lysinibacillus xylanilyticus SR-86, Lactobacillus brevis SR-416 and Bacillus cereus SR-772.*** *Note that the scale for Lactobacillus brevis SR-416 is 10 times higher than that of the other organisms. The final volume of the reaction was 500 µL with 10 mM ACC and 10 mM of the other substrates. The control is protein extract with 10 mM ACC. Sealed 10 mL GC headspace vials were incubated to 16 h at 30 °C, 170 rpm. The amount of ethylene was analyzed using gas chromatography (n=3). The values are given in* ***Tab. A.13****.*

Irrespective of the combinations tested, all organisms showed a similar pattern as far as the production of the ethylene is concerned. No ethylene was produced from α-ketoglutarate, L-arginine and their combination, except in *Lysinibacillus xylanilyticus* SR-86, were very little ethylene was produced from L-arginine and α-ketoglutarate. In all strains, the Efe substrates L-arginine and α-ketoglutarate did not produce any ethylene. However, in *Lysinibacillus xylanilyticus* SR-86, the addition of ACC to α-ketoglutarate yielded in an 15-fold, L-arginine and ACC in 1.3-fold and an 29-fold increase when

all three substrates were combined compared to ACC alone (control). In *Bacillus cereus* SR-772, the addition of ACC to α-ketoglutarate resulted in 4.7 times more ethylene compared to ACC alone. The addition of ACC to L-arginine showed a 2-fold increase and the addition of L-arginine and ACC to α-ketoglutarate showed the highest increase of 54-fold. In *Lactobacillus brevis* SR-416, the highest ethylene production was observed when ACC and α-ketoglutarate were combined being 453-fold compared to ACC alone. The combination of ACC and L-arginine was 407-fold and ACC, L-arginine and α-ketoglutarate were 7.5-fold increased.

3.2.1.4 pH-Dependence of Ethylene Formation from ACC

The pH-dependence of ethylene formation from ACC has been tested for *Bacillus cereus* SR-772 and *Lysinibacillus xylanilyticus* SR-86 (see **Fig. 3.10**). Both organisms were grown for three days at 30 °C at 150–170 rpm in ST1GSE medium. The cells were treated as described in **Section 2.16.1** and 450 µL protein extract and 50 µL 100 mM stock solution ACC was mixed in a head space gas chromatography vial and sealed. The reaction mixture was incubated for 16 h at 30 °C at 150–170 rpm and ethylene production was analyzed (see **Section 2.15.2**).

No activity was detected at pH 5; however, ethylene production increased with increasing pH of the reaction from pH 5 to pH 9 for *Bacillus cereus* SR-772 and pH 3 to pH 11 for *Lysinibacillus xylanilyticus* SR-86 (see **Fig. 3.10**). *Lysinibacillus xylanilyticus* SR-86 showed the highest ethylene formation at pH 11 with 0.763±0.136 pA*min and the lowest at pH 7 with 0.043±0.015 pA*min. *Bacillus cereus* SR-772 also showed the highest ethylene amount at pH 9 with 0.020±0.0032 pA*min, 0.094±0.0162 at pH7 and no ethylene at pH 5.

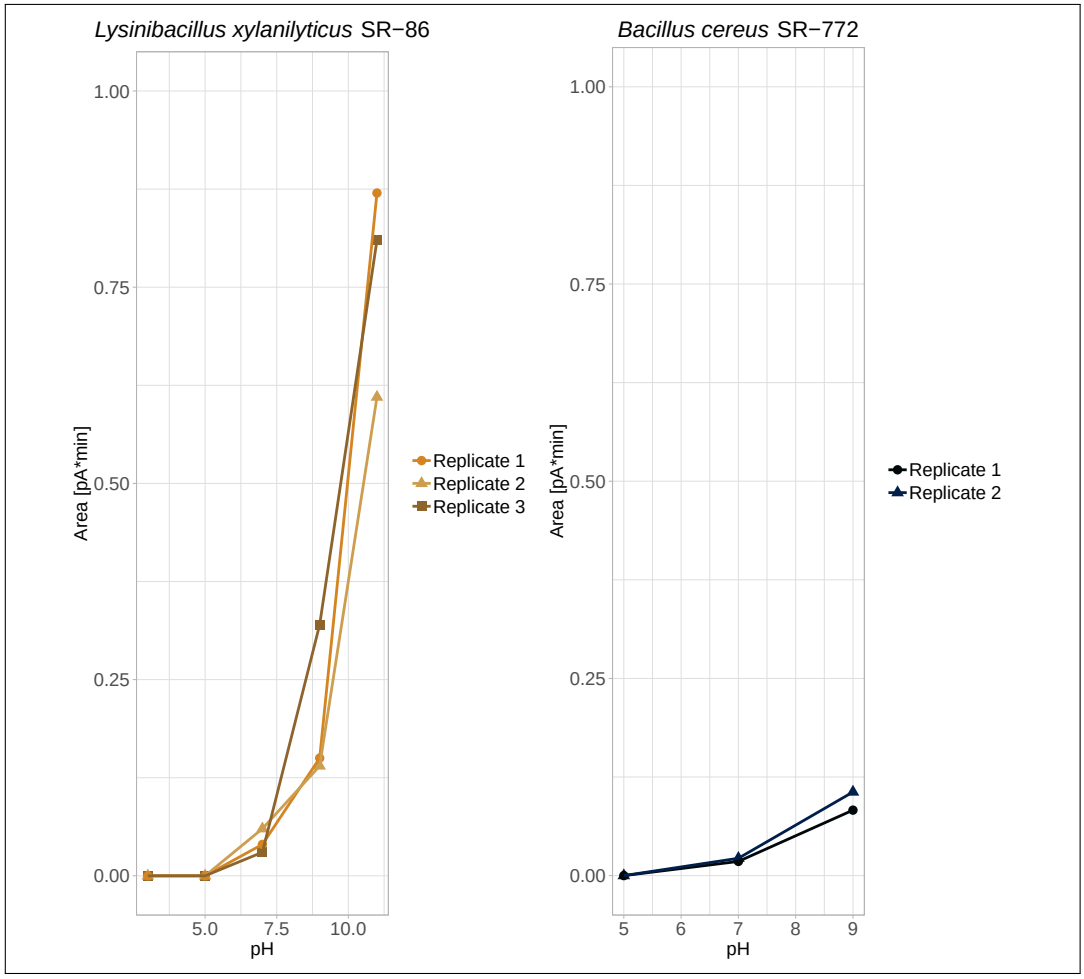

Figure 3.10: ***Effect of pH on ethylene production from ACC in the protein extract of Bacillus cereus SR-772 and Lysinibacillus xylanilyticus SR-86.*** *The effect on ethylene production in the protein extract of Lysinibacillus xylanilyticus SR-86 (n=3) and Bacillus cereus SR-772 (n=2). The final volume of the reaction was 500 µL with 10 mM ACC. Sealed 10 mL GC headspace vials were incubated to 16 h at 30 °C, 170 rpm. The amount of ethylene was analyzed using gas chromatography. The values are given in* ***Tab. A.14****.*

3.2.1.5 Ethylene Formation in Dependence of ACC and α-ketoglutarate Concentration

In order to determine the effect of ACC and α-ketoglutarate on the ethylene formation from the protein extracts of *Lysinibacillus xylanilyticus* SR-86 (2500 µg/mL) and *Lactobacillus brevis* SR-416 (750 µg/mL), the protein extract was incubated with various concentrations of ACC and α-ketoglutarate, in which one was held constant at 10 mM, whereas the other was variable (see **Fig. 3.11**). The sealed 10 mL GC headspace vials were incubated for 16 h at 30 °C at 150–170 rpm (see **Fig. 3.11**).

The amount of ethylene produced in *Lactobacillus brevis* SR-416 was higher than that of *Lysinibacillus xylanilyticus* SR-86. Interestingly, the organisms behave conversely: while the amount of ethylene increased linearly with increasing ACC in *Lactobacillus brevis* SR-416, the opposite is true for *Lysinibacillus xylanilyticus* SR-86 (square). The behaviour was also seen with a variable α-ketoglutarate concentration (rectangle).

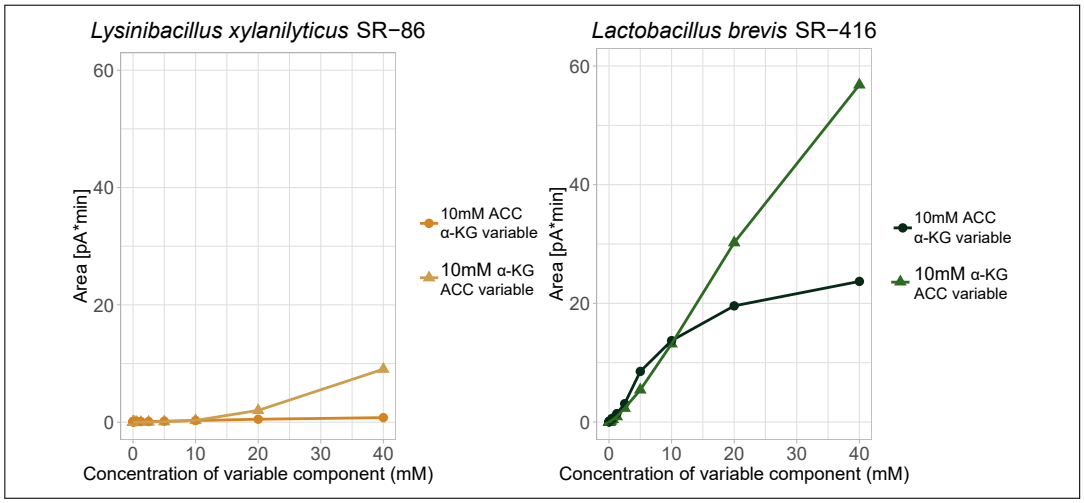

Figure 3.11: ***The effect of ACC and α-ketoglutarate concentration on the ethylene production from ACC in the protein extract of Bacillus cereus SR-772 and Lysinibacillus xylanilyticus SR-86.*** *The protein concentration of Lysinibacillus xylanilyticus SR-86 was 2500 µg/mL and for Lactobacillus brevis SR-416 750 µg/mL. The protein extract was incubated for 16 h at 30 °C and 150–170 rpm in a sealed 10 mL GC head space vial with 450 µL protein extract and 50 µL substrate solution. The values are given in* ***Tab. A.15****.*

No ethylene was produced in either organism when no ACC was added. In *Lactobacillus brevis* SR-416, ethylene production in reaction with a constant α-ketoglutarate concentration of 10 mM was measurable at an ACC concentration of 0.01 mM. Ethylene was detected in *Lysinibacillus xylanilyticus* SR-86 from ACC concentrations of 0.625 mM with constant α-ketoglutarate. With constant ACC at 10 mM and varying α-ketoglutarate concentration, ethylene was measurable from the lowest concentration (0.005 mM) in both organisms.

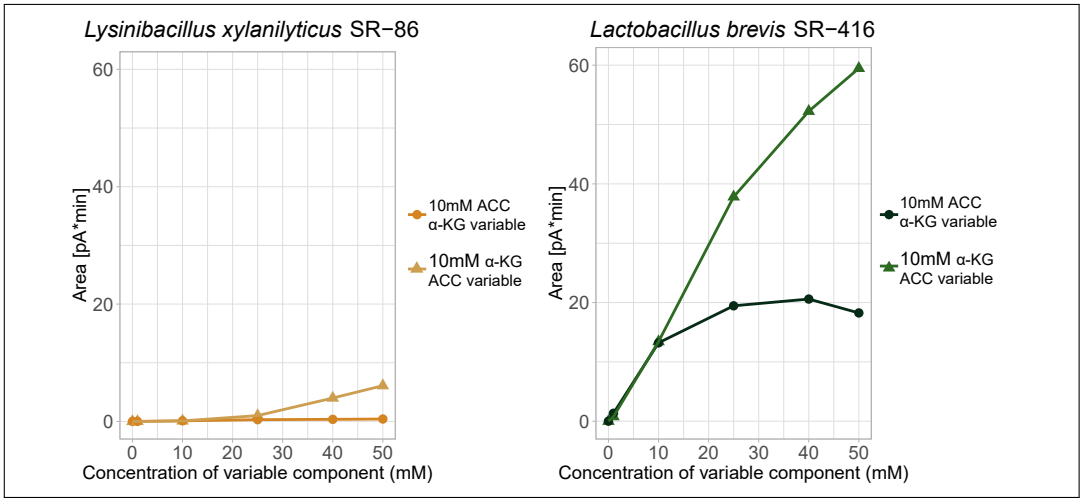

Figure 3.12: ***Effect of ACC and α-ketoglutarate concentration on ethylene production from ACC in the desalted protein extract of Bacillus cereus SR-772 and Lysinibacillus xylanilyticus SR-86.*** *Protein concentration of Lysinibacillus xylanilyticus SR-86 was 2500 µg/mL and for Lactobacillus brevis SR-416 750 µg/mL. The protein extract was incubated for 16 h at 30 °C and 150-170 rpm in a sealed 10 mL GC headspace vial with 450 µL protein extract and 50 µL substrate solution. The values are given in* ***Tab. A.16****.*

The protein extract was desalted as described in **Section 2.16.6** and the experiment was repeated (see **Fig. 3.12**). The curves resemble the graphs seen for the protein extract (see **Fig. 3.11**). When 10 mM α-ketoglutarate was used with varying amounts of ACC, 0.776 pA*min ethylene was detected at 1 mM ACC and increased linearly to 59.469 pA*min at 50 mM. When ACC was constant at 10 mM, ethylene formation was detected even in the absence of α-ketoglutarate with 0.011 pA*min and ended with 18.249 pA*min at 50 mM with a saturation curve. In *Lysinibacillus xylanilyticus* SR-86, ethylene production with constant α-ketoglutarate concentration at 10 mM was detected at 10 mM ACC with 0.09 pA*min and increased to 6.094 pA*min at 50 mM. Ethylene production started from 0.006 pA*min at 1 mM α-ketoglutarate with constant ACC at 10 mM and increased to 0.408 at 50 mM.

When the buffer (50 mM MOPS at pH 7) was checked as a negative control with and without 1% trace elements solution (see **Fig. 3.13**), the same pattern as with the protein extracts was achieved with slightly higher ethylene amounts.

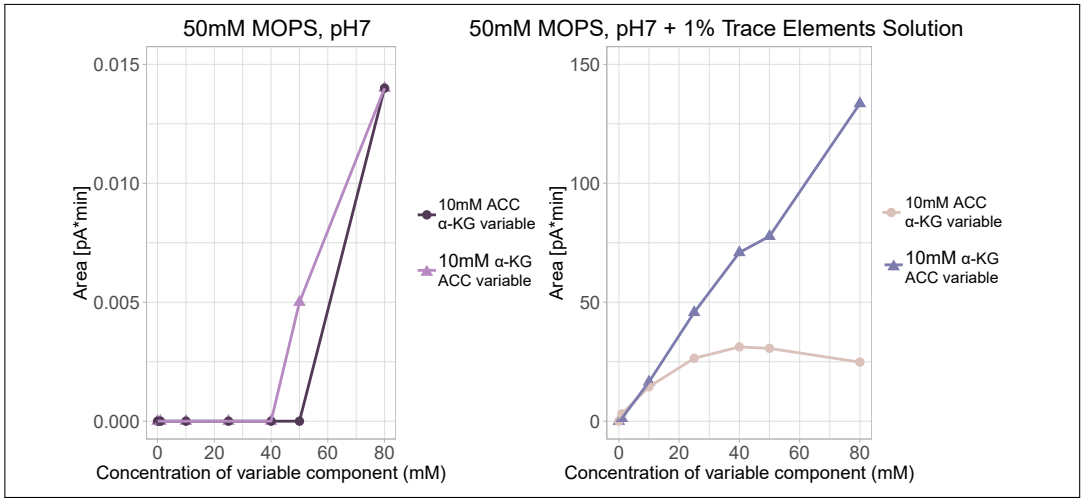

Figure 3.13: ***The effect of ACC and α-ketoglutarate concentration on the ethylene production from ACC in the desalted protein extract of 50 mM MOPS at pH7 and 50 mM MOPS + 1% Trace Elements Solution at pH7.*** *Note that the scale of MOPS + 1% Trace Elements Solution is 10,000 times higher than the scale of MOPS. Buffers were incubated for 16 h at 30 °C and 150–170 rpm in a sealed 10 mL GC head space vial with 450 µL buffer and 50 µL substrate solution. The values are given in* ***Tab. A.17****.*

Little amounts of ethylene were detected in 50 mM MOPS at pH 7.0 when 10 mM α-ketoglutarate and variable concentrations of ACC were incubated for 16 h at 30 °C. At 50 mM ACC, 0.005 pA*min and at 80 mM ACC 0.015 pA*min ethylene were detected. A similar behavior was observed for variable α-ketoglutarate concentrations and 10 mM ACC, with ethylene (0.0014 pA*min) being detected at 80 mM α-ketoglutarate. A comparable curve to that of the protein extracts of *Lactobacillus brevis* SR-416 was observed when the buffer was supplemented with 1% trace elements solution (see **Tab. 2.25**). The ethylene formation started at 1 mM ACC (α-ketoglutarate constant at 10 mM) with 1.199 pA*min and increased linearly to 133.53 pA*min at 80 mM. When ACC was constant at 10 mM, ethylene was detected at 1 mM α-ketoglutarate with 3.101 pA*min and increased in a saturation curve to 24.822 pA*min at 80 mM α-ketoglutarate.

3.2.2 Protein Purification Attempts of the ACC Dependent Ethylene Producing Enzyme Using AEKTA^{TM}

In order to purify the enzyme from the protein extract several conditions (e. g. pH and buffer) and columns were tested. The experiments were partly done for *Bacillus cereus* SR-772 and *Lactobacillus brevis* SR-416, but the main focus was on *Lysinibacillus xylanilyticus* SR-86. The following result represents the best purification achieved (see **Fig. 3.14**).

The protein extract from *Lysinibacillus xylanilyticus* SR-86 was prepared as described in **Section 2.16.1** in 40 mM Glycin NaOH at pH 9.0. The AEKTA^{TM} was operated as described in **Section 2.16.6** with a 10 column volume gradient starting with 0% buffer B (40 mM Glycin NaOH + 1 M NaCl) to 50%B (with buffer A being 40 mM Glycin NaOH). The total volume of fractions was 10 mL. In order to have a higher amount, six runs were performed and pooled for the activity assay (see **Section 2.15.2**).

AEKTA^{TM} fractions A3 and A4 had the highest specific activity and were subsequently used to sequence to determine possible proteins (see **Section 3.2.4**).

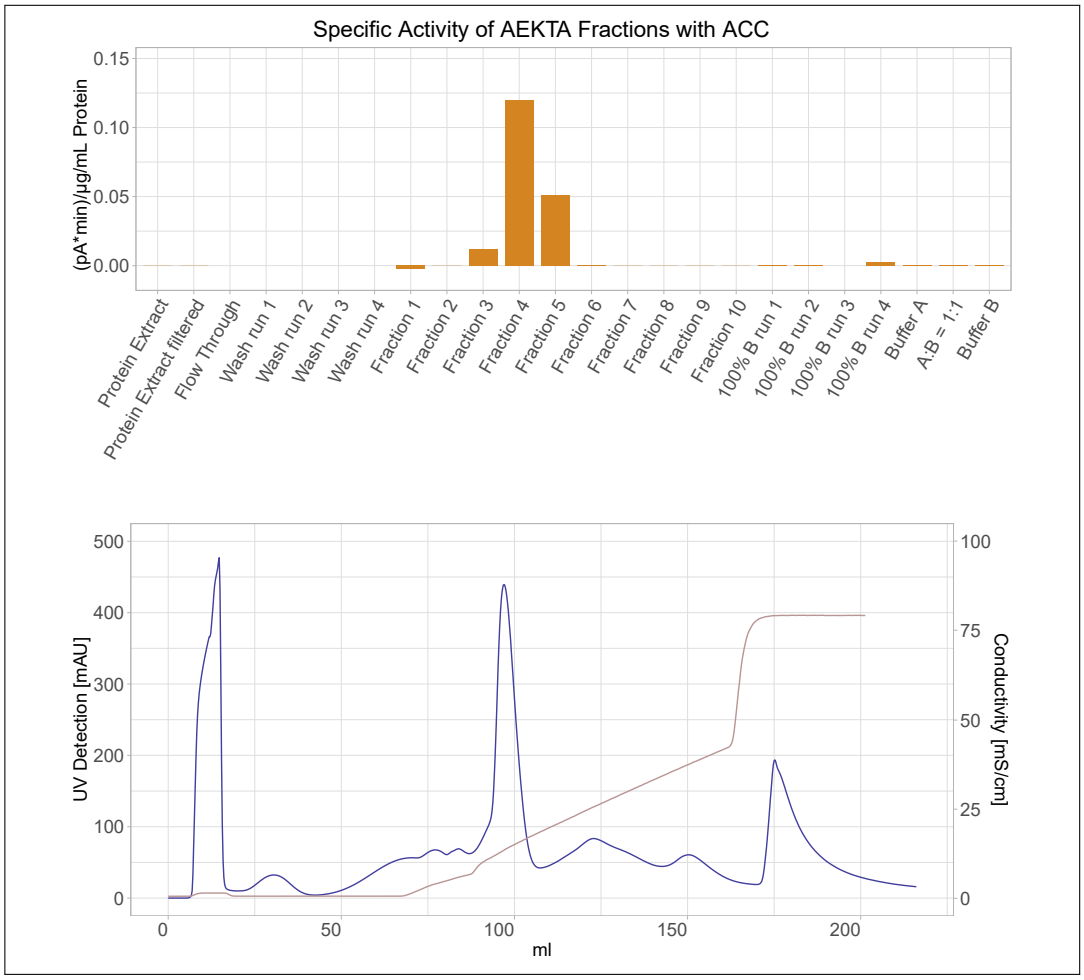

Figure 3.14: ***AEKTA^{TM} fractions of Lysinibacillus xylanilyticus SR-86 protein extract at pH 9 in 40 mM Glycin NaOH buffer from 5 mL QXL and 5 mL DEAE-FF interlocked using ACC as substrate.*** *The specific activity is calculated for six pooled runs. One run is shown as an exemplary UV (blue) and conductivity (purple) profile. Highly active fractions are fractions 3 and 4, which were incubated for 16 h at 30 °C and 150–170 rpm in a sealed 10 mL GC head space vial with 450 µL sample and 50 µL ACC solution (final concentration of 10 mM). The amount of ethylene was analyzed using gas chromatography and the amount of protein was determined using the Bradford assay. The values are given in* ***Tab. A.18****.*

3.2.3 Genome Sequencing of Isolated Microorganisms and Features Found for Ethylene Production

Genomes of *Bacillus cereus* SR-772, *Lysinibacillus xylanilyticus* SR-86 and *Pichia fermentans* SR-265 were sequenced and annotated as described in **Section 2.14.1**. The sequencing for *Lysinibacillus xylanilyticus* SR-86 achieved a high coverage and a low number of contigs, in contrast to the sequencing of *Bacillus cereus* SR-772 and *Pichia fermentans* SR-265 (see **Tab. 3.4**). The genome sizes of the two bacterial species is considerably smaller than that of the eukaryote *Pichia fermentans* SR-265, which correlates with the number of coding sequences being higher in the latter organism.

Table 3.4: *Results of the Genome Sequencing from Environmental Isolates*

Strain	*B. cereus* SR-772	*L. xylanilyticus* SR-86	*P. fermentans* SR-265
No. of Contigs	1,889	49	29,164
Coverage	22	114	3
Size (bp)	7,126,871	5,221,635	20,191,448
GC Content	42.4	36.6	45.9
N50	NA	412,414	8,834
No. of Coding Sequences	7,477	4,791	12,552
No. of Subsystems	517	462	526
No. of Features	7,611	4893	12,848
No. of RNAs	134	102	296

NA: not assigned

In order to find enzymes involved in ethylene biosynthesis, homologue sequences were searched using the BLAST tool and multiple sequence alignment was performed using ClustalΩ as described in **Section 2.17.3**.

The two bacterial species do not posses an ethylene forming enzyme, as revealed by blastp with the Efe protein sequence of *Pseudomonas syringae* pv. *phaseolicola*[1] as the reference. The same was observed for the plant system of ethylene production, with the ACC oxidase from *Malus domestica*[2] as a reference. In both cases, no significant hits were found, as all hits showed an E-value greater than 0.33.

In order to elucidate, if the eukaroytes yeast posses a plant like enzyme, the ACC oxidase of *M. domestica* was used to perform a PSI-BLAST with four iterations in the taxonomy “true yeasts” (taxid: 147537). Significant hits were found in the genus *Pichia* (new name *Komagataella*) and other yeasts (see **Tab. 3.5**). All significant hits were already annotated as ACC oxidase in the database.

[1] Accession number P32021.1
[2] Accession number CAA74328.1

When performing a tblastn search with the internal RAST blast implementation against the genome of *Pichia fermentans* SR-265 using the ACC oxidase of *M. domestica*, three ACC oxidases, a Yang cycle enzyme, were found (see **Tab. 3.6**).

Interestingly, when the Efe of *P. syringae* was blasted against the *Pichia fermentans* SR-265 genome, the same positions occurred as with blasting the ACC oxidase from *M. domestica*. The hits were significant with E-values smaller than 0.001 and the sequences found are annotated as "oxidoreductase" (see **Tab. 3.7**). The found enzymes are considered to be putative ACC oxidases, as the score and the E-value are more significant when using the ACCO of *M. domestica*. Moreover, the guide tree of ClustalΩ shows a closer relationship between the *Pichia fermentans* SR-265 ACC oxidase with that of *M. domestica* than with Efe of *P. syringae*.

Table 3.5: *Results of the searching for homologous ACCO sequences in yeasts using blastp. The ACC oxidase from Malus domestica (CAA74328.1) was used as query for a PSI-BLAST in the taxonomy "true yeasts" (taxid: 147537) with four iterations; in each iteration, only as "ACC oxidase" annotated hits were used in the next iteration. The table shows the results after four iterations.*

Organism	Description	Max score	Total score	Query cover	E-value	Ident	Accession
Wickerhamomyces ciferrii	ACCO 3	342	342	93%	7,00E-115	23%	CCH43516.1
Komagataella pastoris CBS7435	ACCO 3	307	307	86%	3,00E-101	23%	CCA38452.1
Kluyveromyces marxianus	ACCO	297	297	85%	5,00E-97	26%	BAP73289.1
Kluyveromyces marxianus DMKU3-1042	ACCO	297	297	85%	1,00E-96	26%	BAO41860.1
Wickerhamomyces ciferrii	ACCO 3	287	287	85%	2,00E-92	22%	CCH43443.1

The three topmost sequences with E-values smaller than 1e-06 were aligned using ClustalΩ against well-characterized plant ACC oxidases (see **Fig. 3.15**). The facial triad, responsible for the Fe(II) binding (His177, Arg179 and His234) in the ACC oxidase of plants is present in all three predicted *Pichia fermentans* SR-265 ACC oxidases. The bicarbonate binding site (Thr157, Arg244, Ser246) is partially present in all three predicted *Pichia fermentans* SR-265 ACC oxidases with the residues Arg244 and Ser246; Thr157, however, is missing from all three predicted ACC oxidases.

In order to investigate if the predicted ACC oxidases found in *Komagataella pastoris* CBS 7435 and *Pichia fermentans* SR-265 are actual ACC oxidases, the work was

Table 3.6: *Results of the search for homologous ACCO sequences in Pichia fermentans SR-265 using the blast tool on the RAST server. Blast of the ACC oxidase 3 from Malus domestica (CAA74328.1) against the annotated genome of Pichia fermentans SR-265 using RAST.*

Sequences on contig	Score	E-value	Ident
fig|6666666.210804.peg.4016	72	1e-14	23%
fig|6666666.210804.peg.593	61	4e-11	33%
fig|6666666.210804.peg.8391	55	4e-09	24%
fig|6666666.210804.peg.3501	32	0.12	27%
fig|6666666.210804.peg.7300	28	1.9	29%
fig|6666666.210804.peg.6426	28	2.4	25%
fig|6666666.210804.peg.7811	26	4.7	47%
fig|6666666.210804.peg.8722	27	6.7	36%
fig|6666666.210804.peg.11177	27	7.4	23%

Table 3.7: *Results of the search for homologous EFE sequences in Pichia fermentans SR-265 using the blast tool on the RAST server. Blast of the EFE from Pseudomonas syringae pv. phaseolicola (P32021.1) against the annotated genome of Pichia fermentans SR-265 using RAST.*

Sequences.on.contig	Score	E.value	Ident
fig|6666666.210804.peg.8391	39	7e-04	23%
fig|6666666.210804.peg.593	37	0.004	23%
fig|6666666.210804.peg.4016	37	0.006	24%
fig|6666666.210804.peg.1221	30	0.39	28%
fig|6666666.210804.peg.10865	30	0.62	23%
fig|6666666.210804.peg.10773	29	0.95	24%
fig|6666666.210804.peg.10706	28	2.0	30%
fig|6666666.210804.peg.9423	27	7.8	27%

outsourced as a master's thesis[3]. In brief, it was not possible to obtain any experimental evidence if the sequences were actually encoding ACC oxidases. 3D modeling using PyMol indicates that there is a significant similarity between the predicted and the characterized plant ACC oxidases.

[3]Oliver Sarosi (2017). Fermentative Production of Ethylene by Yeast. Master's thesis

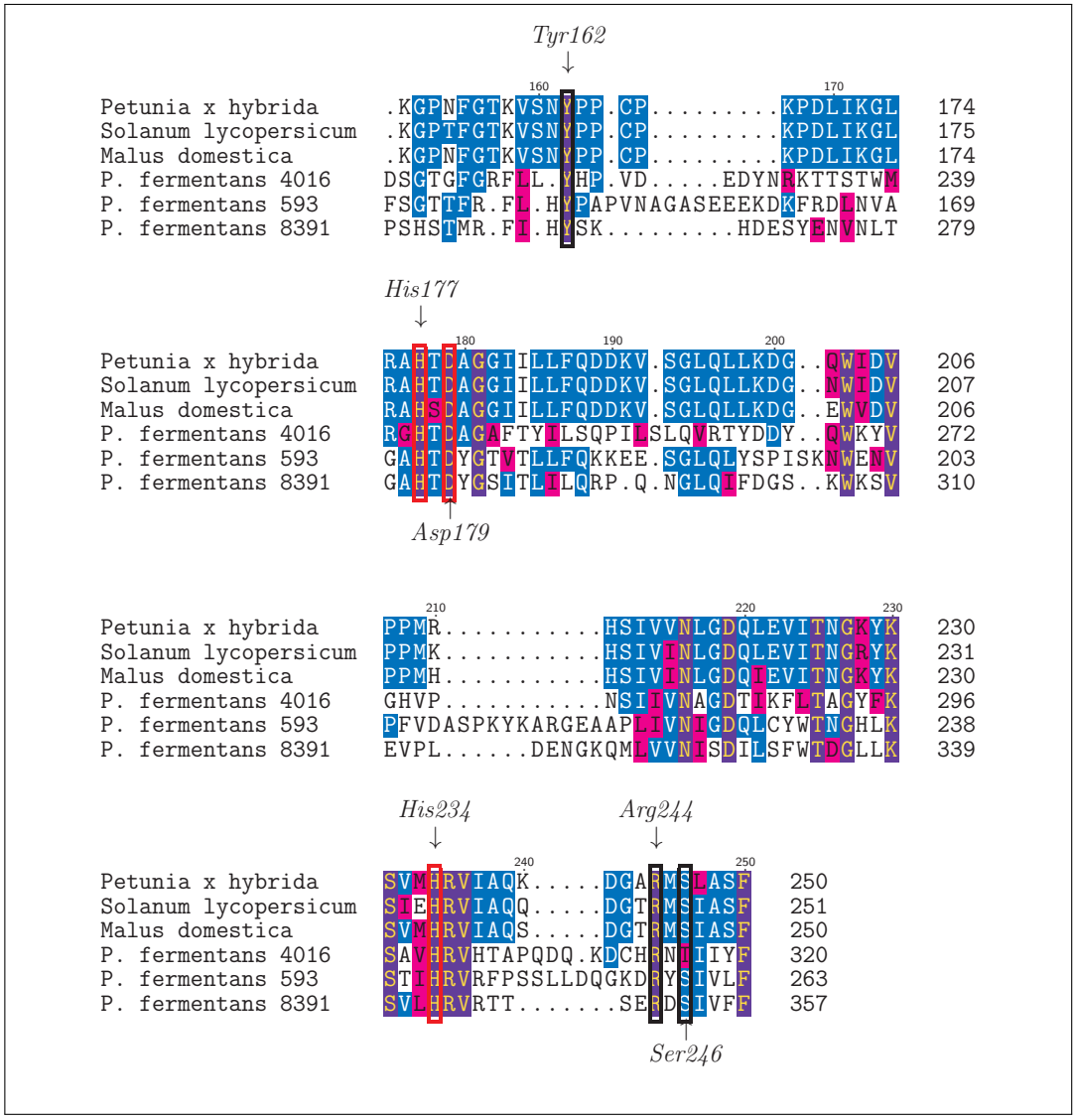

Figure 3.15: ***A multiple sequence alignment of plant and predicted yeast ACC oxidases.*** *ClustalΩ with standard parameters was used to generate this alignment (see* ***Section 2.17.4****). Only the part with the characteristic positions for ACC oxidases is shown. In addition to the ACC oxidase from Malus domestica, the ACC oxidase of Petunia x hybrida was used, as it is the only ACC oxidase with an available 3D structure (PDB database 1WA6). Accession No. from top to bottom are Q08506.1, NP_001233867.2 and CAA74328.1 (Pichia fermentans SR-265 sequences are not annotated, but the number reflects the contigs as listed in* ***Tab. 3.6****). Arrows and red boxes indicate the crucial amino acids for Fe(II) binding (H189, D191, H268) and black boxes for bicarbonate interaction (Y162, R244, S246) in ACC oxidases [46, 82, 227]. The complete sequences are presented in* ***Appendix A.4****.*

3.2.4 Identification and Expression of the Candidate Enzyme "4-Oxalocrotonate Tautomerase" from *Lysinibacillus xylanilyticus* SR-86

3.2.4.1 Peptide Mass Fingerprinting of AEKTA^{TM} fractions

Fraction A3 and A4 (see **Fig. 3.14**) were subjected to peptide mass fingerprinting using HPLC as described in **Section 2.16.8**. Several samples were extracted from the SDS-PAGE gel (see **Fig. 3.16**) and digested by a trypsin digestion, before analyzing the peptide mass.

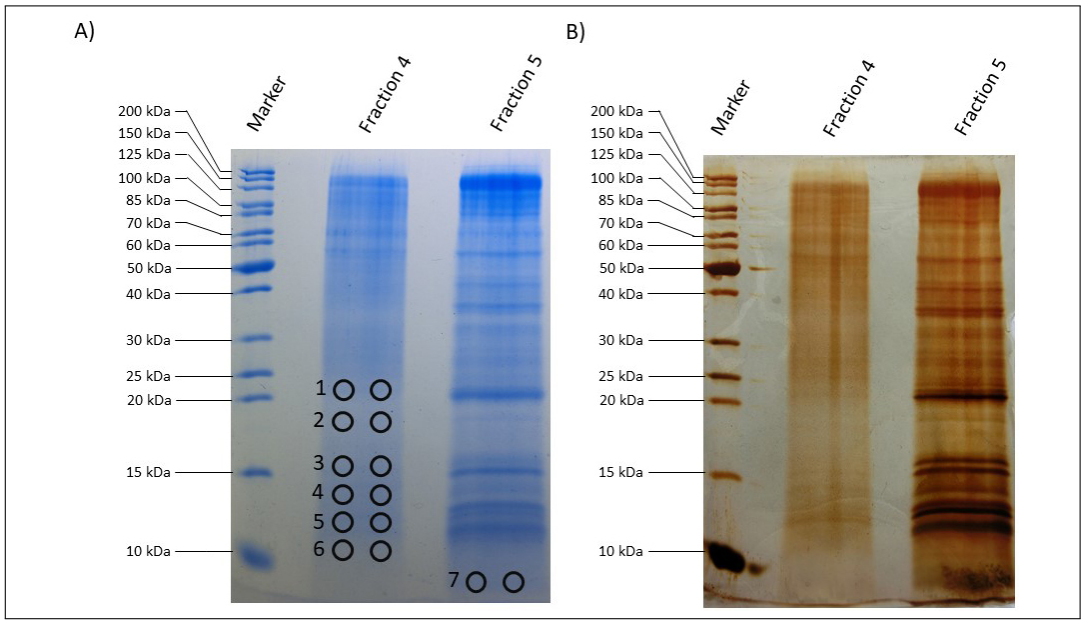

Figure 3.16: ***SDS-PAGE gel of the AEKTA fractions used for peptide mass fingerprinting.*** *A) Coomassie staining and B) silver staining of the AEKTA fraction A3 and A4. Marker: PageRumer^{TM} Unstained Protein Ladder (Thermo Scientific, Waltham, MA, USA). Circles and numbers on A indicate samples taken (two per band).*

After determining the mass of the fragments by HPLC, the results were analyzed with the Mascot database [242]. Among the significant results from all samples (see **Tab. 3.8**), the 4-oxalocrotonate tautomerase was a possible candidate that could produce ethylene.

Table 3.8: *Results of the mass fingerprinting of AEKTA fractions A3 and A4 from Lysinibacillus xylanilyticus SR-86*

Sample	No. 3	No. 7
Query	188	129
Observed	837.4100	994.9100
Mr(expt)	1672.8054	1987.8054
Mr(calc)	1672.8519	1987.9925
Miss	0	0
Expect	0.014	$4.9e-6$
Peptide	K.ELPGSTDLIVTSADQK.T	R.TVNAPAENVTVFIEEMPK.N
Mass (Mr)	118445	6810
emPAI	0.04	0.70
pI (Calculated)	5.45	6.06
Protein	hypothetical protein	4-oxalocrotonate tautomerase

Sample number indicates puncture in SDS-PAGE gel (see **Fig. 3.16**)

3.2.4.2 Expression of the 4-Oxalocrotonate Tautomerase

The 4-oxalocrotonate tautomerase (4-OT) gene of *Lysinibacillus xylanilyticus* SR-86, *Bacillus cereus* SR-772, *Lactobacillus brevis* SR-416 and *E. coli* DH5α was cloned into pET24a vector using restriction enzymes NdeI and XhoI. The fragments were amplified using Phusion DNA polymerase (NEB) with the primers SR-86-4ot-f/SR-86-4ot-r, SR-773-4ot-f/SR-773-4ot-r, SR-416-4ot-f/SR-773-4ot-r and DH5a-4ot-f/DH5a-4ot-r respectively.

The genes were expressed in *E. coli* BL21. An overnight pre-culture was grown in triplicates in 5 mL LB containing 100 µg/mL kanamycin. The main culture, each with 50 mL LB (100 µg/mL kanamycin), was inoculated to an OD_{600} of 0.1 and grown at 37 °C, 170 rpm to an OD_{600} of 0.6. The protein expression was induced by adding IPTG to a concentration of 1 M and grown overnight at 30 °C and 170 rpm. The cells were harvested at 4580 *xg* at 4 °C for 10 minutes in 50 mL tubes. The cells were resuspended in 5 mL 50 mM MOPS buffer at pH 7 and distributed equally in 2 mL reaction tubes. The cells were centrifuged at 21,100 *xg* for 5 minutes at 4 °C. The supernatant was discarded and the cell pellets for each replicate were resuspended to yield an OD_{600} of 20. The cells were disrupted by sonication at cycle 0.5, amplitude 0.8 for 1 minute and 1 minute min on ice three times using a Sonicator UIS250L (Hielscher Ultrasonics GmbH) in 2 mL microreaction tubes. The activity was measured by adding 50 µL ACC 200 mM stock solution to 450 µL protein extract adjusted to 3 mg/mL in a head space vial. After sealing the vial, the reaction mixture was incubated for 16 h at 30 °C and 170 rpm. The

successful overexpression was evaluated by an SDS-PAGE (see **Fig. 3.17**). None of the tested enyzmes showed ethylene formation from ACC.

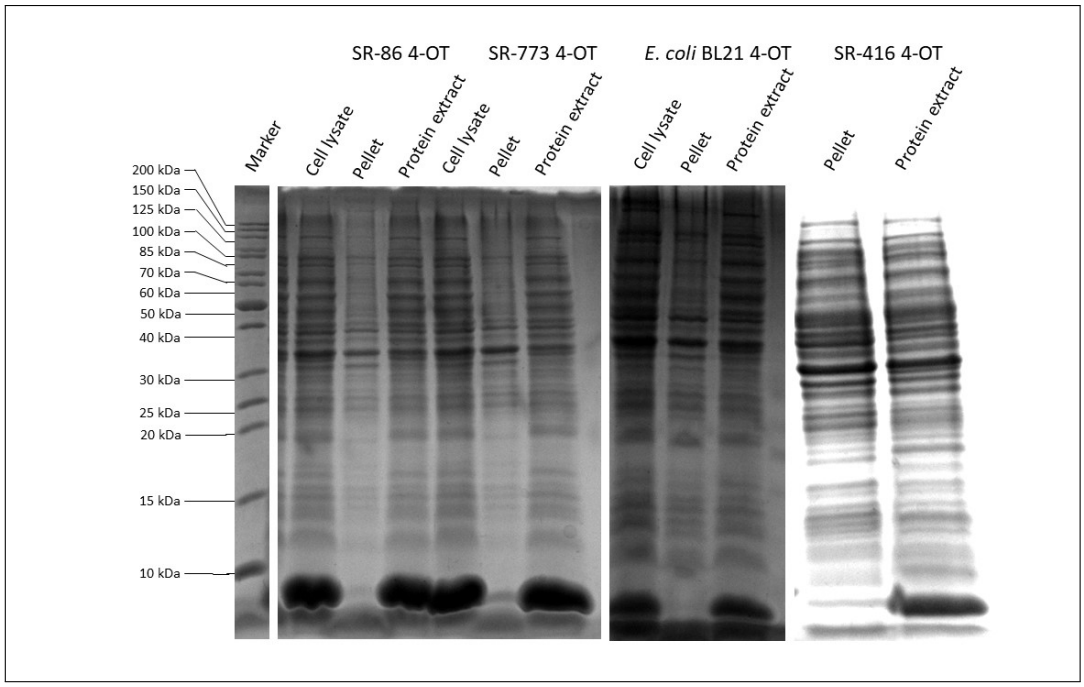

Figure 3.17: ***SDS-PAGE gel of the Expressed 4-OT genes in E. coli BL21.*** *Expression of various 4-OT genes from pET24a in E. coli BL21. Marker: PageRumer™ Unstained Protein Ladder (Thermo Scientific, Waltham, MA, USA).*

3.2.5 Experiments to Determine the Nature of the Ethylene Forming Activity

In order to test if metals are responsible for the effect, 3 mg/mL BSA was tested in 50 mM MOPS at pH 7.0 + 1% trace elements solution with 10 mM ACC and α-ketoglutarate (see **Fig. 3.18**). Ethylene was measured after an incubation of 16 h at 30 °C and 170 rpm in 10 mL GC headspace vials with 500 µL reaction solution.

Buffer with 1% trace elements solution showed ethylene formation of 2.865±0.007 pA*min. The addition of BSA resulted in a decrease to 1.295±0.049 pA*min. When BSA was incubated with proteinase K for 10 minutes before addition of ACC, the ethylene that was formed dropped to 0.060±0.014 pA*min (inactivated proteinase K showed a similar amount formed to BSA with 1.460±0 pA*min).

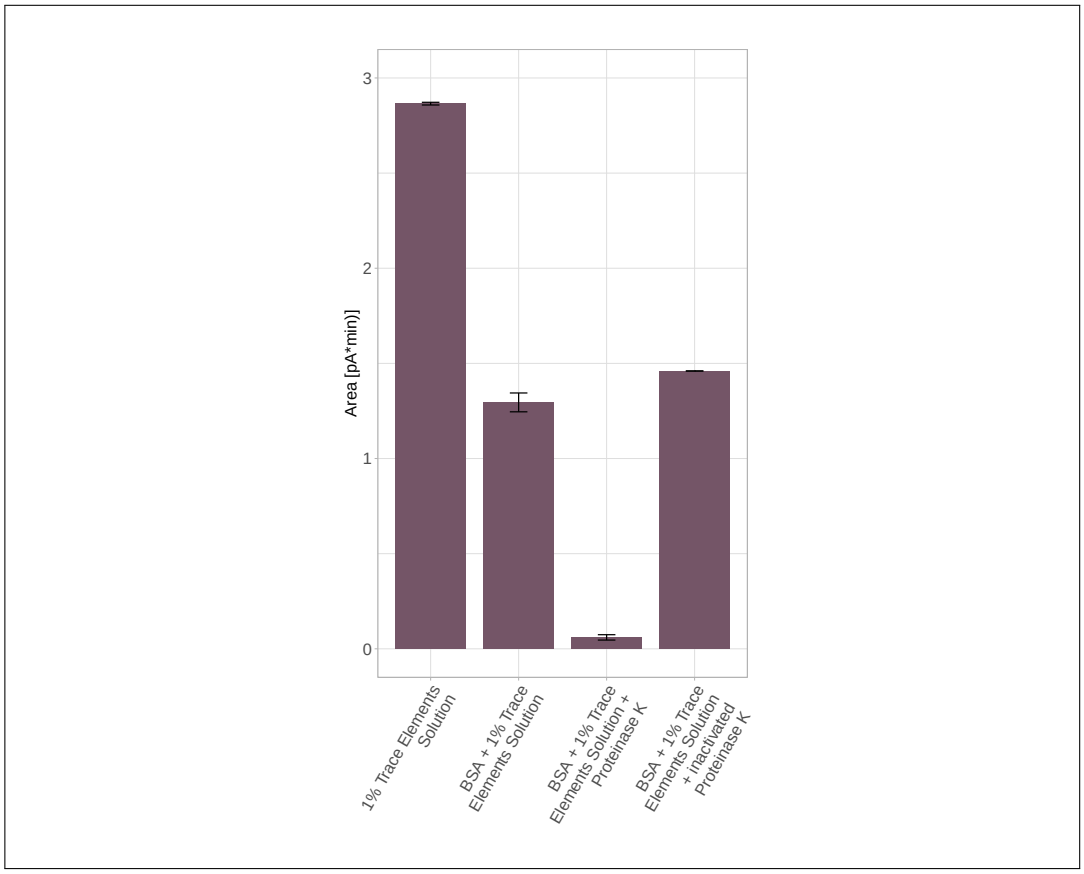

Figure 3.18: ***The effect on protein fragments of ethylene production from 40 mM glycine NaOH + 1% trace elements solution at pH 9.0.*** *3 mg/mL BSA was tested in 40 mM Glycine NaOH at pH 9.0 + 1% trace elements solution with 10 mM ACC incubated for 16 h at 30 °C and 170 rpm in 10 mL GC headspace vials with 500 µL reaction solution and α-ketoglutarate (n=2). The values are given in* ***Tab. A.19****.*

3.2.6 Ethylene Production from KMBA

In order to further characterize the ethylene production from KMBA, various combinations were tested at pH 7 and pH 5. Protein extract (prepared as described in **Section 2.16.1** in 50 mM MOPS at pH 7.0) was diluted 1:1 with 50 mM MOPS at pH 7 for the experiments at pH 7 and with 50 mM acetate buffer at pH 3 to achieve pH 5. The catalase was used at a final activity of 100 U by dissolving 1000 U in buffer and adding 5 µL catalase solution to 450 µL protein extract and 50 µL KMBA + additives. The air in the 10 mL GC headspace vials was replaced with argon by flushing the sealed 10 mL GC headspace vials for 5 minutes after all the components have been added. The

assay was conducted as described in **Section 2.15.2**. *Lysinibacillus xylanilyticus* SR-86 (see **Fig. 3.20**) and *Bacillus cereus* SR-772 (see **Fig. 3.19**) showed higher ethylene production at pH 5. For *Lactobacillus brevis* SR-416 ethylene production was only tested at pH 7 (see **Fig. 3.21**).

The following shows the numbers of the ethylene production according to the protein extract with KMBA.

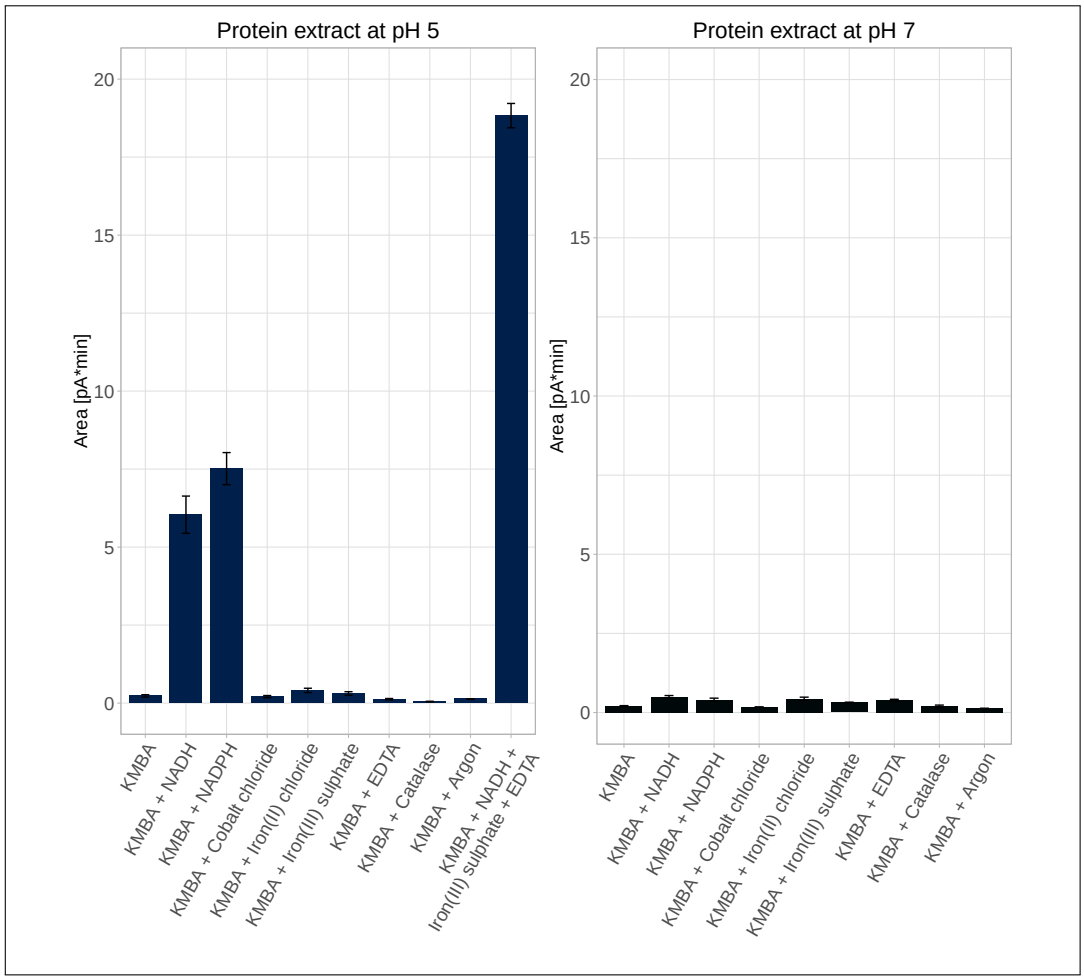

Figure 3.19: ***The ethylene production from KMBA in the protein extract of Bacillus cereus SR-772 at pH 5 and 7.*** *1,250 µg/mL protein extract was used to assess the ethylene produced from KMBA. Sealed 10 mL GC headspace vials were incubated for 16 h at 30 °C and 150–170 rpm (n=3). The values are given in* ***Tab. A.20****.*

The highest amounts of ethylene from *Bacillus cereus* SR-772 protein extract were produced when NADH (2.5-fold) and NADPH (2-fold) were added at pH 7 (see **Fig. 3.19**). Fe(II), Fe(III) and EDTA increased ethylene production. The addition of Co(II) and the replacement of air in the head space with argon resulted in a decrease of the ethylene production, while the addition of the catalase showed no significant effect. The effect of

NADH and NADPH was more significant at pH 5 (**Fig. 3.19**) being 26-fold and 33-fold, respectively. EDTA, the catalase and the replacement of the air in the head space with argon and Co(II) decreased the ethylene production. The strongest effect on the ethylene production from KMBA had the combination of NADH, FeIII and EDTA, by increasing 81-fold.

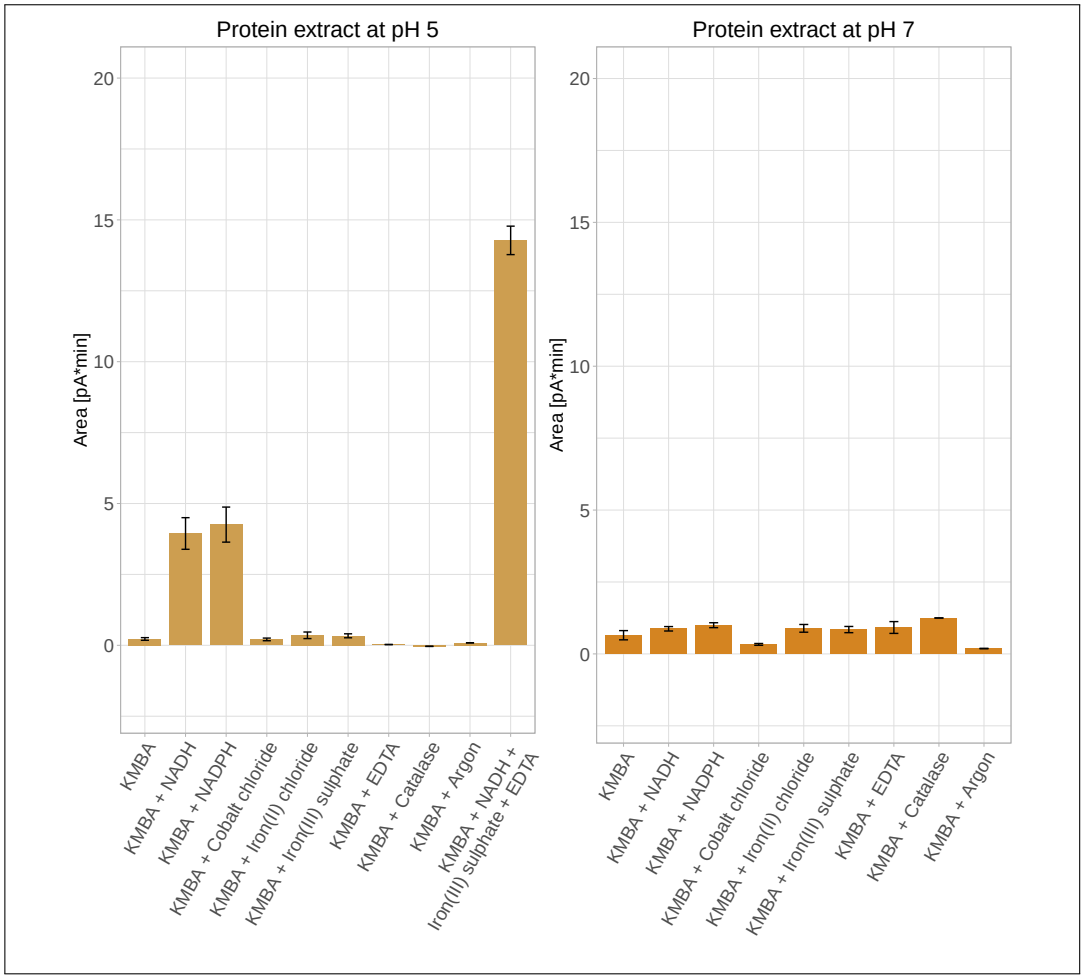

Figure 3.20: ***The ethylene production from KMBA in the protein extract of Lysinibacillus xylanilyticus SR-86 at pH 5 and 7.*** *1,250 µg/mL protein extract was used to assess the ethylene produced from KMBA. Sealed 10 mL GC headspace vials were incubated for 16 h at 30 °C and 150–170 rpm (n=3). The values are given in* ***Tab. A.21****.*

The protein extract of *Lysinibacillus xylanilyticus* SR-86 showed a similar behavior at both pH values. At pH 7, the addition of the catalase, EDTA, FeII, FeIII, NADH and NADPH increased the ethylene produced. For the last two, the ethylene production was 1.3 and 1.5-fold higher, respectively. The ethylene amount decreased when O_2 in the head space was replaced with argon and when Co(II) was added. At pH 5, the ethylene production decreased when the air was replaced with argon in the head space, and

when catalase, Co(II) and EDTA were added. The ethylene amount increased when Fe(II) and Fe(III) were added. Ethylene production increased 18-fold when NADH and 19-fold when NADPH were added. The addition of NADH, Fe(III) and EDTA resulted in the most significant increase by 64-fold.

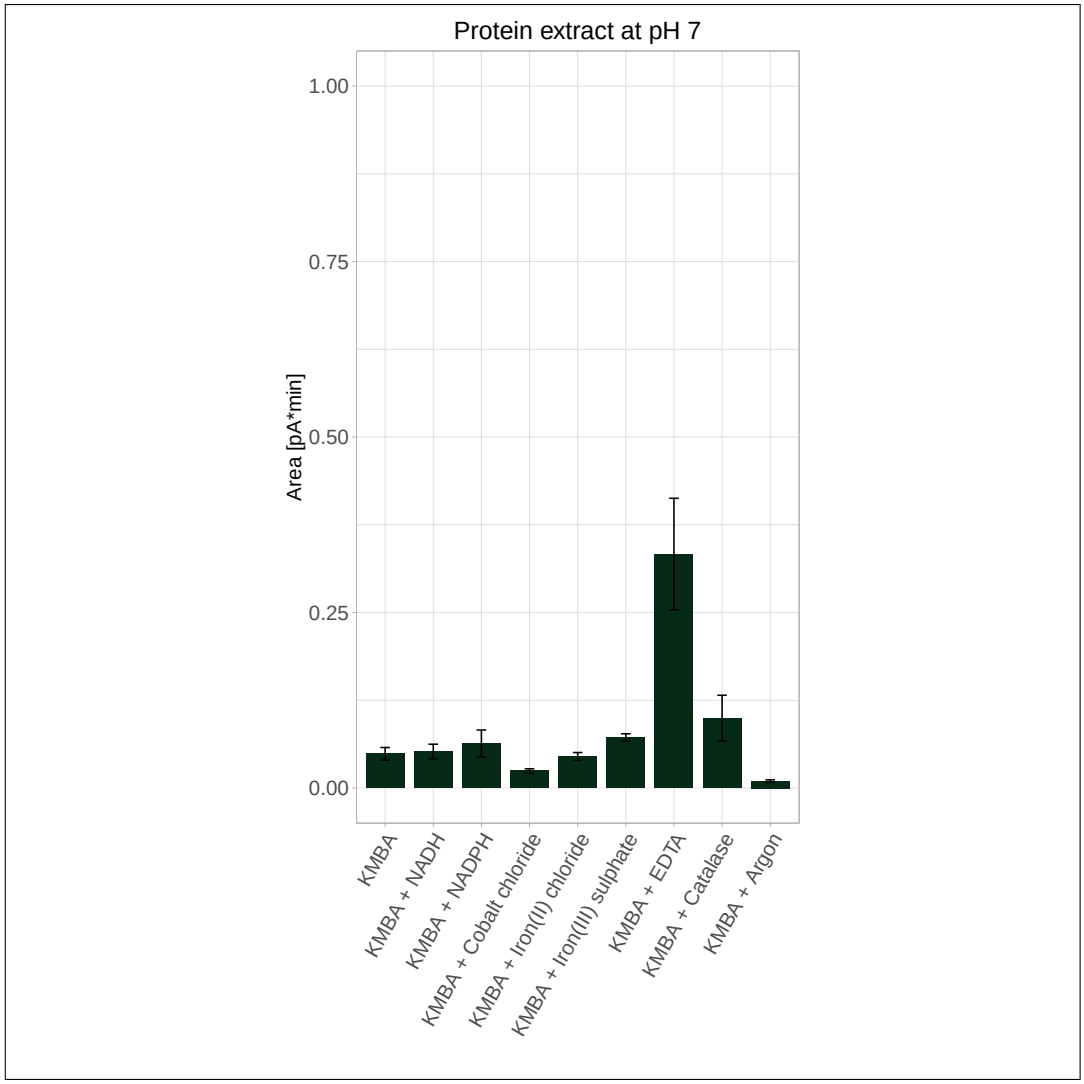

Figure 3.21: ***The ethylene production from KMBA in the protein extract of Lactobacillus brevis SR-416 at pH 5 and 7.*** *375 µg/mL protein extract was used to assess the ethylene produced from KMBA. Sealed 10 mL GC headspace vials were incubated for 16 h at 30 °C and 150-170 rpm (n=3). The values are given in* ***Tab. A.22****.*

Lactobacillus brevis SR-416 produced showed only minor changes at pH 7, except when EDTA was added, which resulted in a 7-fold increase of the ethylene produced. Addition of Ar and Co(II) resulted in a decrease of the ethylene production. The catalase, EDTA, Fe(III), NADH and NADPH slightly increased the ethylene amount.

3.2.6.1 pH-Dependence of Ethylene Production from KMBA

The production of ethylene from KMBA is pH-dependent (see **Fig. 3.22**). The highest values were achieved at pH 3 (4.179±0.570 pA*min) and the lowest at pH 11 (0.033±0.007 pA*min). There seems to be a local minimum of the ethylene production around pH 7 (0.46±0.271 pA*min), which increases slightly when the pH is slightly acidic (0.901±0.308 pA*min at pH 5) or basic (0.554±0.358 pA*min at pH 9) (only shown for *Lysinibacillus xylanilyticus* SR-86).

In total, *Bacillus cereus* SR-772 produced less ethylene (see **Fig. 3.22**). The lowest ethylene amount was at pH 7 with 0.172±0.018 pA*min. Almost equal amounts were produced at pH 5 and 9 with 0.340±0.115 pA*min and 0.371±0.190 pA*min.

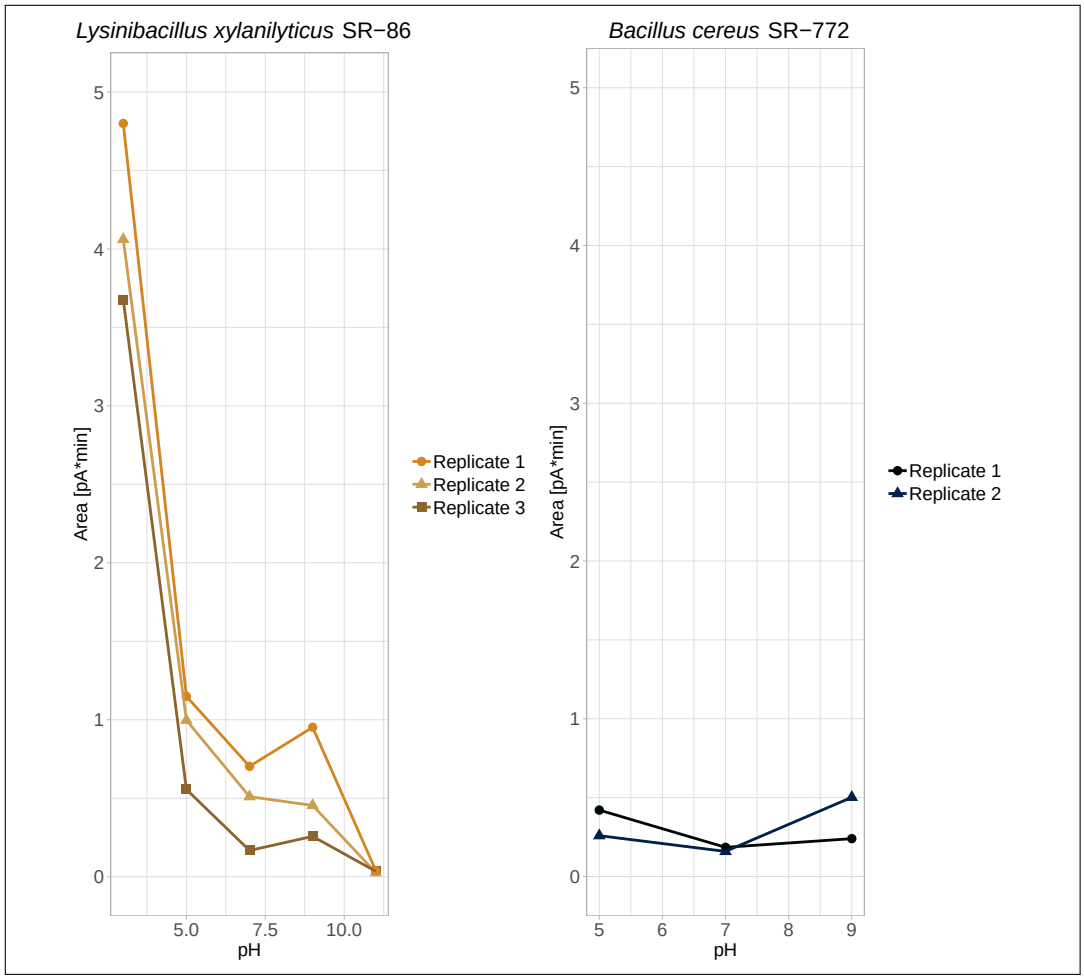

Figure 3.22: ***pH-dependance of the ethylene production from KMBA in Lysinibacillus xylanilyticus SR-86 and Bacillus cereus SR-772.*** *3000 µg/mL protein extract of Bacillus cereus SR-772 and 2500 µg/mL protein extract of Lysinibacillus xylanilyticus SR-86 was used to assess the ethylene produced from KMBA at various pH values. Sealed 10 mL GC headspace vials were incubated for 16 h at 30 °C and 150–170 rpm (n=3). The values are given in* ***Tab. A.23****.*

3.2.6.2 Purification of the Ethylene Producing KMBA-dependent Enzyme

In this section, only enzyme purification attempts regarding *Bacillus cereus* SR-772 are described, as they were the most promising.

3.2.6.2.1 Ammonium Sulphate Precipitation

As a first purification step, an ammonium sulphate precipitation was done in 5% steps (v/w) starting from 5% to 50% $(NH_4)_2SO_4$. The cells were harvested from a three-day culture concentrated to OD_{600} = 100 and disrupted. The protein extract in 20 mM MOPS at pH 7.0 was prepared from *Bacillus cereus* SR-772, as described in **Section 2.16.1**. Solid $(NH_4)_2SO_4$ was pestled to obtain fine powder. The powder was added to 2 mL of the protein extract in a time course of 5 minutes at 5 °C and stirred for 2 h at 4 °C. After the precipitation was completed, the samples were centrifuged. The supernatant was transferred to a 6–8 kDa dialysis tube and the pellet was resuspended in 2 mL 20 mM MOPS at pH 7.0 and also transferred. The tubes were put in 5 L 20 mM MOPS at pH 7.0 for 15 h and the buffer was changed two times. For more information, see **Section 2.16.5**.

After the dialysis was completed, the protein concentration was measured (see **Section 2.16.4**) and the ethylene assay was conducted (see **Section 2.15.2**) with 10 mM KMBA. Sealed 10 mL GC headspace vials were incubated at 30 °C at 150 rpm for 16 h with a total reaction volume of 500 µL.

The protein extract had a specific activity of ethylene of 0.0034 pA*min/µg/mL protein. The highest purification factor was about 2-fold at 30% $(NH_4)_2SO_4$ supernatant of 0.0075 pA*min/µg/mL protein. After this concentration, the major portion of activity was detected in the pellet.

As a next purification step, the separation by hydrophobic interaction was tested.

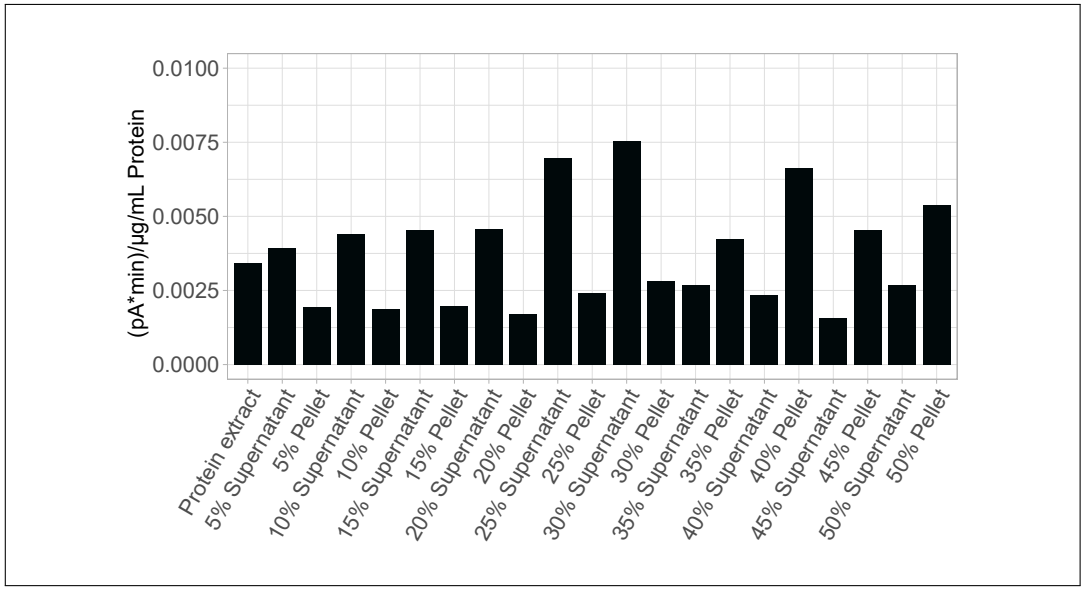

Figure 3.23: ***The ammonium sulphate precipitation of the protein extract of Bacillus cereus SR-772 in 20 mM MOPS at pH 7.0.*** *The appropriate amount of pestled was added to 2 mL protein extract (3000 mug/mL) of Bacillus cereus SR-772 (w/v) and stirred for 2 h at 4 °C. The samples were centrifuged and dialysed in a 6–8 kDa dialysis tube against 3x2,L 20 mM MOPS at pH 7.0. To 450 µL dialysed sample, 50 µL of a 100 mM KMBA-solution was added (final concentration 10 mM). The sealed 10 mL GC headspace vials with 500 µL reaction mixture were incubated at 30 °C at 150 rpm for 16 h (n=1). The values are given in* ***Tab. A.24****.*

3.2.6.2.2 AEKTA™

Several columns were tested at various pH values. None were sufficient to bind the enzyme. As the ammonium sulphate precipitation showed good results (see above), hydrophobic interaction columns (HIC) were favored in the next purification step. The ammonium sulphate precipitation was carried out as mentioned in the above paragraph at a larger volume and 20% $(NH_4)_2SO_4$ (w/v). The supernatant was injected onto the columns using the AEKTA™ system (the method is described in **Section 2.16.6**).

Three columns were tested in order to further purify the KMBA-dependent enzyme after the ammonium sulphate precipitation step. The cells were harvested from a three-day culture of *Bacillus cereus* SR-772 in ST1GSE (see **Section 2.16.1**) and concentrated to OD_{600} = 100. As the KMBA-dependent enzyme started to precipitate around 30% $(NH_4)_2SO_4$ (v/w), 20% were chosen with a margin that allowed naturally occurring variations. The columns were prepared and operated as described in **Section 2.16.6** and ethylene was assayed as described in **Section 2.15.2**.

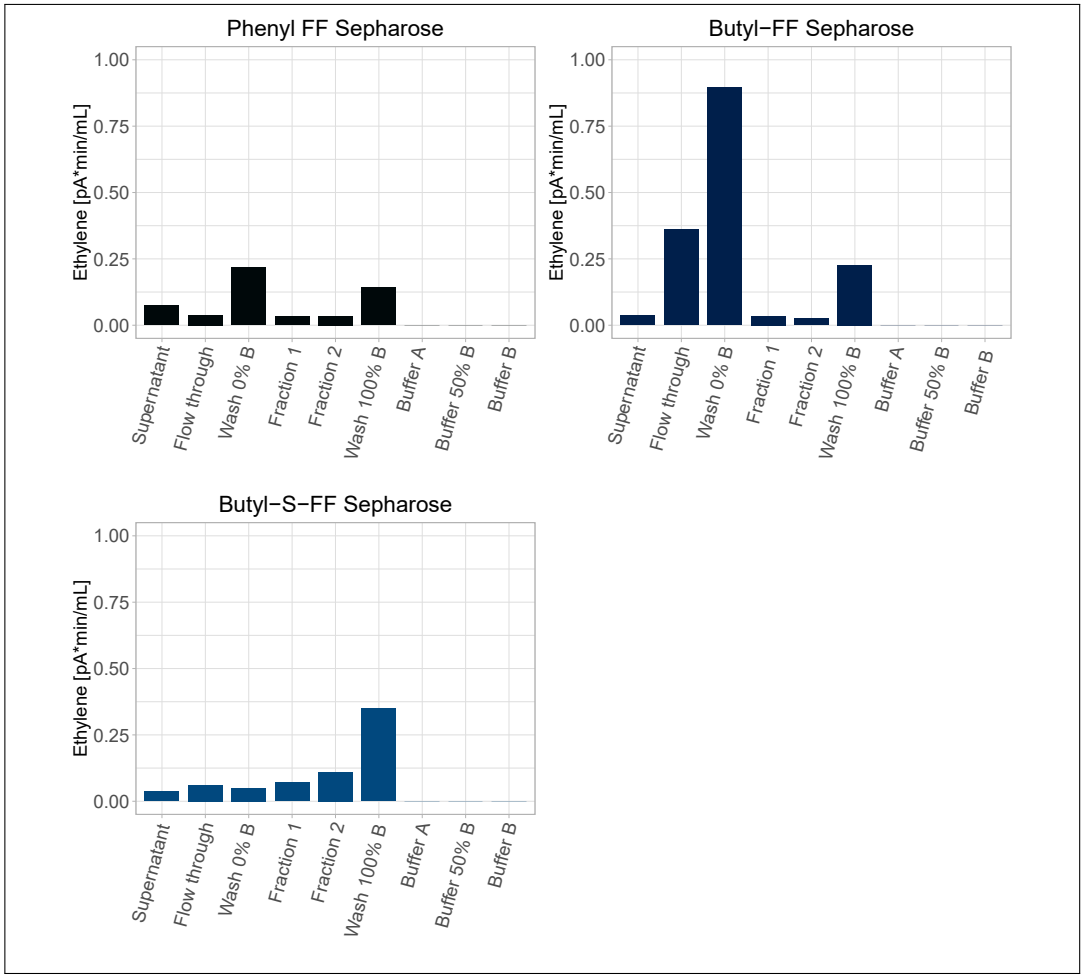

Figure 3.24: ***The protein purification of the KMBA-dependent enzyme from the protein extract of Bacillus cereus SR-772 after ammonium sulphate precipitation using various HIC columns.*** *The protein extract from Bacillus cereus SR-772 in 20 mM MOPS at pH 7.0 was treated with 20% $(NH_4)_2SO_4$ (v/w). The supernatant was injected onto three different HIC columns using AEKTA^{TM}. The ethylene amount was measured by detecting ethylene in sealed 10 mL GC headspace vials with 500 µL reaction mixture (450 µL fraction + 50 µL substrate). The samples were incubated at 30 °C at 150 rpm for 16 h (n=1). The area was normalized using the volume of the fractions. The values are given in* ***Tab. A.25****.*

It seems that the enzyme was bound too tightly on the phenyl ff sepharose column, as ethylene was found in the 100% B wash fraction and 0% B wash fraction. The most promising column was the butyl-S-FF sepharose (the weakest HIC column from the ones tested), to which the activity still bound tightly, but could be eluted. In the next steps, the $(NH_4)_2SO_4$ concentration was varied in order to find the optimal parameters to which the activity is bound on butyl-S-FF sepharose.

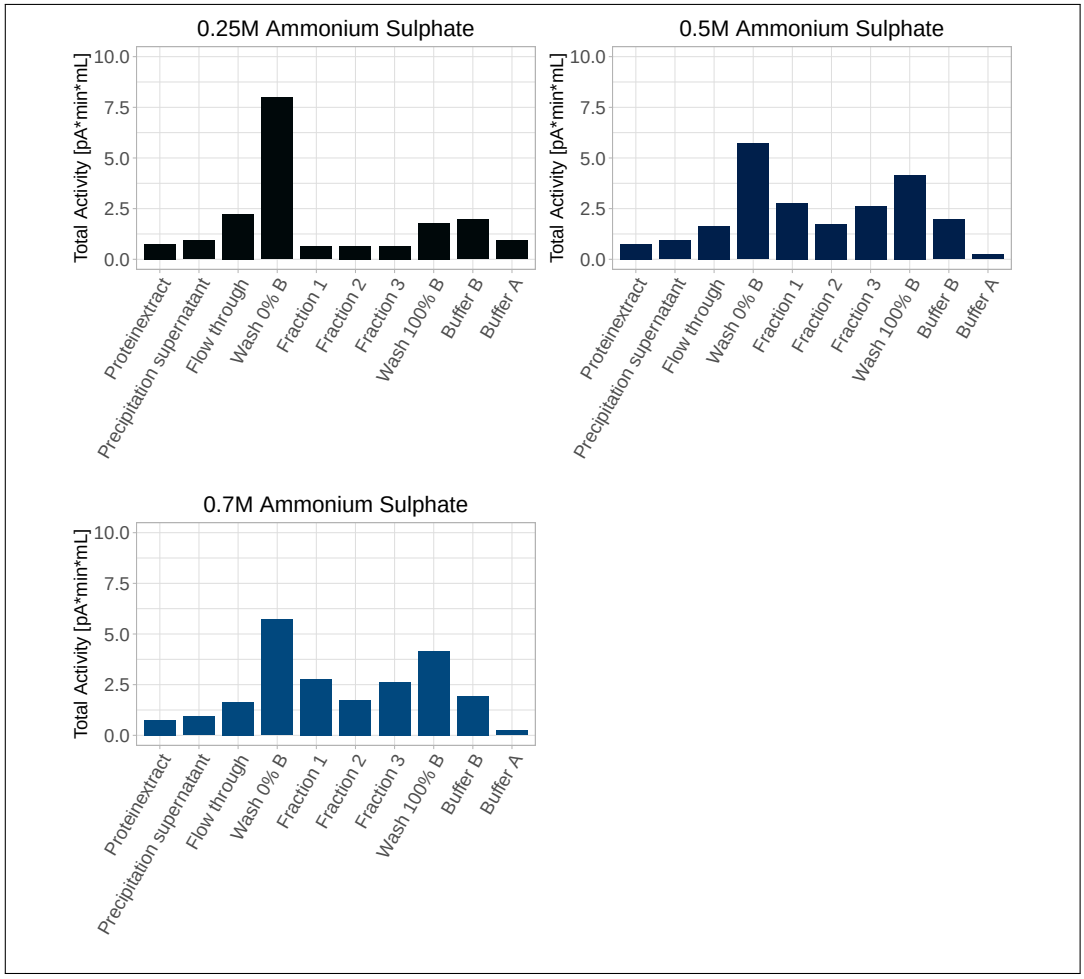

Figure 3.25: ***The HIC-purification of the KMBA-dependent enzyme of Bacillus cereus SR-772 with various $(NH_4)_2SO_4$-concentations using Butyl-S FF.*** *The protein extract from Bacillus cereus SR-772 was treated with various concentrations of $(NH_4)_2SO_4$. The supernatant was injected onto a Butyl-S FF Sepharose column. The ethylene amount was measured by detecting ethylene in sealed 10 mL GC headspace vials with 500 µL reaction mixture (450 µL fraction + 50 µL substrate). The samples were incubated at 30 °C at 150 rpm for 16 h (n=1). The values are given in* ***Tab. A.26****.*

To optimize binding conditions, various concentrations of $(NH_4)_2SO_4$ were added to the protein extract. When treated with 0.25 M $(NH_4)_2SO_4$, most of the activity was found in the washing fraction with the binding buffer (Wash 0% B), which means that the protein did not bind to the column matrix (see **Fig. 3.25**). Increasing the $(NH_4)_2SO_4$ concentration to 0.5 M showed better binding of the protein, but not satisfyingly enough. No significant improvement was achieved by increasing the $(NH_4)_2SO_4$ concentration to 0.7 M. Further increase of the $(NH_4)_2SO_4$ concentration was not tested, as most of the activity was found in the pellet at concentrations greater than 0.7 M (about 30% (w/v)) (see **Fig. 3.23**).

3.3 Isoprene

3.3.1 Enhancing Isoprene Production in *Pichia pastoris* X-33

3.3.1.1 Cloning of the Kudzu Isoprene Synthase into the Integrative Expression Vector pPICZ B

The sequence for all primers used for cloning and sequencing are given in **Tab. 2.5**. In order to establish isoprene production in *Pichia pastoris* X-33 (Thermo Fisher Scientific, Waltham, MA, USA), the isoprene synthase gene from *Pueraria montana* (Kudzu) was cloned into the integrative expression vector pPICZ B (Thermo Fisher Scientific, Waltham, MA, USA).

The isoprene synthase from *Pueraria montana* pBA2kIKmA2 was received as a plasmid from Anastasios Melis (Addgene #39213). It was modified by deleting the chloroplast transit seqeuence [188]. The isoprene synthase (hereafter abbreviated kIspS) was amplified using the primers kIspS-pPICZB-EcoRI and kIspS-pPICZB-KpnI with the restriction sites EcoRI and KpnI-HF, respectively. In the PCR mix for Phusion (see **Tab. 2.45**), 1 µL of purified pBA2kIKmA2 with 422 ng/µL was added to 40 µL of the reaction mixture. The program was used as described in **Tab. 2.47**, with an annealing temperature of 60 °C and an elongation time of 1 minute. The vector pPICZ B was purified from *E. coli* XL10, as described in **Section 2.14.6.5**. 1 µg kIspS and the vector were digested using KpnI-HF and EcoRI in CutSmart buffer for 1 h at 37 °C and inactivated for 20 minutes at 60 °C. The fragments were then separated by electrophoresis (see **Section 2.14.5**), the bands were cut out and purified as described in **Section 2.14.6.6**. Both fragments were ligated using T4 DNA ligase (NEB) at 16 °C overnight with 40 ng of cut vector DNA and kIspS in 3x excess. *E. coli* DH5α was transformed with 5 µL of the ligation using the chemical compentent cells method (see **Paragraph 2.14.6.10.2**). The cells were spread on LB-low agar plates containing 100 µg/mL Zeocin™ (Thermo Fisher Scientific) and incubated at 37 °C overnight. After colonies were formed, they were screened for the integrated insert using restriction digestion of purified plasmid (see **Paragraph 2.14.6.11.3**). Colonies were picked and cultivated in 10 mL LB-low with Zeocin (100 µg/µL) at 37 °C at 150 rpm overnight. Plasmids were purified as described in **Section 2.14.6.5** and digested in a 10 µL reaction using KpnI-HF (NEB) and EcoRI (NEB) (see **Paragraph 2.14.6.11.3**). Positive clones were selected for DNA sequencing (see **Section 2.14.8**) using the primers ColPCR-pPICZB-f and ColPCR-pPICZB-r. One clone with the correct sequence was used for the transformation of *Pichia pastoris* X-33.

In order to transform *Pichia pastoris* X-33, the plasmid needs to be linearized [310]. Hence, 6 µg of plasmid DNA was linearized using PmeI (NEB) in a 40 µL reaction with

CutSmart buffer at 37 °C for 105 minutes and inactivated for 25 minutes at 65 °C. The DNA was then concentrated using the butanol precipitation protocol (see **Section 2.14.3**) to 270 ng/ µL (pPICZB-kIspS) and 300 ng/ µL (pPICZB). The competent *Pichia pastoris* X-33 cells were generated as described in **Section 2.14.6.4** and transformed with 5 µL pPICZB-kIspS and pPICZB as described in **Paragraph 2.14.6.10.3** and spread on YPDS agar plates containing 100 µg/ mL Zeocin™. Five positive clones were identified (see **Paragraph 2.14.6.11.2**) and used for the activity measurement in the following section.

3.3.1.1.1 Activity Assay for Isoprene Using Gas Chromatography

The activity was measured in minimal medium with 1% methanol (see **Tab. 2.21**) as sole carbon source and inducer. As a comparison, the activity was also measured on YPD after 24 h. The gas chromatographic measurement and the calibration of the isoprene concentration is described in **Section 2.15.1**.

The *Pichia pastoris* X-33 strains were inoculated in 2 mL glucose minimal medium containing 2% glucose in deep well plates to an OD_{600} of 0.1 and were incubated at 30 °C and 900 rpm for 24 h. The main culture was inoculated to an OD_{600} = 0.1 in a final volume of 2 mL. All media tested contained methanol at an end concentration of 0.5% and were incubated at 30 °C for 1–6 days at 150 rpm. The OD_{600} was measured after the isoprene production was assessed. The transformant X-33 pPICZB-kIspS #2 and #4 showed little isoprene production of 0.011 pA*min and 0.004 pA*min at an OD_{600} of 1.7 and 1.0 on methanol minimal medium (see **Fig. 3.26** a). No isoprene production was detected after 1 day on either YPD or methanol minimal medium, both induced with 1% methanol (see **Fig. 3.26** b)

The clones were also tested in buffered minimal medium (see **Tab. 2.22**), as there might have been be a higher activity [310]. The clones were grown in 800 µL buffered minimal medium at 900 rpm, 30 °C for 72 h. 500 µL of the culture were transferred into a 10 mL GC vial, 5 µL methanol was added and the vials were sealed. The tested clones showed very little isoprene production after 18 h. (see **Fig. 3.27**). The OD_{600} was measured as described under **Section 2.13.2** with 10 µL cells diluted with 290 µL PBS. Only transformant #2 showed in two of three replicates an isoprene production of maximum 0.005 pA*min and 0.004 pA*min isoprene. The transformants with the empty vector as well as the wildtype showed no production of isoprene.

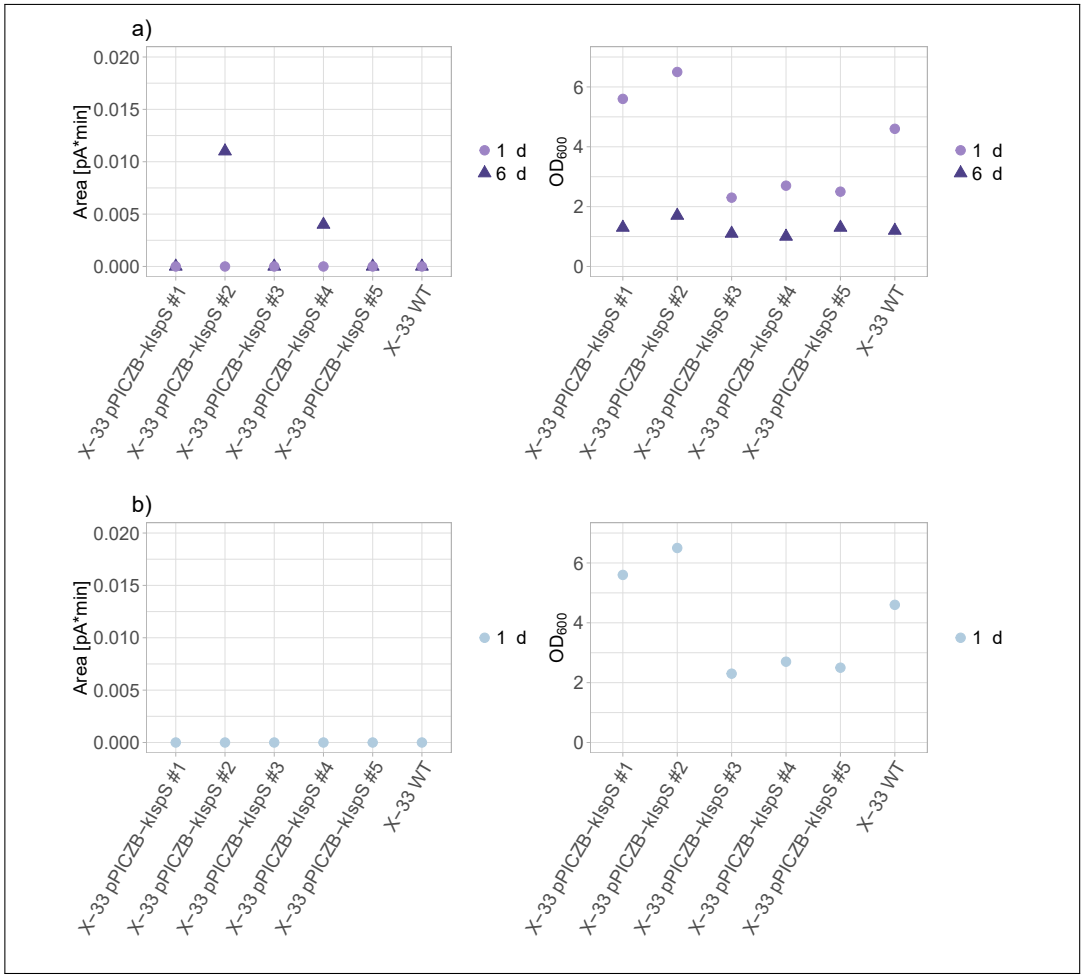

Figure 3.26: ***The isoprene production of Pichia pastoris X-33 on YPD and methanol minimal medium.*** *A) The isoprene production of X-33 transformants on methanol minimal medium after 1 day and 6 days. B) OD_{600} of Pichia pastoris X-33 transformants on methanol minimal medium after two time points. C) T isoprene production of X-33 transformands on 0.5% methanol YPD after 1 day. D) OD_{600} X-33 transformands on 0.5% methanol YPD after 1 day. The cultures were incubated in 10 mL GC headspace vials at 30 °C at 150 rpm containing 2 mL medium. A 1 mL sample of the headspace was withdrawn and analyzyed for the isoprene production. Red circle: 1 day, turquoise triangle: 6 days, X-33: Pichia pastoris X-33, WT: wild type, X-33 pPICZB-klspS #1: X-33 with integrated klspS and its number. The values are given in* ***Tab. A.27****.*

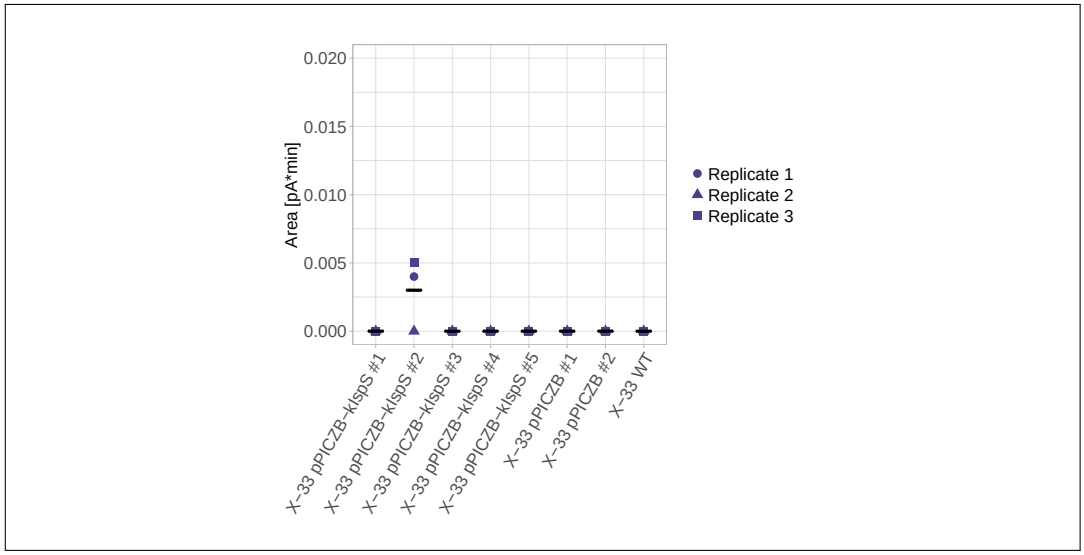

Figure 3.27: ***The isoprene production of Pichia pastoris X-33 klspS after six days on buffered methanol minimal medium.*** *The color of the circles indicates the replicate, the bar represents the mean. A) Isoprene production. The cultures were incubated in 10 mL GC headspace vials at 30 °C at 150 rpm containing 0.5 mL pre-culture and 1% methanol freshly added for 18 h. A 1 mL sample of the headspace was withdrawn and analyzed for isoprene production using gas chromatography. B) The growth of the Pichia pastoris X-33 pre-cultures in the buffered minimal medium.* OD_{600} *was measured after 24 h. The values are given in* ***Tab. A.28****.*

3.3.2 Enhancing Isoprene Production in *Cupriavidus necator*

The overall strategy to enhance the isoprene production in *Cupriavidus necator* was, firstly, to find vectors, which could be used for heterologous gene expression in *Cupriavidus necator*, and, secondly, overexpressing genes involved in isoprene production. In order to achieve this, a literature search was made for plasmids capable of expression in *C. necator* and cross-checked with the existing institute's plasmid collection. The strategy was as follows: once a suitable vector was found and the isoprene production was enhanced, the expressing construct from the vector was transferred to pKnock-Cm, an integrative plasmid, to construct a *C. necator* strain for the isoprene production.

3.3.2.1 Selecting an Expression Vector Using GFP/eGFP

As shown in **Tab. 3.9**, it is evident that the department's plasmid collection had a good variety of host-range vectors at its disposal that were based on the same vector backbones and were used to start tests for protein expression. For all other plasmids, *gfp* or *egfp* was cloned into the vector to evaluate the expression strength (see **Paragraph 3.3.2.1.2**).

Table 3.9: *Vectors for C. necator described in the literature and available at the laboratory's plasmid collection*

Name	Relevant Elements	Resistance	Reference
pME6032	P_{tac}	Tetracycline	[128]
pKnock-Cm	none	Chloramphenicol	[1]
pJeMTcR	P_{rha}	Tetracycline	[297]
pMS137	P_{chnB}	Kanamycin	[292]
pBBR1MCS-3	P_{lac}	Tetracycline	[168]

3.3.2.1.1 Antibiogram

In order to investigate which plasmid is possible to use, an antibiogram was performed (see **Section 2.13.4**).

For *C. necator* DSM531, 100 µL of an overnight culture in DSMZ-1 medium were spread on DSMZ-1 medium agar plates containing filter paper with 5 µL antibiotic solution. The plates were incubated for 3 days at 30 °C (see **Fig. 3.29**). For *C. necator* DSM428, 100 µL of an overnight culture in NR medium were spread on a gradient NR agar plate containing antibiotics and incubated for two days at 30 °C (see **Fig. 3.28**). For *C. necator* DSM531 the antibiotics carbenicillin, gentamycin, ampicillin and neomycinsulfate showed no inhibitory effect on growth. Kanamycin at 0.5 mg/mL, tetracyclin at 62.5 µg/mL and chloramphenicol at 0.05 mg/mL showed inhibition of growth. *C. necator* DSM428 is sensitive to 0.5 mg/mL kanamycin, 1 µg/mL tetracycline, 125 µg/mL and 42.5 µg/mL chloramphenicol.

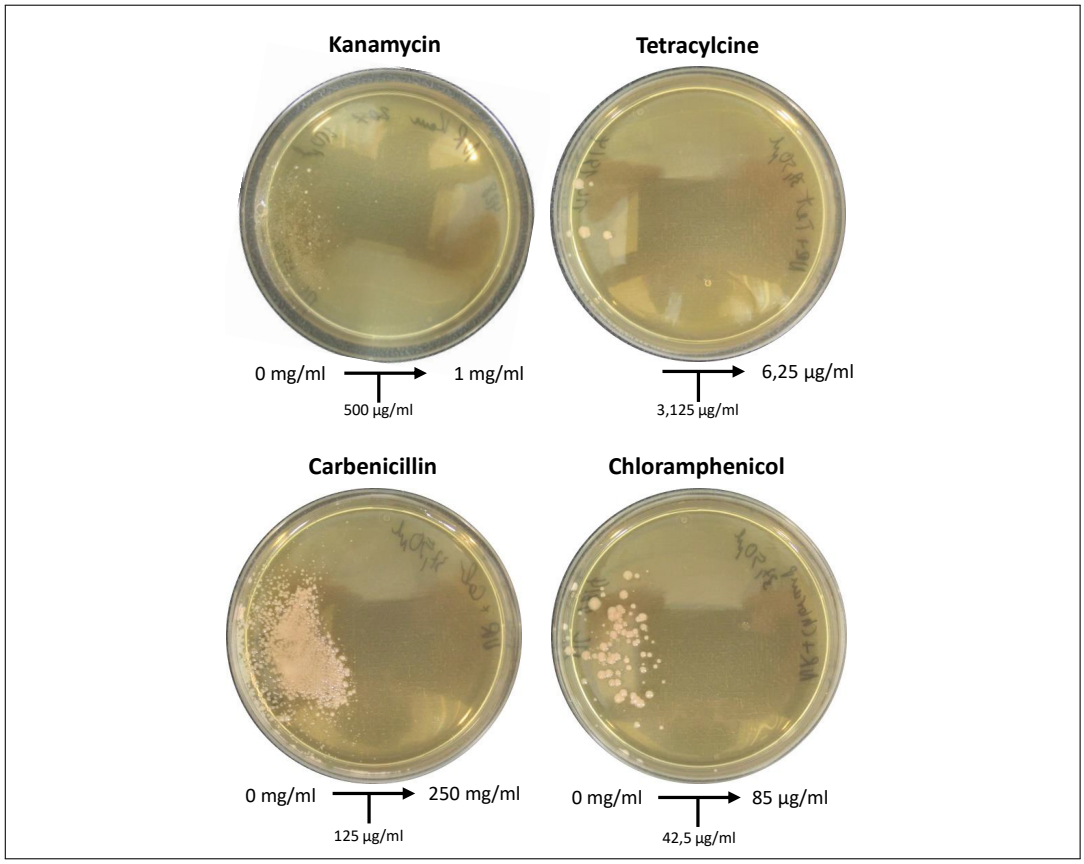

Figure 3.28: ***Antibiogram of C. necator DSM428 on gradient plates.*** *Gradient plates were incubated for two days at 30 °C. The appropriate antibiotoc concentration was estimated from the growth zone.*

Both strains were incubated on medium containing the lowest antibiotic concentration which produced a zone of inhibition to verify, if the antibiogram was reliable. Additionally, they were spread on agar plates containing the standard dilution in the laboratory, which is 1:1000 of the stock solution's concentration. Based on these results, when transformed with plasmids carrying corresponding markers, *C. necator* DSM531 and DSM428 were incubated on a medium containing 12.5 µg/ mL tetracycline, 500 µg/ mL kanamycine or 34 µg/ mL chloramphenicol.

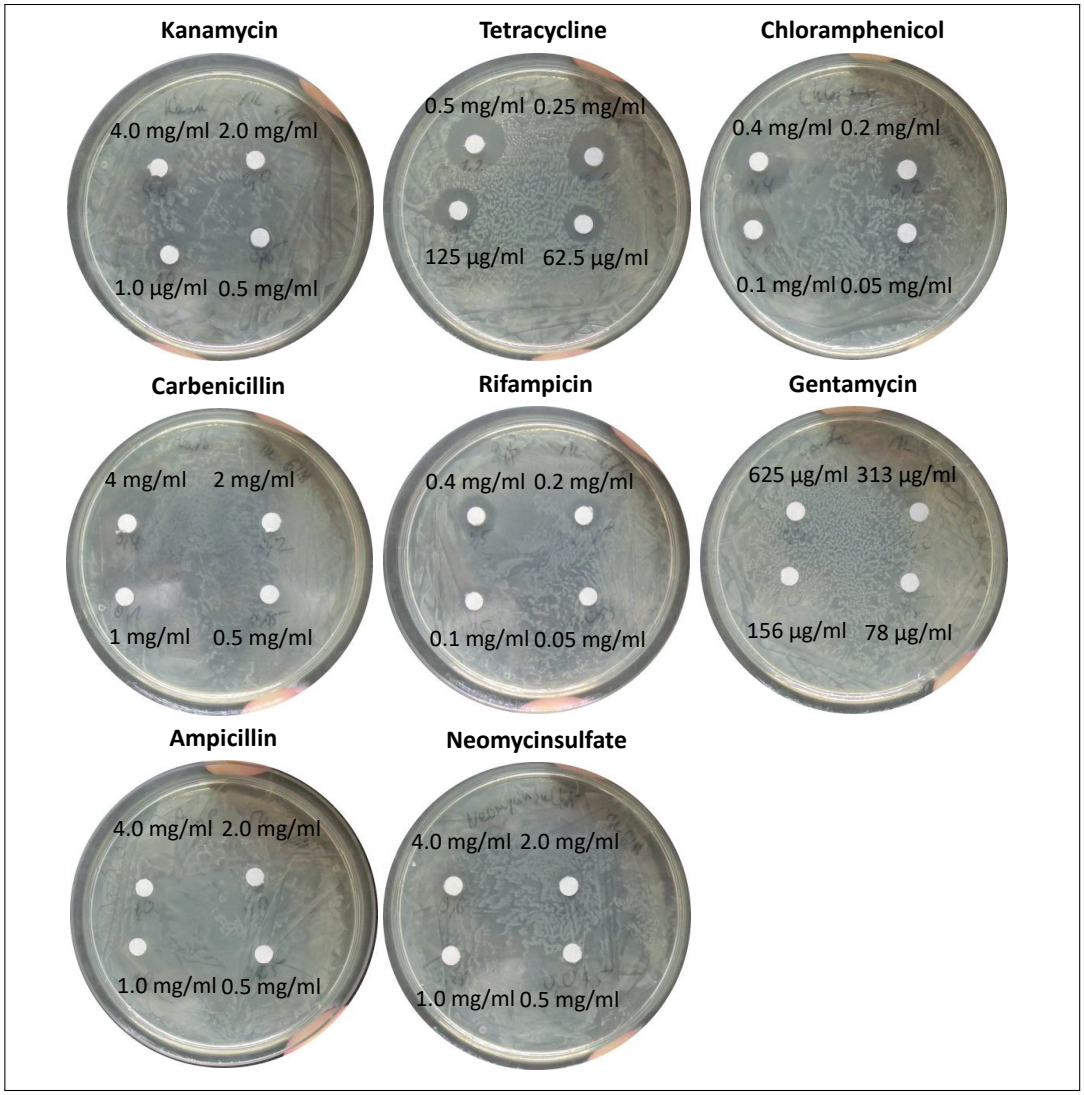

Figure 3.29: ***Antibiogram of C. necator DSM428 on DSMZ-1 agar medium.*** *Agar plates were incubated for three days at 30° C. The appropriate antibiotoc concentration was estimated from the growth zone.*

3.3.2.1.2 Cloning and Expression of *gfp* and *egfp*

For all plasmids listed in **Tab. 3.9**, *gfp* or *egfp* was cloned into the vectors in order to have a fast, reliable and easy reporter to evaluate the expression. The cloning is described in **Section 2.14.6.12**.

The *gfp* gene was cloned into pME6032 by amplifying the gen using Phusion-HF with primers gfp-pME6032-f and gfp-pME6032-r from pUC-gfp by the EcoRI and KpnI sites. It was not possible to detect any increase in fluorescence.

The expression of pJeM1TcR was tested in *C. necator* DSM531 and DSM428 as well as in *E. coli* BL21 (DE3) (see **Fig. 3.30**). In all three microorganisms, pJeM1TcR carrying *egfp* showed fluorescence when induced and little when not induced. With induction *E. coli* BL21 showed the highest signal of 606±42 induced. Both *Cupriavidus necator* strains showed less fluorescence as *E. coli* BL21 with 58±7 for *C. necator* DSM428 and 12±4 for *C. necator* DSM531 induced. The expression in *C. necator* DSM428 is higher than in *C. necator* DSM531, but the expression from *efgp* works in both strains.

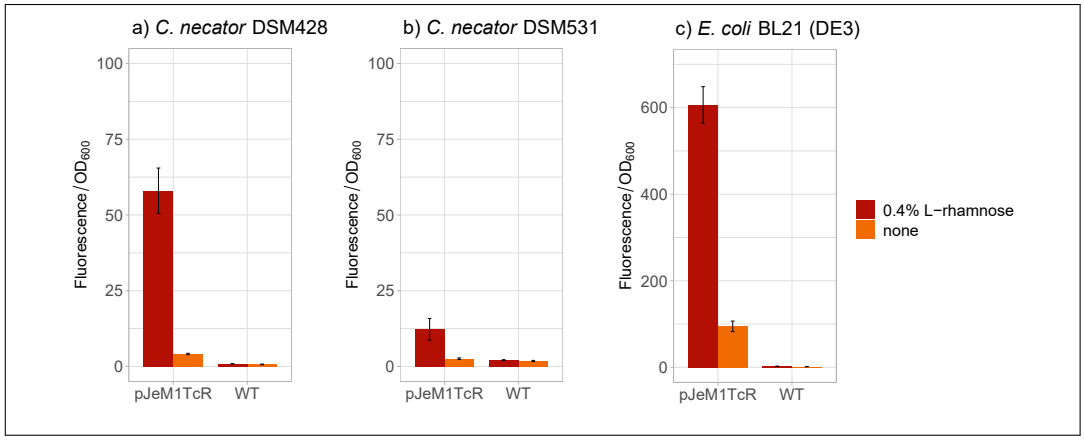

Figure 3.30: ***The expression of egfp from the plasmid pJeM1TcR in C. necator DSM531, 428 and E. coli BL21.*** *egfp expression is given as arbitrary unit. a) C. necator DSM428 b) C. necator DSM531 c) E. coli BL21. Note the axis of E. coli BL21 being 6 times higher. All cultures were inocculated from an overnight culture of 1 mL LB incubated at 30 °C at 800 rpm. 10 µL of the pre-culture were inoculated in 1 mL LB. If induced, L-rhamnose was added to a concentration of 0.4% from a 40% stock solution. If necessary, tetracycline was added to a final concentration of 12.5 µg/µL. The culture was incubated at 30 °C, 800 rpm overnight in 96 deep well plates. The fluorescence was measured with 485 nm excitation wavelength and detected at 535 nm (see* ***Section 2.16.2****). The values are given in* ***Tab. A.31****.*

The expression of eGFP from the plasmid pBBR1MCS-3, bearing the *lac* promoter showed only fluorescence when expressed in *E. coli* BL21 but not in either *Cupriavidus necator* strains (see **Fig. 3.31**), although a slight increase in fluorescence was present compared to the wildtype and the vector without insert. *E. coli* BL21 (see **Fig. 3.31** c) with and without pBBR1MCS-3 showed little fluorescence ranging from 1.7 to 2.5. The

uninduced strain bearing *egfp* showed fluorescence of 45.5±3.2 with induction showing a maximum of 55.9±3.2 at 1 mM. The fluorescence decreased slightly when induced with higher concentrations of IPTG to 50.1±2.6 with 10 mM IPTG. *C. necator* DSM531 (**Fig. 3.31** b) showed no significant increase in fluorescence with values ranging from 1.2±0.1 to 2.4±0.4. *C. necator* DSM428 (see (see **Fig. 3.31**) a) showed no significant increase in fluorescence with values ranging from 0.9±0.1 to 1.8±0.3 for the three strains.

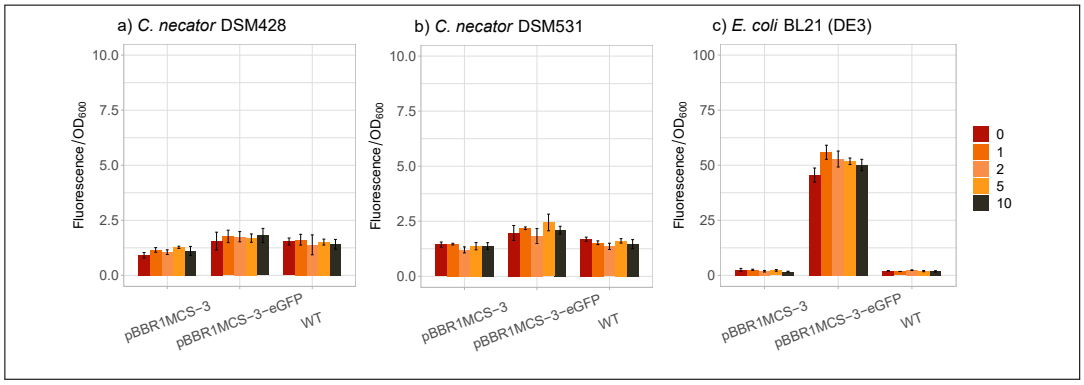

Figure 3.31: ***The expression of egfp from the plasmid pBBR1MCS-3 in C. necator DSM531 and 428 and E. coli BL21.*** *egfp expression is given as arbitrary unit. a) C. necator DSM428 b) C. necator DSM531 c) E. coli BL21. Note the axis of E. coli BL21 being 10 times higher. All cultures were inoculated from an overnight culture of 1 mL LB incubated at 30 °C at 800 rpm. 10 µL of the pre-culture were inoculated in 1 mL LB. If induced, IPTG was added to a concentration from a 1 M stock solution. If necessary, tetracycline was added to a final concentration of 12.5 µg/µL. The culture was incubated at 30 °C, 800 rpm overnight in 96 deep well plates. The fluorescence was measured with 485 nm excitation wavelength and detected at 535 nm (see* ***Section 2.16.2****). The values are given in* ***Tab. A.32****.*

The plasmid pMS137-eGFP was tested in *E. coli* BL21, *C. necator* DSM428 and additionally in *E. coli* DH5α. It was not possible to express *egfp* in either *E. coli* strain, but it was possible to detect it in *C. necator* DSM428 (see **Fig. 3.32**). Here, the uninduced expression resulted in a fluorescence per OD_{600} of 165±13 and was 1.45-fold higher when induced with 1 mM cyclohexanone with 239±10. The wildtype strain showed little fluoresence between 0.9 and 1.6.

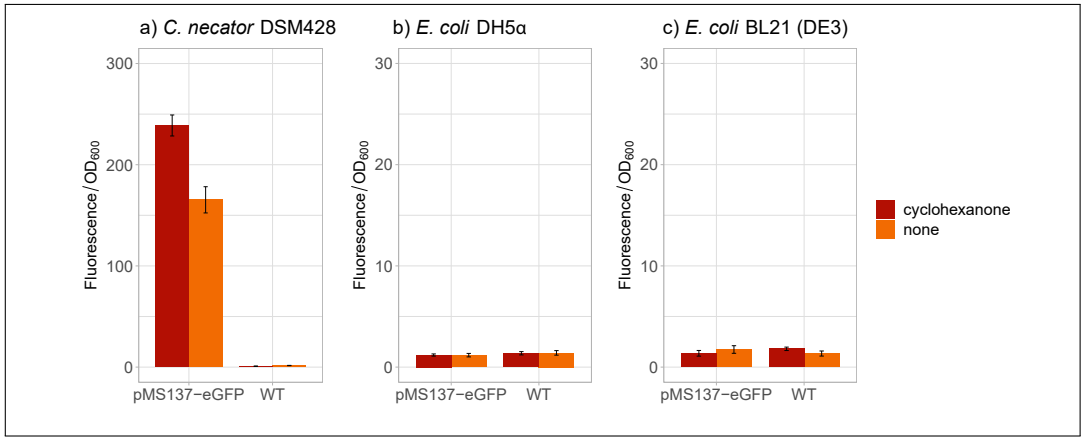

Figure 3.32: ***The expression of egfp from the plasmid pMS137 in C. necator DSM428, E. coli DH5α and E. coli BL21.*** *egfp expression is given as arbitrary unit. a) C. necator DSM428 b) E. coli DH5α c) E. coli BL21. Note the axis of E. coli BL21 being 10 times higher. All cultures were inoculated from an overnight culture of 1 mL LB incubated at 30 °C at 800 rpm. 10 µL of the pre-culture were inoculated in 1 mL LB. If induced, cyclohexanone was added to a concentration on 1 mM. If necessary, tetracycline was added to a final concentration of 12.5 µg/µL. The culture was incubated at 30 °C, 800 rpm overnight in 96 deep well plates. The fluorescence was measured with 485 nm excitation wavelength and detected at 535 nm (see* ***Section 2.16.2****). The values are given in* ***Tab. A.33****.*

In order to determine the cyclohexanone concentration for optimal expression, *C. necator* DSM428 was incubated with various concentrations overnight in 96 well deep plates at 30 °C at 800 rpm in LB and minimal medium with gluconate as sole carbon source (see **Fig. 3.33**). On LB (see **Fig. 3.33** a), the wildtype and the empty vector pMS137 showed a basic level of fluorescence around 0.7. The strain containing *egfp* (pMS137-eGFP), showed a steady increase in fluorescence with increasing cyclohexanone concentration starting from 96±9 at 0 mM to 183±20 at 10 mM. A similar picture was seen on the minimal medium with gluconate (see **Fig. 3.33** b) with the wildtype and pMS137 having a basal fluorescence level at around 1.1. The strain containing *egfp* showed an increased fluorescence starting from 12.0±0.9 at 0 mM and 25.0±1.8 at 10 mM. However, the increase was not as straight-forward as on LB.

Based on the expression tests in this section, the plasmids pJeM1TcR and pMS137 were selected to express genes to enhance the isoprene production in *C. necator*.

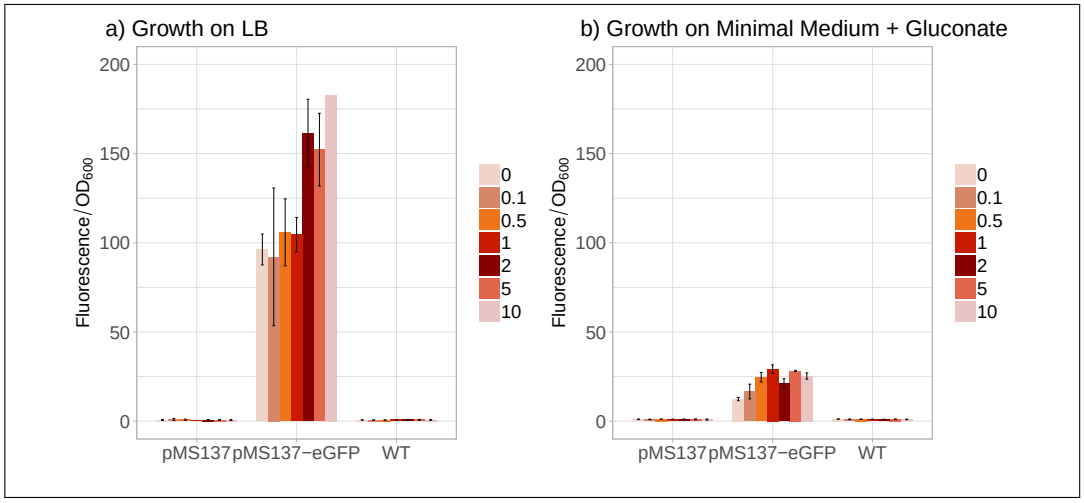

Figure 3.33: ***The expression of egfp from the plasmid pMS137 in C. necator DSM428 with various cyclohexanone concentrations.*** *egfp expression is given as arbitrary unit. a) LB b) minimal medium with 2% gluconate. All cultures were inoculated from an overnight culture of 1 mL medium incubated at 30 °C at 800 rpm. 10 µL of the pre-culture were inoculated in 1 mL medium. If induced, cyclohexanone was added as noted in the figure in millimolar. If necessary, tetracycline was added to a final concentration of 12.5 µg/µL. The culture was incubated at 30 °C, 800 rpm overnight in 96 deep well plates. The fluorescence was measured with 485 nm excitation wavelength and detected at 535 nm (see* ***Section 2.16.2****). The values are given in* ***Tab. A.34****.*

3.3.2.2 Enhancing Isoprene Production of *C. necator* Using *kispS* and the *mev*-Operon

The wild type strain of *Cupriavidus necator* H16 produces isoprene (see **Fig. 3.34**). Four different media were tested in order to asses the differences in the isoprene production. The media tested were DSMZ medium 1, a complex medium, minimal media with either gluconate or fructose as the sole carbon source, and 10% silage press juice (v/v, diluted with water). The growth conditions for the different media were the same: a 1 mL overnight pre-culture in the medium of the culture in a 96 well deep well plate, incubated at 30 °C at 800 rpm, was used to inoculate a 2 mL culture at an OD_{600} = 0.2 in a GC headspace vial. The vial was sealed and incubated at 30 °C at 170 rpm. Isoprene was subsequently measured (for the method, see **Section 2.15.2**). The growth curves and isoprene production were similar on DSMZ medium 1 and 10% silage press juice, and on both variants of the minimal medium. The maximum OD_{600} on DSMZ medium 1 was 3.42±0.25 after 32 h and on 10% silage press juice 5.41±0.33 at 30.5 h. The corresponding isoprene produced was 0.03±0.01 pA*min and 0.03±0 pA*min. When grown on a minimal medium, *Cupriavidus necator* reached a higher OD_{600} with 6.77±0.15 (fructose) and 8.81±0.14 (gluconate). The isoprene

produced was higher with 0.05±0 pA*min after 46.5 h on fructose and 0.05±0 pA*min on gluconate at 32 h. On all media which were examined, the production rate of isoprene decreased towards the end of the growth curve and was the highest during the exponential phase.

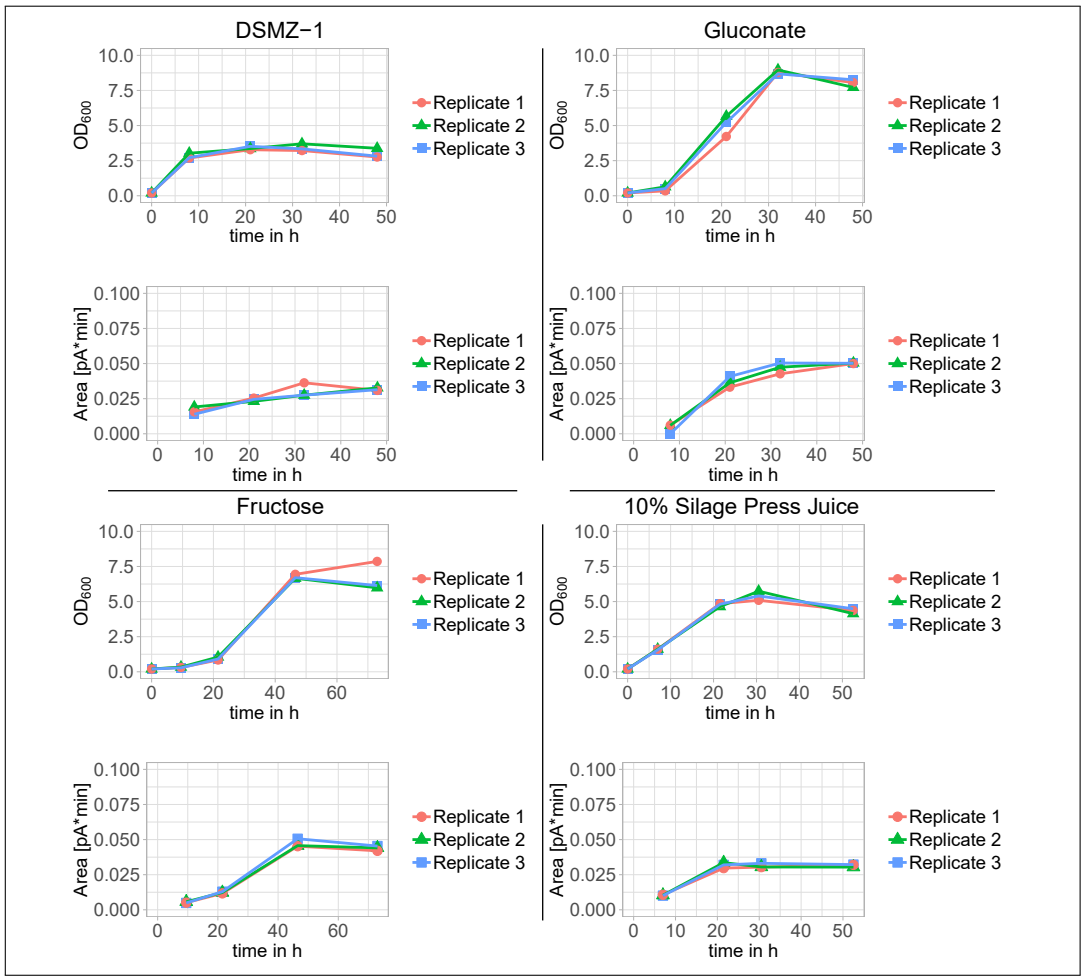

Figure 3.34: ***The isoprene production of C. necator DSM428 wildtype on various media.*** *All cultures were incubated in sealed gas chromatography headspace vials at 30 °C at 170 rpm starting with OD_{600} = 0.2. The values are given in* ***Tab. A.29****.*

In order to enhance isoprene production, isoprene synthase from *Populus alba* kudzu or the *mev*-operon was desired to be cloned into the plasmids listed in **Tab. 3.9**.

Both the *kispS* and the *mev*-operon were aspired to be cloned into pBBR1MCS-3 using golden gate cloning (see **Section 2.14.4.1**). The vector backbone was amplified using PCR, as described in the previous section, and *kisps* was amplified using Phusion-HF (NEB) with primers kIspS-MCS3-gg-f and kIspS-MCS3-gg-r. The *mev*-operon was amplified using Phusion-HF (NEB) with primers Mev-MCS3-gg-f and Mev-MCS3-gg-r. Cloning of *kispS* was successful, whereas cloning of the *mev*-operon was unsuccessful,

as only the first half of the operon could be inserted into the vector. This led to the strategy of splitting the *mev*-operon by PCR into two fragments using Phusion-HF and the primer pairs Mev-MCS3-gg-f/Mev-MCS3-mid-gg-r and Mev-MCS3-mid-gg-f/Mev-MCS3-gg-r. This attempt was also unsuccessful, as the same half of the *mev*-operon, in this case a individual fragment, was not inserted into the vector.

As making the constructs using pBBR1MCS-3 as vector was unsuccessful for the *mev*-operon, pMS137 was used as an alternative for cloning the isoprene synthase and the *mev*-operon. The *kispS* was amplified using Taq DNA polymerase (NEB) with primers kIspS-pMS137-f and kIspS-pMS137-r and inserted into pMS137 over the PciI and KpnI site. The *mev*-operon was amplified using Phusion-HF (NEB) with primers Mev-pMS137-f and Mev-pMS137-r and inserted over the PciI and KpnI site.

Cupriavidus necator was only transformed (method see **Paragraph 2.14.6.10.5**) with the designed pMS137 constructs and the isoprene forming capacity was examined as described in the following paragraph.

To examine the isoprene production of the transformants, pre-cultures inoculated from cryo stock in LB medium were incubated overnight at 30 °C at 150–170 rpm. 10 mL GC headspace vials containing 2 mL LB medium were inoculated to an OD_{600} = 0.2, induced with 1% cyclohexanone, sealed and incubated for 16 h at 30 °C, 150–170 rpm. The isoprene amount was measured as described in **Section 2.15.1**. The isoprene production was nearly identical for all strains bearing different constructs (see **Fig. 3.35**).

During the course of the experiments, the growth of *C. necator* DSM428 with pMS137 derivatives was not uniform. In some cases, the strains started to grow after a lag phase of 24 h. Confusingly, this was not consistent within the same strain. Plasmids remained stable within the strains even after 48 h of incubation as investigated using colony PCR to determine the presence of the plasmid (data not shown).

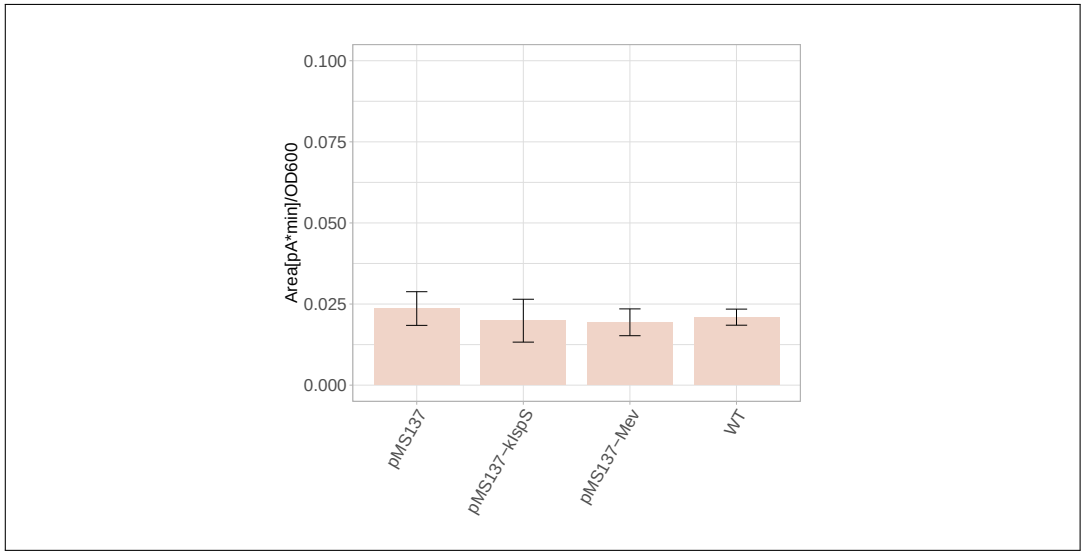

Figure 3.35: ***The isoprene production of C. necator DSM428 bearing various genes on pMS137.*** *Isoprene production of various genes expressed on pMS137 in C. necator DSM428 for 16 h at 30 °C, 150–170 rpm in sealed 10 mL GC headspace vials, induced with 1% cyclohexanone. Isoprene amount is normalized to the OD_{600} . WT: wild type The values are given in* ***Tab. A.30****.*

3.3.2.3 Codon Usage of *Cupriavidus necator*

The codon composition of the genes used varies widely in comparison to the codon usage of *Cupriavidus necator* (see **Fig. 3.36**). The number of rare codons present in *kispS* is greater than that of rare codons in *egfp*, which can also be seen by the CAI value (see **Fig. 3.37**). Codon usage and CAI values were determined as described in **Section 2.17.5**.

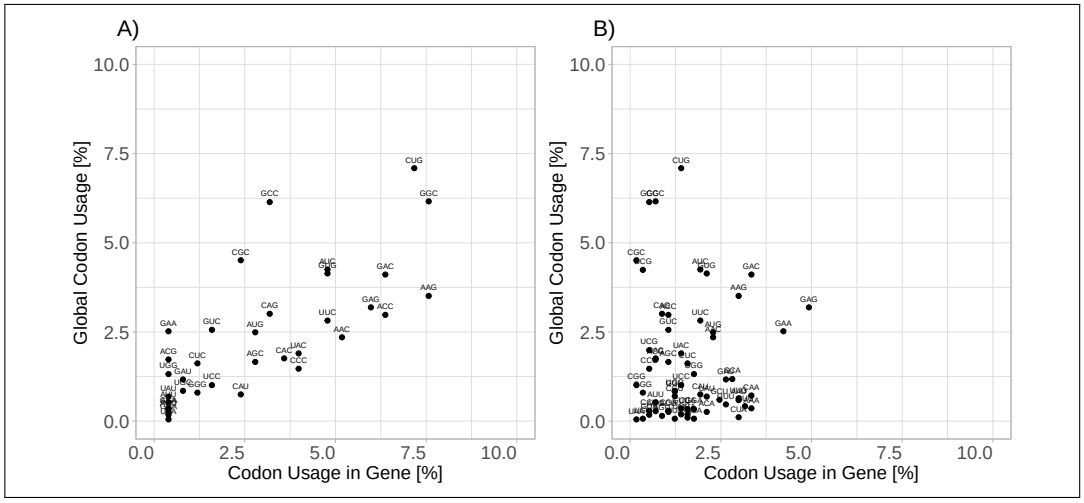

Figure 3.36: ***Codon usage of expressed genes in Cupriavidus necator of A) egfp and B) kispS compared to the global codon usage of Cupriavidus necator.*** *Codon usage is expressed in percent.*

Figure 3.37: *CAI values for genes expressed in Cupriavidus necator*

Gene	CAI value
egfp	0.69
kispS	0.11
Mevalonate kinase	0.38
HMG CoA synthase	0.39
HMG CoA reductase	0.41
Acetyl-CoA acyltransferase	0.43
Phosphomevalonate kinase	0.35
Diphosphomevalonate decarboxylase	0.37

CAI values were calculated using the online tool http://www.jcat.de using the closest organism provided: *Cupriavidus necator* JMP134 (as *Ralstonia eutropha* JMP134)

4 Discussion

4.1 Ethylene and Propylene

4.1.1 Screening

4.1.1.1 Isolation of Microorganisms from Environmental Samples

Ethylene is an important bulk chemical that is almost entirely produced from petroleum. Although small-scale plants producing ethylene from sugar cane (via ethanol generation and chemical dehydration of the same) have been established (see **Section 1.1.3.2**), a one-step fermentation process was required to match the strategy of the farm-stead biorefinery. In order to achieve this new approach, a screening was made to find enzymatic reactions or pathways which produce ethylene from cheap substrates or with a higher stoichiometric efficiency (see **Section 3.1**).

The focus was on microorganisms, as the conversion of the substrates in the farm-stead biorefinery was designed to be performed by microorganisms. From various environmental sampling sites, microorganisms were isolated and cultured in different media in order to increase diversity. Chitin agar medium was used as a selective medium on which only microorganisms possessing a chitinase were able to grow (mainly *Streptomyces*). Media favored by yeasts (YPD) and *Lactobacillus* species (MRS and aMRS) were used as well. The phylogenetic diversity of the organisms producing ethylene showed *Bacillus* strains to be predominant. It was expected to find this genus (among other *Firmicutes*, such as *Paenibacillus* and *Brevibacillus*), as they are abundant in soil and are known to produce ethylene [21, 34, 194, 202]. Ethylene production is also known from some *Proteobacteria*, such as *E. coli* [138, 202], *P. syringae* and the yeast *Penicillium citrinum* [61, 103, 343].

4.1.1.2 Screening Methodology and Analytics

The screening method was first conducted with 1 mL culture volume in 96 well plates for high throughput. Here, eight strains – one column – were pooled in 1 mL buffer. However, the amount was too little to detect any ethylene formation from 145 (SR-761 to SR-809 and SR-903 to SR-998) isolates tested. It was decided to change the assay by using 10 mL culture volume, harvested and resuspended in a 1 mL buffer (for details, see **Section 2.13.5**), in order to increase the overall protein amount and concentration. With this change, 16 ethylene producers were found among the same 145 previously tested strains. The remaining SR culture collection – comprising a total of 524 strains during

that time – was screened with 10 mL culture volume. After washing and cell disruption, substrates were added and the 10 mL GC headspace vial was sealed. The samples were incubated for 4 h at 30 °C, which was derived from literature [97, 192].

The cells applied to the screening were grown for three days, in order to ensure high cell mass for the ethylene assay. Additionally, as the environmental isolates were identified only if ethylene was produced, the generation times were uncertain. It is not uncommon to extend the growth phase, which was also done by Billington, Golding, and Primrose [34] with a two day growth. For *Bacillus cereus* SR-772 and *Lysinibacillus xylanilyticus* SR-86, ethylene production from cells harvested after overnight growth was comparable to that after three days (data not shown).

Ethylene-producing microorganisms have been discovered by screenings performed directly from the flask's atmosphere from 50 mL to 500 mL flask [34, 70, 192, 304]. This method is somewhat slow and requires manual injection of the gas samples into the gas chromatograph, as there is no auto sampler available for those vessels. Cristescu et al. [69] reported the measurement of ethylene directly in the culture vessel using a laser. However, this setup was not available at the institute. Fukuda, Fujii, and Ogawa [97] performed a screening, in which 2 mL of a pre-culture were transferred into a test tube. Ethylene was analyzed after 5–10 hours of incubation. All the above-mentioned screenings detected ethylene production from substrates present in the media. In our screening, cells were washed in order to get rid of the media components, as specific substrates were used. However, it can be speculated that ethylene production was detected from the remaining substrates in the cytosol of the cells (see **Tab. 3.2**).

Fujii, Ogawa, and Fukuda [96] developed a colorimetric assay for the detection of ethylene based on the reaction of molybdenum with olefins and a palladium catalyst, and utilizing olefin-using microorganisms. The chemical screening method is less sensitive compared to the microbiological method. Both methods are, however, less sensitive than the detection of olefins by gas chromatography.

A review of the mostly used ethylene detection methods is given by Cristescu et al. [68].

4.1.1.3 Ethylene and Propylene Production

No propylene producers were found. Other screenings in the literature identified propylene producers [71, 98]. The screening performed in this thesis differed from them by not using alive cells growing in their medium but partially disrupted cells provided with specific substrates (see **Section 2.13.5**).

Thirty one strains showed ethylene production, but the effect of the substrates (β-Alanine and 3-phosphonooxy-propanoic acid) was very little and, in some cases,

not reproducible. Hence, a fraction of the strains – ten – were further characterized (see **Sections 3.2.1.1** and **3.2.1.2**) using substrates for the three reported ethylene forming pathways (see **Paragraph 1.1.1.1.1** and **Sections 1.1.1.2** and **1.1.1.3**).

It has been reported by Grichko and Glick [117] and los Milagros Orberá Ratón et al. [194] that between 22% and 30% of aerobic endospore forming bacteria isolated from soil are found to produce ethylene. In this screening, 6% of the screened microorganisms showed ethylene production. This difference can be explained by two factors: (1) as the aim was to increase the phylogenetic diversity, no heating step in isolation from soil samples was performed in order to only get endospore-forming bacteria (as described in [194]); (2) the screening was not performed with typical ethylene precursors (KMBA, L-Met, ACC and α-KG), instead, β-Ala and 3-phosphonooxy propanoic acid were used. In another screening performed by Fukuda, Fujii, and Ogawa [97], 30% of the tested microorganisms showed ethylene production as well. They also used intact cells for the ethylene assay without any substrate, incubating the samples 5 to 10 hours.

Strains producing ethylene were categorized into yeasts and bacteria based on their morphology. Based on these observations, the 16S rRNA gene was amplified or a part of the fungal rRNA gene (see **Section 2.14.7**) and sequenced (see **Sections 2.14.8** and **2.17.1**). The primers used are well described in the literature: the primer pair for identification of the yeasts, targets parts of the 5.8S rDNA, the spacer region between the 5.8S rDNA and 25S rDNA, and parts of the 25S rDNA [330]. Bacteria were identified with the standard primer pair 27f and 1525r [94, 177, 326]. Typically, also the reverse primer 1492r is used, but yields in a shorter fragment. Some samples were prepared twice or more before successful sequencing occurred. The reason is unknown, but there were probably impurities in the samples. However, as the sample preparation was repeated in the same way with a successful sequencing, there might have been additional problems. This is also the reason why sequences which might be considered rather short and being not at the 3'-end were used for identification. The sequence length ranged from 324 bp (160 bp trimmed) to 1419 bp (1222 bp trimmed). For most (clinical) samples, 500 bp length at the 5'-end is sufficient in most cases [157]. However, there are differences in the species level to consider with 500 bp, but the genus level is resolved [63]. It has to be noted that the strains *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus anthracis* are genetically very closely related [129] but distinguishable with their 16S rDNA sequence [129, 266, 312]. Considering the time and money spent for each sample, it was also decided to use the sequences of length shorter than 500 bp. At least, they provide identification on the genus level. Wilck et al. [350] reported that 200 bp sequences sufficed in identification at the species level, whereas Okubo et al. [236] reported that especially in the genus *Bradyrhizobium* and *Methylosinus* it was necessary to use sequence length of greater than 400 bp. In only one case, a length

of the forward read was shorter than 200 bp. The result could be verified by the result of the reverse sequence, which gave the same genus with a sequence length of 840 bp (trimmed) (*Stenotrophomonas*, see **Appendix A.2.2**).

4.1.2 Characterization of Ethylene Producers Based on Substrate Combinations

A total of ten strains were assigned to the three published ethylene pathways based on the combination of substrates from these pathways (see **Sections 3.2.1.1** and **3.2.1.2**). The combinations of L-Met, KMBA, ATP, SAM and ACC are related to the ACC pathway, while L-Met, α-ketoglutarate and KMBA are related to the KMBA pathway and α-ketoglutarate to the Efe pathway. According to the combinations of substrates, the strains reported in **Section 3.2.1.1** can be assigned to the KMBA pathway. Moreover, *Lactobacillus brevis* SR-416 reported in **Section 3.2.1.2** makes ethylene from KMBA as well. Lactobacilli are known to form CH_3SH from KMBA [341, p. 252ff.], which is supposed to be formed in the ethylene reaction [138]. According to Fukuda et al. [106] and Ince and Knowles [138], KMBA is converted to CH_3SH, CO_2 and ethylene. According to the proposed pathway (see **Fig. 1.4**), addition of α-KG to L-Met should result in more ethylene. This was observed for *Lysinibacillus xylanilyticus* SR-86, *Lactobacillus brevis* SR-416 and *Bacillus cereus* SR-772. The addition of KMBA supports this observation (see **Fig. 3.4**). So far, there is no record of *Providencia* sp., a γ-*Proteobacterium*, producing ethylene (see **Section 3.2.1.1**) . So far, the species *Bacillus luti* and *Bacillus thuringiensis*, *Bacillus cereus* and *Lysinibacillus xylanilyticus* have also not been reported to produce ethylene (see **Section 3.2.1.1**). Close relatives of the strains reported to produce ethylene include *Bacillus mycoides*, *Bacillus* sp. ALK-7 and *Bacillus subtilis* [21, 34, 202]. The inhibitory effect of ATP on ethylene synthesis from L-Met has not been reported so far. However, it was shown that phosphate has an inhibitory effect on the ethylene production from α-ketoglutarate (Efe reaction), tested with various concentrations of the phosphate buffer. No phosphate in the buffer resulted in a decreased ethylene production as well, with the optimum being 0.08 mM to 0.008 mM phosphate [115].

Interestingly, all strains showed the production of ethylene from ACC, which is the precursor of ethylene in plants (see **Paragraph 1.1.1.1.1**). The production of ethylene from ACC has been reported for *Bacillus* sp. ALK-7 by Bae and Kim [21]. Moreover, several studies showed that microorganisms in soil samples were responsible for the ethylene production from ACC [14, 142, 158, 228, 229]. It seems to be a single reaction rather than the pathway occurring in plants. If ethylene was generated with the Yang

cycle, addition of ATP to the reaction with L-Met should have resulted in an increase of ethylene, but the opposite was observed in *Lactobacillus brevis* SR-416 (see **Fig. 3.2**), *Bacillus cereus* SR-772 and *Lysinibacillus xylanilyticus* SR-86 (see **Fig. 3.3**). In order to further elucidate the mechanism, additional experiments and protein purification was performed (see **Sections 3.2.1.2**, **3.2.2** to **3.2.4** and **3.2.4.2**). Results are discussed in **Section 4.1.3**.

Interestingly, in *Pichia fermentans* SR-265, ethylene production was observed when ATP (with or without L-Met) was added (see **Fig. 3.3**), which was in contrast to the other organisms. This would indicate the presence of the Yang cycle in *Pichia fermentans* SR-265. The addition of 3.2 mM SAM showed very little ethylene production (see **Fig. 3.3**). A higher concentration would have been necessary to verify the ethylene production, but higher concentrations of SAM were too expensive. An additional clue was provided by homologous sequences of the ACC oxidase in the genome of *Pichia fermentans* SR-265 (see **Fig. 3.15**). This has been considered for the slime mold *Dictyostelium mucoroides* [6] and the fungus *Penicillium cintrinum* [146]. The expression of the putative ACC oxidases in *Pichia pastoris* X-33 [271] were inconclusive and more experimental work has to be done. Next steps would include purification of the enzyme by FPLC, identifying other enzymes of the Yang cycle (see **Chapter 5**). Ethylene formation from ACC was little, which could be explained by 0.3 μm cobalt in the medium (see **Tab. 2.25**). It has been shown, that Co(II) at 10 μM inhibited ethylene formation by the ACC oxidase of up to 70% in mung bean [334, 368]. Although cobalt was 33 times more concentrated in the reports, the enzyme was also different and, therefore, it was not possible to rule it out.

No organisms were found producing ethylene via the Efe-pathway, which could be related to the reaction being inhibited by high-phosphate concentrations [115]. As we used sodium phosphate buffer (NaPi) for the reaction (see **Section 2.13.5**), this might have inhibited the reaction. Up to now, this ethylene-forming enzyme has only been found in *Pseudomonas* sp. and *Penicillium* sp., and should also be present in soil samples. The characterization of the enzyme was also made only for these two microorganisms. However, bioinformatic analyses showed that it was also present in other organisms, such as *Ralstonia solanacearum* [321] and *Xanthomonas oryzae* [82], among others. The ethylene amount produced via the KMBA-dependent pathway was less than that of the Efe-pathway, as shown to differ in *Ralstonia solanacearum* compared to *Pseudomonas syringae* pv. *pisi* [344].

4.1.3 Further Characterization of Ethylene Production from ACC

4.1.3.1 Effect on Metals and Co-substrates on Ethylene Production from ACC

In order to further characterize the ethylene production from ACC, various possible cofactors were investigated. In contrast to the ethylene formation from ACC in soils as reported by Nazli et al. [229], the addition of Iron(II), Iron(III) and Nickel(II) showed no strong effect on the ethylene formation in *Lysinibacillus xylanilyticus* SR-86, *Bacillus cereus* SR-772 and *Lactobacillus brevis* SR-416. The same was found for the addition of glucose and mannitol (see **Fig. 3.5**). A concurring finding was the increase of ethylene when Mn(II), Co(II) and Zn(II) was added (with the exception of *Lactobacillus brevis* SR-416, showing a slight decline when Zn(II) was added) (see **Fig. 3.5**). The addition of EDTA to the protein extract of the three organisms tested showed a similar reaction than the soil samples. The effect of EDTA and Co(II) was stronger in the protein extracts. Deviations from the effect seen in this thesis to Nazli et al. [229] might occur due to the different specimen types used, as Nazli et al. [229] used soil samples, and protein extracts were used in this thesis.

Nazli et al. [229] showed the biogenic origin of ethylene formation by the addition of antibiotics. The digestion of the protein extract from *Lactobacillus brevis* SR-416, *Lysinibacillus xylanilyticus* SR-86 and *Bacillus cereus* SR-772 showed a decrease in the production of ethylene, indicating a biogenic source – in this case an enzymatic – origin of the reaction (see **Fig. 3.8**). The activity decrease of the protein extract heated to 95 °C for 10 minutes for *Lysinibacillus xylanilyticus* SR-86 and *Lactobacillus brevis* SR-416 indicated an enzymatic origin. The remaining activity in the protein extract heated to 95 °C (see **Fig. 3.8**) did not exclude an enzymatic origin, as enzymes from mesophilic organisms showed to be active after heating and autoclaving, e. g. a copper/zinc superoxide dismutase [171–173, 187, 191]. Moreover, the mechanism of inactivation by heat also depends on destroying the enzyme's structure. Refolding of enzymes can restore its activity, as shown for many enzymes, e. g. horseradish peroxidase [196]. A radical mechanism could be involved, as the exchange of the air in the headspace with argon showed a reduction in the ethylene production in the protein extract of *Lysinibacillus xylanilyticus* SR-86 and *Bacillus cereus* SR-772 (see **Fig. 3.8**). The decline which occurred after the catalase was added in *Lysinibacillus xylanilyticus* SR-86 and *Lactobacillus brevis* SR-416 supports this hypothesis, but is contra-indicated by a slight increase in *Bacillus cereus* SR-772 and no effect of argon in *Lactobacillus brevis* SR-416.

In order to investigate the co-substrates stimulating or inhibiting ethylene production, various amino acids and other organic acids were tested (see **Fig. 3.6**). α-ketoglutarate showed the strongest effect in all three microorganisms tested, with the activity increase in *Lactobacillus brevis* SR-416 being the highest. The addition of α-keto acids showed an increase in the ethylene production from ACC in the protein extract of *Lysinibacillus xylanilyticus* SR-86 as well, but not as potent (see **Fig. 3.7**). The possibility of ethylene production being caused by the ethylene-forming enzyme was ruled out by testing the substrate combination of α-ketoglutarate and L-arginine (see **Fig. 3.9**). Ethylene was produced only when ACC was present in the protein extracts of *Lysinibacillus xylanilyticus* SR-86, *Bacillus cereus* SR-772 and *Lactobacillus brevis* SR-416.

Moderate activity increase was shown in the protein extracts of all three microorganisms for L-arginine, L-glutamate, L-lysine and succinate. A strong inhibitory effect was shown by the addition of ascorbate. This indicated another co-substrate for the ethylene production from ACC as is known from plants, as the co-substrate in plants is ascorbate [149]. In addition, Fe(II) is an essential component [40], and no strong effect on the formation of ethylene was shown in the three strains (see **Fig. 3.5**). The other co-substrates showed a differing, but minor effect on the strains tested.

Increased activity with α-ketoglutarate might also indicate an enzyme belonging to the same family as the ACCO from plants, the 2-OG oxygenases (see **Paragraph 1.1.1.1.1**). However, some family members, such as the Isopenicillin N synthase (EC 1.21.3.1), do not rely on α-ketoglutarate as a cofactor, which is also seen for plant ACC oxidases [46, 66].

4.1.3.2 Protein Purification

Several columns and buffers were tested in order to find conditions to bind and purify the enzyme which was responsible for the production of ethylene from ACC. DEAE FF sepharose and QXL columns proved to be successful and were used stacked on top of each other, beginning with the QXL, see **Section 3.2.2**. As the enzyme did not bind to other columns tested, the active fractions of this one-step purification were used in order to identify proteins responsible for the ethylene production, see **Section 3.2.4**. The fractions were combined and concentrated using lyophilisation, as the activity was only partly retained by a 3 kDa filter, see **Fig. A.3**. Only two proteins were significantly detected with the mass fingerprinting, see **Tab. 3.8**. The 4-oxalocrotonate tautomerase (4-OT) was thought to be the responsible protein to generate ethylene from ACC, as it is one of the smallest proteins known with ca. 62 amino acids (ca. 6 kDa) containing subunits [216]. Its mechanism is to translocate the H^+ from the C2 to the C5 of its substrate, the oxalocrotonate [349]. For the catalysis, the Pro-1 is

very important. Evidence that the 4-oxalocrotonate tautomerase was the responsible enzyme was also provided by the ethylene formation from protein extract of *Lysinibacillus xylanilyticus* SR-86, which decreases with lower pH, see **Fig. 3.10**. The optimal pH for the 4-oxalocrotonate tautomerase is reported to be around pH 8 with a drop in activity at pH 6 and 10, because of the Pro-1 being protonated at pH 6, which would make the enzyme incapable of abstracting the proton from the C2 and the Arg-11 being deprotonated at pH 10 [296, 349] and not being able to interact with the C5 and C6 [125]. There are several reports on this enzyme to be promiscuous in its substrate acceptance [218, 248, 370]. Although being a hexamer at physiological conditions [349], we did not rule out the possibility of monomers being catalytically active, or being re-assembled. The increase of ethylene activity by adding various organic compounds and metals was a counter-indicator to 4-OT being responsible for the ethylene formation, as it does not need a cofactor. This was a contradiction to the finding, that α-ketoglutarate and various metals increased ethylene production, see **Fig. 3.5** and **3.6**.

The reaction favored a higher pH in the protein extract of *Lysinibacillus xylanilyticus* SR-86 and *Bacillus cereus* SR-772, with no reaction taking place from pH 5 and lower, see **Fig. 3.10**. The pH profile, the partial restraining of the activity by a 3 kDa filter and the 4-oxalocrotonate tautomerase being present in the fraction with the highest activity, see **Fig. 3.14** and **3.16** lead to the further investigation of the 4-oxalocrotonate tautomerase as the source of ethylene formation, see **Section 3.2.4.2**.

It did not seem far-fetched that α-ketoglutarate might stimulate ethylene production from an unknown enzyme, as the two known proteins that produce ethylene – Efe and ACC oxidase – were 2OG-dependent oxygenases. However, the ACC oxidase is α-ketoglutarate-independent [46]. Various concentrations of α-ketoglutarate combined with 10 mM ACC in the protein extracts of *Lysinibacillus xylanilyticus* SR-86 and *Lactobacillus brevis* SR-416 (see **Fig. 3.12** and **3.13**) revealed that one part of the reaction was linear, whereas the other was saturated, indicating a chemical and possibly an enzymatic reaction.

As no ethylene could be detected from the 4-OTs when expressed in *E. coli* BL21, the results pointed more and more in the direction of a chemical reaction taking place. By checking the dependency of the ethylene production from the concentration of α-ketoglutarate and ACC in buffer supplemented with 1% trace elements solution (v/v) (see **Fig. 3.13**), it was revealed that the same pattern was observed. The effect of proteinase K on reducing the ethylene formation in the protein extracts of all three species could also be elucidated by a complexing effect of protein fragments from digestion of BSA in a 3 mg/mL BSA solution, see **Fig. 3.18**. Similar results were found by Boller, Herner, and Kende [37], with the addition of BSA reducing the ethylene to 67% with 0.2% and 37% with 2% BSA.

Although ACC has been reported to induce ethylene production in soil microbes [229] and is responsible for ethylene production in *Bacillus* ALK-7 [21], we identified the cause of ethylene production from our samples to be of a chemical origin, and not of biological, see **Fig. 3.13** and **3.18**.

Confusingly, the Mn(II) bound and was eluted from the anionic exchangers, to which only negatively charged particles bind directly. However, it seems, that Mn(II) co-eluted with other particles in very distinct fractions (see **Fig. 3.14**) and was restrained partly by a 3 kDa filter, see **Fig. A.3**. Sun et al. [302] showed for *Helicobacter pylori* that its 4-OT interacted with Nickel in an Ni-affinity chromatography and was reproducibly bound to the column and eluted. It seemed likely that 4-OT can also bind Mn(II), as other enzymes have been reported to also bind metals other than their preferred ones [93, 238, 314]. The 4-OT could have bound to the sepharose independent of Nickel, which was then released during the assay for ethylene production. Moreover, Nickel and Manganese have the (preferred) oxidation number of $2+$ [133]. As there are also other proteins in the fractions examined (see **Fig. 3.16**), the same could apply to each of them.

Although ethylene formation from ACC in a *Bacillus* strain has already been reported [21], considering the fact that a glycine NaOH buffer at pH 10.3 was used, the activity reported by this paper is caused by a chemical reaction. As a final conclusion, the ethylene formed from ACC was a chemical reaction catalyzed by Mn(II), as reported by [65] and [37]. The increase of ethylene from ACC when α-ketoglutarate is added (see **Section 3.2.1.5**), can be explained by the reaction of Mn^{2+} with amino acids and α-ketoglutarate [151, 253].

It must be noted that not all microorganisms applied to the screening produced ethylene from ACC, despite growing in media containing trace elements (with Mn(II)), see **Tab. 3.2** and **3.3**. The ability of the microorganisms to capture $MnCl_2$ seems to be responsible for the positive results of ethylene production from ACC in bacteria in this screening.

4.1.4 Ethylene from KMBA

4.1.4.1 Effect of Various Factors on Ethylene Production from KMBA

Ethylene formation from KMBA has been established by several studies reporting it from soil samples [14, 158] and microorganisms [105, 106, 138, 250]. It has been reported that the formation of ethylene from KMBA can also be (photo-)chemically achieved [363] and works best at a pH of 3.5 [34, 54]. The same behavior was seen for *Lysinibacillus xylanilyticus* SR-86, see **Fig. 3.22**. The profile for *Bacillus cereus* SR-772

is inconclusive, as it shows an increase in both, more acidic and more alkaline pH values, see **Fig. 3.22**. To resolve the tendency, more pH values have to be tested.

The enzyme responsible for the ethylene production from KMBA was purified from *Cryptococcus albidus* [106], but no sequence has been published so far. The results indicate that the production of ethylene from KMBA in *Bacillus cereus* SR-772 and *Lysinibacillus xylanilyticus* SR-86 are of enzymatic origin, see **Fig. 3.19**, **3.20**, **3.23** and **3.25**.The proposed mechanism of ethylene formation from KMBA starts with the generation of the hydroxide radical. Basically, every system generating (hydroxyl) radicals is able to convert KMBA into ethylene [234].

The reaction is highly dependent on NADH/NADPH and Fe(III) in *Lysinibacillus xylanilyticus* SR-86 and *Bacillus cereus* SR-772, see **Fig. 3.19** and **3.20**. There were no experiments run for *Lactobacillus brevis* SR-416 at pH 5. It is known that *Lactobacillus* produces CH_3SH from KMBA, when this is converted to ethylene, see **Section 4.1.2**.

The experiments for *Lysinibacillus xylanilyticus* SR-86 and *Bacillus cereus* SR-772 show a similar behavior as Fukuda et al. [106] showed for *Cryptococcus albidus*.

In contrast to the results of Arshad et al. [14] for soil samples, Fe(II) had no significant stimulatory effect, and Fe(III) had no inhibitory effect in *Lysinibacillus xylanilyticus* SR-86 and *Bacillus cereus* SR-772, see **Fig. 3.19** and **3.20**. Co(II) had a inhibitory effect on *Lysinibacillus xylanilyticus* SR-86 and *Bacillus cereus* SR-772, in contrast to a stimulatory effect on soil samples. EDTA had an inhibitory effect at pH 5 on *Lysinibacillus xylanilyticus* SR-86 and *Bacillus cereus* SR-772, as was also reported by Arshad et al. [14]. It did, however, have a stimulating effect at pH 7, see **Fig. 3.19** to **3.21**. For the KMBA pathway, it also seems to be typical that microorganisms with a high catalase activity showed less ethylene production [363].

Billington, Golding, and Primrose [34] and Fukuda et al. [106] reported that catalase had an inhibitory effect, showing the reactions radical mechanistic nature. *Lysinibacillus xylanilyticus* SR-86 and *Bacillus cereus* SR-772 showed the same behavior, but catalase inhibited only the reaction at pH 5, not at pH 7, see **Fig. 3.19** and **3.20**. The highest ethylene production was seen when EDTA, Fe(III) and NADH were added to KMBA, concurring with the findings by Fukuda et al. [105]. This supports the conclusion that *Lysinibacillus xylanilyticus* SR-86, *Lactobacillus brevis* SR-416 and *Bacillus cereus* SR-772 produce ethylene via the KMBA-pathway. The replacement of oxygen with argon in the headspace of 10 mL GC headspace vial indicates that the reaction is O_2-dependent, see **Section 3.2.6**. This supports the observation that the mechanism is radical-based. As the other microorganisms showed a similar pattern (see **Fig. 3.1** and **3.2**), it is very likely that they produce ethylene via the same pathway, except for *Pichia fermentans* SR-265.

This conversion is linked to nitrogen limitation [282], as the amino group from L-Met is transferred to α-ketoglutarate by an aromatic amino acid aminotransaminase [109]. KMBA formation has been reported for many organisms, including *Lactobacillus*, which degrades into methanethiol and possibly to CO_2 and ethylene [106, 109, 139].

4.1.4.2 Protein Purification of the Enzyme Producing Ethylene from KMBA

Attempts to purify the enzyme from the protein extract using various columns (HiTrap Blue, cation and anion exchange columns) have been unsuccessful. Mainly *Lysinibacillus xylanilyticus* SR-86 and *Bacillus cereus* SR-772 have been used, as their ethylene-forming activity was higher than that of *Lactobacillus brevis* SR-416. The best strategy available was to treat the protein extract of *Bacillus cereus* SR-772 with 20% ammonium sulphate (w/v), and then apply the protein extract to a HIC column (best results with Butyl-S FF, see **Fig. 3.24**). Further effort in order to increase the purification degree by optimizing the ammonium sulphate concentration remained unsuccessful, see **Fig. 3.25**. This strategy was pursued, as a similar first step was successful in purifying the KMBA-dependent enzyme from *Cryptococcus albidus* by subsequent purification over HIC [106].

One attempt was made to further purify the protein solution treated with 20% (v/w) ammonium sulphate precipitation gel filtration (Sephadex 26/10). The ethylene formation was seen in three areas of the elution, but by checking the fractions on an SDS-PAGE, a wide range of protein size was seen, with no chance of gaining more information.

4.1.5 Genome Sequencing

The genomes of *Bacillus cereus* SR-772, *Pichia fermentans* SR-265 and *Lysinibacillus xylanilyticus* SR-86 [195] were sequenced (see **Tab. 3.4**) in order to identify possible genes involved in the production of ethylene. The sequence quality of *Pichia fermentans* SR-265 and *Bacillus cereus* SR-772 were too poor to be published. One reason for *Pichia fermentans* SR-265 could be that the sequencing platform was not optimized for large genomes, as yeast genomes are, compared to bacterial genomes. The poor sequence quality of *Bacillus cereus* SR-772 might be related to its poor sequencing depth (>1000 contigs). As the main strains investigated were *Lysinibacillus xylanilyticus* SR-86 and *Bacillus cereus* SR-772, both showing very similar characteristics in their substrate use for ethylene production, the sequencing was not repeated for the latter organism.

4.2 Isoprene

Isoprene is a promising bulk chemical in the sense of bioeconomy [41, 60]. It has the advantage of being gaseous at 34 °C and is hence a good candidate as a product for the farm-stead biorefinery. The strains *Cupriavidus necator* and *Pichia pastoris* X-33 were used, as both were ideal candidates for chemical production with industrial interest and could use low-cost substrates, such as CO_2 and methanol [67, 123, 145, 185, 369]. Additionally, both were able to grow on silage press juice, the substrate in the farm-stead biorefinery for fermentation processes [276].

4.2.1 Enhancing the Isoprene Production in *Pichia pastoris* X-33

The first attempt to produce isoprene showed very little isoprene amounts. Hence, the cells were grown, harvested and used as whole cell catalysts to produce isoprene from methanol, see **Fig. 3.26**. It has been shown for *Bacillus subtilis* that it produces isoprene in growing and non-growing phases [89].

Isoprene production in engineered *Saccheromyces cerevisiae* showed very low production of isoprene [131, 329]. With a codon optimized IspS from *P. montana*, the isoprene and derivates detectable in the headspace produced were about 500 µg/L culture. Another attempt with a truncated IspS from *Mucuna bracteata* resulted in 16.1 µg/L [127, 329]. Other fungi that were engineered include *Yarrowia lipolytica* with 0.5-1.0 µg/L in the headspace and *Trichoderma reesei* culture with 0.5 µg/L [42, 329]. Till now, fungi are considered to be bad isoprene producers compared to bacteria, especially *E. coli* [329].

Possible strategies to enhance the isoprene production could be codon optimization, which led to a 2-fold improvement in *S. cerevisiae* [131] or multiple copies. This has been proven successful for the production of amorphadiene, an isoprenoid, in *S. cerevisiae* by increasing the carbon flux into the MVA pathway by increasing the level of acetyl-CoA, the precursor in the MVA pathway [281].

On considering the project goals, the expression system used with methanol as inducer was not suitable for generating isoprene when using silage press juice as the medium. Sugar compromises protein expression [310] and in silage press juice sugars are abundant [276].

As the yield of isoprene with 0.018 µM was rather low (especially considering the better result with the same gene expressed in *Bacillus subtilis* [114]), and the fact that a patent

has already been filed for the production of isoprene using *Pichia pastoris* [338], this project was abandoned.

4.2.2 Enhancing Isoprene in *C. necator*

GFP was not expressed from pBBR1MCS-3 either in *C. necator* DSM531 and *C. necator* DSM428, which was not anticipated as it has been reported to work with pBBR1MCS-2 [107]. The vector pBBR1MCS-2 used in [107] and pBBR1MCS-3 were identical in the *lac*-promoter and *lacZ*-region. Differences exist in the marker region among other regions [168]. However, these differences should not lead to a different expression behavior. *egfp* could not be expressed in *E. coli* BL21 and *E. coli* DH5α, but was successfully expressed in *C. necator* DSM428 from pMS137, see **Fig. 3.32**. As *egfp* was also successfully expressed from pBBR1MCS-3 in *E. coli* BL21 (see **Fig. 3.31**) and pJeM1TcR (see **Fig. 3.30**), it could be possible that the *chnB* promoter did not work in *E. coli*, although being proven functional in *E. coli* DH5α and *Pseudomonas fluorescense* and *E. coli* DH5α [292].

The P_{lac} is non inducible in *C. necator*, as there is no *lac* operon found in the genome and IPTG cannot be transported passively into the cell. However, it has been shown that the *lac*-promoter is acting as a constitutive promoter in *C. necator* [107].

It has already been reported that the production of isoprenoids differ according to the strain of *E. coli* used. For example, *E. coli* BL21(λDE3) produces 10 times higher titers than *E. coli* FM5 [36, 53, 329]. Intermediates of the MVA pathway are known to interfere with the cell metabolism [259].

It seems that *C. necator* does not have an isopentenyl-diphosphate Δ-isomerase, as a blast of Idi from *E. coli* did not reveal significant hits. Moreover, two species from the same genus do not possess an *idi* [181]. It is assumed that organisms having the MEP pathway do not necessarily need an Idi [33], which *C. necator* does [181]. Therefore, the isopentenyl di-phosphate is accumulated and not converted into dimethylallyl pyrophosphate. This could also lead to an inhibition of the isoprene synthase, as demonstrated in *Synechococcus elongatus* [110]. A general cytotoxicity of prenyl diphosphates (IPP and DMAPP) was also observed in *Bacillus subtilis* [288, 356]. It is possible to overcome this toxicity by converting these two precursors into terpenes (as shown in *E. coli* [356]) or into isoprene in plants [33, 45]. The lack of an IPP isomerase in *C. neactor* could also be the reason, that the overexpression of the *mev*-operon showed no increased isoprene production , see **Fig. 3.35**. The IPP generated from the MEP pathway could not be converted to DMAPP, the isoprene precursor, in this scenario.

Overexpression of the enzyme Dxs in *B. subtilis* showed a 40% increase in isoprene, whereas overexpression of the Dxr enzyme had no effect [359]. Moreover, in *E. coli* overexpression of the native *dxs* also resulted in an increase. The highest production was found when the genes *dxs*, *dxr* and *idi* were overexpressed [198].

The expression of the *idi* gene showed in *Synechococcus elongatus* PCC 7942 a 130-fold increase in the intracellular DMAPP/IPP ratio. The isoprene production was increased by 65% [110]. The Idi plays an important role in the delivery of the isoprene precursor DMAPP. It is proposed to be a key in regulating isoprene production, as the ratio of IPP and DMAPP from the MEP pathway is 5:1, limiting the excess of DMAPP [198]. It would also be the limiting step in the MVA pathway, as only IPP is produced, see **Fig. 1.5**.

It could also be possible that *Cupriaividus necator* does not posses an isoprene synthase, but the isoprene is synthesized by its IspH [112]. In that case, increasing the precursors DMAPP and IPP would not affect the isoprene level.

There are two main reasons to choose the MVA pathway to increase isoprenoid production. First, the carbon flux in the MEP pathway is highly regulated and its feedback loops are still not fully understood. Secondly, the MVA pathway is understood in more detail [328, 329]. Expressing the MVA pathway in *Cupriavidus necator* without an isoprene synthase is possible, as *Cupriavidus necator* produces isoprene, see **Fig. 3.34** by an unknown mechanism.

The overexpression of the *kispS* showed no enhanced isoprene expression (see **Section 3.3.2.2**). The codons of the gene were not optimized for the use in *Cupriavidus necator*. In some studies, a correlation between the codon usage and enhancement of the isoprene production has been shown [131, 328]. In general, the codon usage can act on the efficiency of transcription (gene expression), stability of mRNA [249] and translation. For the transcription of DNA, the codon optimization plays a role in stability of the mRNA and excess of the ribosome by folding [247]. On the contrary, it has also been shown that slow translation of proteins, especially large proteins, gives the proteins time to fold and a codon optimization is, therefore, not favorable [372]. Concerning highly expressed genes, a better codon adaptation to the tRNA pool positively effects translation elongation rates [291]. The protein stability also seems to be affected by the codon adaptation, assuming a different folding. The faster the polypeptide can exit the ribosome, the faster, and possible better, can the protein fold [291]. It seems that the opposite is also true [161, 269, 367]. On the one hand, it seems that the expression of individual genes does not necessarily depend on its codon usage [170]. On the other hand, individual codons and combination of adjacent codons can affect the translation efficiency (shown in yeast) [108]. The same gene was used in our laboratory to successfully enhance the isoprene production of *Bacillus subtilis* DSM 402 [114]. The

comparison of the codon usages in both organisms shows that the *kispS* has a codon adaptation index (CAI) value of 0.11 in *Cupriavidus necator* and 0.38 in *Bacillus subtilis* DSM 168, see **Section 3.3.2.3**. This means that the translation of the mRNA is less efficient in *Cupriavidus necator* than in *Bacillus subtilis*, which could lead to the observed result due to degraded proteins, decreased mRNA stability and premature termination of translation [120].

Overexpressed protein bands could not be detected in SDS-PAGE gels for kIspS (see **Fig. A.5**) or for eGFP (see **Fig. A.4**), although eGFP was expressed as seen by fluorescence measurement, see **Fig. 3.32**. Thus, detecting a protein band corresponding to kIspS as prove of successful expression might not be suitable.

Besides the possibility that *kispS* is not expressed, it could also not fold properly, be inhibited or might not be able to pull detectable amounts of DMAPP from the MEP pathway of *C. necator* DSM428.

5 Future Perspectives

Gaseous products were selected for the farm-stead biorefinery, as their purification from the fermentation is very simple and cheap. However, the efficiency by which ethylene and propylene are produced by biosynthesis is rather low. One strategy to overcome this could be to specialize in fine chemicals, depending on the purification process. Adding other short-chain olefins to the repertoire, such as butene and pentene, might be suitable as well. A step in this direction was to add isoprene.

The aim to produce ethylene from renewable resources by fermentation is worth pursuing. It would be desirable to develop a high-throughput screening method to screen for more substrates and environmental isolates within a short time. One possible colorimetric assay could involve the Ellman's reagent which detects free SH groups. As CH_3SH is generated alongside ethylene from KMBA as substrate [106], a pre-screening could be made for organisms producing CH_3SH. Positive hits would then be tested for ethylene formation by headspace gas chromatography. For NADH/NADPH-dependent enzymes, it could be possible to use a commercially available colorimetric assay.

In order to purify the putative fungal ACC oxidases, it is necessary to investigate various buffers and pH values. Next, it is necessary to find cofactors, in order to be able to detect ethylene forming activity in diluted samples from AEKTA^{TM} runs. The sequences can be cloned into expression vectors for *E. coli* or yeasts, and be purified from there alongside. This has the advantage of enough protein and therefore activity, even without further optimizing the reaction in the wildtype by finding cofactors. Additionally, sequences for putative ACC oxidases from other yeasts are to be investigated, as they might be more active or stable, and give further insight into the evolution of this putative protein.

In order to purify the enzyme responsible for the production of ethylene from KMBA, the columns (with possible changes to the pH) used by Fukuda et al. [106] would be a reasonable point to proceed. It would also be interesting to try purification of the enzyme from various other microorganisms, as slight changes in the sequence could alter the properties of the enzyme, which in turn could favor enzyme purification.

It might be possible to enhance the isoprene production in *Cupriavidus necator* by expressing a codon-optimized version of the kIspS from various organisms. Another attempt should be the expression of an *idi*, to generate more DMAPP for the conversion to isoprene. Yet, another strategy could be the overexpression of the native MEP pathway. *E. coli* was successfully engineered using this strategy to produce protoilludene 49-fold. The redox reactions were optimized by overexpressing NADPH producing enzymes and deleting possible non-essential NADPH-dependent enzymes [378]. In

order to determine if the expression of the *mev*-operon and isoprene synthase were successful, the next step would be to tag the proteins with a hexahistidine tag and detect them on a western-blot. In this work, adding a hexahistidine tag was omitted, as it has been shown that an N-terminal hexahistidine tag lowers the isoprene synthase activity by 90% and a C-terminal hexahistidine tag decreases the solubility of the recombinant protein [381].

A very promising microorganism for isoprene production is *Bacillus subtilis*, as it produced relatively large amounts of isoprene and showed increased isoprene production by simply bringing a non-codon optimized version of the Kudzu isoprene synthase into it [114, 150, 289, 337, 359]. This strain could be further engineered by adding the *mev*-operon to the isoprene synthase.

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A Appendix

A.1 Calibration Curves for Gases

A.1.1 Calibration of Ethylene

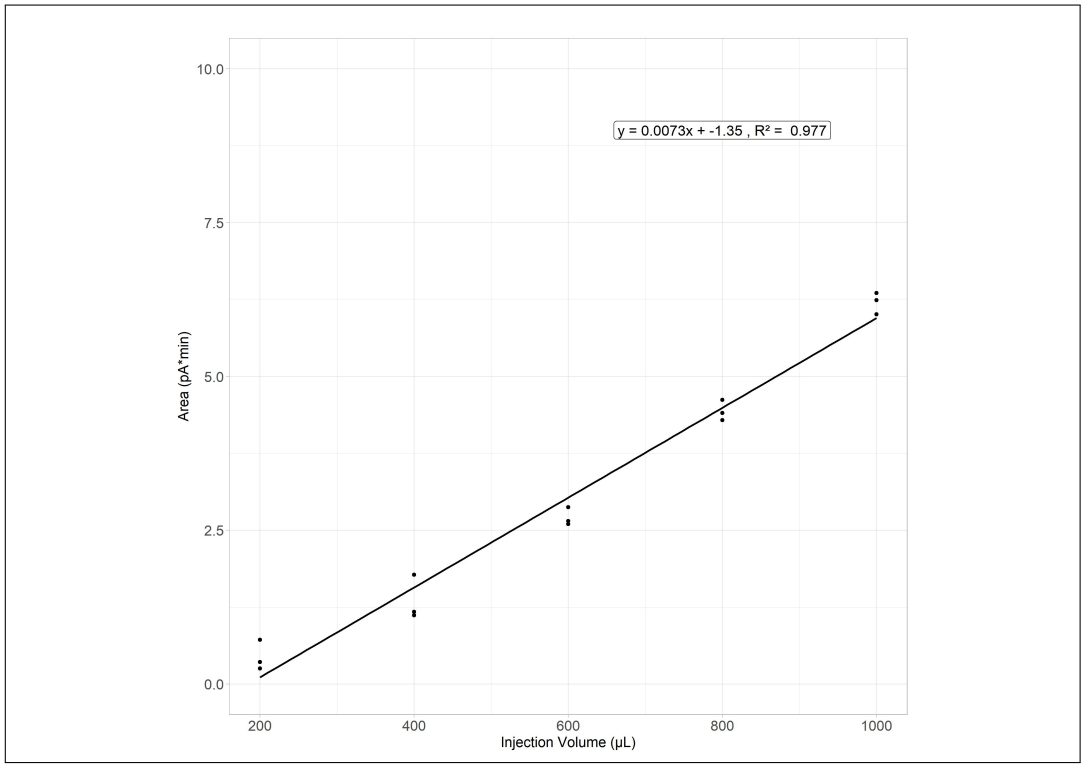

Figure A.1: ***Calibration of Ethylene.*** *Calibration of ethylene was done by injecting various volumes of a standard concentration of 100 ppm in N_2 and additional olefins (Restek GmbH, Germany).*

A.1.2 Calibration of Isoprene

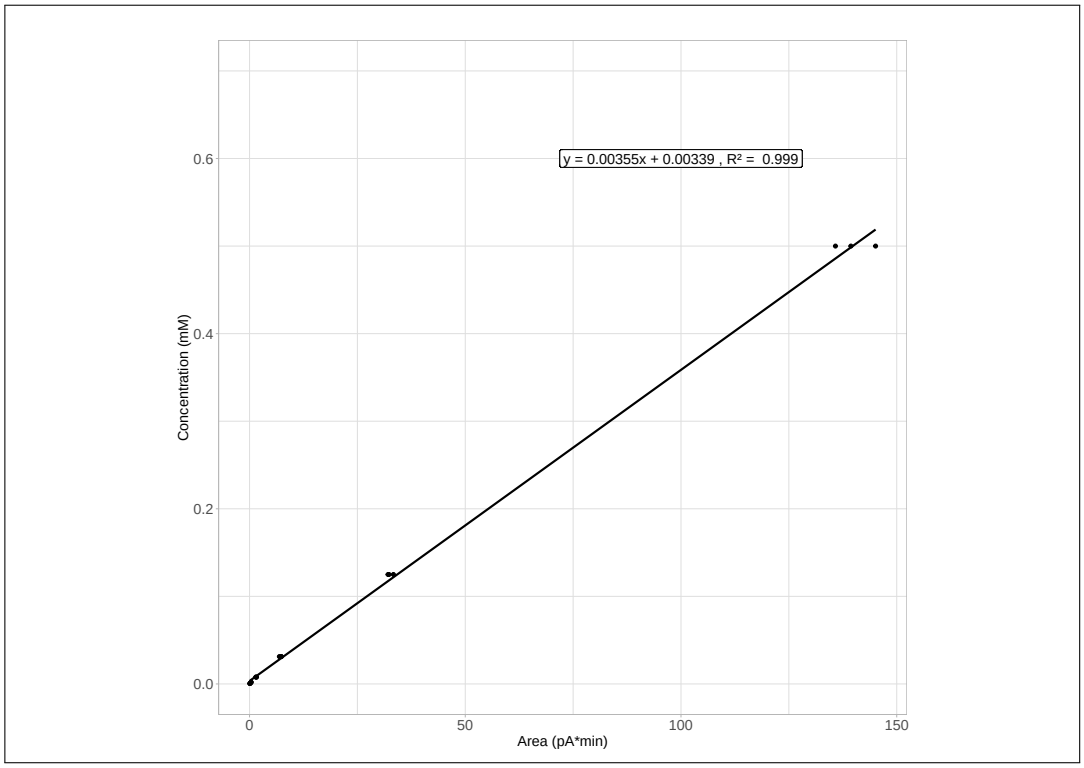

Figure A.2: ***Calibration of Isoprene.*** *Calibration of ethylene was done by injecting various concentrations of isoprene diluted in ethyl acetate.*

A.2 Screening

A.2.1 Growth of Isolated Strains for Ethylene Analysis

Table A.1: *Number of strains grown on specific media*

Medium	Number of Strains
AMASE	11
DSMZ medium 1	17
GYMSE	8
LBSE	10
Marine Broth SE	2
ST1SE	406
YEPDSE	70

A.2.2 Sequencing Data for the 16S/18S rDNA Sequencing of the Ethylene Producers

Table A.2: *16S/fungal sequencing details for ethylene producers: forward or reverse reads*

Read	Length raw sequence	Length trimmed sequence	Accession #	Similarity (%)	Difference	Taxonomy
SR-19-rv	1015	811	LXEP01000074	99.38	5/810	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacterales; Enterobacteriaceae; Buttiauxella
SR-261-rv	1419	1222	NG_055109.1	99	10/1219	Eukaryota; Opisthokonta; Fungi; Dikarya; Ascomycota; saccharomyceta; Saccharomycotina; Saccharomycetes; Saccharomycetales; Pichiaceae; Pichia
SR-263-rv	1076	900	NG_055109.1	99	2/899	Eukaryota; Opisthokonta; Fungi; Dikarya; Ascomycota; saccharomyceta; Saccharomycotina; Saccharomycetes; Saccharomycetales; Pichiaceae; Pichia
SR-266-rv	1384	1168	KM029994.1	99	12/1154	Eukaryota; Opisthokonta; Fungi; Dikarya; Ascomycota; saccharomyceta; Saccharomycotina; Saccharomycetes; Saccharomycetales; Pichiaceae; Pichia
SR-268-rv	1472	1180	KM029994.1	99	14/1175	Eukaryota; Opisthokonta; Fungi; Dikarya; Ascomycota; saccharomyceta; Saccharomycotina; Saccharomycetes; Saccharomycetales; Pichiaceae; Pichia
SR-36-fw	598	572	KJ812430	96.84	18/570	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus

Continued on next page

Read	Length raw sequence	Length trimmed sequence	Accession #	Similarity (%)	Difference	Taxonomy
SR-73-rv	524	458	JH792148	98.25	8/458	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus
SR-786-rv	1050	773	HM640295	99.01	7/705	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacterales; Enterobacteriaceae; HM640295_g
SR-788-rv	1250	875	CP006579	100.00	0/875	Bacteria; Proteobacteria; Gammaproteobacteria; Aeromonadales; Aeromonadaceae; Aeromonas
SR-794-rv	1140	731	CP006579	99.73	2/731	Bacteria; Proteobacteria; Gammaproteobacteria; Aeromonadales; Aeromonadaceae; Aeromonas
SR-932-rv	1021	906	JH792148	99.78	2/904	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus

Table A.3: *16S/fungal sequencing details for ethylene producers: forward and reverse reads*

Read	Length raw sequence	Length trimmed sequence	Accession #	Similarity (%)	Difference	Taxonomy
SR-12-fw	1130	873	NA	NA	NA	NA
SR-12-rv	1559	1038	NA	NA	NA	NA
SR-16-fw	324	160	FJ347998	99.38	1/160	Bacteria; Proteobacteria; Gammaproteobacteria; Lysobacterales; Lysobacteraceae; Stenotrophomonas
SR-16-rv	1235	840	CP001111	100.00	0/840	Bacteria; Proteobacteria; Gammaproteobacteria; Lysobacterales; Lysobacteraceae; Stenotrophomonas
SR-228-fw	996	849	AJ251467	100.00	0/845	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacterales; Enterobacteriaceae; Raoultella
SR-228-rv	1065	372	KF308333	100.00	0/366	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacterales; Enterobacteriaceae; Gibbsiella
SR-234-fw	1085	769	NA	NA	NA	NA
SR-234-rv	1026	840	NA	NA	NA	NA
SR-24-fw	632	514	NA	NA	NA	NA
SR-24-rv	1189	891	NA	NA	NA	NA

Continued on next page

Read	Length raw sequence	Length trimmed sequence	Accession #	Similarity (%)	Difference	Taxonomy
SR-256-fw	1026	168	KM029994.1	99	1/166	Eukaryota; Opisthokonta; Fungi; Dikarya; Ascomycota; saccharomyceta; Saccharomycotina; Saccharomycetes; Saccharomycetales; Pichiaceae; Pichia
SR-256-rv	1110	979	KM029994.1	100	0/941	Eukaryota; Opisthokonta; Fungi; Dikarya; Ascomycota; saccharomyceta; Saccharomycotina; Saccharomycetes; Saccharomycetales; Pichiaceae; Pichia
SR-25-fw	948	706	NA	NA	NA	NA
SR-25-rv	1036	845	NA	NA	NA	NA
SR-265-fw	1125	165	NA	NA	NA	NA
SR-265-rv	1328	941	NA	NA	NA	NA
SR-26-fw	1059	686	NA	NA	NA	NA
SR-26-rv	1046	792	NA	NA	NA	NA
SR-27-fw	983	770	NA	NA	NA	NA
SR-27-rv	1165	829	NA	NA	NA	NA
SR-30-fw	949	696	NA	NA	NA	NA
SR-30-rv	1118	833	NA	NA	NA	NA
SR-4-fw	1369	1121	NA	NA	NA	NA
SR-4-rv	1244	1009	NA	NA	NA	NA
SR-416-fw	1211	1077	NA	NA	NA	NA
SR-416-rv	1281	1010	NA	NA	NA	NA
SR-41-fw	860	42	NA	NA	NA	NA

Continued on next page

Read	Length raw sequence	Length trimmed sequence	Accession #	Similarity (%)	Difference	Taxonomy
SR-41-rv	874	616	PDFK01000008	100.00	0/615	Bacteria; Firmicutes; Bacilli; Bacillales; Planococcaceae; Lysinibacillus
SR-44-fw	907	819	NA	NA	NA	NA
SR-44-rv	1007	701	NA	NA	NA	NA
SR-45-fw	1143	968	NA	NA	NA	NA
SR-45-rv	1515	1049	NA	NA	NA	NA
SR-58-fw	806	530	AMXN01000021	99.81	1/530	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus subtilis
SR-58-rv	1053	523	AMXN01000021	99.24	4/523	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus subtilis
SR-59-fw	1032	704	NA	NA	NA	NA
SR-59-rv	1604	971	NA	NA	NA	NA
SR-70-fw	1260	964	NA	NA	NA	NA
SR-70-rv	1119	946	NA	NA	NA	NA
SR-71-fw	1025	633	JH792383	100.00	0/633	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus
SR-71-rv	719	439	CP013274	100.00	0/439	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus
SR-763-fw	1473	961	NA	NA	NA	NA
SR-763-rv	1403	1086	NA	NA	NA	NA
SR-768-fw	526	362	CP013274	99.45	2/362	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus

Continued on next page

Read	Length raw sequence	Length trimmed sequence	Accession #	Similarity (%)	Difference	Taxonomy
SR-768-rv	659	442	CM000733	99.77	1/442	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus
SR-769-fw	983	715	NA	NA	NA	NA
SR-769-rv	1116	872	NA	NA	NA	NA
SR-76-fw	1205	1092	NA	NA	NA	NA
SR-76-rv	1365	1036	NA	NA	NA	NA
SR-772-fw	1220	732	KJ812430	100.00	0/732	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus
SR-772-rv	815	203	JH792148	100.00	0/203	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus
SR-778-fw	1022	773	NA	NA	NA	NA
SR-778-rv	1152	979	NA	NA	NA	NA
SR-780-fw	1162	963	NA	NA	NA	NA
SR-780-rv	1171	962	NA	NA	NA	NA
SR-782-fw	1076	286	AZYZ01000028	99.65	1/286	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacterales; Morganellaceae; Providencia
SR-782-rv	1064	510	AM040489	99.80	1/510	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacterales; Morganellaceae; Providencia
SR-783-fw	1383	693	NA	NA	NA	NA
SR-783-rv	1266	862	NA	NA	NA	NA

Continued on next page

Read	Length raw sequence	Length trimmed sequence	Accession #	Similarity (%)	Difference	Taxonomy
SR-784-fw	1124	843	NA	NA	NA	NA
SR-784-rv	917	614	NA	NA	NA	NA
SR-78-fw	961	714	NA	NA	NA	NA
SR-78-rv	1217	1015	NA	NA	NA	NA
SR-790-fw	1047	586	NA	NA	NA	NA
SR-790-rv	1053	715	NA	NA	NA	NA
SR-792-fw	1335	773	NA	NA	NA	NA
SR-792-rv	1733	1033	NA	NA	NA	NA
SR-795-fw	1199	846	NA	NA	NA	NA
SR-795-rv	1136	844	NA	NA	NA	NA
SR-796-fw	1401	463	CP006863	100.00	0/463	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus
SR-796-rv	1230	773	CM000733	100.00	0/772	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus
SR-798-fw	793	668	NA	NA	NA	NA
SR-798-rv	866	777	NA	NA	NA	NA
SR-801-fw	761	416	KJ812430	100.00	0/416	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus
SR-801-rv	1029	583	CM000733	100.00	0/582	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus
SR-85-fw	962	765	NA	NA	NA	NA
SR-85-rv	1016	858	NA	NA	NA	NA
SR-86-fw	1152	850	NA	NA	NA	NA

Continued on next page

Read	Length raw sequence	Length trimmed sequence	Accession #	Similarity (%)	Difference	Taxonomy
SR-86-rv	1296	823	NA	NA	NA	NA
SR-88-fw	658	485	jgi.1107767	100.00	0/485	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Myroides
SR-88-rv	823	590	CP010817	100.00	0/590	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Myroides
SR-89-fw	628	472	jgi.1107767	100.00	0/472	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Myroides
SR-89-rv	1117	771	CP010817	99.87	1/771	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Myroides
SR-90-fw	875	489	jgi.1107767	100.00	0/489	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Myroides
SR-90-rv	1023	484	CP010817	100.00	0/484	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Myroides
SR-92-fw	1242	842	NA	NA	NA	NA
SR-92-rv	1197	851	NA	NA	NA	NA
SR-955-fw	631	521	KJ812430	100.00	0/521	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus
SR-955-rv	520	390	CM000733	100.00	0/389	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus

Continued on next page

Read	Length raw sequence	Length trimmed sequence	Accession #	Similarity (%)	Difference	Taxonomy
SR-974-fw	1147	565	NA	NA	NA	NA
SR-974-rv	1106	839	NA	NA	NA	NA

NA: not available

Table A.4: *16S/fungal sequencing details for ethylene producers: merged forward and reverse reads*

Read	Accession #	Similarity (%)	Difference	Taxonomy	Length sequence
SR-12	AY970951	99.64	5/1379	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	1380
SR-234	BBJT01000029	99.06	13/1376	Bacteria; Firmicutes; Bacilli; Bacillales; Paenibacillaceae; Paenibacillus	1377
SR-24	CP009048	99.64	5/1394	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	1395
SR-25	ACMU01000002	99.86	2/1408	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	1408
SR-265	KM029994.1	99	6/1557	Eukaryota; Opisthokonta; Fungi; Dikarya; Ascomycota; saccharomyceta; Saccharomycotina; Saccharomycetes; Saccharomycetales; Pichiaceae; Pichia	1555
SR-26	MACI01000041	99.93	1/1389	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	1389
SR-27	MACI01000041	100.00	0/1377	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	1377
SR-30	PDFK01000008	100.00	0/1376	Bacteria; Firmicutes; Bacilli; Bacillales; Planococcaceae; Lysinibacillus	1376

Continued on next page

Read	Accession #	Similarity (%)	Difference	Taxonomy	Length sequence
SR-4	LT222224	99.58	6/1417	Bacteria; Proteobacteria; Gammaproteobacteria; Lysobacterales; Lysobacteraceae; Stenotrophomonas	1417
SR-416	KI271266	99.86	2/1436	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	1436
SR-44	NVLJ0100002	899.71	4/1378	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	1379
SR-45	MACI0100004	199.93	1/1423	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	1423
SR-59	AE017333	99.65	5/1433	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	1433
SR-70	JH792383	99.93	1/1426	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	1426
SR-763	AE016877	100.00	0/1421	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	1421
SR-769	ACNF0100015	699.93	1/1420	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	1420
SR-76	NVLJ0100002	8100.00	0/1420	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	1420
SR-778	ACNF0100015	699.79	3/1438	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	1438
SR-780	ACNF0100015	699.93	1/1438	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	1438

Continued on next page

Read	Accession #	Similarity (%)	Difference	Taxonomy	Length sequence
SR-783	MACI01000041	99.93	1/1353	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	1353
SR-784	Y17654	99.34	9/1363	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacterales; Enterobacteriaceae; Klebsiella; Klebsiella pneumoniae	1367
SR-78	ACNF01000156	99.86	2/1426	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	1426
SR-790	KY930948	99.71	2/700	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacterales; Morganellaceae; Proteus	1293
SR-792	NVLJ01000028	99.86	2/1438	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	1438
SR-795	L37599	100.00	0/1379	Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Staphylococcus; Staphylococcus capitis	1379
SR-798	L37605	99.85	2/1373	Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Staphylococcus	1373
SR-85	MACI01000041	99.93	1/1423	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	1423
SR-86	MDDN01000041	100.00	0/1375	Bacteria; Firmicutes; Bacilli; Bacillales; Planococcaceae; Lysinibacillus	1375
SR-92	CDBZ01000012	99.93	1/1368	Bacteria; Proteobacteria; Gammaproteobacteria; Aeromonadales; Aeromonadaceae; Aeromonas	1368

Continued on next page

Read	Accession #	Similarity (%)	Difference	Taxonomy	Length sequence
SR-974	AE016877	100.00	0/1377	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	1377

A.3 DNA Sequences

A.3.1 DNA Sequence of the *Pueraria montana* Isoprene Synthase (kIspS)

The native kIspS gene from *P. montana* used to enhance isoprene production in *C. necator* and *P. pastoris* X-33. GenBank accession no AY316691 [280].

ATGGCAACCAACCTTTTATGCTTGTCTAATAAATTATCGTCCCCCACACCAACACCAAGTACTAGATTTCCACAAAGTAA
GAACTTCATCACACAAAAAACATCTCTTGCCAATCCCAAACCTTGGCGAGTTATTTGTGCTACGAGCTCTCAATTTACCC
AAATAACAGAACATAATAGTCGGCGTTCAGCTAATTACCAGCCAAACCTCTGGAATTTTGAATTTCTGCAGTCTCTGGAA
AATGACCTTAAGGTGGAAAAACTAGAAGAGAAGGCAACAAAGCTAGAGGAGGAGGTACGATGCATGATCAACAGAGTAGA
CACACAACCATTAAGCTTACTAGAATTGATCGACGATGTCCAGCGTCTAGGATTGACCTACAAGTTTGAGAAGGACATAA
TCAAAGCCCTTGAGAATATTGTTTTGCTGGATGAGAATAAGAAAAATAAAAGTGACCTCCATGCTACTGCTCTCAGCTTC
CGTTTACTTAGACAACATGGCTTTGAGGTTTCCCAAGATGTGTTGAGAGATTTAAGGACAAGGAGGGAGGTTTCAGTGGT
GAACTTAAAGGTGATGTGCAAGGGTTGCTGAGTCTATATGAAGCATCCTATCTTGGCTTTGAGGGAGAAAATCTCTTGGA
GGAGGCAAGGACATTTTCAATAACACATCTCAAGAACAACCTAAAAGAAGGAATAAACACCAAAGTGGCAGAACAAGTTA
GTCATGCACTGGAACTTCCCTATCATCAAAGATTGCATAGACTAGAAGCACGATGGTTCCTTGACAAATATGAACCAAAG
GAACCCCACCATCAGTTACTACTCGAGCTTGCAAAGCTAGATTTCAATATGGTGCAAACATTGCACCAGAAAGAACTGCA
AGACCTGTCAAGGTGGTGGACGGAGATGGGGCTAGCAAGCAAGCTAGACTTTGTCCGAGACAGATTAATGGAAGTGTATT
TTTGGGCGTTGGGAATGGCACCTGATCCTCAATTCGGTGAATGTCGTAAAGCTGTCACTAAATGTTTGGATTGGTCACCA
TCATCGATGATGTATATGACGTTTATGGTACTTTGGATGAGCTACAACTCTTCACTGATGCTGTTGAGAGATGGGACGTG
AATGCCATAAACACACTTCCAGACTACATGAAGTTGTGCTTCCTAGCACTTTATAACACCGTCAATGACACGTCTTATAG
CATCCTTAAAGAAAAAGGACACAACAACCTTTCCTATTTGACAAAATCTTGGCGTGAGTTATGCAAAGCATTCCTTCAAG
AAGCAAAATGGTCGAACAACAAAATCATTCCAGCATTTAGCAAGTACCTGGAAAATGCATCGGTGTCCTCCTCCGGTGTG
GCTTTGCTTGCTCCTTCCTACTTCTCAGTGTGCCAACAACAAGAAGATATCTCAGACCATGCTCTTCGTTCTTTAACTGA
TTTCCATGGCCTTGTGCGCTCCTCATGCGTCATTTTCAGACTCTGCAATGATTTGGCTACCTCAGCGGCTGAGCTAGAGA
GGGGTGAGACGACAAATTCAATAATATCTTATATGCATGAGAATGACGGCACTTCTGAAGAGCAAGCACGTGAGGAGTTG
AGAAAATTGATCGATGCAGAGTGGAAGAAGATGAACCGAGAGCGAGTTTCAGATTCTACACTACTCCCAAAAGCTTTTAT
GGAAATAGCTGTTAACATGGCTCGAGTTTCGCATTGCACATACCAATATGGAGACGGACTTGGAAGGCCAGACTACGCCA
CAGAGAATAGAATCAAGTTGCTACTTATAGACCCCTTTCCAATCAATCAACTAATGTACGTGTAA

A.3.2 DNA Sequence of the *mev*-Operon (Synthetic Genes)

ATGACCCGTAAAGGCTATGGCGAAAGCACCGGTAAAATCATTCTGATTGGCGAACATGCGGTGACCTTTGGCGAACCGGC
GATTGCGGTGCCGTTTAACGCGGGCAAAATTAAAGTGCTGATCGAAGCGCTGGAAAGCGGCAACTATAGCAGCATTAAAA
GCGATGTGTATGATGGCATGCTGTATGATGCGCCGGATCATCTGAAAAGCCTGGTGAACCGTTTTGTGGAACTGAACAAC
ATTACCGAACCGCTGGCCGTGACCATTCAGACCAACCTGCCGCCGAGCCGTGGTCTGGGTAGCAGCGCGGCGGTTGCGGT

TGCGTTTGTGCGTGCGAGCTATGATTTTCTGGGCAAAAGCCTGACCAAAGAAGAACTGATCGAAAAAGCGAACTGGGCGG
AACAGATTGCGCATGGCAAACCGAGCGGCATTGATACCCAGACCATTGTGAGCGGCAAACCGGTGTGGTTTCAGAAAGGC
CATGCGGAAACCCTGAAAACCCTGAGCCTGGATGGCTATATGGTGGTGATTGATACCGGCGTGAAAGGCAGCACCCGTCA
GGCGGTGGAAGATGTGCATAAACTGTGCGAAGATCCGCAGTATATGAGCCATGTGAAACATATTGGCAAACTGGTGCTGC
GTGCGAGCGATGTGATTGAACATCATAACTTTGAAGCGCTGGCCGACATCTTTAACGAATGCCACGCGGATCTGAAAGCG
CTGACCGTGAGCCATGATAAAATTGAACAGCTGATGAAAATCGGCAAAGAAAACGGCGCGATTGCGGGCAAACTGACCGG
TGCGGGCCGTGGTGGTAGCATGCTGCTGCTGGCCAAAGATCTGCCGACCGCGAAAAACATTGTGAAAGCGGTGGAAAAAG
CGGGTGCGGCGCATACCTGGATTGAAAACCTGGGCGGCTAATTAATTAACGGATCCAGAAGGAGATATACAATGACCATC
GGCATTGATAAAATCAACTTCTATGTGCCGAAATACTATGTGGATATGGCGAAACTGGCCGAAGCGCGTCAGGTTGATCC
GAACAAATTTCTGATTGGCATTGGCCAGACCGAAATGGCGGTGAGCCCGGTGAACCAGGATATTGTGAGCATGGGCGCGA
ACGCGGCGAAAGATATTATTACCGATGAAGATAAAAAAAAAATCGGCATGGTGATTGTGGCGACCGAAAGCGCGGTGGAT
GCGGCGAAAGCGGCGGCGGTGCAGATTCATAACCTGCTGGGCATTCAGCCGTTTGCGCGTTGCTTTGAAATGAAAGAAGC
GTGCTACGCGGCGACCCCGGCGATTCAGCTGGCCAAAGATTATCTGGCCACCCGTCCGAACGAAAAAGTGCTGGTGATTG
CGACCGATACCGCGCGTTATGGCCTGAACAGCGGCGGTGAACCGACCCAGGGTGCGGGTGCGGTGGCGATGGTGATTGCG
CATAATCCGAGCATTCTGGCCCTGAACGAAGATGCGGTGGCGTATACCGAAGATGTGTACGATTTTTGGCGTCCGACCGG
CCATAAATATCCGCTGGTTGATGGCGCCCTGAGCAAAGATGCGTATATTCGTAGCTTTCAGCAGAGCTGGAACGAATATG
CGAAACGTCAGGGTAAAAGCCTGGCCGATTTTGCGAGCCTGTGCTTTCATGTGCCGTTTACCAAAATGGGCAAAAAAGCC
CTGGAAAGCATTATTGATAACGCGGATGAAACCACCCAGGAACGTCTGCGTAGCGGCTATGAAGATGCGGTTGATTATAA
CCGTTACGTGGGTAACATTTATACCGGCAGCCTGTATCTGAGCCTGATTAGCCTGCTGGAAAACCGTGATCTGCAGGCGG
GCGAAACCATTGGCCTGTTTAGCTATGGCAGCGGCAGCGTGGGCGAATTTTATAGCGCGACCCTGGTGGAAGGCTATAAA
GATCATCTGGATCAGGCGGCGCATAAAGCGCTGCTGAACAACCGTACCGAAGTGAGCGTGGATGCGTATGAAACCTTTTT
TAAACGCTTCGATGATGTGGAATTTGATGAAGAACAGGATGCGGTGCATGAAGATCGCCATATCTTCTATCTGAGCAACA
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AAAAACTTTCGTCATCTGAGCCGTCAGCAGAAACTGCAGCAGCTGGTTGATAAACAGTGGCTGTCTGAAGATCAGTTTGA
TATCCTGCTGAACCATCCGCTGATTGATGAAGAAGTGGCGAACAGCCTGATTGAAAACGTGATTGCGCAGGGCGCGCTGC
CGGTTGGTCTGCTGCCGAACATTATCGTGGATGATAAAGCGTATGTGGTGCCGATGATGGTGGAAGAACCGAGCGTTGTT
GCGGCGGCGAGCTATGGCGCGAAACTGGTGAACCAGACCGGCGGCTTTAAAACCGTGAGCAGCGAACGTATTATGATTGG
CCAGATCGTGTTTGATGGCGTGGATGATACCGAAAAACTGAGCGCGGATATTAAAGCGCTGGAAAAACAGATTCACAAAA
TCGCGGATGAAGCGTATCCGAGCATTAAAGCGCGTGGCGGCGGTTATCAGCGTATTGCGATTGATACCTTTCCGGAACAG
CAGCTGCTGTCTCTGAAAGTGTTTGTGGATACCAAAGATGCGATGGGCGCGAATATGCTGAACACCATTCTGGAAGCGAT
TACCGCGTTTCTGAAAAACGAAAGCCCGCAGAGCGATATTCTGATGAGCATTCTGAGCAACCATGCGACCGCGAGCGTGG
TGAAAGTGCAGGGCGAAATTGATGTGAAAGATCTGGCCCGTGGCGAACGTACCGGCGAGGAAGTGGCCAAACGTATGGAA
CGTGCGAGCGTGCTGGCCCAGGTGGATATTCATCGTGCGGCGACCCATAACAAAGGCGTGATGAACGGCATTCATGCGGT
GGTGCTGGCCACCGGCAACGATACCCGTGGTGCGGAAGCGAGCGCGCATGCGTATGCGAGCCGTGATGGCCAGTATCGTG
GCATTGCGACCTGGCGTTATGATCAGAAACGTCAGCGTCTGATTGGCACCATTGAAGTGCCGATGACCCTGGCCATTGTG
GGCGGTGGCACCAAAGTGCTGCCGATTGCGAAAGCGAGCCTGGAACTGCTGAACGTGGATAGCGCGCAGGAACTGGGCCA
CGTTGTTGCCGCCGTTGGCCTGGCCCAGAACTTTGCGGCGTGCCGTGCGCTGGTGAGCGAAGGCATTCAGCAGGGCCATA
TGAGCCTGCAGTACAAAAGCCTGGCCATCGTGGTGGGCGCGAAAGGCGATGAAATTGCGCAGGTGGCGGAAGCGCTGAAA

CAGGAACCGCGCGCGAACACCCAGGTGGCCGAACGTATTCTGCAGGAAATTCGTCAGCAGTAAACTAGTAGCGGCCGCAG
AAGGAGATATACAATGACCCGCGTGGTTCTGGCCGCCGCGTATCGTACCCCGATTGGCGTGTTTGGCGGTGCGTTTAAAG
ATGTGCCGGCGTATGATCTGGGCGCGACGCTGATTGAACATATCATCAAAGAAACCGGCCTGAATCCGAGCGAAATTGAT
GAAGTGATTATCGGCAACGTGCTGCAGGCGGGTCAGGGTCAGAATCCGGCGCGTATTGCGGCGATGAAAGGCGGCCTGCC
GGAAACCGTTCCGGCGTTTACCGTGAACAAAGTGTGCGGCAGCGGCCTGAAAAGCATCCAGCTGGCCTATCAGAGCATTG
TGACCGGCGAAAACGATATTGTGCTGGCCGGTGGCATGGAAAACATGAGCCAGAGCCCGATGCTGGTGAACAACAGCCGT
TTTGGCTTTAAAATGGGCCATCAGAGCATGGTGGATAGCATGGTGTATGATGGTCTGACCGATGTGTTTAACCAGTATCA
TATGGGCATTACCGCCGAAAACCTGGTGGAACAGTATGGCATTAGCCGCGAAGAACAGGATACCTTTGCGGTGAACAGCC
AGCAGAAAGCGGTGCGTGCGCAGCAGAACGGCGAATTTGATAGCGAAATTGTGCCGGTGAGCATTCCGCAGCGTAAAGGC
GAACCGATTGTGGTGACCAAAGATGAAGGCGTGCGTGAAAACGTGAGCGTGGAAAAACTGAGCCGTCTGCGTCCGGCGTT
TAAAAAAGATGGCACCGTGACCGCCGGTAACGCGAGCGGCATTAACGATGGTGCGGCGATGATGCTGGTGATGAGCGAAG
ATAAAGCGAAAGAACTGAACATTGAACCGCTGGCCGTTCTGGATGGCTTTGGCAGCCATGGCGTTGATCCGAGCATCATG
GGCATTGCGCCGGTGGGCGCGGTTGAAAAAGCCCTGAAACGCAGCAAAAAAGAACTGAGCGATATTGATGTGTTCGAACT
GAACGAAGCGTTTGCGGCGCAGAGCCTGGCCGTGGATCGTGAACTGAAACTGCCGCCGGAAAAAGTGAACGTGAAAGGCG
GTGCGATTGCGCTGGGCCATCCGATTGGTGCGAGCGGTGCGCGTGTGCTGGTGACCCTGCTGCATCAGCTGAACGATGAA
GTGGAAACCGGTCTGACCAGCCTGTGCATTGGCGGCGGTCAGGCGATTGCGGCGGTGGTGAGCAAATATAAATAAATCGA
TGGGCCCAGAAGGAGATATACAATGATCCAGGTGAAAGCGCCGGGTAAACTGTATATTGCGGGCGAATATGCGGTTACCG
AACCGGGCTATAAAAGCGTGCTGATCGCGCTGGATCGTTTTGTGACCGCGACCATTGAAGAAGCGGATCAGTATAAAGGC
ACCATTCATAGCAAAGCGCTGCACCATAATCCGGTGACCTTTAGCCGTGATGAAGATAGCATTGTGATTAGCGATCCGCA
TGCGGCGAAACAGCTGAACTATGTGGTGACCGCGATTGAAATCTTTGAACAGTACGCGAAAAGCTGCGATATTGCGATGA
AACATTTCCACCTGACCATTGATAGCAACCTGGATGATAGCAACGGTCATAAATATGGCCTGGGCAGCAGCGCCGCCGTT
CTGGTGAGCGTGATCAAAGTGCTGAACGAATTTTATGATATGAAACTGTCTAACCTGTACATTTATAAACTGGCCGTGAT
CGCCAATATGAAACTGCAGAGCCTGAGCAGCTGCGGCGATATTGCGGTGAGCGTGTATAGCGGCTGGCTGGCCTATAGCA
CCTTTGATCACGAATGGGTGAAACATCAGATTGAAGATACCACCGTGGAAGAAGTGCTGATTAAAAACTGGCCGGGTCTG
CATATCGAACCGCTGCAGGCGCCGGAAAACATGGAAGTTCTGATTGGCTGGACCGGTAGCCCGGCGAGCAGCCCGCATTT
TGTGAGCGAAGTGAAACGTCTGAAAAGCGATCCGAGCTTTTATGGCGATTTTCTGGAAGATAGCCATCGTTGCGTTGAAA
AACTGATCCATGCGTTCAAAACCAACAACATTAAAGGCGTGCAGAAAATGGTGCGTCAGAACCGTACCATTATTCAGCGC
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GAGCAAAACCAGCGGTGCGGGTGGCGGCGATTGTGGCATTACCATCATCAACAAAGATGTGGATAAAGAAAAAATCTATG
ATGAATGGACCAAACATGGCATTAAACCGCTGAAATTCAACATTTATCATGGCCAGTAACTCGAGAAGCTTAGAAGGAGA
TATACAATGATTAAAAGCGGCAAAGCGCGTGCGCATACCAACATTGCGCTGATTAAATATTGGGGCAAAAAAGATGAAGC
GCTGATTATTCCGATGAACAACAGCATTAGCGTGACCCTGGAAAAATTTTATACCGAAACCAAAGTGACCTTTAACGATC
AGCTGACCCAGGATCAGTTTTGGCTGAACGGCGAAAAAGTGAGCGGTAAAGAACTGGAAAAAATCAGCAAATATATGGAT
ATTGTGCGTAACCGTGCGGGCATTGATTGGTATGCGGAAATCGAAAGCGATAACTTTGTGCCGACCGCCGCCGGTCTGGC
CAGCAGCGCGAGCGCGTATGCGGCGCTGGCCGCCGCCTGTAATCAGGCGCTGGATATGCAGCTGTCTGATAAAGATCTGA
GCCGCCTGGCCCGTATTGGTAGCGGTAGCGCGAGCCGTAGCATTTATGGCGGCTTTGCGGAATGGGAAAAAGGCTATAGC
GATGAAACCAGCTATGCGGTGCCGCTGGAAAGCAACCATTTTGAAGATGATCTGGCCATGATTTTTGTGGTGATCAACCA
GCATAGCAAAAAAGTGCCGAGCCGTTATGGCATGAGCCTGACCCGTAACACCAGCCGTTTTTATCAGTATTGGCTGGATC

ACATTGATGAAGATCTGGCCGAAGCCAAAGCGGCGATTCAGGATAAAGACTTTAAACGTCTGGGCGAAGTGATTGAAGAA
AACGGCCTGCGTATGCATGCGACCAACCTGGGTAGCACGCCGCCGTTTACCTATCTGGTGCAAGAAAGCTATGACGTGAT
GGCGCTGGTGCATGAATGCCGTGAAGCGGGCTATCCGTGCTATTTCACCATGGATGCGGGTCCGAACGTCAAAATTCTGG
TGGAAAAAAAAAACAAACAGCAGATCATTGATAAACTGCTGACCCAGTTTGATAACAACCAGATTATTGATAGCGATATC
ATTGCGACCGGCATCGAAATCATCGAATAA

A.3.3 DNA Sequence of the GFP Genes

gfp from pUC-gfp

ATGAGTAAAGGAGAAGAACTTTTCACCGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATT
TTCTGTCAGTGGAGAGGGTGAAGGTGATGCAACATACGGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAACTAC
CTGTTCCATGGCCAACACTTGTCACTACTTTCTCTTATGGTGTTCAATGCTTTTCAAGATACCCAGATCATATGAAACGG
CATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAAAGAACTATATTTTTCAAAGATGACGGGAACTACAA
GACACGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATAGAATCGAGTTAAAAGGTATTGATTTTAAAGAAGATG
GAAACATTCTTGGACACAAATTGGAATACAACTATAACTCACACAATGTATACATCATGGCAGACAAACAAAAGAATGGA
ATCAAAGTTAACTTCAAAATTAGACACAACATTGAAGATGGAAGCGTTCAACTAGCAGACCATTATCAACAAAATACTCC
AATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGATCCCAACGAAA
AGAGAGACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAACTATACAAATAG

gfp from pJeM1TcR

ATGACCATGATTACGCATCATCATCATCATCATGGATCCAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCT
GGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGC
TGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTG
CAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGA
GCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACC
GCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCAC
AACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAG
CGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGA
GCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGG
ATCACTCTCGGCATGGACGAGCTGTACAAGTAA

A.3.4 Sequences of the Cloned 4-Oxalocrotonate Tautomerase Genes

4-OT of *Bacillus cereus* SR-772

ATGCCATACGTAACAGTGAAAATGCTAGAAGGACGCACAGAAGAACAAAAGAAAGCTCTTGCTGAGAAAGTAACAGCAGC
AGTAAGCGAAACAACTGGTGCTCCTGAAGAAAACATCGTTGTTTTCATCGAAGAAATGTCTAAAAACCATTATGCAGTTG
GCGGAAAACGCTTAAGCGACAAATAA

4-OT of *Lactobacillus brevis* SR-416

ATGCCAATCGTAAACATCGACTTAATTGCTGGTCGTTCACAAGACCAACTCAAGGCTTTAGTTCAAGACGTGACGACCGC
TGTGACTAAAAATACAGGGGCCCCTGCTGAACACGTCCACGTTATTTTACGTGAAATGCAACCTAACCGTTACGGCGTGG
CTGGCGTTTTGAAGAGCGACGAAAAATAA

4-OT of *E. coli* DH5α

ATGCCGCACATCGACATTAAATGTTTTCCGCGTGAACTGGACGAACAACAAAAAGCAGCACTTGCTGCAGATATTACCGA
CGTTATTATTCGTCATCTGAACAGTAAAGACAGTTCGATAAGCATTGCTCTACAGCAGATTCAACCAGAATCTTGGCAAG
CTATCTGGGATGCCGAAATCGCGCCCCAAATGGAGGCTTTGATAAAGAAACCTGGTTATAGCATGAATGCTTAA

4-OT of *Lysinibacillus xylanilyticus* SR-86

ATGCCATACGTAACAGTGAAAATGCTTGAAGGTCGTACAGACGAACAAAAACGCGCACTAGTAAAAGAAGTAACAGAAGC
CGTTTCTCGTACAGTTAACGCGCCAGCAGAAAACGTAACAGTCTTTATCGAAGAAATGCCTAAAAACCATTACGGCGTTG
CAGGCGTGCTCCACAGTGATAAATAA

A.4 Protein Sequences

Petunia x hybrida ACCO, Q08506.1

MENFPIISLDKVNGVERAATMEMIKDACENWGFFELVNHGIPREVMDTVEKMTKGHYKKCMEQRFKELVASKALEGVQAE
VTDMDWESTFFLKHLPISNISEVPDLDEEYREVMRDFAKRLEKLAEELLDLLCENLGLEKGYLKNAFYGSKGPNFGTKVS
NYPPCPKPDLIKGLRAHTDAGGIILLFQDDKVSGLQLLKDGQWIDVPPMRHSIVVNLGDQLEVITNGKYKSVMHRVIAQK
DGARMSLASFYNPGSDAVIYPAPALVEKEAEENKQVYPKFVFDDYMKLYAGLKFQAKEPRFEAMKAMETDVKMDPIATV

Solanum lycopersicum ACCO, NP_001233867.2

MESNFPVVDMGLLQTEKRPEAMDKIKDACENWGFFELVNHGISHELLDTVENLTKGHYKKCMEQRFKEMVASKGLEAVQT
EIDDLDWESTFFLKHLPVSNVYEVPDLDDEYRKVMKDFALKLEKLAENLLDLLCENLGLEKGYLKKAFYGSKGPTFGTKV
SNYPPCPKPDLIKGLRAHTDAGGIILLFQDDKVSGLQLLKDGNWIDVPPMKHSIVINLGDQLEVITNGRYKSIEHRVIAQ
QDGTRMSIASFYNPGSDAVIFPAPELIEKTEEDNKLKYPKFVFEDYMKLYAGLKFQAKEPRFEAMKAVETTVNLGPIETV

Malus domestica ACCO, CAA74328.1

MATFPVVDLSLVNGEERAATLEKINDACENWGFFELVNHGMSTELLDTVEKMTKDHYKKTMEQRFKEMVAAKGLDDVQSE
IHDLDWESTFFLRHLPSSNISEIPDLEEEYRKTMKEFAVELEKLAEKLLDLLCENLGLEKGYLKKVFYGSKGPNFGTKVS
NYPPCPKPDLIKGLRAHSDAGGIILLFQDDKVSGLQLLKDGEWVDVPPMHHSIVINLGDQIEVITNGKYKSVMHRVIAQS
DGTRMSIASFYNPGNDSFISPAPAVLEKKTEDAPTYPKFVFDDYMKLYSGLKFQAKEPRFEAMKAKESTPVATA

Pichia fermentans SR-265 ACCO, 4016

MTTVATNTTKHQKMDQTKLEIIDLEDTSSDNAKKIVHASSTQGFLMFEGHGFTEQEIDLLFQYSHDYFKLSVDEKNKCVI
QTDNAGYTGMGVENLEEDDLDRGYGDPKEGFNFANFDLKTGIPKQPIPEFWQDKMEVISATVLKLRDALKKALRLMAVGL
EIEGDEGLDENWFVQRHHDDDFSGTTFRFLHYPAPVNAGASEEEKDKFRDLNVAGAHTDYGTVTLLFQKKEESGLQLYSP
ISKNWENVPFVDASPKYKARGEAAPLIVNIGDQLCYWTNGHLKSTIHRVRFPSSLLDQGKDRYSIVLFAHPGDETHLEPV
PSKFIKEIKGRGASHYMEKHGVSQTAGQHLTNRLNSTYGWKY

Pichia fermentans SR-265 ACCO, 593

MISPEEKQAIREKYNYRPFKEAPPSKAEVELLPLETIDLSKYDEGPEARKQLAQRLEKALTEYGFFRLVNHGFSPEFLET
MKSISQSTFETSEEVKAKFLAGKNNIELDDGLELGVIRGTGYKPRGYWTYTNETKDNVEFFNVRHFNFHEVFNSIEHPEF
VKANLDDIEFYFTYLHQQIQRKVLNLIDIILGLPEGEMYTKYFRVEDNDPNDSGTGFGRFLLYHPVDEDYNRKTTSTWMR
GHTDAGAFTYILSQPILSLQVRTYDDYQWKYVGHVPNSIIVNAGDTIKFLTAGYFKSAVHRVHTAPQDQKDCHRNTIIYF
ASPKLDINMDPETLNSPKLTEKGYTRDKSIPKVTIREWDEAKGKFFNKKSANWVTNIVILGRECVGSLIGEKAIKT

***Pichia fermentans* SR-265 ACCO, 8391**

MTMTTTPDAIPVIDLSDDSPKNCKRLIEAAIKSGCLFLKNHLLEEDEIDKLFDISSQFFRLPTEVKNQYPITNKNAGYVA
PFVEDLQEDGTGAGDDKEAFNMCRINLSTFYPDQNLPQVFAENMPFISFCMRKHYVMLHMICRMLAQGLEVKDNRGFLDP
YFFASAHALDHPSHSTMRFIHYSKHDESYENVNLTGAHTDYGSITLILQRPQNGLQIFDGSKWKSVEVPLDENGKQMLVV
NISDILSFWTDGLLKSVLHRVRTTSERDSIVFFCHPADDVLLVPVNSELVKSYNGKCFSLNKNGLPLTALDHLRLRLNEG
YKRKD

A.5 Gas Chromatography Data for Ethylene Assays

Table A.5: *Ethylene production (Area [pA*min]) from various substrate combinations of eight chosen positive hits from the screening. Corresponding figure:* ***Fig. 3.1*** *and* ***3.2***

Strain	Replicate	Substrate	Ethylene (Area [pA*min])
SR-782	Replciate 1	10 mM L-Met, 10 mM ATP	0.037
SR-782	Replciate 1	10 L-Met, 20 mM ATP	0
SR-782	Replciate 1	20 mM L-Met, 10 mM ATP	0.045
SR-782	Replciate 1	20 mM L-Met, 20 mM ATP	0.005
SR-782	Replciate 1	10 mM L-Met	0.004
SR-782	Replciate 1	20 mM L-Met	0.006
SR-782	Replciate 1	10 mM ATP	0.006
SR-782	Replciate 1	20 mM ATP	0
SR-782	Replciate 1	10 mM L-Met, 10 mM α-KG	0.04
SR-782	Replciate 1	10 L-Met, 20 mM α-KG	0.039
SR-782	Replciate 1	20 mM L-Met, 10 mM α-KG	0.038
SR-782	Replciate 1	20 mM L-Met, 20 mM α-KG	0.055
SR-782	Replciate 1	10 mM α-KG	0.004
SR-782	Replciate 1	20 mM α-KG	0
SR-782	Replciate 1	10 mM ACC	0
SR-782	Replciate 1	H_2O	0
SR-782	Replciate 2	10 mM L-Met, 10 mM ATP	0.008
SR-782	Replciate 2	10 L-Met, 20 mM ATP	0
SR-782	Replciate 2	20 mM L-Met, 10 mM ATP	0.008
SR-782	Replciate 2	20 mM L-Met, 20 mM ATP	0
SR-782	Replciate 2	10 mM L-Met	0
SR-782	Replciate 2	20 mM L-Met	0

Continued on next page

Strain	Replicate	Substrate	Ethylene (Area [pA*min])
SR-782	Replciate 2	10 mM ATP	0.003
SR-782	Replciate 2	20 mM ATP	0
SR-782	Replciate 2	10 mM L-Met, 10 mM α-KG	0.011
SR-782	Replciate 2	10 L-Met, 20 mM α-KG	0.018
SR-782	Replciate 2	20 mM L-Met, 10 mM α-KG	0.01
SR-782	Replciate 2	20 mM L-Met, 20 mM α-KG	0.024
SR-782	Replciate 2	10 mM α-KG	0
SR-782	Replciate 2	20 mM α-KG	0.002
SR-782	Replciate 2	10 mM ACC	0.123
SR-782	Replciate 2	H_2O	0
SR-782	Replciate 3	10 mM L-Met, 10 mM ATP	0.028
SR-782	Replciate 3	10 L-Met, 20 mM ATP	0.004
SR-782	Replciate 3	20 mM L-Met, 10 mM ATP	0.029
SR-782	Replciate 3	20 mM L-Met, 20 mM ATP	0.005
SR-782	Replciate 3	10 mM L-Met	0.004
SR-782	Replciate 3	20 mM L-Met	0.004
SR-782	Replciate 3	10 mM ATP	0.006
SR-782	Replciate 3	20 mM ATP	0
SR-782	Replciate 3	10 mM L-Met, 10 mM α-KG	0
SR-782	Replciate 3	10 L-Met, 20 mM α-KG	0.014
SR-782	Replciate 3	20 mM L-Met, 10 mM α-KG	0.012
SR-782	Replciate 3	20 mM L-Met, 20 mM α-KG	0.017
SR-782	Replciate 3	10 mM α-KG	0
SR-782	Replciate 3	20 mM α-KG	0
SR-782	Replciate 3	10 mM ACC	0.187
SR-782	Replciate 3	H_2O	0
SR-783	Replciate 1	10 mM L-Met, 10 mM ATP	0.014
SR-783	Replciate 1	10 L-Met, 20 mM ATP	0
SR-783	Replciate 1	20 mM L-Met, 10 mM ATP	0.01
SR-783	Replciate 1	20 mM L-Met, 20 mM ATP	0
SR-783	Replciate 1	10 mM L-Met	0
SR-783	Replciate 1	20 mM L-Met	0
SR-783	Replciate 1	10 mM ATP	0.004
SR-783	Replciate 1	20 mM ATP	0
SR-783	Replciate 1	10 mM L-Met, 10 mM α-KG	0.008
SR-783	Replciate 1	10 L-Met, 20 mM α-KG	0.015

Continued on next page

Strain	Replicate	Substrate	Ethylene (Area [pA*min])
SR-783	Replciate 1	20 mM L-Met, 10 mM α-KG	0.009
SR-783	Replciate 1	20 mM L-Met, 20 mM α-KG	0.021
SR-783	Replciate 1	10 mM α-KG	0
SR-783	Replciate 1	20 mM α-KG	0.003
SR-783	Replciate 1	10 mM ACC	0.519
SR-783	Replciate 1	H_2O	0
SR-783	Replciate 2	10 mM L-Met, 10 mM ATP	0
SR-783	Replciate 2	10 L-Met, 20 mM ATP	0
SR-783	Replciate 2	20 mM L-Met, 10 mM ATP	0
SR-783	Replciate 2	20 mM L-Met, 20 mM ATP	0
SR-783	Replciate 2	10 mM L-Met	0
SR-783	Replciate 2	20 mM L-Met	0
SR-783	Replciate 2	10 mM ATP	0
SR-783	Replciate 2	20 mM ATP	0
SR-783	Replciate 2	10 mM L-Met, 10 mM α-KG	0.008
SR-783	Replciate 2	10 L-Met, 20 mM α-KG	0.008
SR-783	Replciate 2	20 mM L-Met, 10 mM α-KG	0.008
SR-783	Replciate 2	20 mM L-Met, 20 mM α-KG	0.009
SR-783	Replciate 2	10 mM α-KG	0.003
SR-783	Replciate 2	20 mM α-KG	0.003
SR-783	Replciate 2	10 mM ACC	0.799
SR-783	Replciate 2	H_2O	0
SR-783	Replciate 3	10 mM L-Met, 10 mM ATP	0.1
SR-783	Replciate 3	10 L-Met, 20 mM ATP	0.052
SR-783	Replciate 3	20 mM L-Met, 10 mM ATP	0.102
SR-783	Replciate 3	20 mM L-Met, 20 mM ATP	0.059
SR-783	Replciate 3	10 mM L-Met	0.012
SR-783	Replciate 3	20 mM L-Met	0.031
SR-783	Replciate 3	10 mM ATP	0.006
SR-783	Replciate 3	20 mM ATP	0
SR-783	Replciate 3	10 mM L-Met, 10 mM α-KG	0.216
SR-783	Replciate 3	10 L-Met, 20 mM α-KG	0.231
SR-783	Replciate 3	20 mM L-Met, 10 mM α-KG	0.205
SR-783	Replciate 3	20 mM L-Met, 20 mM α-KG	0.252
SR-783	Replciate 3	10 mM α-KG	0.007
SR-783	Replciate 3	20 mM α-KG	0.007

Continued on next page

Strain	Replicate	Substrate	Ethylene (Area [pA*min])
SR-783	Replciate 3	10 mM ACC	0.303
SR-783	Replciate 3	H_2O	0
SR-86	Replciate 1	10 mM L-Met, 10 mM ATP	0.012
SR-86	Replciate 1	10 L-Met, 20 mM ATP	0
SR-86	Replciate 1	20 mM L-Met, 10 mM ATP	0.009
SR-86	Replciate 1	20 mM L-Met, 20 mM ATP	0
SR-86	Replciate 1	10 mM L-Met	0.019
SR-86	Replciate 1	20 mM L-Met	0.018
SR-86	Replciate 1	10 mM ATP	0
SR-86	Replciate 1	20 mM ATP	0
SR-86	Replciate 1	10 mM L-Met, 10 mM α-KG	0.094
SR-86	Replciate 1	10 L-Met, 20 mM α-KG	0.121
SR-86	Replciate 1	20 mM L-Met, 10 mM α-KG	0.082
SR-86	Replciate 1	20 mM L-Met, 20 mM α-KG	0.116
SR-86	Replciate 1	10 mM α-KG	0
SR-86	Replciate 1	20 mM α-KG	0
SR-86	Replciate 1	10 mM ACC	0.087
SR-86	Replciate 1	H_2O	0.005
SR-86	Replciate 2	10 mM L-Met, 10 mM ATP	0.01
SR-86	Replciate 2	10 L-Met, 20 mM ATP	0.003
SR-86	Replciate 2	20 mM L-Met, 10 mM ATP	0.007
SR-86	Replciate 2	20 mM L-Met, 20 mM ATP	0.004
SR-86	Replciate 2	10 mM L-Met	0.017
SR-86	Replciate 2	20 mM L-Met	0.016
SR-86	Replciate 2	10 mM ATP	0
SR-86	Replciate 2	20 mM ATP	0
SR-86	Replciate 2	10 mM L-Met, 10 mM α-KG	0.104
SR-86	Replciate 2	10 L-Met, 20 mM α-KG	0.102
SR-86	Replciate 2	20 mM L-Met, 10 mM α-KG	0.095
SR-86	Replciate 2	20 mM L-Met, 20 mM α-KG	0.111
SR-86	Replciate 2	10 mM α-KG	0
SR-86	Replciate 2	20 mM α-KG	0.004
SR-86	Replciate 2	10 mM ACC	0.14
SR-86	Replciate 2	H_2O	0.005
SR-86	Replciate 3	10 mM L-Met, 10 mM ATP	0.008
SR-86	Replciate 3	10 L-Met, 20 mM ATP	0

Continued on next page

Strain	Replicate	Substrate	Ethylene (Area [pA*min])
SR-86	Replciate 3	20 mM L-Met, 10 mM ATP	0
SR-86	Replciate 3	20 mM L-Met, 20 mM ATP	0
SR-86	Replciate 3	10 mM L-Met	0
SR-86	Replciate 3	20 mM L-Met	0.015
SR-86	Replciate 3	10 mM ATP	0
SR-86	Replciate 3	20 mM ATP	0
SR-86	Replciate 3	10 mM L-Met, 10 mM α-KG	0.092
SR-86	Replciate 3	10 L-Met, 20 mM α-KG	0.111
SR-86	Replciate 3	20 mM L-Met, 10 mM α-KG	0.09
SR-86	Replciate 3	20 mM L-Met, 20 mM α-KG	0.125
SR-86	Replciate 3	10 mM α-KG	0.003
SR-86	Replciate 3	20 mM α-KG	0
SR-86	Replciate 3	10 mM ACC	0.128
SR-86	Replciate 3	H_2O	0.004
SR-792	Replciate 1	10 mM L-Met, 10 mM ATP	0.005
SR-792	Replciate 1	10 L-Met, 20 mM ATP	0
SR-792	Replciate 1	20 mM L-Met, 10 mM ATP	0.005
SR-792	Replciate 1	20 mM L-Met, 20 mM ATP	0
SR-792	Replciate 1	10 mM L-Met	0
SR-792	Replciate 1	20 mM L-Met	0
SR-792	Replciate 1	10 mM ATP	0
SR-792	Replciate 1	20 mM ATP	0
SR-792	Replciate 1	10 mM L-Met, 10 mM α-KG	0
SR-792	Replciate 1	10 L-Met, 20 mM α-KG	0
SR-792	Replciate 1	20 mM L-Met, 10 mM α-KG	0
SR-792	Replciate 1	20 mM L-Met, 20 mM α-KG	0.003
SR-792	Replciate 1	10 mM α-KG	0
SR-792	Replciate 1	20 mM α-KG	0
SR-792	Replciate 1	10 mM ACC	0.142
SR-792	Replciate 1	H_2O	0
SR-792	Replciate 2	10 mM L-Met, 10 mM ATP	0.004
SR-792	Replciate 2	10 L-Met, 20 mM ATP	0
SR-792	Replciate 2	20 mM L-Met, 10 mM ATP	0.004
SR-792	Replciate 2	20 mM L-Met, 20 mM ATP	0
SR-792	Replciate 2	10 mM L-Met	0.003
SR-792	Replciate 2	20 mM L-Met	0.005

Continued on next page

Strain	Replicate	Substrate	Ethylene (Area [pA*min])
SR-792	Replciate 2	10 mM ATP	0
SR-792	Replciate 2	20 mM ATP	0
SR-792	Replciate 2	10 mM L-Met, 10 mM α-KG	0.009
SR-792	Replciate 2	10 L-Met, 20 mM α-KG	0.01
SR-792	Replciate 2	20 mM L-Met, 10 mM α-KG	0.013
SR-792	Replciate 2	20 mM L-Met, 20 mM α-KG	0.014
SR-792	Replciate 2	10 mM α-KG	0.004
SR-792	Replciate 2	20 mM α-KG	0.004
SR-792	Replciate 2	10 mM ACC	0.42
SR-792	Replciate 2	H_2O	0
SR-792	Replciate 3	10 mM L-Met, 10 mM ATP	0
SR-792	Replciate 3	10 L-Met, 20 mM ATP	0
SR-792	Replciate 3	20 mM L-Met, 10 mM ATP	0
SR-792	Replciate 3	20 mM L-Met, 20 mM ATP	0
SR-792	Replciate 3	10 mM L-Met	0
SR-792	Replciate 3	20 mM L-Met	0.005
SR-792	Replciate 3	10 mM ATP	0
SR-792	Replciate 3	20 mM ATP	0
SR-792	Replciate 3	10 mM L-Met, 10 mM α-KG	0.008
SR-792	Replciate 3	10 L-Met, 20 mM α-KG	0.009
SR-792	Replciate 3	20 mM L-Met, 10 mM α-KG	0.01
SR-792	Replciate 3	20 mM L-Met, 20 mM α-KG	0.008
SR-792	Replciate 3	10 mM α-KG	0.005
SR-792	Replciate 3	20 mM α-KG	0.006
SR-792	Replciate 3	10 mM ACC	0.409
SR-792	Replciate 3	H_2O	0
SR-769	Replciate 1	10 mM L-Met, 10 mM ATP	0.057
SR-769	Replciate 1	10 L-Met, 20 mM ATP	0.004
SR-769	Replciate 1	20 mM L-Met, 10 mM ATP	0.051
SR-769	Replciate 1	20 mM L-Met, 20 mM ATP	0.005
SR-769	Replciate 1	10 mM L-Met	0.125
SR-769	Replciate 1	20 mM L-Met	0.117
SR-769	Replciate 1	10 mM ATP	0.005
SR-769	Replciate 1	20 mM ATP	0
SR-769	Replciate 1	10 mM L-Met, 10 mM α-KG	0.291
SR-769	Replciate 1	10 L-Met, 20 mM α-KG	0.281

Continued on next page

Strain	Replicate	Substrate	Ethylene (Area [pA*min])
SR-769	Replciate 1	20 mM L-Met, 10 mM α-KG	0.282
SR-769	Replciate 1	20 mM L-Met, 20 mM α-KG	0.287
SR-769	Replciate 1	10 mM α-KG	0.029
SR-769	Replciate 1	20 mM α-KG	0.024
SR-769	Replciate 1	10 mM ACC	0.086
SR-769	Replciate 1	H_2O	0.015
SR-769	Replciate 2	10 mM L-Met, 10 mM ATP	0.031
SR-769	Replciate 2	10 L-Met, 20 mM ATP	0
SR-769	Replciate 2	20 mM L-Met, 10 mM ATP	0.036
SR-769	Replciate 2	20 mM L-Met, 20 mM ATP	0
SR-769	Replciate 2	10 mM L-Met	0.008
SR-769	Replciate 2	20 mM L-Met	0.012
SR-769	Replciate 2	10 mM ATP	0.004
SR-769	Replciate 2	20 mM ATP	0
SR-769	Replciate 2	10 mM L-Met, 10 mM α-KG	0.017
SR-769	Replciate 2	10 L-Met, 20 mM α-KG	0.018
SR-769	Replciate 2	20 mM L-Met, 10 mM α-KG	0.024
SR-769	Replciate 2	20 mM L-Met, 20 mM α-KG	0.022
SR-769	Replciate 2	10 mM α-KG	0.005
SR-769	Replciate 2	20 mM α-KG	0.003
SR-769	Replciate 2	10 mM ACC	0.183
SR-769	Replciate 2	H_2O	0
SR-769	Replciate 3	10 mM L-Met, 10 mM ATP	0.092
SR-769	Replciate 3	10 L-Met, 20 mM ATP	0.005
SR-769	Replciate 3	20 mM L-Met, 10 mM ATP	0.111
SR-769	Replciate 3	20 mM L-Met, 20 mM ATP	0.007
SR-769	Replciate 3	10 mM L-Met	0.087
SR-769	Replciate 3	20 mM L-Met	0.105
SR-769	Replciate 3	10 mM ATP	0.008
SR-769	Replciate 3	20 mM ATP	0
SR-769	Replciate 3	10 mM L-Met, 10 mM α-KG	0.417
SR-769	Replciate 3	10 L-Met, 20 mM α-KG	0.445
SR-769	Replciate 3	20 mM L-Met, 10 mM α-KG	0.516
SR-769	Replciate 3	20 mM L-Met, 20 mM α-KG	0
SR-769	Replciate 3	10 mM α-KG	0.025
SR-769	Replciate 3	20 mM α-KG	0.03

Continued on next page

Strain	Replicate	Substrate	Ethylene (Area [pA*min])
SR-769	Replciate 3	10 mM ACC	0.151
SR-769	Replciate 3	H_2O	0.007
SR-772	Replciate 1	10 mM L-Met, 10 mM ATP	0.074
SR-772	Replciate 1	10 L-Met, 20 mM ATP	0
SR-772	Replciate 1	20 mM L-Met, 10 mM ATP	0.058
SR-772	Replciate 1	20 mM L-Met, 20 mM ATP	0.005
SR-772	Replciate 1	10 mM L-Met	0.101
SR-772	Replciate 1	20 mM L-Met	0.09
SR-772	Replciate 1	10 mM ATP	0
SR-772	Replciate 1	20 mM ATP	0
SR-772	Replciate 1	10 mM L-Met, 10 mM α-KG	0.41
SR-772	Replciate 1	10 L-Met, 20 mM α-KG	0.404
SR-772	Replciate 1	20 mM L-Met, 10 mM α-KG	0
SR-772	Replciate 1	20 mM L-Met, 20 mM α-KG	0
SR-772	Replciate 1	10 mM α-KG	0.054
SR-772	Replciate 1	20 mM α-KG	0.041
SR-772	Replciate 1	10 mM ACC	0.187
SR-772	Replciate 1	H_2O	0.034
SR-772	Replciate 2	10 mM L-Met, 10 mM ATP	0.086
SR-772	Replciate 2	10 L-Met, 20 mM ATP	0.004
SR-772	Replciate 2	20 mM L-Met, 10 mM ATP	0.068
SR-772	Replciate 2	20 mM L-Met, 20 mM ATP	0.005
SR-772	Replciate 2	10 mM L-Met	0.133
SR-772	Replciate 2	20 mM L-Met	0.134
SR-772	Replciate 2	10 mM ATP	0.009
SR-772	Replciate 2	20 mM ATP	0
SR-772	Replciate 2	10 mM L-Met, 10 mM α-KG	0.612
SR-772	Replciate 2	10 L-Met, 20 mM α-KG	0.611
SR-772	Replciate 2	20 mM L-Met, 10 mM α-KG	0.552
SR-772	Replciate 2	20 mM L-Met, 20 mM α-KG	0.518
SR-772	Replciate 2	10 mM α-KG	0.053
SR-772	Replciate 2	20 mM α-KG	0.054
SR-772	Replciate 2	10 mM ACC	0.249
SR-772	Replciate 2	H_2O	0.046
SR-772	Replciate 3	10 mM L-Met, 10 mM ATP	0.085
SR-772	Replciate 3	10 L-Met, 20 mM ATP	0.009

Continued on next page

Strain	Replicate	Substrate	Ethylene (Area [pA*min])
SR-772	Replciate 3	20 mM L-Met, 10 mM ATP	0.083
SR-772	Replciate 3	20 mM L-Met, 20 mM ATP	0.022
SR-772	Replciate 3	10 mM L-Met	0.149
SR-772	Replciate 3	20 mM L-Met	0.148
SR-772	Replciate 3	10 mM ATP	0
SR-772	Replciate 3	20 mM ATP	0
SR-772	Replciate 3	10 mM L-Met, 10 mM α-KG	0.233
SR-772	Replciate 3	10 L-Met, 20 mM α-KG	0.292
SR-772	Replciate 3	20 mM L-Met, 10 mM α-KG	0.316
SR-772	Replciate 3	20 mM L-Met, 20 mM α-KG	0.355
SR-772	Replciate 3	10 mM α-KG	0.024
SR-772	Replciate 3	20 mM α-KG	0.02
SR-772	Replciate 3	10 mM ACC	0.102
SR-772	Replciate 3	H_2O	0.017
SR-778	Replciate 1	10 mM L-Met, 10 mM ATP	0.011
SR-778	Replciate 1	10 L-Met, 20 mM ATP	0
SR-778	Replciate 1	20 mM L-Met, 10 mM ATP	0.016
SR-778	Replciate 1	20 mM L-Met, 20 mM ATP	0
SR-778	Replciate 1	10 mM L-Met	0
SR-778	Replciate 1	20 mM L-Met	0
SR-778	Replciate 1	10 mM ATP	0
SR-778	Replciate 1	20 mM ATP	0
SR-778	Replciate 1	10 mM L-Met, 10 mM α-KG	0.007
SR-778	Replciate 1	10 L-Met, 20 mM α-KG	0.006
SR-778	Replciate 1	20 mM L-Met, 10 mM α-KG	0.009
SR-778	Replciate 1	20 mM L-Met, 20 mM α-KG	0.008
SR-778	Replciate 1	10 mM α-KG	0
SR-778	Replciate 1	20 mM α-KG	0
SR-778	Replciate 1	10 mM ACC	0.175
SR-778	Replciate 1	H_2O	0
SR-778	Replciate 2	10 mM L-Met, 10 mM ATP	0.018
SR-778	Replciate 2	10 L-Met, 20 mM ATP	0
SR-778	Replciate 2	20 mM L-Met, 10 mM ATP	0.019
SR-778	Replciate 2	20 mM L-Met, 20 mM ATP	0
SR-778	Replciate 2	10 mM L-Met	0
SR-778	Replciate 2	20 mM L-Met	0

Continued on next page

Strain	Replicate	Substrate	Ethylene (Area [pA*min])
SR-778	Replciate 2	10 mM ATP	0
SR-778	Replciate 2	20 mM ATP	0
SR-778	Replciate 2	10 mM L-Met, 10 mM α-KG	0.005
SR-778	Replciate 2	10 L-Met, 20 mM α-KG	0.006
SR-778	Replciate 2	20 mM L-Met, 10 mM α-KG	0.008
SR-778	Replciate 2	20 mM L-Met, 20 mM α-KG	0.008
SR-778	Replciate 2	10 mM α-KG	0
SR-778	Replciate 2	20 mM α-KG	0
SR-778	Replciate 2	10 mM ACC	0.103
SR-778	Replciate 2	H_2O	0
SR-778	Replciate 3	10 mM L-Met, 10 mM ATP	0.023
SR-778	Replciate 3	10 L-Met, 20 mM ATP	0
SR-778	Replciate 3	20 mM L-Met, 10 mM ATP	0.022
SR-778	Replciate 3	20 mM L-Met, 20 mM ATP	0
SR-778	Replciate 3	10 mM L-Met	0.005
SR-778	Replciate 3	20 mM L-Met	0.006
SR-778	Replciate 3	10 mM ATP	0
SR-778	Replciate 3	20 mM ATP	0
SR-778	Replciate 3	10 mM L-Met, 10 mM α-KG	0.024
SR-778	Replciate 3	10 L-Met, 20 mM α-KG	0.025
SR-778	Replciate 3	20 mM L-Met, 10 mM α-KG	0.032
SR-778	Replciate 3	20 mM L-Met, 20 mM α-KG	0.042
SR-778	Replciate 3	10 mM α-KG	0
SR-778	Replciate 3	20 mM α-KG	0
SR-778	Replciate 3	10 mM ACC	0.106
SR-778	Replciate 3	H_2O	0
SR-780	Replciate 1	10 mM L-Met, 10 mM ATP	0.011
SR-780	Replciate 1	10 L-Met, 20 mM ATP	0
SR-780	Replciate 1	20 mM L-Met, 10 mM ATP	0.016
SR-780	Replciate 1	20 mM L-Met, 20 mM ATP	0
SR-780	Replciate 1	10 mM L-Met	0
SR-780	Replciate 1	20 mM L-Met	0
SR-780	Replciate 1	10 mM ATP	0
SR-780	Replciate 1	20 mM ATP	0
SR-780	Replciate 1	10 mM L-Met, 10 mM α-KG	0.007
SR-780	Replciate 1	10 L-Met, 20 mM α-KG	0

Continued on next page

Strain	Replicate	Substrate	Ethylene (Area [pA*min])
SR-780	Replciate 1	20 mM L-Met, 10 mM α-KG	0.009
SR-780	Replciate 1	20 mM L-Met, 20 mM α-KG	0.007
SR-780	Replciate 1	10 mM α-KG	0
SR-780	Replciate 1	20 mM α-KG	0
SR-780	Replciate 1	10 mM ACC	0.309
SR-780	Replciate 1	H_2O	0
SR-780	Replciate 2	10 mM L-Met, 10 mM ATP	0.159
SR-780	Replciate 2	10 L-Met, 20 mM ATP	0.01
SR-780	Replciate 2	20 mM L-Met, 10 mM ATP	0.121
SR-780	Replciate 2	20 mM L-Met, 20 mM ATP	0.016
SR-780	Replciate 2	10 mM L-Met	0.135
SR-780	Replciate 2	20 mM L-Met	0.117
SR-780	Replciate 2	10 mM ATP	0.015
SR-780	Replciate 2	20 mM ATP	0
SR-780	Replciate 2	10 mM L-Met, 10 mM α-KG	0.423
SR-780	Replciate 2	10 L-Met, 20 mM α-KG	0.412
SR-780	Replciate 2	20 mM L-Met, 10 mM α-KG	0.44
SR-780	Replciate 2	20 mM L-Met, 20 mM α-KG	0.366
SR-780	Replciate 2	10 mM α-KG	0.022
SR-780	Replciate 2	20 mM α-KG	0.02
SR-780	Replciate 2	10 mM ACC	0.127
SR-780	Replciate 2	H_2O	0.02
SR-780	Replciate 3	10 mM L-Met, 10 mM ATP	0.162
SR-780	Replciate 3	10 L-Met, 20 mM ATP	0.014
SR-780	Replciate 3	20 mM L-Met, 10 mM ATP	0.149
SR-780	Replciate 3	20 mM L-Met, 20 mM ATP	0.017
SR-780	Replciate 3	10 mM L-Met	0.198
SR-780	Replciate 3	20 mM L-Met	0.186
SR-780	Replciate 3	10 mM ATP	0.018
SR-780	Replciate 3	20 mM ATP	0
SR-780	Replciate 3	10 mM L-Met, 10 mM α-KG	0.344
SR-780	Replciate 3	10 L-Met, 20 mM α-KG	0.224
SR-780	Replciate 3	20 mM L-Met, 10 mM α-KG	0.306
SR-780	Replciate 3	20 mM L-Met, 20 mM α-KG	0.184
SR-780	Replciate 3	10 mM α-KG	0.022
SR-780	Replciate 3	20 mM α-KG	0.02

Continued on next page

Strain	Replicate	Substrate	Ethylene (Area [pA*min])
SR-780	Replciate 3	10 mM ACC	0.114
SR-780	Replciate 3	H_2O	0.025

Table A.6: *Corresponding OD_{600} values to the experiments in **Tab. A.5***

Strain	OD_{600}
SR-782	94
SR-783	78
SR-792	124
SR-769	100
SR-772	100
SR-778	100
SR-780	100
SR-86	81

Table A.7: *Ethylene production (Area [pA*min]) from substrate combinations identifying the ACC pathway. Corresponding figure: **Fig. 3.3***

Strain	Substrate	Ethylene (Area [pA*min])
B. cereus SR-772	10mM L-Met, 10mM ATP	0.2 $\pm$0.17
B. cereus SR-772	10mM L-Met, 20mM ATP	0.084 $\pm$0.076
B. cereus SR-7724	20mM L-Met, 10mM ATP	0.2 $\pm$0.15
B. cereus SR-772	20mM L-Met, 20mM ATP	0.091 $\pm$0.074
B. cereus SR-772	10mM L-Met	0.072 $\pm$0.068
B. cereus SR-772	20mM L-Met	0.06 $\pm$0.056
B. cereus SR-772	10mM ATP	0.014 $\pm$0.019
B. cereus SR-772	20mM ATP	0.007 $\pm$0.0066
B. cereus SR-772	10mM ACC	0.076 $\pm$0.044
B. cereus SR-772	20mM ACC	0.17 $\pm$0.028
B. cereus SR-772	1.6mM SAM	0.0093 $\pm$0.0081
B. cereus SR-772	3.2mM SAM	0.01 $\pm$0.009
B. cereus SR-772	Water	0.0097 $\pm$0.011
P. fermantans SR-265	10mM L-Met, 10mM ATP	0.036 $\pm$0.003
P. fermantans SR-265	10mM L-Met, 20mM ATP	0.11 $\pm$0.059
P. fermantans SR-265	20mM L-Met, 10mM ATP	0.041 $\pm$0.0076
P. fermantans SR-265	20mM L-Met, 20mM ATP	0.12 $\pm$0.067

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Strain	Substrate	Ethylene (Area [pA*min])
P. fermantans SR-2653	10mM L-Met	0.013 ±0.012
P. fermantans SR-265	20mM L-Met	0.012 ±0.011
P. fermantans SR-2653	10mM ATP	0.031 ±0.0023
P. fermantans SR-265	20mM ATP	0.06 ±0.012
P. fermantans SR-265	10mM ACC	0.04 ±0.012
P. fermantans SR-265	20mM ACC	0.095 ±0.054
P. fermantans SR-265	1.6mM SAM	0.009 ±0.0085
P. fermantans SR-265	3.2mM SAM	0.013 ±0.0047
P. fermantans SR-265	Water	0.019 ±0.0083
L. brevis SR-416	10mM L-Met, 10mM ATP	0 ±0
L. brevis SR-416	10mM L-Met, 20mM ATP	0 ±0
L. brevis SR-4165	20mM L-Met, 10mM ATP	0 ±0
L. brevis SR-4165	20mM L-Met, 20mM ATP	0 ±0
L. brevis SR-4165	10mM L-Met	0 ±0
L. brevis SR-416	20mM L-Met	0 ±0
L. brevis SR-4165	10mM ATP	0 ±0
L. brevis SR-416	20mM ATP	0 ±0
L. brevis SR-416	10mM ACC	0.23 ±0.11
L. brevis SR-416	20mM ACC	0.71 ±0.31
L. brevis SR-416	1.6mM SAM	0.00067 ±0.0012
L. brevis SR-416	3.2mM SAM	0 ±0
L. brevis SR-416	Water	0 ±0
L. xylanilyticus SR-86	10mM L-Met, 10mM ATP	0.023 ±0.0038
L. xylanilyticus SR-86	10mM L-Met, 20mM ATP	0.003 ±0.001
L. xylanilyticus SR-86	20mM L-Met, 10mM ATP	0.018 ±0.0052
L. xylanilyticus SR-86	20mM L-Met, 20mM ATP	0.0027 ±0.0012
L. xylanilyticus SR-86	10mM L-Met	0.043 ±0.0062
L. xylanilyticus SR-86	20mM L-Met	0.043 ±0.0065
L. xylanilyticus SR-86	10mM ATP	0.0043 ±0.00058
L. xylanilyticus SR-86	20mM ATP	0 ±0
L. xylanilyticus SR-86	10mM ACC	0.2 ±0.014
L. xylanilyticus SR-86	20mM ACC	0.36 ±0.062
L. xylanilyticus SR-86	1.6mM SAM	0.0053 ±0.00058
L. xylanilyticus SR-86	3.2mM SAM	0.0063 ±0.00058
L. xylanilyticus SR-86	Water	0.005 ±0.001

Table A.8: *Ethylene production (Area [pA*min]) from substrate combinations identifying the KMBA pathway. Corresponding figure:* ***Fig. 3.3***

Strain	Substrate	Ethylene (Area [pA*min])
B. cereus SR-772	10mM L-Met	0.072 ±0.068
B. cereus SR-772	20mM L-Met	0.06 ±0.056
B. cereus SR-772	10mM α-ketoglutarate 10mM L-Met	0.21 ±0.22
B. cereus SR-772	10mM α-ketoglutarate-KG, 20mM L-Met	0.3 ±0.26
B. cereus SR-772	20mM α-ketoglutarate-KG, 10mM L-Met	0.19 ±0.17
B. cereus SR-772	20mM α-ketoglutarate-KG, 20mM L-Met	0.28 ±0.25
B. cereus SR-772	10mM α-ketoglutarate-KG	0.012 ±0.014
B. cereus SR-772	20mM α-ketoglutarate-KG	0.01 ±0.011
B. cereus SR-772	10mM KMBA	2.5 ±2.3
B. cereus SR-772	20mM KMBA	2.8 ±2.3
B. cereus SR-772	H_2O	0.0097 ±0.011
P. fermentans SR-265	10mM L-Met	0.013 ±0.012
P. fermentans SR-265	20mM L-Met	0.012 ±0.011
P. fermentans SR-2653	10mM α-ketoglutarate 10mM L-Met	0.011 ±0.019
P. fermentans SR-2653	10mM α-ketoglutarate-KG, 20mM L-Met	0.0097 ±0.017
P. fermentans SR-265	20mM α-ketoglutarate-KG, 10mM L-Met	0.014 ±0.015
P. fermentans SR-265	20mM α-ketoglutarate-KG, 20mM L-Met	0.013 ±0.014
P. fermentans SR-265	10mM α-ketoglutarate-KG	0.019 ±0.012
P. fermentans SR-265	20mM α-ketoglutarate-KG	0.015 ±0.012
P. fermentans SR-265	10mM KMBA	0.083 ±0.05
P. fermentans SR-265	20mM KMBA	0.18 ±0.098
P. fermentans SR-265	H_2O	0.019 ±0.0083
L. brvis SR-416	10mM L-Met	0 ±0
L. brvis SR-416	20mM L-Met	0 ±0
L. brvis SR-416 5	10mM α-ketoglutarate 10mM L-Met	0 ±0
L. brvis SR-416 5	10mM α-ketoglutarate-KG, 20mM L-Met	0 ±0
L. brvis SR-416	20mM α-ketoglutarate-KG, 10mM L-Met	0 ±0
L. brvis SR-416 5	20mM α-ketoglutarate-KG, 20mM L-Met	0 ±0
L. brvis SR-416	10mM α-ketoglutarate-KG	0 ±0
L. brvis SR-416	20mM α-ketoglutarate-KG	0 ±0
L. brvis SR-416	10mM KMBA	0.24 ±0.0055
L. brvis SR-416	20mM KMBA	0.45 ±0.063
L. brvis SR-416	H_2O	0 ±0
L. xylanilyticus SR-86	10mM L-Met	0.043 ±0.0062

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Strain	Substrate	Ethylene (Area [pA*min])
L. xylanilyticus SR-86	20mM L-Met	0.043 ±0.0065
L. xylanilyticus SR-862	10mM α-ketoglutarate 10mM L-Met	0.16 ±0.042
L. xylanilyticus SR-86	10mM α-ketoglutarate-KG, 20mM L-Met	0.25 ±0.075
L. xylanilyticus SR-86	20mM α-ketoglutarate-KG, 10mM L-Met	0.16 ±0.021
L. xylanilyticus SR-86	20mM α-ketoglutarate-KG, 20mM L-Met	0.29 ±0.068
L. xylanilyticus SR-86	10mM α-ketoglutarate-KG	0.0083 ±0.0023
L. xylanilyticus SR-86	20mM α-ketoglutarate-KG	0.01 ±0.0032
L. xylanilyticus SR-86	10mM KMBA	0.75 ±0.17
L. xylanilyticus SR-86	20mM KMBA	1.4 ±0.22
L. xylanilyticus SR-86	H_2O	0.005 ±0.001

Table A.9: *Ethylene production (Area [pA*min]) with added metals and electron complexes. Corresponding figure:* ***Fig. 3.5***

Substrate	*L. xylanilyticus* SR-86	*B. cereus* SR-773	*L. brevis* SR-416
Control	0.073 ±0.019	0.016 ±0.0026	0.029 ±0.021
Zink chloride	0.095 ±0.023	0.033 ±0.018	0.028 ±0.018
Cobalt chlroide	1.1 ±0.52	0.37 ±0.033	0.069 ±0.033
Nickel sulpahte	0.085 ±0.0084	0.016 ±0.0021	0.028 ±0.016
Manganese chloride	0.16 ±0.037	0.078 ±0.017	0.064 ±0
Mannitol (10 mM)	0.066 ±0.017	0.014 ±0.0012	0.041 ±0.012
Boric acid	0.068 ±0.017	0.015 ±0.0021	0.033 ±0.018
Glucose (10 mM)	0.068 ±0.016	0.015 ±0.0015	0.03 ±0.0092
Iron(III) sulphate	0.074 ±0.012	0.022 ±0.0031	0.035 ±0.013
Iron(II) chlroide	0.073 ±0.013	0.018 ±0.0012	0.029 ±0.023
EDTA	0.019 ±0.018	0 ±0	0.007 ±0.0017
Copper sulphate	0.047 ±0.018	0.014 ±0.0023	0.018 ±0.004
Molybdate	0.07 ±0.015	0.016 ±0.003	0.04 ±0.019
Sodium tartrate	0.07 ±0.015	0.015 ±0.0031	0.031 ±0.014
Vitamine B12	0.075 ±0.02	0.015 ±0.0017	0.029 ±0.013
Calcium chloride	0.07 ±0.015	0.016 ±0.0021	0.041 ±0.02
Magnesium sulphate	0.072 ±0.016	0.016 ±0.0035	0.046 ±0.0064
Sodium chloride	0.071 ±0.02	0.016 ±0.0025	0.037 ±0.014

Table A.10: *Ethylene production (Area [pA*min]) with added metals and electron complexes. Corresponding figure:* ***Fig. 3.6***

Substrate	*L. xylanilyticus* SR-86	*B. cereus* SR-773	*L. brevis* SR-416
Control	0.5 ±0.15	0.015 ±0.0021	0.043 ±0.013
β-Alanine	0.47 ±0.19	0.015 ±0.004	0.016 ±0.017
D-Serine	0.44 ±0.14	0.019 ±0.0042	0.061 ±0.02
Iso-Leucine	0.35 ±0.1	0.016 ±0.0031	0.05 ±0.031
L-Alanine	0.42 ±0.13	0.017 ±0.0032	0.045 ±0.018
L-Arginine	0.72 ±0.05	0.032 ±0.0038	0.32 ±0.18
L-Cysteine	0.38 ±0.062	0.026 ±0.0025	0.0093 ±0.0015
L-Glutamate	1.3 ±0.31	0.019 ±0.0026	0.055 ±0.013
L-Glutamine	0.71 ±0.19	0.017 ±0.0023	0.038 ±0.019
L-Glycine	0.46 ±0.11	0.016 ±0.0015	0.047 ±0.014
L-Leucine	0.37 ±0.087	0.018 ±0.0035	0.038 ±0.019
L-Lysine	0.63 ±0.19	0.028 ±0.0059	0.075 ±0.042
L-Methionine	0.39 ±0.072	0.014 ±0.0021	0.058 ±0.021
L-Proline	0.5 ±0.14	0.018 ±0.001	0.018 ±0.019
L-Tyrosine	0.23 ±0.063	0.015 ±0.0026	0.018 ±0.0066
L-Valine	0.42 ±0.08	0.018 ±0.0012	0.044 ±0.0068
α-KG	5.2 ±5.4	0.071 ±0.02	19 ±0.826
Cas amino acids	0.96 ±0.077	0.028 ±0.0055	0.012 ±0.012
Ascorbate	0.059 ±0.04	0.0063 ±0.0035	0.005 ±0.005
Tryptophane	0.16 ±0.055	0.019 ±0.0078	0.043 ±0.014
Succinate	0.96 ±0.36	0.033 ±0.0075	0.066 ±0.019

Table A.11: *Ethylene production of Lysinibacillus xylanilyticus SR-86 from ACC in combination with various α-keto acids. Corresponding figure:* ***Fig. 3.7***

Substrate	Ethylene production (Area [pA*min])
ACC	0.042
α-aminoisobutyric acid	0
α-Ketobutyric acid	0
α-Ketoglutarate	0
ACC + α-aminoisobutyric acid	0.069
ACC + α-Ketobutyric acid	0.095
ACC + α-Ketoglutarate	0.212

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Substrate	Ethylene production (Area [pA*min])
α-Ketobglutarate + a-aminoisobutyric acid	0

Table A.12: *Effect on ethylene production (area [pA*min]) of Lysinibacillus xylanilyticus SR-86, Bacillus cereus SR-772 and Lactobacillus brevis SR-416 from ACC of Argon, catalase, proteinase K and heat (95°C). Corresponding figure:* ***Fig. 3.8***

Substrate	*L. xylanilyticus* SR-86	*B. cereus* SR-773	*L. brevis* SR-416
ACC	0.073 $\pm$0.019	0.016 $\pm$0.0026	0.035 $\pm$0.023
37 °C	0.078 $\pm$0.017	0.017 $\pm$0.0017	0.037 $\pm$0.025
Proteinase K	0.026 $\pm$0.013	0.0087 $\pm$0.0012	0.02 $\pm$0.016
Catalase	0.032 $\pm$0.013	0.02 $\pm$0.0026	0.024 $\pm$0.0071
Argon	0.011 $\pm$0.0029	0.0087 $\pm$0.0021	0.031 $\pm$0.019
95 °C	0.013 $\pm$0.0082	0.03 $\pm$0.0026	0.028 $\pm$0.0047

Table A.13: *Effect on ethylene production (area [pA*min]) from the Efe-pathway substrates L-arginine and α-ketoglutarate in Lysinibacillus xylanilyticus SR-86, Bacillus cereus SR-772 and Lactobacillus brevis SR-416. Corresponding figure:* ***Fig. 3.9***

Substrate	*L. xylanilyticus* SR-86	*B. cereus* SR-773	*L. brevis* SR-416
ACC	0.073 $\pm$0.019	0.015 $\pm$0.0021	0.043 $\pm$0.013
L-arginine + Water	0 $\pm$0	0 $\pm$0	0 $\pm$0
L-arginine + ACC	0.094 $\pm$0.0064	0.032 $\pm$0.0038	0.32 $\pm$0.18
α-KG + Water	0 $\pm$0	0 $\pm$0	0 $\pm$0
α-KG + ACC	1.1 $\pm$0.59	0.071 $\pm$0.02	19 $\pm$0.82
L-arginie + α-KG	0.0023 $\pm$0.0021	0 $\pm$0	0 $\pm$0
L-arginine + α-KG + ACC	2.1 $\pm$0.57	0.81 $\pm$0.21	18 $\pm$15

Table A.14: *Effect of pH on ethylene production (area [pA*min]) of Lysinibacillus xylanilyticus SR-86 and Bacillus cereus SR-772 as average and standard deviation from three individual experiments. Corresponding figure:* ***Fig. 3.10***

pH	*L. xylanilyticus* SR-86	*B. cereus* SR-772
3	0 $\pm$0	NA $\pm$NA
5	0 $\pm$0	0 $\pm$0

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pH	*L. xylanilyticus* SR-86	*B. cereus* SR-772
7	0.04 ±0.02	0.02 ±0
9	0.2 ±0.1	0.09 ±0.02
11	0.76 ±0.14	NA ±NA

Table A.15: *Effect of variable ACC and α-KG concentrations on ethylene production (area [pA*min] of protein extracts from Lysinibacillus xylanilyticus SR-86 and Lactobacillus brevis SR-416 (n=1). Corresponding figure:* ***Fig. 3.11****)*

Substrate	Concentration [mM]	*L. xylanilyticus* SR-86	*L. brevis* SR-416
[α-KG] = variable, [ACC] = 10 mM	0	0	0
	0.005	0	0
	0.01	0	0.005
	0.02	0	0.01
	0.04	0	0.021
	0.08	0	0.046
	0.156	0	0.091
	0.313	0	0.186
	0.625	0.005	0.425
	1.25	0.011	0.911
	2.5	0.027	2.329
	5	0.083	5.42
	10	0.306	13.178
	20	2.017	30.227
	40	9.044	56.822
[ACC] = variable, [α-KG] = 10 mM	0	0.081	0.044
	0.005	0.087	0.05
	0.01	0.075	0.058
	0.02	0.089	0.064
	0.04	0.086	0.088
	0.08	0.082	0.118
	0.156	0.087	0.177
	0.313	0.097	0.34
	0.625	0.106	0.658
	1.25	0.117	1.422
	2.5	0.126	3.087
	5	0.183	8.527
	10	0.288	13.72
	20	0.508	19.576
	40	0.786	23.695

Table A.16: *Effect of variable ACC and α-KG concentrations on ethylene production (area [pA*min]) of desalted protein extracts from Lysinibacillus xylanilyticus SR-86 and Lactobacillus brevis SR-416 (n=1). Corresponding figure:* ***Fig. 3.12***

Substrate	Concentration [mM]	*L. xylanilyticus* SR-86	*L. brevis* SR-416
[α-KG] = variable, [ACC] = 10 mM	0	0	0.011
	1	0	1.339
	10	0.09	13.209
	25	0.995	19.451
	40	3.983	20.587
	50	6.094	18.249
[ACC] = variable, [α-KG] = 10 mM	0	0	0
	1	0	0.776
	10	0.09	13.414
	25	0.995	37.811
	40	3.983	52.233
	50	6.094	59.469

Table A.17: *Effect of variable ACC and α-KG concentrations on ethylene formation (area [pA*min]) from 20 mM MOPS pH7 with and without 0.1% trace elements (n=1). Corresponding figure:* ***Fig. 3.13***

Substrate	Concentration [mM]	20 mM MOPS pH7	20 mM MOPS pH7 0.1% trace elements
[α-KG] = variable, [ACC] = 10 mM	0	0	0.011
	1	0	1.339
	10	0.09	13.209
	25	0.995	19.451
	40	3.983	20.587
	50	6.094	18.249
[ACC] = variable, [α-KG] = 10 mM	0	0	0
	1	0	0.776
	10	0.09	13.414
	25	0.995	37.811
	40	3.983	52.233
	50	6.094	59.469

Table A.18: *Raw data for the protein purification from Lysinibacillus xylanilyticus SR-86. Ethylene was measured from six pooled runs. Corresponding figure:* ***Fig. 3.14***

Fraction	Area [pA*min]	Volume [mL]	Total activity	c(protein)	Protein amount	Specific activity
Protein Extract	0.225	12	6	2450	29400	0.000204
Protein Extract filtered	0.146	12	3.89	NA	NA	NA
Flow Through	0.013	48	1.39	272	13100	0.000106
Wash run 1	0.101	50	11.2	79.1	3960	0.00284
Wash run 2	0.01	50	1.11	78.9	3950	0.000282
Wash run 3	0.009	50	1	82	4100	0.000244
Wash run 4	0.005	50	0.556	79.2	3960	0.00014
Fraction 1	0.012	10	0.267	-10	-100	-0.00266
Fraction 2	0.06	10	1.33	10.8	108	0.0123
Fraction 3	1.09	10	24.2	20.3	203	0.12
Fraction 4	1.61	10	35.9	69.9	699	0.0513
Fraction 5	0.004	10	0.0889	144	1440	6.19e-05
Fraction 6	0	10	0	200	2000	0
Fraction 7	0	10	0	204	2040	0
Fraction 8	0	10	0	136	1360	0
Fraction 9	0	10	0	76.4	764	0
Fraction 10	0	10	0	45.4	454	0
100100100100Buffer A	0.009	12	0.24	NA	NA	NA
A:B = 1:1	0.033	10	0.733	NA	NA	NA

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Fraction	Area [pA*min]	Volume [mL]	Total activity	c(protein)	Protein amount	Specific activity
Buffer B	0.05	50	5.56	NA	NA	NA

NA: not available

Table A.19: *Effect of 3 mg/mL BSA in Glycine NaOH at pH 9.0 on ethylene formation from the 1% trace elements solution (v/v). Corresponding figure:* ***Fig. 3.18***

Components	Ethylene production (Area [pA*min])
1% Trace Elements Solution	2.9 ±0.0071
BSA + 1% Trace Elements Solution	1.3 ±0.049
BSA + 1% Trace Elements Solution + inactivated Proteinase K	1.5 ±0
BSA + 1% Trace Elements Solution + Proteinase K	0.06 ±0.014

Table A.20: *Effect of various compounds on the ethylene production of Bacillus cereus SR-772 from KMBA at pH 7 and pH 5. Corresponding figure:* ***Fig. 3.19***

	Ethylene production (Area [pA*min])	
Substrate	pH 7	pH 5
KMBA	0.20 ±0.0188	0.231 ±0.0377
KMBA + Argon	0.13 ±0.0075	0.134 ±0.0035
KMBA + Catalase	0.20 ±0.0370	0.049 ±0.0090
KMBA + Cobalt chloride	0.17 ±0.0045	0.208 ±0.0340
KMBA + EDTA	0.38 ±0.0325	0.125 ±0.0216
KMBA + Iron(II) chloride	0.43 ±0.0568	0.406 ±0.0699
KMBA + Iron(III) sulphate	0.32 ±0.0107	0.307 ±0.0571
KMBA + NADH	0.50 ±0.0372	6.039 ±0.5966
KMBA + NADH + Iron(III) sulphate + EDTA	NA ±NA	18.830 ±0.3900
KMBA + NADPH	0.41 ±0.0441	7.515 ±0.5140

Table A.21: *Effect of various compounds on the ethylene production of Lysinibacillus xylanilyticus SR-86 from KMBA at pH 7 and pH 5. Corresponding figure:* ***Fig. 3.20***

	Ethylene production (Area [pA*min])	
Substrate	pH 7	pH 5
KMBA	0.65 ±0.1604	0.224 ±0.0470
KMBA + Argon	0.19 ±0.0082	0.088 ±0.0038
KMBA + Catalase	1.25 ±0.0017	-0.036 ±0.0044
KMBA + Cobalt chloride	0.33 ±0.0318	0.208 ±0.0467
KMBA + EDTA	0.92 ±0.2050	0.027 ±0.0035
KMBA + Iron(II) chloride	0.89 ±0.1346	0.353 ±0.1159

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Substrate	Ethylene production (Area [pA*min]) pH 7	pH 5
KMBA + Iron(III) sulphate	0.85 ±0.1080	0.335 ±0.0703
KMBA + NADH	0.87 ±0.0772	3.943 ±0.5576
KMBA + NADH + Iron(III) sulphate + EDTA	NA ±NA	14.277 ±0.5016
KMBA + NADPH	1.00 ±0.0854	4.256 ±0.6178

Table A.22: *Effect of various compounds on the ethylene production of Lactobacillus brevis SR-416 from KMBA at pH 7. Corresponding figure:* ***Fig. 3.21***

Substrate	Ethylene production (Area [pA*min])
KMBA	0.20 ±0.0188
KMBA + Argon	0.13 ±0.0075
KMBA + Catalase	0.20 ±0.0370
KMBA + Cobalt chloride	0.17 ±0.0045
KMBA + EDTA	0.38 ±0.0325
KMBA + Iron(II) chloride	0.43 ±0.0568
KMBA + Iron(III) sulphate	0.32 ±0.0107
KMBA + NADH	0.50 ±0.0372
KMBA + NADH + Iron(III) sulphate + EDTA	NA ±NA
KMBA + NADPH	0.41 ±0.0441

Table A.23: *Effect of pH on ethylene production (area [pA*min]) of Lysinibacillus xylanilyticus SR-86 (n = 3) and Bacillus cereus SR-772 (n = 2). Given is the average and the standard deviation. Corresponding figure:* ***Fig. 3.22***

pH	*L. xylanilyticus* SR-86	*B. cereus* SR-773
3	32.26 ±4.4	NA ±NA
5	6.95 ±2.38	2.62 ±0.88
7	3.55 ±2.1	1.33 ±0.14
9	4.28 ±2.77	2.86 ±1.43
11	0.25 ±0.05	NA ±NA

NA: not available.

Table A.24: *Ethylene production (Area [pA*min]) of fractions of ammonium sulphate precipitation of protein extract from Bacillus cereus SR-772. Ethylene was measured from six pooled runs. Corresponding figure:* ***Fig. 3.23***

Fraction	Area [pA*min]	Protein concentration [mg/mL]	Specific activity [pA*min/ μg/mL]
Protein extract	0.855	249	0.00343
5% Supernatant	0.713	181	0.00393
5% Pellet	0.032	16.6	0.00193
10% Supernatant	0.73	165	0.00441
10% Pellet	0.04	21.2	0.00189
15% Supernatant	0.647	142	0.00455
15% Pellet	0.065	32.8	0.00198
20% Supernatant	0.626	137	0.00457
20% Pellet	0.082	47.7	0.00172
25% Supernatant	0.577	83	0.00695
25% Pellet	0.181	75.4	0.0024
30% Supernatant	0.45	59.8	0.00753
30% Pellet	0.324	115	0.00283
35% Supernatant	0.086	32.1	0.00268
35% Pellet	0.534	126	0.00423
40% Supernatant	0.044	18.7	0.00235
40% Pellet	0.786	119	0.00663
45% Supernatant	0.025	16	0.00156
45% Pellet	0.611	135	0.00453
50% Supernatant	0.039	14.6	0.00268
50% Pellet	0.81	151	0.00538

NA: not available

Table A.25: *Ethylene production (Area [pA*min]) of fractions of HIC column screening for purification of the KMBA-dependent enzyme of Bacillus cereus SR-772. Ethylene was measured from six pooled runs. Corresponding figure:* ***Fig. 3.24***

HIC column	Fraction	Area [pA*min]	Volume [mL]	Ethylene/mL [pA*min/mL]
sepharose	Supernatant	0.084	2.5	0.074
sepharose	Flow through	0.044	2.5	0.039
sepharose	Wash 0% B	0.495	5	0.22
sepharose	Fraction 1	0.077	5	0.034

Continued on next page

HIC column	Fraction	Area [pA*min]	Volume [mL]	Ethylene/mL [pA*min/mL]
sepharose	Fraction 2	0.078	5	0.034
sepharose	Wash 100% B	0.324	5	0.14
sepharose	Buffer A	0	0.45	0
sepharose	Buffer 50% B	0	0.45	0
sepharose	Buffer B	0	0.45	0
butyl-ff	Supernatant	0.084	5	0.037
butyl-ff	Flow through	0.821	5	0.36
butyl-ff	Wash 0% B	2.035	5	0.9
butyl-ff	Fraction 1	0.081	5	0.036
butyl-ff	Fraction 2	0.057	5	0.025
butyl-ff	Wash 100% B	0.519	5	0.23
butyl-ff	Buffer A	0	0.45	0
butyl-ff	Buffer 50% B	0	0.45	0
butyl-ff	Buffer B	0	0.45	0
butyl-s-ff	Supernatant	0.084	5	0.037
butyl-s-ff	Flow through	0.14	5	0.062
butyl-s-ff	Wash 0% B	0.115	5	0.051
butyl-s-ff	Fraction 1	0.162	5	0.071
butyl-s-ff	Fraction 2	0.252	5	0.11
butyl-s-ff	Wash 100% B	0.794	5	0.35
butyl-s-ff	Buffer A	0	0.45	0
butyl-s-ff	Buffer 50% B	0	0.45	0
butyl-s-ff	Buffer B	0	0.45	0

NA: not available

Table A.26: *Ethylene production (Area [pA*min]) of fractions of Butyl-S FF Sepharose from protein extracts treated with varying concentrations of ammonium sulphate for purification of the KMBA-dependent enzyme of Bacillus cereus SR-772. Ethylene was measured from six pooled runs. Corresponding figure:* ***Fig. 3.25***

Ammonium sulphate concentration	Fraction	Area [pA*min]	Volume [mL]	Total activity [pA*min*mL]
0.25M	Proteinextract	0.138	2.5	0.77
0.25M	Precipitation supernatant	0.084	5	0.93
0.25M	Flow through	0.201	5	2.2

Continued on next page

Ammonium sulphate concentration	Fraction	Area [pA*min]	Volume [mL]	Total activity [pA*min*mL]
0.25M	Wash 0% B	0.359	10	8
0.25M	Fraction 1	0.08	3.5	0.62
0.25M	Fraction 2	0.085	3.5	0.66
0.25M	Fraction 3	0.085	3.5	0.66
0.25M	Wash 100% B	0.081	10	1.8
0.25M	Buffer B	0.088	10	2
0.25M	Buffer A	0.084	5	0.93
0.5M	Proteinextract	0.138	2.5	0.77
0.5M	Precipitation supernatant	0.084	5	0.93
0.5M	Flow through	0.149	5	1.7
0.5M	Wash 0% B	0.257	10	5.7
0.5M	Fraction 1	0.357	3.5	2.8
0.5M	Fraction 2	0.224	3.5	1.7
0.5M	Fraction 3	0.338	3.5	2.6
0.5M	Wash 100% B	0.187	10	4.2
0.5M	Buffer B	0.088	10	2
0.5M	Buffer A	0.022	5	0.24
0.7M	Proteinextract	0.099	2.5	0.55
0.7M	Precipitation supernatant	0.09	5	1
0.7M	Flow through	0	5	0
0.7M	Wash 0% B	0.148	10	3.3
0.7M	Fraction 1	0.199	3.5	1.5
0.7M	Fraction 2	0.45	3.5	3.5
0.7M	Fraction 3	1.762	3.5	14
0.7M	Wash 100% B	0.198	10	4.4
0.7M	Buffer A	0.071	5	0.79
0.7M	Buffer B	0.034	10	0.76

NA: not available

A.6 Gas Chromatograpyh Data for Isoprene Production

Table A.27: *Isoprene production from Pichia pastoris X-33 harboring the kisps gene. Corresponding figure:* ***Fig. 3.26***

Strain	Incubation time	Medium	Area [pA*min]	OD_{600}
X-33 WT	1 d	YPD	0	4.6
X-33 pPICZB-kIspS #1	1 d	YPD	0	5.6
X-33 pPICZB-kIspS #2	1 d	YPD	0	6.5
X-33 pPICZB-kIspS #3	1 d	YPD	0	2.3
X-33 pPICZB-kIspS #4	1 d	YPD	0	2.7
X-33 pPICZB-kIspS #5	1 d	YPD	0	2.5
X-33 WT	1 d	Methanol Minimal Medium	0	0.57
X-33 pPICZB-kIspS #1	1 d	Methanol Minimal Medium	0	0.27
X-33 pPICZB-kIspS #2	1 d	Methanol Minimal Medium	0	0.61
X-33 pPICZB-kIspS #3	1 d	Methanol Minimal Medium	0	0.28
X-33 pPICZB-kIspS #4	1 d	Methanol Minimal Medium	0	0.37
X-33 pPICZB-kIspS #5	1 d	Methanol Minimal Medium	0	0.6
X-33 WT	6 d	Methanol Minimal Medium	0	1.2
X-33 pPICZB-kIspS #1	6 d	Methanol Minimal Medium	0	1.3
X-33 pPICZB-kIspS #2	6 d	Methanol Minimal Medium	0.011	1.7
X-33 pPICZB-kIspS #3	6 d	Methanol Minimal Medium	0	1.1
X-33 pPICZB-kIspS #4	6 d	Methanol Minimal Medium	0.004	1
X-33 pPICZB-kIspS #5	6 d	Methanol Minimal Medium	0	1.3

NA: not available

Table A.28: *Isoprene production from Pichia pastoris X-33 harboring the kisps gene after 6 days. Corresponding figure:* ***Fig. 3.27***

Strain	Replicate	Area [pA*min]
X-33 pPICZB-kIspS #1	Replicate 1	0
X-33 pPICZB-kIspS #2	Replicate 1	0.004
X-33 pPICZB-kIspS #3	Replicate 1	0
X-33 pPICZB-kIspS #4	Replicate 1	0
X-33 pPICZB-kIspS #5	Replicate 1	0
X-33 pPICZB #1	Replicate 1	0
X-33 pPICZB #2	Replicate 1	0
X-33 WT	Replicate 1	0
X-33 pPICZB-kIspS #1	Replicate 2	0

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	Strain	Replicate	Area [pA*min]
	X-33 pPICZB-kIspS #2	Replicate 2	0
	X-33 pPICZB-kIspS #3	Replicate 2	0
	X-33 pPICZB-kIspS #4	Replicate 2	0
	X-33 pPICZB-kIspS #5	Replicate 2	0
	X-33 pPICZB #1	Replicate 2	0
	X-33 pPICZB #2	Replicate 2	0
	X-33 WT	Replicate 2	0
	X-33 pPICZB-kIspS #1	Replicate 3	0
	X-33 pPICZB-kIspS #2	Replicate 3	0.005
	X-33 pPICZB-kIspS #3	Replicate 3	0
	X-33 pPICZB-kIspS #4	Replicate 3	0
	X-33 pPICZB-kIspS #5	Replicate 3	0
	X-33 pPICZB #1	Replicate 3	0
	X-33 pPICZB #2	Replicate 3	0
	X-33 WT	Replicate 3	0

NA: not available

Table A.29: *Isoprene production and growth of C. necator DSM428 on various carbon sources (n=3) with standard deviation. Corresponding figure:* ***Fig. 3.34***

Medium/C-source	Time [h]	OD_{600} ±Isoprene [pA*min]	±
DSMZ-1	0	0.2 ±0	NA ±NA
DSMZ-1	8	2.81 ±0.19	0.02 ±0
DSMZ-1	21	3.39 ±0.12	0.02 ±0
DSMZ-1	32	3.42 ±0.25	0.03 ±0.01
DSMZ-1	48	2.99 ±0.34	0.03 ±0
Fructose	0	0.2 ±0	NA ±NA
Fructose	9.5	0.31 ±0.03	0.01 ±0
Fructose	21.5	0.93 ±0.11	0.01 ±0
Fructose	46.5	6.77 ±0.15	0.05 ±0
Fructose	73	6.66 ±1.04	0.04 ±0
Gluconate	0	0.2 ±0	NA ±NA
Gluconate	8	0.5 ±0.14	0 ±0
Gluconate	21	5.03 ±0.74	0.04 ±0
Gluconate	32	8.81 ±0.14	0.05 ±0
Gluconate	48	8.01 ±0.27	0.05 ±0

Continued on next page

Medium/C-source	Time [h]	OD_{600} ±Isoprene [pA*min]	±
Silage press juice	0	0.2 ±0	NA ±NA
Silage press juice	7	1.59 ±0.03	0.01 ±0
Silage press juice	21.5	4.77 ±0.11	0.03 ±0
Silage press juice	30.5	5.41 ±0.33	0.03 ±0
Silage press juice	52.5	4.33 ±0.18	0.03 ±0

NA: not available

Table A.30: *Isoprene production after 16 h at 30 °C of C. necator DSM428 bearing various constructs (n=3) with standard deviation. Corresponding figure:* ***Fig. 3.35***

Construct	Isoprene [pA*min]/OD_{600}
pMS137	0.024 ±0.005
pMS137-kIspS	0.02 ±0.007
pMS137-Mev	0.019 ±0.004
Wild Type	0.021 ±0.002

NA: not available

A.7 Fluorescence Measurement Data for eGFP Activity in *C. necator*

Table A.31: *Expression of egfp from the plasmid pJeM1TcR in C. necator DSM531, 428 and E. coli BL21 (n=3) with standard deviation. Corresponding figure:* ***Fig. 3.30***

Strain	Phenotype	induced	Fluorescence [Arbitrary unit/OD_{600}]
BL21	pJeM1TcR	0.4% L-rhamnose	606,224 ±42,172
BL21	pJeM1TcR	none	94,727 ±12,179
BL21	WT	0.4% L-rhamnose	2,48 ±0,396
BL21	WT	none	1,585 ±0,157
DSM428	pJeM1TcR	0.4% L-rhamnose	58,009 ±7,484
DSM428	pJeM1TcR	none	4,122 ±0,223
DSM428	WT	0.4% L-rhamnose	0,869 ±0,04
DSM428	WT	none	0,671 ±0,123
DSM531	pJeM1TcR	0.4% L-rhamnose	12,249 ±3,567
DSM531	pJeM1TcR	none	2,573 ±0,253
DSM531	WT	0.4% L-rhamnose	2,083 ±0,158

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Strain	Phenotype	induced	Fluorescence [Arbitrary unit/OD_{600}]
DSM531	WT	none	1,805 ±0,2

NA: not available

Table A.32: *Expression of egfp from the plasmid pMS137 in C. necator DSM428, E. coli DH5α and E. coli BL21 (n=3) with standard deviation. Corresponding figure:* ***Fig. 3.31***

Strain	Phenotype	[IPTG] (mM)	Fluorescence [Arbitrary unit/OD_{600}]
BL21	pBBR1MCS-3	0	2.5 ±0.67
BL21	pBBR1MCS-3	1	2.5 ±0.25
BL21	pBBR1MCS-3	2	1.9 ±0.35
BL21	pBBR1MCS-3	5	2.3 ±0.37
BL21	pBBR1MCS-3	10	1.7 ±0.18
BL21	pBBR1MCS-3-eGFP	0	46 ±3.2
BL21	pBBR1MCS-3-eGFP	1	56 ±3.2
BL21	pBBR1MCS-3-eGFP	2	53 ±3.6
BL21	pBBR1MCS-3-eGFP	5	52 ±1.5
BL21	pBBR1MCS-3-eGFP	10	50 ±2.6
BL21	WT	0	2.1 ±0.072
BL21	WT	1	1.8 ±0.029
BL21	WT	2	2.4 ±0.14
BL21	WT	5	1.9 ±0.28
BL21	WT	10	1.8 ±0.3
DSM428	pBBR1MCS-3	0	0.91 ±0.13
DSM428	pBBR1MCS-3	1	1.2 ±0.1
DSM428	pBBR1MCS-3	2	1.1 ±0.11
DSM428	pBBR1MCS-3	5	1.3 ±0.051
DSM428	pBBR1MCS-3	10	1.1 ±0.21
DSM428	pBBR1MCS-3-eGFP	0	1.6 ±0.4
DSM428	pBBR1MCS-3-eGFP	1	1.8 ±0.28
DSM428	pBBR1MCS-3-eGFP	2	1.8 ±0.23
DSM428	pBBR1MCS-3-eGFP	5	1.7 ±0.19
DSM428	pBBR1MCS-3-eGFP	10	1.8 ±0.32
DSM428	WT	0	1.5 ±0.16
DSM428	WT	1	1.6 ±0.24
DSM428	WT	2	1.4 ±0.45
DSM428	WT	5	1.5 ±0.13

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Strain	Phenotype	[IPTG] (mM)	Fluorescence [Arbitrary unit/OD_{600}]
DSM428	WT	10	1.4 ±0.22
DSM531	pBBR1MCS-3	0	1.4 ±0.12
DSM531	pBBR1MCS-3	1	1.5 ±0.038
DSM531	pBBR1MCS-3	2	1.2 ±0.14
DSM531	pBBR1MCS-3	5	1.4 ±0.16
DSM531	pBBR1MCS-3	10	1.4 ±0.15
DSM531	pBBR1MCS-3-eGFP	0	2 ±0.34
DSM531	pBBR1MCS-3-eGFP	1	2.2 ±0.06
DSM531	pBBR1MCS-3-eGFP	2	1.8 ±0.34
DSM531	pBBR1MCS-3-eGFP	5	2.4 ±0.38
DSM531	pBBR1MCS-3-eGFP	10	2.1 ±0.18
DSM531	WT	0	1.7 ±0.094
DSM531	WT	1	1.5 ±0.084
DSM531	WT	2	1.4 ±0.14
DSM531	WT	5	1.6 ±0.1
DSM531	WT	10	1.5 ±0.2

NA: not available

Table A.33: *Expression of egfp from the plasmid pMS137 in C. necator DSM428, E. coli DH5α and E. coli BL21 (n=3) with standard deviation. Corresponding figure:* ***Fig. 3.32***

Strain	Phenotype	induced	Fluorescence [Arbitrary unit/OD_{600}]
BL21	pMS137-eGFP	cyclohexanone	1,365 ±0,281
BL21	pMS137-eGFP	none	1,74 ±0,375
BL21	WT	cyclohexanone	1,808 ±0,169
BL21	WT	none	1,342 ±0,251
DH5α	pMS137-eGFP	cyclohexanone	1,196 ±0,107
DH5α	pMS137-eGFP	none	1,181 ±0,172
DH5α	WT	cyclohexanone	1,368 ±0,163
DH5α	WT	none	1,406 ±0,225
DSM428	pMS137-eGFP	cyclohexanone	238,78 ±10,368
DSM428	pMS137-eGFP	none	165,315 ±12,958
DSM428	WT	cyclohexanone	0,886 ±0,236
DSM428	WT	none	1,584 ±0,134

NA: not available

Table A.34: *Expression of egfp from the plasmid pMS137 in C. necator DSM428, E. coli DH5α and E. coli BL21 (n=3) with standard deviation. MM+G: minimal medium with 2% gluconate. Corresponding figure:* ***Fig. 3.33***

Medium	Phenotype	[Cyclohexanone] (mM)	Fluorescence [Arbitrary unit/OD_{600}]
LB	pMS137	0	0,722 ±0,277
LB	pMS137	0.1	0,976 ±0,604
LB	pMS137	0.5	0,77 ±0,38
LB	pMS137	1	0,504 ±0,568
LB	pMS137	2	0,646 ±0,307
LB	pMS137	5	0,673 ±0,306
LB	pMS137	10	0,627 ±0,226
LB	pMS137-eGFP	0	96,222 ±8,645
LB	pMS137-eGFP	0.1	92,106 ±38,596
LB	pMS137-eGFP	0.5	105,866 ±18,748
LB	pMS137-eGFP	1	104,598 ±9,598
LB	pMS137-eGFP	2	161,226 ±19,198
LB	pMS137-eGFP	5	152,191 ±20,378
LB	pMS137-eGFP	10	182,833 ±19,964
LB	WT	0	0,699 ±0,027
LB	WT	0.1	0,668 ±0,019
LB	WT	0.5	0,656 ±0,055
LB	WT	1	0,803 ±0,108
LB	WT	2	0,785 ±0,118
LB	WT	5	0,777 ±0,222
LB	WT	10	0,724 ±0,18
MM+G	pMS137	0	1,04 ±0,162
MM+G	pMS137	0.1	0,978 ±0,143
MM+G	pMS137	0.5	1,154 ±0,042
MM+G	pMS137	1	1,05 ±0,078
MM+G	pMS137	2	1,053 ±0,183
MM+G	pMS137	5	1,007 ±0,267
MM+G	pMS137	10	0,922 ±0,298
MM+G	pMS137-eGFP	0	12,4 ±0,93
MM+G	pMS137-eGFP	0.1	16,625 ±4,12
MM+G	pMS137-eGFP	0.5	24,654 ±2,673
MM+G	pMS137-eGFP	1	29,238 ±2,34

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Medium	Phenotype	[Cyclohexanone] (mM)	Fluorescence [Arbitrary unit/OD_{600}]
MM+G	pMS137-eGFP	2	21,259 ±2,525
MM+G	pMS137-eGFP	5	28,137 ±0,355
MM+G	pMS137-eGFP	10	25,329 ±1,761
MM+G	WT	0	1,166 ±0,08
MM+G	WT	0.1	1,044 ±0,212
MM+G	WT	0.5	1,124 ±0,135
MM+G	WT	1	1,026 ±0,132
MM+G	WT	2	1,016 ±0,125
MM+G	WT	5	1,125 ±0,103
MM+G	WT	10	0,973 ±0,179

NA: not available

A.8 PCR Parameters for Construction of the Plasmids Used in *C. necator*

Table A.35: *PCR parameters for cloning reactions for the constructs related to isoprene enhancement in C. necator DSM428*

Vector	Fragment	Annelaing/Elongation	Template	Primers	Polymerase
pME6032	*egfp*	60 °C, 30 s/72 °C, 1 min	CBR_P_15	38/39	Phusion-HF
	kIspS	60 °C, 30 s/72 °C, 1 min	pBA2kIKmA2	34/35	Phusion-HF
pBBR1MCS-3	pBBR1MCS-3 1	58 °C, 30 s/72 °C, 6 min	pBBR1MCS-3	204/205	Phusion-HF (NEB)
	pBBR1MCS-3 2	58 °C, 30 s/72 °C, 6 min	pBBR1MCS-3	206/207	Phusion-HF (NEB)
	T7 terminator	58 °C, 30 s/72 °C, 20 s	pET28a	208/209	Phusion-HF (NEB)
	egfp	58 °C, 30 s/72 °C, 6 min	pJeM1TcR	210/211	Phusion-HF (NEB)
	kIspS	58 °C, 30 s/72 °C, 6 min	pBA2kIKmA2	212/213	Phusion-HF (NEB)
	mev-operon	58 °C, 30 s/72 °C, 6 min	pGA1-Mev	214/215	Phusion-HF (NEB)
	mev-operon 2 parts	58 °C, 30 s/72 °C, 8 min (3% DMSO)	pGA1-Mev	214/232 & 231/215	Accuzyme (BioLine)
pMS137	*egfp*	60 °C, 30 s/72 °C, 1 min (3% DMSO)	pJeM1TcR	159/160	Phusion-HF (NEB)
	kIspS	60 °C, 30 s/68 °C, 2 min	pBA2kIKmA2	157/102	Taq (NEB)
	mev operon	60 °C, 30 s/72 °C, 6 min	pGA1-Mev	216/217	Phusion-HF (NEB)
pJeM1TcR	*pJeM1TcR 1*	60 °C, 30 s/72 °C, 2:10 min	pJeM1TcR	150/154	Phusion-HF (NEB)
	pJeM1TcR 2	60 °C, 30 s/72 °C, 4 min	pJeM1TcR	151/155	Taq (NEB)
	kIspS	60 °C, 30 s/72 °C, 2:10 min	pBA2kIKmA2	152/153	Phusion-HF (NEB)

A.9 Supplementary Information

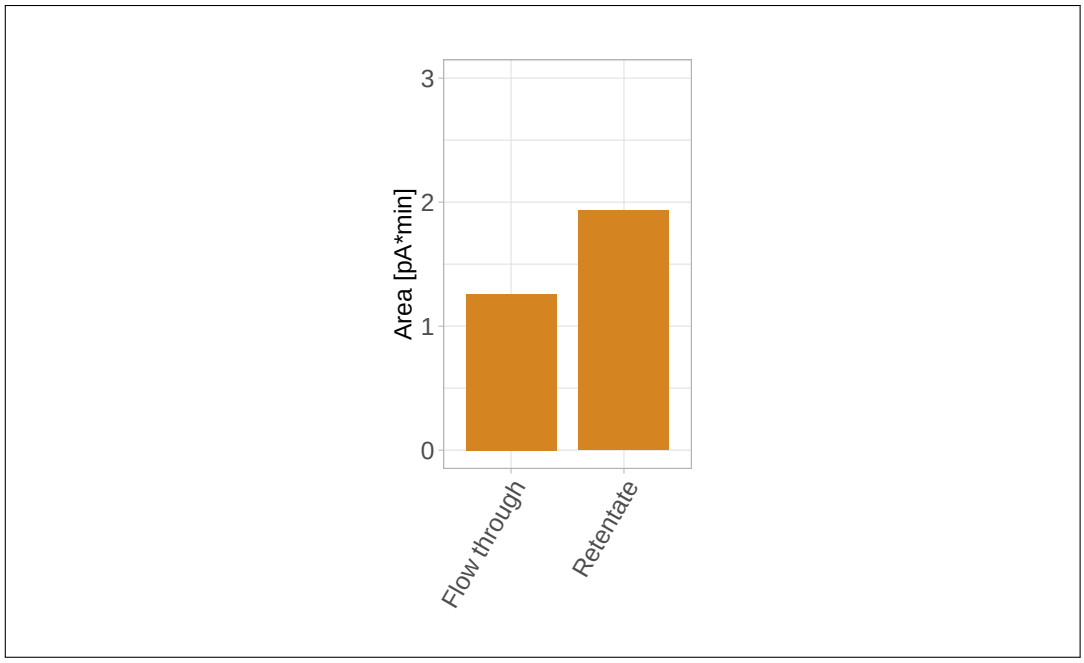

Figure A.3: ***Attempted concentration of an active AEKTA^{TM} fraction using a 3 kDa filter from Lysinibacillus xylanilyticus SR-86.*** *A portion of the AEKTA^{TM} fraction with the highest activity was attempted to concentrate using a 3 kDa filter and the Amicon ultra filtration unit. Fractions were incubated for 16 h at 30 °C and 150-170 rpm in a sealed 10 mL GC head space vial with 450 µL sample and 50 µL substrate solution (final concentration of 10 mM). The amount of ethylene was analyzed using gas chromatography ($n = 1$).*

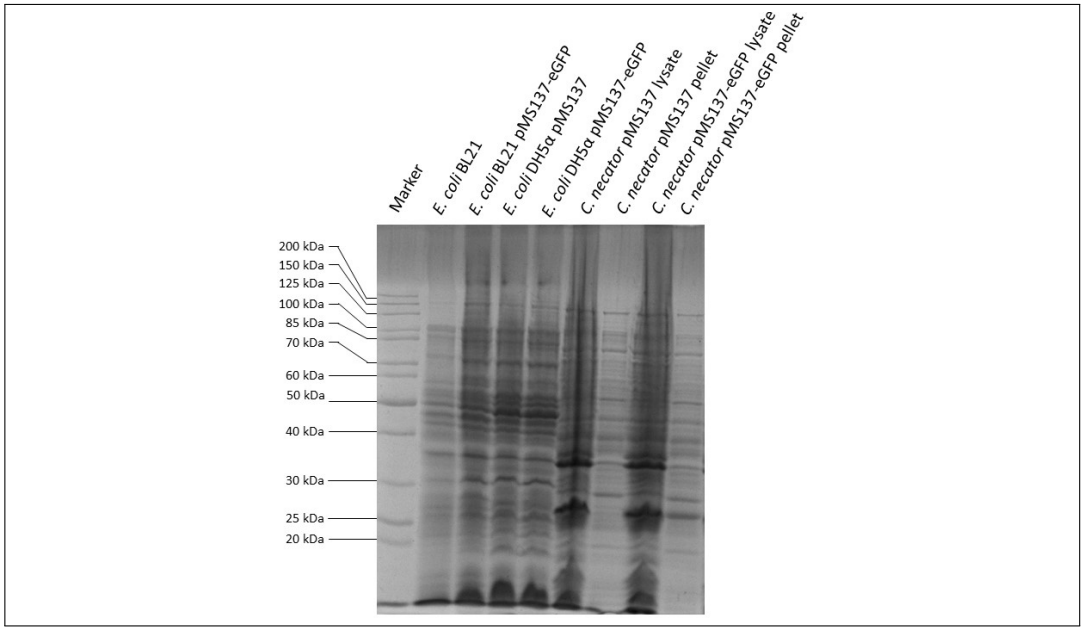

Figure A.4: ***SDS-PAGE of E. coli and C. necator expressing egfp.*** *eGFP has a size of 28.3 kDa.*

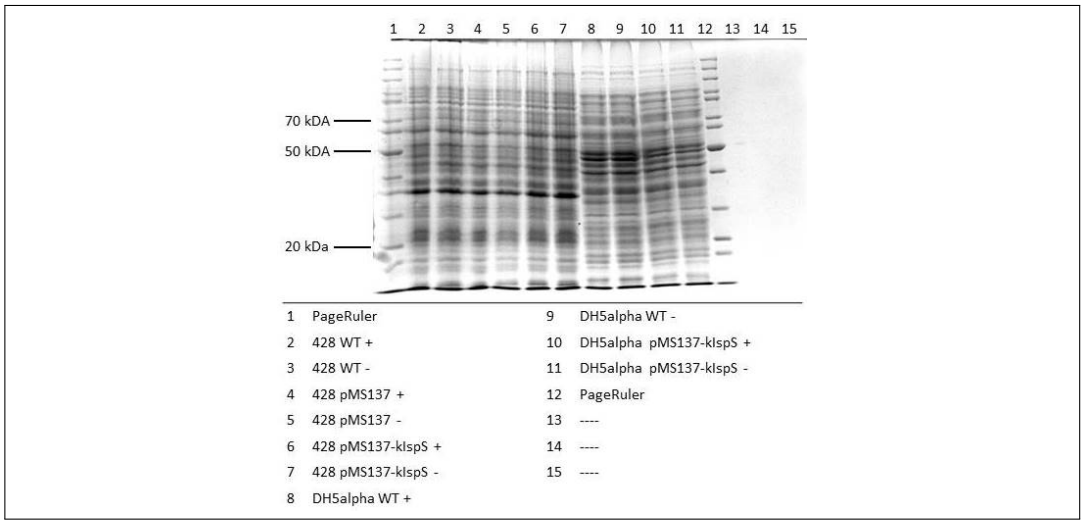

Figure A.5: ***SDS-PAGE of expressed kisps from C. necator DSM428 and E. coli.*** *+ induced, − not induced. kIspS has a size of 65.6 kDa.*

A.10 List of Abbreviations

4-OT	4-oxalocrotonate tautomerase
A	Adenosine
AACT	acetyl-coenzyme A C-acetyltransferase
ACC	1-Aminocyclopropanecarboxylic acid
ACCO	1-aminocyclopropane-1-cyrboxylate oxidase
AIB	2-Aminoisobutyric acid
AMA	Marine Medium
AMASE	Marine Medium and trace elements
aMRS	acidified MRS Bouillon
aMRSSE	acidified MRS Bouillon with trace elements
AOX1	Alcohol oxidase 1
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BMM	Buffered methanol minimal medium
bp	base pairs
C	Cytosine
CamA	Putidaredoxin reductase
CamB	Putidaredoxin
CBR	Chair for Chemistry of Biogenic Resources
CoA	Coenzyme A
CoA	Coenzyme A
Da	Dalton
DMAPP	Dimethylallyl pyrophosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Nucleoside triphosphate
DSMZ	Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen
NA	und Zellkulturen GmbH
DSMZ-1	DSMZ Medium 1
DSMZ-1SE	DSMZ Medium 1 with trace elements
DTT	Dithiothreitol
DXR	1-deoxy-D-xylulose-5-phosphate reductoisomerase
DXS	1-deoxyxylulose-5-phosphate synthase
E. coli	*Escherichia coli*
e.g.	Exempli gratia
EC number	Enzyme commission number
EDTA	Ethylenediaminetetraacetic acid
Efe	Ethylene-forming enzyme
egfp	Enhanced green fluorescent protein gene
EPS	Exopolysaccharides
ESI	Electrospray ionization

FMN	Flavin mononucleotide
fw	Forward
G	Guanosine
GC	Gas chromatography
gDNA	Genomic DNA
gfp	Green fluorescent protein gene
GYM	Glucose Yeast Malt Medium
GYMSE	Glucose Yeast Malt Medium with trace elements
HDR	hydroxymethylbutenyl diphosphatereductase
HDS	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIC	Hydrophobic interaction chromatography
HMG-CoA	Hydroxymethylglutaryl-CoA
HMGR	HMG reductase
HMGS	HMG synthase
HPLC	High-performance liquid chromatography
IEA	International Energy Agency
IEX	Ion Exchange
IPNS	Isopenicillin N-synthase
IPP	Isopentenyl pyrophosphate
IPPI	IPP-Δ-isomerase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IspS	Isoprene synthase
IUPAC	International Union of Pure and Applied Chemistry
kIspS	Kudzu isoprene synthase
KMBA	2-keto-4-methylthiobutyrate
LB	Lysogeny broth [31]
LBSE	Lysogeny broth [31] + trace elements
L-Met	L-methionine
LPG	Liquified petroleum gas
MEP	2*C*-methyl-D-erythritol-4-phosphate
Mev	Mevanolate Operon
MK	mevalonate kinase
MMM	Methanol minimal medium
MRS	MRS Bouillon
MRSSE	MRS Bouillon with trace elements
MS	Mass spectrometry
MVA	mevalonic acid
NA	Not available
NAD^+	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NaPi	Sodium phosphate buffer

NaPiSE	Sodium phosphate buffer with trace elements
NaPiSE-TPP	Sodium phosphate buffer with trace elements and TPP
NCBI	National Center for Biotechnology Information
NGL	Natural gas liquids
NMPDR	National microbial pathogen data resource
NREL	National Renewable Energy Laboratory
nt	nucleotides
OD	Optical density
OleT	terminal olefin producing cytochrome P450
OT	4-oxalocrotonate tautomerase
P. fermentans	*Pichia fermentans*
P. hybrida	*Petunia hybrida*
P. pastoris	*Pichia pastoris*
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PDB	protein data base
PE	polyethylene
PET	Polyethylene terephthalate
PMK	phospho-mevalonate kinase
PP	Polypropylene
PS	polystyrole
RAST	Rapid annotation using subsystem technology
rpm	Revolutions per minute
rv	Reverse
SAM	S-adenosyl methionine
SDS	sodium dodecyl sulphate
SE	Spurenelemente (Trace Elements)
SOB	Super Optimal Broth
SOC	Super Optimal Broth with Catabolite Repression
ST1	Standard Medium 1
ST1	Standard Medium 1
ST1GSE	Standard Medium 1 with glucose and trace elements
T	Thymidine
TAE buffer	Tris-acetate-EDTA buffer
TB buffer	Tris-Borat buffer
TFB	Transformation buffer
TPP	Thiamine pyrophosphate
Tris	tris(hydroxymethyl)aminomethane
WT	wild type
Y(E)PD	Yeast extract peptone dextrose
Y(E)PDSE	Yeast extract peptone dextrose with trace elements
YPDS	Yeast extract peptone dextrose sorbitol
α-KG	α-ketoglutarate

Acknowledgements

An dieser Stelle möchte ich meinen besonderen Dank nachstehenden Personen entgegen bringen, ohne deren Mithilfe die Anfertigung dieser Dissertation nicht zustande gekommen wäre:

Allen voran gilt zunächst Herrn Prof. Dr. Volker Sieber, meinem Doktorvater, für die Betreuung dieser Arbeit mein besonderer Dank. Ebenso für die Aufnahme in sein Team und das Angebot der Doktorarbeit. Für seine äußerst kompetenten und intellektuellen Gespräche auf fachlicher, aber auch persönlicher Ebene. Sie werden mir immer als bereichernd und konstruktiv, aber auch menschlich in Erinnerung bleiben. Ich habe unsere Dialoge stets als sehr gewinnbringend empfunden.

Dr. Jochen Schmid möchte ich für die hilfsbereite und wissenschaftliche Betreuung danken. Für die langen Gespräche und Diskussionen und für das Korrekturlesen meiner Arbeit.

Herrn Prof. Dr. Robert Huber danke ich für die Übernahme der Mentorenschaft, sowie für die anregenden Diskussionen und Ideen.

Des Weiteren möchte ich mich bei Frau Petra Lommes bedanken, dass sie mir mit Rat und Tat im Labor zur Seite stand und mich in das Thema eingearbeitet hat.

Ebenfalls möchte ich mich bei Frau Elisabeth Aichner bedanken, welche mich immer tatkräftig bei der Erledigung bürokratischer Angelegenheiten unterstützt hat, vor allem in den Endzügen dieser Arbeit.

Bei Frau Marlit Köstler möchte ich mich für die Hilfe, Unterstützung und Betreuung des Gaschromatographen bedanken, an dem ich sehr viele Stunden messen konnte.

Auch danke ich Frau Dr. Marina Zapilko für ihr offenes Ohr in Bezug auf alle Angelegenheiten, uneingeschränkte Hilfe und persönliches Engagement.

Auch danke ich meinen Kollegen am Lehrstuhl für Chemie Biogener Rohstoffe, bei Fraunhofer und in Straubing dafür, dass immer jemand da war (auch am Wochenende), mit dem man fachlich und persönlich reden konnte. Vielen Dank für die Unterstützung im Labor. Das Arbeitsklima war herrlich und es haben sich intensive Freundschaften

gebildet. Meinen Büro-Mitbewohnern möchte ich auch für das ausgezeichnete Klima und Miteinander danken.

Für das Korrekturlesen meiner Arbeit möchte ich mich bei Herrn Dr. Sumanth Ranganathan und Herrn Dr. Steven Koenig bedanken. Vielen Dank, dass ihr euch diese umfangreiche Arbeit durchgelesen habt und sie mit Anmerkungen versehen habt.

Mein außerordentlicher Dank gilt meinen Eltern Josef und Karola, dass sie mich während der Zeit im Labor und auch danach unterstützt haben, ohne deren Geduld und finanzielle Unterstützung diese Arbeit nie hätte gelingen können.

Auch möchte ich meinem Bruder Franz sowie meiner Tante Brigitte und ihrem Mann Franco für ihre stete Akzeptanz und klaren Worte in schwierigen Situationen danken.

Weiterhin geht ein sehr großer Dank an Familie Kaschner/Wirth. Allem voran Frau Stefanie Kaschner, ohne deren mühevolle Geduld und liebevolles Verständnis in dieser schwierigen Zeit ein solcher Arbeitsumfang niemals hätte gelingen können. Mehrfache Durchsicht, kritische Betrachtungsweise, differenzierte Anmerkungen, sowie vor allem aber ihr moralischer Beistand und der menschliche Halt durch die Aufnahme in ihre Familie, haben mir Kraft und Mut gegeben. An dieser Stelle sei auch aus den gleichen Gründen, sowie für oftmaliges Korrekturlesen und Layout-Tips, Dominik Wirth gedankt. Zudem möchte ich mich von Herzen bei Ihren gemeinsamen Kindern Lauritz, Marlitt und Malte dafür bedanken, dass sie selbstlos, bereitwillig und in hohem Maße geduldig ihren Eltern den Freiraum gegeben haben mir zu helfen. Wegen Eurer besonderen persönlichen Bindung und Unterstützung gebührt Euch allen mein besonderer Dank.

Tief verbunden und dankbar bin ich meinen Zwillingssöhnen Jannis und Kilian, die mir immer wieder einen Spiegel vorhalten und zeigen, was wirklich wichtig im Leben ist, und die der Grund sind, dass diese Arbeit nun vollendet ist. Ich liebe euch beide von ganzem Herzen und widme Euch daher auch diese Arbeit.

Allen namentlich und nicht namentlich aufgeführten Personen danke ich dafür, dass Sie für mich da sind.

Ihr macht das Leben lebenswert!