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„Biotransformation of aromatic compounds by recombinant bacteria, expressing prenyltransferases“

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List of abbreviations

1,6-DHN	1,6-dihydroxy naphthalene
2,7-DHN	2,7-dihydroxy naphthalene
4-HPP	4-hydroxyphenylpyruvate
ABBA	alpha-beta-beta-alpha prenyltransferase
Am	apramycin
Amp	ampicillin
ATP	adenosine triphosphate
BGCs	biosynthetic gene clusters
Cml	chloramphenicol
COX-2	cyclooxygenase-2
ddsH2O	double-distilled sterile water
DHNs	dihydroxy naphthalenes
DMAPP	dimethylallyl pyrophosphate
DNA	deoxyribonucleic acid
FPP	farnesyl pyrophosphate
G3P	glyceraldehyde-3-phosphate
GFPP	geranylfarnesyl pyrophosphate
GGPP	geranylgeranyl pyrophosphate
GPP	geranyl pyrophosphate
HPLC	high performance liquid chromatography
Hsp90	90 kDa heat shock protein
IPP	isopentenyl pyrophosphate
Kana	kanamycin
LC-MS	liquid chromatography-mass spectrometry
MEP	2-C-methyl D-erythritol-4-phosphate pathway
MIC	minimal inhibitory concentration
MTPs	meroterpenoids
MVA	mevalonate pathway
NPs	natural products
PPH	prenylated phenazines
S1	juglone
S2	2-hydroxy-1,4-naphthoquinone
S3	2-methoxy-1,4-naphthoquinone
S4	1,4-naphthoquinone
S5	purpurin
THN	1,3,6,8-tetrahydroxynaphthalene

1 Zusammenfassung

Die ständige Zunahme an bakteriellen Resistenzen stellt mittlerweile eine globale Gefahr dar, die es zu bekämpfen gilt. Nachdem die Forschung & Entwicklung neuer Antibiotika einige Jahrzehnte lang stagnierte, nimmt sie zum Glück in den letzten Jahren wieder an Fahrt auf. Dabei dienen natürliche Quellen wie Pflanzen, Pilze und Bakterien, speziell *Streptomyces* sp. weiterhin als wertvolle Ausgangspunkte. Vielversprechende biosynthetische Gencluster (BGC), die für Sekundärmetabolite wie Meroterpenoide (MTP) codieren, wurden bereits in *Streptomyces* sp. identifiziert. MTP sind prenylierte aromatische Verbindungen, die aktuell als multipotente Arzneimittelkandidaten diskutiert werden. Die enzymatisch katalysierte Prenylierung stellt dabei einen wesentlichen Erfolgsfaktor für die Wirkung im Zielorganismus dar.

Das Hauptziel dieser Masterarbeit war es, rekombinante *Streptomyces venezuelae spp.* Stämme zu erzeugen und anhand Fermentation und Zugabe entsprechender chemischer Vorstufen (Substrate S1 bis S5) deren Biotransformation, speziell die Prenylierung, zu induzieren. Die verwendeten Gene stammten aus dem BGC von *Streptomyces* sp. S4.7, der das Genrepertoire für die Biosynthese von Meroterpenoiden enthält. Dabei sind Enzyme wie Farnesylpyrophosphat-Synthase, Geranylpyrophosphat-Synthase, *O*-Methyltransferase und aromatische Prenyltransferasen beteiligt.

Mittels homologer Klonierung wurden die jeweiligen Gene in 6 Vektoren, pF, pF_mfqW, pF_mfqWH, pG, pG_mfqM, und pG_mfqMH zusammengefasst und anschließend in *S. venezuelae* T1-4D2 transferiert. Die geeignetsten Fermentationsmedien und die minimalen Hemmkonzentrationen (MIC) der zugesetzten Substrate S1 bis S5 wurden im Vorfeld definiert. Die Arbeit umfasste ebenso den Vergleich unterschiedlicher Extraktionsverfahren und ein Zelllyse-Protokoll. Die daraus gewonnenen Extrakte wurden mittels HPLC und LC-MS analysiert. Ein pF-Extrakt deutete auf eine vermutlich prenylierte Substanz des Substrats 2 (2-Hydroxy-1,4-Naphthoquinon), Lapachol, hin. Trotz mehrerer experimenteller Wiederholungen konnten in den pF-Extrakten weder Lapachol, noch andere Substrat-Reaktionsprodukte nachgewiesen werden. Die Arbeitshypothese wurde demzufolge nicht bestätigt, wenngleich eine finale Aussage wohl nur nach erneuter Begutachtung der pG-Extrakte möglich ist.

2 Abstract

The continuous increase in bacterial resistance has become a global threat that needs to be addressed. After a prolonged period of stagnation in researching and developing new antibiotics, there has been a resurgence in research efforts. In this regard, natural sources such as plants, fungi, and bacteria, particularly *Streptomyces* species, continue to serve as valuable starting points. Promising biosynthetic gene clusters (BGCs) encoding for secondary metabolites such as meroterpenoids (MTPs) have already been identified in *Streptomyces* species. Meroterpenoid compounds are prenylated aromatic NPs currently being discussed as multipotent drug candidates. Enzymatic catalysis of prenylation is a key factor for their efficacy in the target organism.

The main objective of this master's thesis was to generate recombinant strains of *Streptomyces venezuelae* spp. and induce their biotransformation, specifically prenylation, through fermentation and the addition of appropriate chemical precursors (substrates S1 to S5). The employed genes were derived from the BGC of *Streptomyces* sp. S4.7, which encompasses the gene repertoire for the biosynthesis of meroterpenoids, involving enzymes such as farnesyl pyrophosphate synthase, geranyl pyrophosphate synthase, *O*-methyltransferase, and aromatic prenyltransferase. The respective genes were homologously cloned into six vectors, namely pF, pF_mfqW, pF_mfqWH, pG, pG_mfqM, and pG_mfqMH, and transformed heterologously into *Streptomyces venezuelae* T1-4D2. Prior to the experiments, the most suitable fermentation media and the MIC of the added substrates S1 to S5 were defined. The study also involved comparing different extraction methods and a cell lysis protocol. The obtained extracts were analyzed using HPLC and LC-MS. A pF-extract implied the potential prenylation of S2 (2-hydroxy-1,4-naphthoquinone), indicating lapachol. However, analogous experiment repetitions and final LC-MS analysis didn’t prove biotransformation to lapachol or another biotransformation product. The working hypothesis could not be confirmed, although a definitive statement can only be made after re-evaluating the pG-extracts.

3 Introduction

3.1 Pharmaceutically active compounds produced by *Streptomyces* sp.

Streptomyces species' repertoire on natural products includes pharmaceutically relevant substances such as antibiotics, anticancer drugs, antivirals, immunosuppressants, antifungals, anti-obesity medications, enzymes, as well as enzyme inhibitors (Lee et al., 2020a; Deng et al., 2017, Kim et al., 2015). A significant part of these therapeutically relevant compounds are antibiotics, e.g., streptomycin, chloramphenicol, rifampicin, tetracycline, or novobiocin; see *Figure 1* (De Lima Procópio et al., 2012). These natural products (NPs) are secondary metabolites produced by bacteria to have an advantage in their interactions within the ecological niche they dwell in. Secondary metabolites, in contrast to primary metabolites (i.e., amino acids, fatty acids, sugars, etc.), are not essential for the growth and proliferation of bacteria in the laboratory environment while possibly playing an important roles in nature.

Streptomyces species are filamentous, Gram-positive actinobacteria ubiquitous in nature (Lee et al., 2020a; Kieser, 2000). These bacteria have been widely used as model organisms for studying bacterial development and their biosynthesis of bioactive NPs.

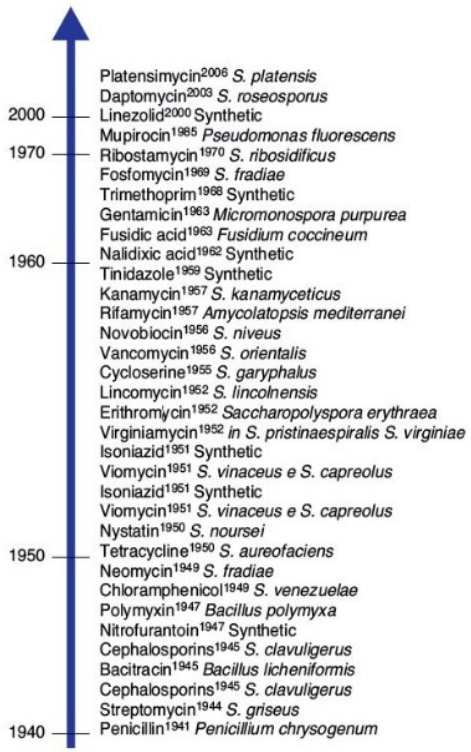

Figure 1: ***Key findings and dates of discovered antibiotics.*** *Streptomyces species mainly produce these compounds (De Lima Procópio et al., 2012, modified by NSCH).*

The number of new antibiotic discoveries steadily decreased after the so-called “Golden Age” of antibiotics that lasted from ca 1940 until ca 1970.

Most of these traditional antibiotics are still in clinical use. However, their effectiveness has been eroded by the rise of antimicrobial resistance, mainly caused by the excessive use of these drugs. This, and the reduced efforts on research, led to the current situation in which bacterial resistance becomes an increasing public health concern (Hutchings et al., 2019).

According to the World Health Organization (WHO), new antibiotics are in development. Still, none are expected to be effective against the most dangerous antibiotic-resistant bacteria. Antibiotic resistance has become a global concern with the ease and frequency of traveling. Finding effective new antibiotics will require international efforts and decisions on many levels (World Health Organization: WHO, 2023).

To address this issue, biotechnological research has been developing new techniques for analyzing *Streptomyces* species or finding new ways of using *Streptomyces* as an effective producer of pharmaceutically relevant compounds. The rise of bioinformatics and genetic engineering methods, especially genome sequencing and mining, analytical methods, and screening, supports research on *Streptomyces* producing pharmacologically relevant NPs (Atanasov et al., 2021; Sekurova et al., 2019; Nepal & Wang, 2019).

3.2 Genome analysis of *Streptomyces* sp.

Revealing the enzymatic pathways for the biosynthesis of secondary metabolites in microbes, particularly discovering specific biosynthetic gene clusters (BGCs), which enable or control the production of secondary metabolites, might be a strategy for further NP discovery in bacteria (Rigali et al., 2018). BGCs are physically clustered groups of genes (*Figure 2*) in a particular microorganism’s genome that encode an enzymatic pathway to produce specialized metabolites, such as the lipopeptide viennamycin, which showed antibacterial activity (Bekiesch et al., 2020; Medema et al., 2015).

Regarding *Streptomyces*, it is known that each species has the genetic potential to produce more than 30 secondary metabolites on average (Lee et al., 2020a). *Streptomyces* possess 25 - 70 BGCs, enabling the biosynthesis of chemically diverse secondary metabolites (Belknap et al., 2020).

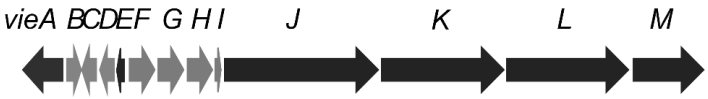

Figure 2: ***Biosynthetic gene cluster (BGC) of Streptomyces sp. S4.7.*** *This BGC contains 13 genes (vieA to vieM) encoding enzymes involved in the biosynthesis of the lipopeptide viennamycin (Bekiesch et al., 2020, modified by NSCH).*

By studying the genes within a BGC and the enzymes encoded by them, researchers may gain insights into the biosynthesis of pharmaceutically relevant secondary metabolites and its regulation. Focusing on the genes and their functionalities also may enable the development of new engineering or modification concepts. The rise of bioinformatics, machine learning, and the steadily increasing knowledge of genetics and BGCs, facilitated *in silico* genome mining, including *Streptomyces* species (Lee et al., 2020a; Atanasov et al., 2021).

Genome mining is a valuable method for identifying and characterizing bacterial BGCs responsible for producing specific metabolites, such as antibiotics or other natural products with potential therapeutic applications. It involves searching for similarities between the bacterial genome of interest and known biosynthetic pathways. Screening is performed with specialized software and various approaches, e.g., phylogeny-based mining. By utilizing genome mining, unique features of bacterial genomes that may play a role in natural product biosynthesis may also be identified (Atanasov et al., 2021; Belknap et al., 2020; Lee et al., 2020a; Lee et al., 2020b; Sekurova et al., 2019).

With genome analyses, researchers can gain a more comprehensive understanding of the biosynthetic potential of bacterial species such as *Streptomyces* spp. and discover new natural products with potential therapeutic applications.

3.3 Prenylated natural products produced by *Streptomyces* spp.

Prenyl moieties are composed of single or multiple condensated C_5 isoprene/hemiterpene units attached to a particular molecule, for example, a protein or NP. All isoprenoids are derived from prenyl diphosphate precursors such as isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Vranová et al., 2013).

Isoprenoids or terpenes are biosynthesized by two independent pathways in bacteria, green microalgae, and plants, named the mevalonate (MVA) pathway or 2-C-methyl D-erythritol-4-phosphate (MEP) pathway. The more common MVA pathway utilizes acetyl-CoA as the primary precursor for IPP and DMAPP, whereas the MEP pathway promotes glyceraldehyde-3-phosphate (G3P) and pyruvate as precursors, as shown in *Figure 3* (Sekurova et al., 2019; Kuzuyama, 2017; Niu et al., 2017; Winkelblech et al., 2015; Bentley et al., 2014; Vranová et al., 2013).

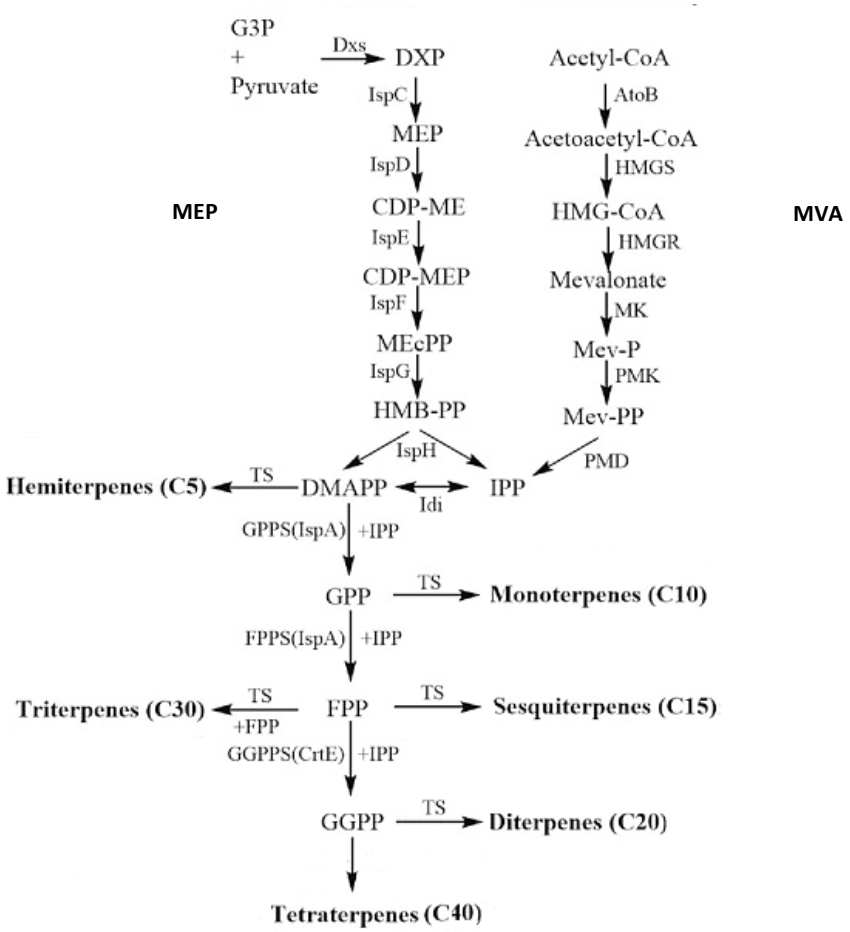

Figure 3: ***Isoprenoid or terpene biosynthetic pathways in plants, green microalgae, and bacteria, 2-C-methyl D-erythritol-4-phosphate (MEP) or mevalonate (MVA).*** *In the first step of MEP, glyceraldehyde 3-phosphate (G3P) and pyruvate are converted into 1-deoxy-d-xylulose-5-phosphate (DXP) and further enzymatically processed to isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). The first step in the MVA pathways involves the transformation of Acetyl-CoA into IPP and DMAPP. Both primary precursors, IPP and DMAPP, are C_5 units or hemiterpenes and can enzymatically be processed to mono-, sesqui, tri, di- and tetraterpenes. Intermediates are geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP). Involved enzymes are: Dxs: 1-deoxy-d-xylulose-5-phosphate synthase; IspC: 1-D-deoxy-d-xylulose 5-phosphate reduction-isomerase; IspD: 2-C-methyl-D-erythritol-4-phosphate cytidylyltransferase; IspE: 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; IspF: 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase; IspG: 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase; IspH: 4-hydroxy-3-methyl-2-(E)-butenyl-4-diphosphate reductase; Idi: Isopentenyl diphosphate isomerase; AtoB: acetoacetyl-CoA synthase; HMGS: hydroxymethylglutaryl-CoA synthase; HMGR: hydroxymethylglutaryl-CoA reductase; MK: mevalonate kinase; PMK: phosphomevalonate kinase; PMD: mevalonate diphosphate decarboxylase; GPPS: GPP synthase; FPPS: FPP synthase; GGPPS: GGPP synthase.; TS: terpene synthase (Niu et al., 2017, modified by NSCH).*

Isoprenoids are chemically and structurally diverse NPs. They derive from C_5 isoprene units such as IPP and DMAPP and undergo various biosynthetic mechanisms, including prenyltransfers, regio- and stereoselective cyclizations, skeleton rearrangements, attachments to host molecules, and tailoring reactions. Various enzymes, e.g., prenyltransferases, are involved in the biosynthesis of the bacterial terpenom (Rudolf et al., 2021).

Prenyltransferases are crucial in NP biosynthesis (Winkelblech et al., 2015). They transfer terpene moieties such as IPP/DMAPP to acceptor molecules and also catalyze terpene condensation, leading to the elongation of allylic pyrophosphate substrates into geranyl pyrophosphate GPP (C_{10}), farnesyl pyrophosphate FPP (C_{15}), and geranylgeranyl pyrophosphate (GGPP), and geranylfarnesyl pyrophosphate (GFPP), in a head-to-tail manner; see *Figure 4*.

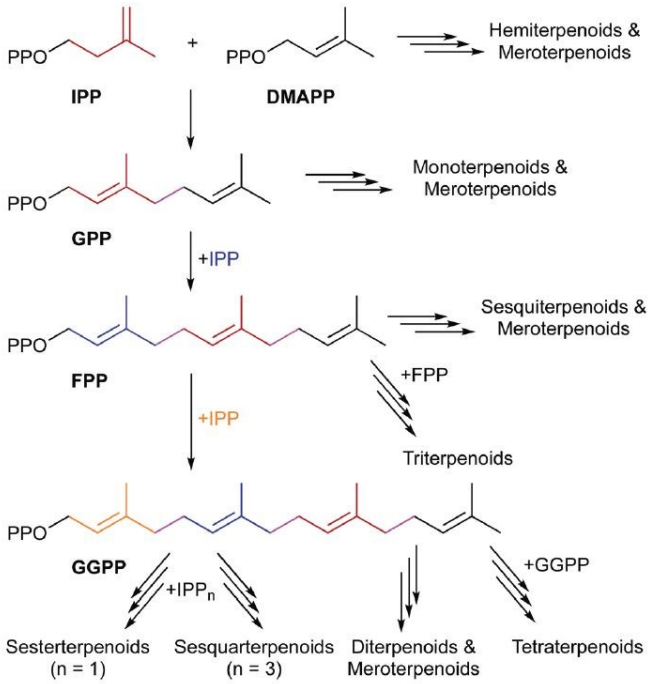

*Figure 4: **The biosynthesis of terpenoids**. IPP: isopentenyl pyrophosphate; DMAPP: dimethylallyl pyrophosphate; GPP: C_{10} geranyl pyrophosphate; FPP: C_{15} farnesyl pyrophosphate; GGPP: C_{20} geranylgeranyl pyrophosphate (Rudolf et al., 2021).*

These longer-chain compounds further undergo cyclization reactions catalyzed by terpene synthases that can utilize the acyclic precursors, GPP, FPP, GGPP, GFPP, or FFPP, to synthesize structurally diverse terpene skeletons or terpenoids (Hu et al., 2023).

Overall, IPP/DMAPP are also considered prenyl donors for peptide-, protein-, and transfer-ribonucleic acid (tRNA) prenyltransferases or aromatic prenyltransferases (Niu et al., 2017; Winkelblech et al., 2015).

Prenylated aromatic compounds in plants, fungi, and bacteria are natural products containing an aromatic ring with an attached terpene group; see *Figure 5*. Aromatic, membrane-bound prenyltransferases catalyze the process of prenylation. They usually catalyze the formation of C-C, C-O, or C-N bonds between the prenyl group's carbon, the aromatic substrates' carbon, or their functional groups (Winkelblech et al., 2015).

Prenyl acceptors are predominantly flavonoids, lignanoids, quinones, indole alkaloids, hydroxynaphthalenes, and coumarins (Mori, 2020; Winkelblech et al., 2015).

These secondary metabolites are biosynthesized in plants, fungi, and bacteria. They have a wide range of biological activities and may be developed into novel therapeutics with anticancer, antioxidative, and antimicrobial effects (Winkelblech et al., 2015).

Recently, the antiviral and anti-inflammatory effects of prenylated aromatic compounds *in vitro* and *in vivo* also have been discussed (Hasan et al., 2022; Su et al., 2021; Brezani et al., 2017).

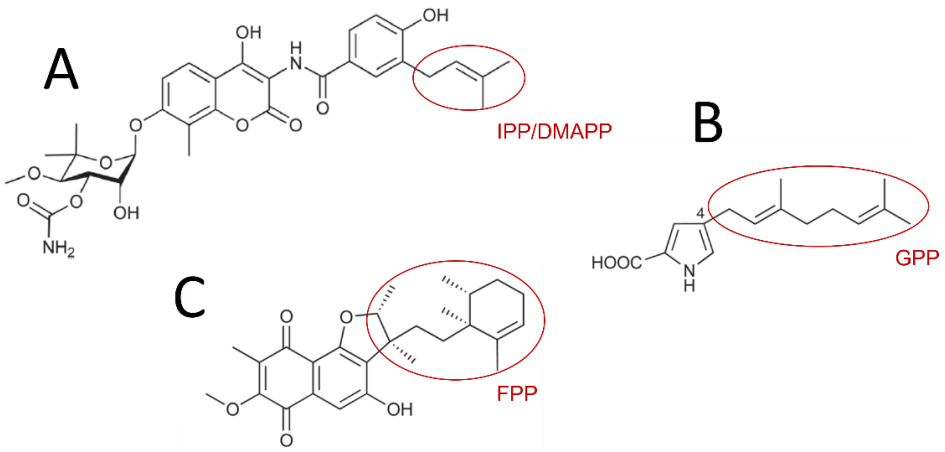

Figure 5: ***Examples of prenylated aromatic compounds biosynthesized in Streptomyces spp.*** *Prenyl units are highlighted in red. A: Novobiocin, prenylated with a C_5 IPP/DMAPP; B: Pyrrolostatin, prenylated with a linear C_{10} GPP; C: Neomarinone, prenylated with a cyclized C_{15} FPP; IPP: isopentenyl pyrophosphate; DMAPP: dimethylallyl pyrophosphate; GPP: geranyl pyrophosphate; FPP: farnesyl pyrophosphate (Rudolf et al., 2021, modified by NSCH).*

Research on prenylation occurring in *Streptomyces* species has evolved in the past twenty years. It is now known and confirmed that the biosynthesis of isoprene units in *Streptomyces* sp. is also done within an MVA or MEP pathway, leading to IPP/DMAPP (Saleh et al., 2009; Dairi, 2005; Kuzuyama et al., 2002).

The prenyltransferases catalyze the transfer of prenyl moieties to aromatic acceptor molecules, forming C-C bonds between C-1 or C-3 of the isoprenoid substrate and one of the aromatic carbons of the acceptor substrate (Kuzuyama, 2017; Winkelblech et al., 2015; Zocher et al., 2012; Saleh et al.,

2009). ABBA-type aromatic prenyltransferases catalyze the attachment of single and condensated IPP/DMAPP moieties to aromatic compounds in bacteria and fungi (Zocher et al., 2012; Saleh et al., 2009).

3.4 Novobiocin, a prenylated antibiotic produced by *Streptomyces* spp.

Novobiocin belongs to the coumarin class of compounds (aminocoumarins). This prenylated aromatic compound with antibacterial activity was first described in the mid of the 20th century. This NP binds to type II topoisomerases, including deoxyribonucleic acid (DNA) gyrase. It inhibits the enzyme-catalyzed hydrolysis of adenosine triphosphate (ATP) and, thus, the functioning of the enzyme, leading to DNA replication arrest and death of a bacterial cell. Novobiocin is biosynthesized by *Streptomyces spheroides*[1] and *Streptomyces niveus* (El-Naggar, 2021; Huang & Blagg, 2007).

Recently, the chemical analogs of novobiocin re-gained the scientific community's attention due to cytotoxic effects on human cancer cells and neuroprotective activity in preclinical tests (Sanchez et al., 2020; Bickel & Gohlke, 2019; De Lima Procópio et al., 2012).

It has been confirmed that novobiocin blocks the ATP-binding site of DNA gyrase and 90 kDa heat shock protein (Hsp90) (Sanchez et al., 2020; Bickel & Gohlke, 2019). Hsp90 is a molecular chaperone that ensures cellular proteostasis by maintaining folding, stabilization, activation, and degradation of client proteins. Hsp90 is critical for routine protein maintenance in healthy cells and states of cellular stress, such as cancer and neurodegenerative diseases (Sanchez et al., 2020; Bickel & Gohlke, 2019; Hyun et al., 2018; Długosz & Janecka, 2017).

Usually, Hsp90 inhibitors exert their activity by binding to the *N*-terminal ATP binding pocket of the enzyme (Blagg & Kerr, 2006). Today it is known that aminocoumarin antibiotics such as novobiocin also act on eukaryotic cells by blocking the *C*-terminal ATP binding site of Hsp90. This induces ubiquitination of the client protein and, therefore, apoptosis (Sanchez et al., 2020).

[1] *Streptomyces spheroides* is also known as *S. spheroids*. References use both names.

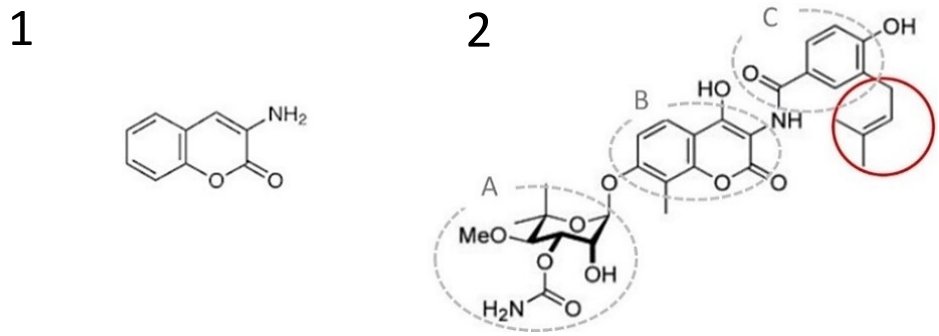

Figure 6: ***Chemical structures of coumarin and novobiocin and their similarities****. 3-aminocoumarin (1) is a heterocyclic compound that contains a coumarin ring system (a benzopyrone fused to a benzene ring) with an amino group (-NH_2) substituted at the C-3-position of the coumarin ring. Novobiocin (2) includes the amino sugar L-noviose (ring A), methyl and hydroxy moieties on its aminocoumarin substructure (ring B), and an L-tyrosine derivative (ring C). The prenyl unit is highlighted in red (Huang & Blagg, 2007, modified by NSCH).*

Novobiocin is prenylated at its aromatic ring, as shown in *Figure 6*. Generally, prenylation is essential for the pharmacological properties of natural products. Prenylation may also increase the lipophilicity and/or binding ability to its target protein. Furthermore, it improves membrane affinity compared to the non-prenylated forms (Chen & Abe, 2021; Mori, 2020; Zocher et al., 2012). The addition of a prenyl moiety to a non-prenylated NP may change its pharmacological properties and thus can be considered as a possible route for new drug discovery or optimization.

3.5 Biotransformation by ABBA-prenyltransferases expressed in *Streptomyces* spp.

According to the IUPAC Goldbook, "biotransformation is any chemical conversion of substances that is mediated by living organisms or enzyme preparations derived therefrom ("Biotransformation," 2017)." Biotransformation in bacteria refers to the ability to convert compounds into different chemical structures using endogenous enzymes and metabolic pathways.

The process of biotransformation in bacteria such as *Streptomyces* spp. has been studied intensively over the last decades. With the steady improvement of genetic engineering methods in molecular biology, *in vivo* and *in vitro* biotransformation by bacteria can now be studied intensively.

Genes encoding bioconverting enzymes can be identified, synthesized, and cloned into host organisms, such as *Streptomyces* spp., which are considered as "power horses" for producing NPs (Xu et al., 2022). Today, whole-cell-, enzyme- and in vitro- biotransformations are carried out in biotechnology, synthesizing complex natural products or modifying existing molecules (Del Carratore et al., 2022).

In *Streptomyces* species, ABBA-prenyltransferases play a crucial role in the biosynthesis of pharmaceutically relevant natural products.

Structurally, ABBA-prenyltransferases consist of beta/alpha folds with antiparallel beta strands. Due to the α-β-β-α architecture of the fold, this group of enzymes was classified as ABBA-prenyltransferases (Mori, 2020).

Today, more and more ABBA-prenyltransferase subtypes are discovered in various organisms, including bacteria, plants, and fungi. ABBA-prenyltransferases identified in several *Streptomyces* strains are involved in the biosynthesis of aminocoumarin antibiotics, meroterpenoids (prenylated polyketides), and prenylated phenazines (Liu et al., 2022; Mori, 2020; Saleh et al., 2009).

ABBA prenyltransferase CloQ prenylates 4-hydroxyphenyl pyruvate (4-HPP), (iso-)flavonoids, and stilbenoids in *Streptomyces roseochromogenes*. The subtype NovQ in *Streptomyces spheroides* attaches IPP/DMAPP onto phenylpropanoids, dihydroxy naphthalenes), and flavonoids (Mori, 2020). Both CloQ and NovQ are 3-dimethylallyl-4-hydroxyphenyl pyruvate transferases, as *Figure 7* indicates. These enzymes are involved in the biosynthesis of novobiocin or clorobiocin (Saleh et al., 2009).

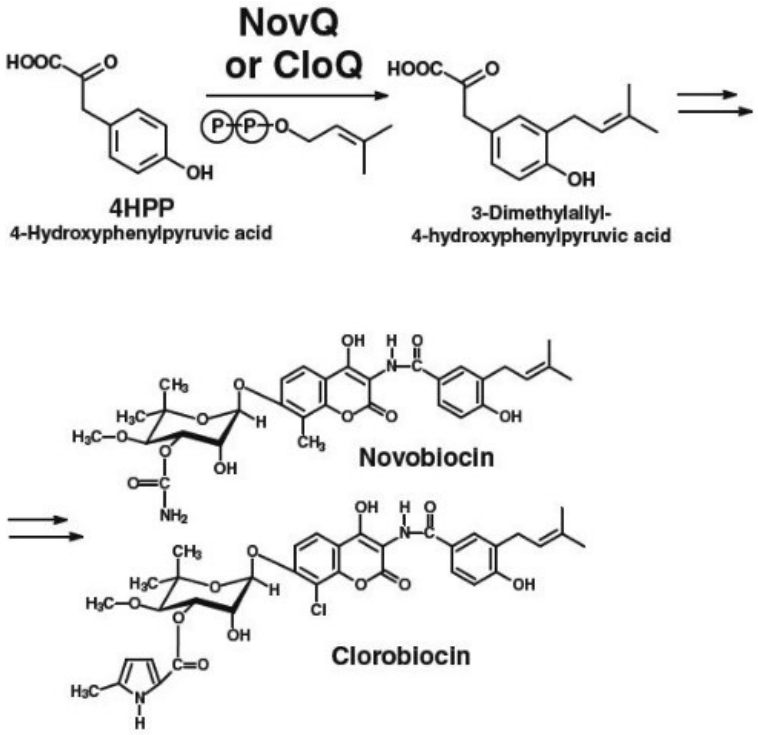

Figure 7: ***Biosynthetic pathway of novobiocin and clorobiocin, two secondary metabolites produced in Streptomyces sp.*** *The metabolites are catalyzed by ABBA-prenyltransferase subtypes NovQ and/or CloQ. The pathway starts with the prenylation of the aromatic compound 4-hydroxyphenyl pyruvic acid (4HPP). C_5 isopentenyl pyrophosphate (IPP) or dimethylallyl pyrophosphate (DMAPP) are considered prenyl donors. The intermediate 3-dimethylallyl-4-hydroxyphenyl pyruvic acid is further processed to novobiocin or clorobiocin in Streptomyces species (Saleh et al., 2009, modified by NSCH).*

Besides NovQ and CloQ, recent studies also focused on revealing the capabilities of the ABBA-prenyltransferase subtype NphB. Acceptable substrates for NphB include 1,6-dihydroxy naphthalene (1,6-DHN), 2,7-DHN, and 4-hydroxyphenylpyruvate (4-HPP), as well as a variety of plant polyphenols, such as naringenin, daidzein, genistein, resveratrol, and olivetol. NphB was first discovered in *Streptomyces* sp. CL190 (Mori, 2020; Kuzuyama, 2017; Winkelblech et al., 2015).

In 2020, the genome of *Streptomyces* sp. S4.7 was sequenced, identifying similarities concerning the size and composition of its genome to that of *Streptomyces niveus* SCSIO, a producer of prenylated polyketides, e.g., marfuraquinocins and prenylated phenazines (Bekiesch et al., 2020). Subsequent research, including this master thesis project, focused on confirming the BGC presumed to be involved in the biosynthesis of prenylated NPs and its gene functions, especially genes that encode aromatic prenyltransferases, targeting meroterpenoids; see *Figure 8*.

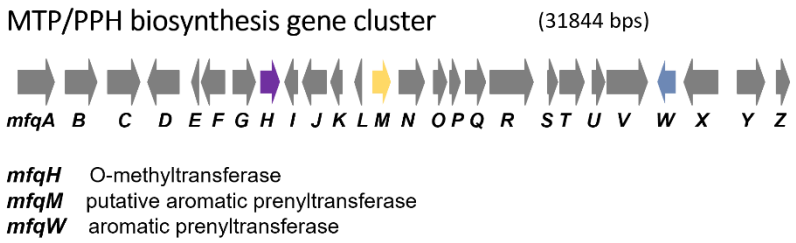

Figure 8: ***The putative gene cluster for the biosynthesis of meroterpenoids (MTP) and prenylated phenazines (PPH) in Streptomyces venezuelae T1-4D2. Data not shown****. Genes mfqH, mfqM, and mfqW (colored arrows) were cloned into vectors and heterologously expressed in S. venezuelae (Zotchev & Schneider, 2022, modified by NSCH).*

3.6 Biosynthesis of prenylated polyketides in *Streptomyces* spp.

Meroterpenoids (MTPs) are organic compounds synthesized by unicellular organisms such as bacteria and multicellular ones, e.g., fungi, plants, and animals, including those of marine origin (Fuloria et al., 2022). They are a structurally and functionally diverse chemical class consisting of terpenoid building blocks attached to mainly aromatic compounds, such as polyketides and phenols, but also to alkaloids and amino acids (Murray et al., 2020).

Polyketide synthases, ketoreductases, aromatases, and cyclases biosynthesize the polyaromatic carbon skeletons of polyketides. Subsequently, the products undergo structural modifications in tailoring steps, including prenylation, oxygenation, dimerization, glycosylation, methylation, and more (Lü et al., 2015). The ABBA-prenyltransferases NphB and SCO7190 catalyze the biotransformation of MTPs by attaching prenyl moieties to polyketides (Mori, 2020).

Prenylated polyketides such as marfuraquinocin display a wide range of biological activities, including antibacterial, cytotoxic, anticholinesterase-, COX-2 inhibitory-, antileishmanial-, antidiabetic-, antioxidative, anti-inflammatory, antineoplastic, antimalarial, antiviral, anti-obesity, and insecticidal effects (Fuloria et al., 2022; Song et al., 2013).

First studies of *Streptomyces niveus* SCSIO 3406 isolated from the deep South China Sea discussed the biosynthesized meroterpenoid marfuraquinocin and its congeners; see *Figure 9.*

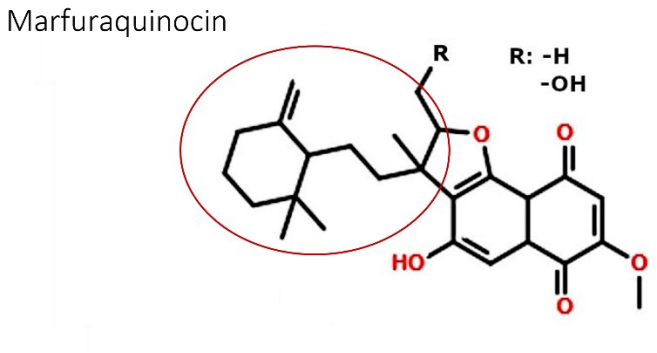

Figure 9: ***Chemical structure of meroterpenoid (MTP) or prenylated polyketide marfuraquinocin****. Prenyl moiety is highlighted in red and appears as cyclized sesquiterpenoid or C_{15} farnesyl pyrophosphate. Marfuraquinocin is an enantiomer (Zhu et al., 2021, modified by NSCH).*

Marfuraquinocins with a cyclized C_{15} farnesyl moiety demonstrate antibacterial activity against *Staphylococcus aureus* ATCC 29213 or methicillin-resistant *Staphylococcus epidermidis* and also had inhibitory effects on an NCI-H460 cancer cell line. Since these compounds show relevant pharmaceutical properties, research has focused on the relevant biosynthetic gene cluster (Song et al., 2013).

Recent studies identified the bacterial gene clusters for polyketide synthase and 1,3,6,8-tetrahydroxynaphthalene (THN) monooxygenase, responsible for the formation and modification of THN to generate THN moiety of marfuraquinocin in *Streptomyces niveus* SCSIO 3406 (Zhu et al., 2021).

4 Aims of the project

The project aimed to validate the hypothesis that recombinant *Streptomyces venezuelae* strains expressing prenyltransferases from the marfuroquinocin BGC of *Streptomyces* sp. S4.7 enable the prenylation of externally added aromatic compounds (biotransformation).

The project strategy is based on constructing the plasmids harboring genes deemed necessary for prenylation in *Escherichia coli* and genes encoding synthases for producing prenyl building blocks, geranyl- or farnesyl pyrophosphates. Within this project, the plasmids were transferred into *Streptomyces venezuelae,* genetically engineered to eliminate their own terpene synthases.

The project also aimed to explore the impact of added aromatic compounds on the recombinant host strain *Streptomyces venezuelae* T1-4D2. Therefore, the substrates' minimal inhibitory concentration (MIC) had to be determined.

Lastly, it was planned to perform an HPLC analysis of the extracts and implement LC-MS analysis to determine whether potential biotransformation has occurred.

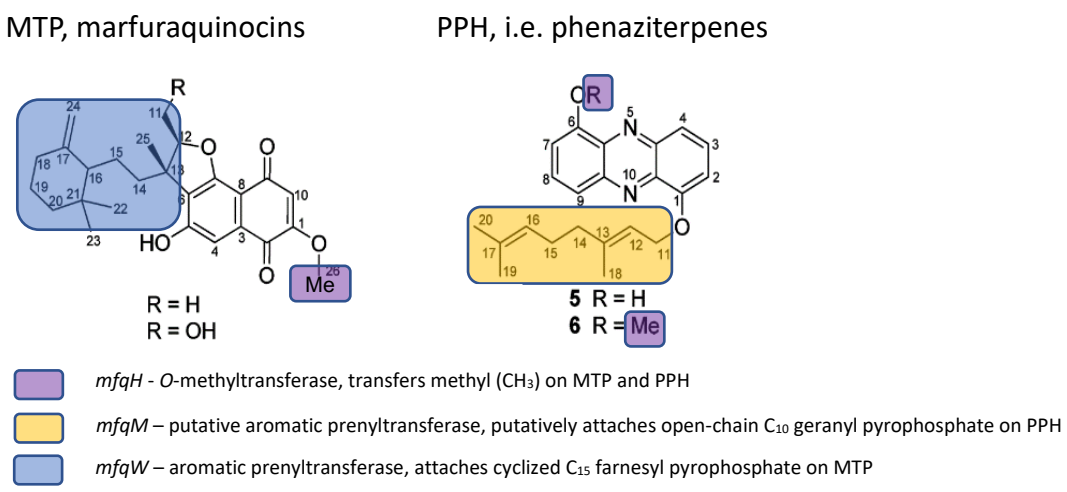

Figure 10: ***Functions of the projects genes mfqW, mfqH, and mfqM on MTP and PPH, respectively. Data not shown.*** *Genes mfqW and putatively mfqM encode for aromatic prenyltransferases that catalyze the transfer of prenyl moieties on their acceptor molecules. Encoding gene mfqH leads to an O-methyltransferase for MTP and PPH. Genes fpps for farnesyl pyrophosphate synthase and gpps for geranyl pyrophosphate synthase were also cloned. Fpps synthesizes the C_{15} prenyl moieties (blue box) and gpps C_{10} prenyl moieties (yellow box) (Zotchev & Schneider, 2022, modified by NSCH).*

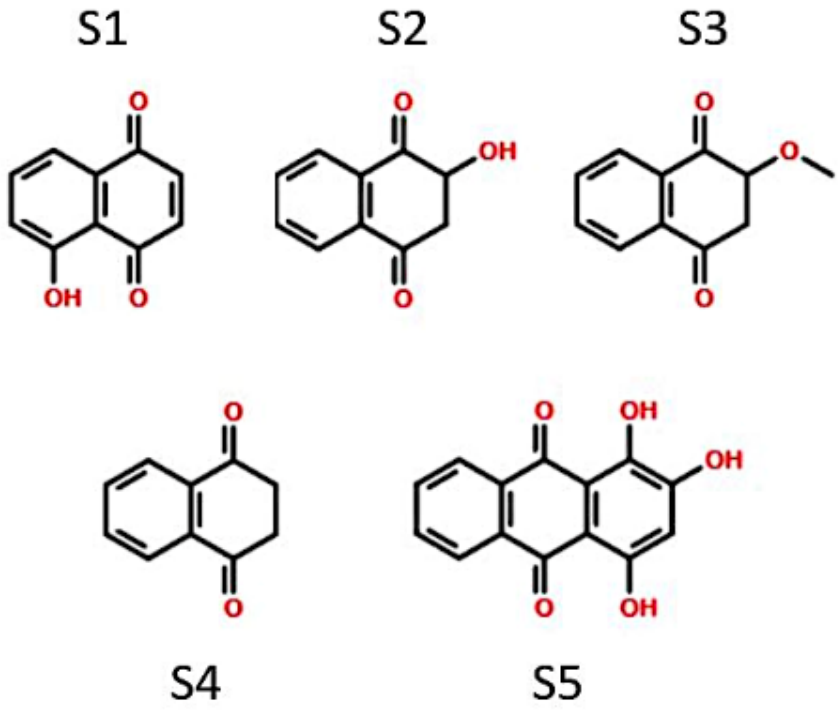

Figure 11: ***Aromatic compounds used in this master thesis project as precursors for prenylation induction.*** *S1 juglone, S2 2-Hydroxy-1,4-naphthoquinone, S3 2-Methoxy-1,4-naphthoquinone, S4-1,4-Naphthoquinone, and S5 purpurin (Chemical Structure Search, 2022, modified by NSCH).*

5 Results

5.1 Genes of interest

The biosynthesis gene cluster of meroterpene (MTP) and prenylated phenazines (PPH) in *Streptomyces* sp. S4.7 spans approximately 32 kbp and consists of 26 genes.

The final vectors containing the genes *fpps (pF), gpps (pG), mfqM, mfqW,* and *mfqH* (see *Figure 12*) were constructed as described in *Chapters 8 & 9* and successfully transformed into *Streptomyces venezuelae* T1-4D. Gene *mfqM* putatively encodes an aromatic prenyltransferase specific for phenazines. Gene *mfqH* encodes an *O*-methyltransferase, and gene *mfqW* leads to an aromatic prenyltransferase, specific for meroterpenoids. To increase the chances for biotransformation, the project also included genes *fpps* and *gpps* for farnesyl- and geranyl synthases (FPPS, GPPS). These enzymes synthesize C_{15} (farnesyl) and C_{10} (geranyl) prenyl moieties, which are subsequently attached to the chemical MTP framework. The vectors contain ermEp and P21 promotors. Both are strong promoters that can deliver a high-level expression of transgenes in *Streptomyces* sp. (Siegl et al., 2013; Bibb et al., 1985).

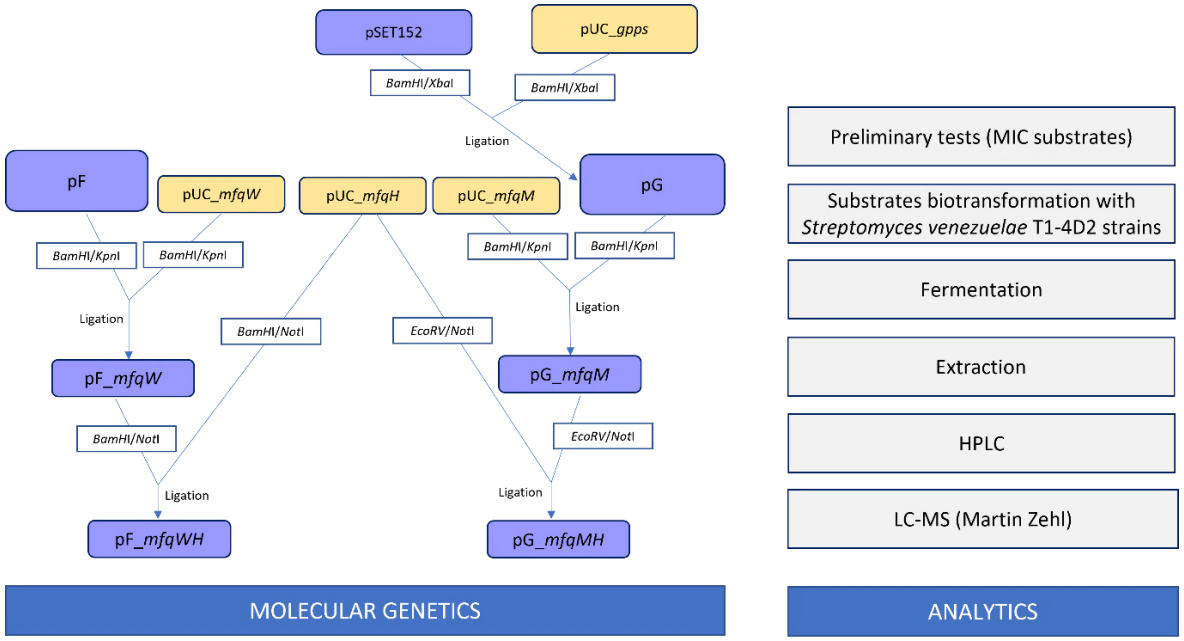

Figure 12: ***Workflow of the master thesis project.*** *The first part focused on molecular genetics and constructing the final vectors pF_mfqWH and pG_mfqMH. The second part, analytics, involved several steps, e.g., preliminary tests for the MIC of substrates and their biotransformation with S. venezuelae T1-4D2 strains, fermentation and extraction, and HPLC or LC-MS analysis, respectively.*

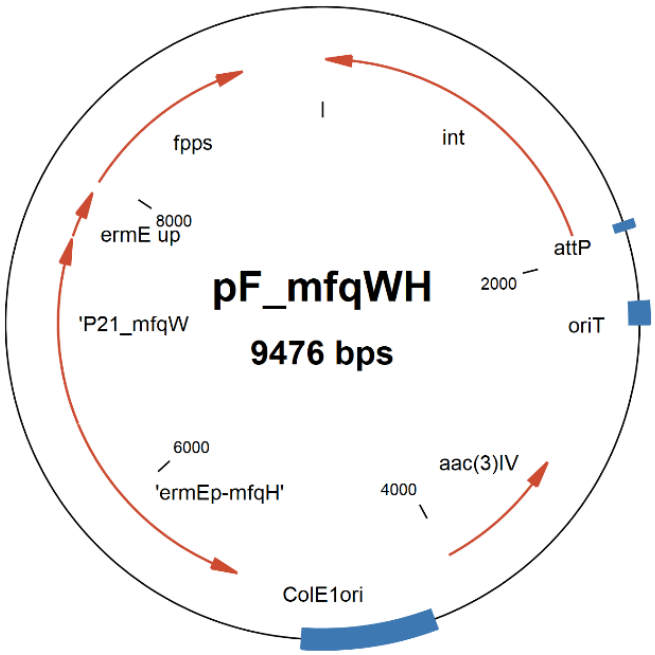

Figure 13: ***Vector map of pF_mfqWH.*** *pF_mfqWH was conjugated with S. venezuelae T1-4D2. The vector includes the DNA fragments fpps, ermEp-mfqH, and P21_mfqW. The vector has a size of 9476 bps. ErmE: a strong promotor. aac(3)IV: apramycin resistance. Int: integrase, ColE1ori: replication origin for Escherichia coli. AttP: attachment site.*

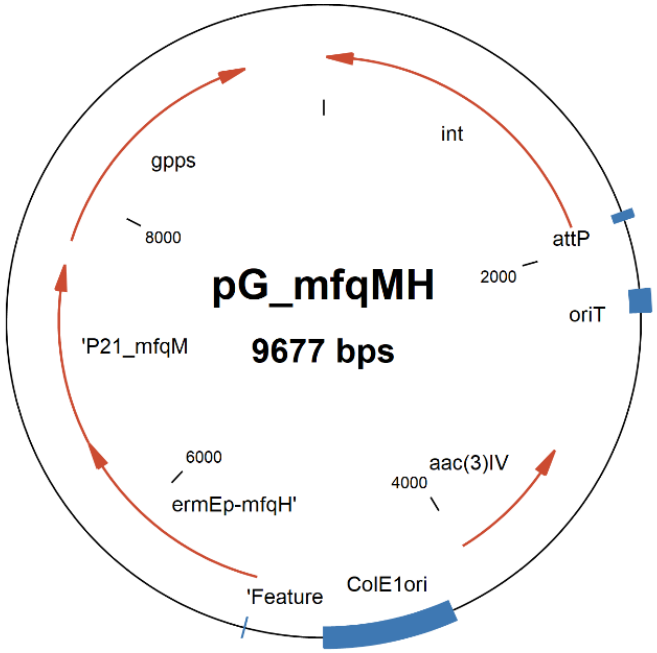

Figure 14: ***Vector map of pG_mfqMH.*** *pG_mfqMH was conjugated with S. venezuelae T1-4D2. The vector includes the DNA fragments gpps, ermEp-mfqH, and P21_mfqM. The vector's final size was 9677 bps. ErmE: a strong promotor. aac(3)IV: apramycin resistance. Int: integrase, ColE1ori: replication origin for Escherichia coli. AttP: attachment site.*

5.2 pF_mfqWH and its gene functions

The final plasmid vector pF_mfqWH contained three genes, i.e., *fpps* (FPP synthase), *mfqH* (*O*-methyltransferase), and *mfqW* (aromatic prenyltransferase), which should induce the farnesyl-bioproduction, and prenylation and/or methylation of the added MTP precursors (substrates S1 to S5). The corresponding vectors are displayed in *Figure 15*.

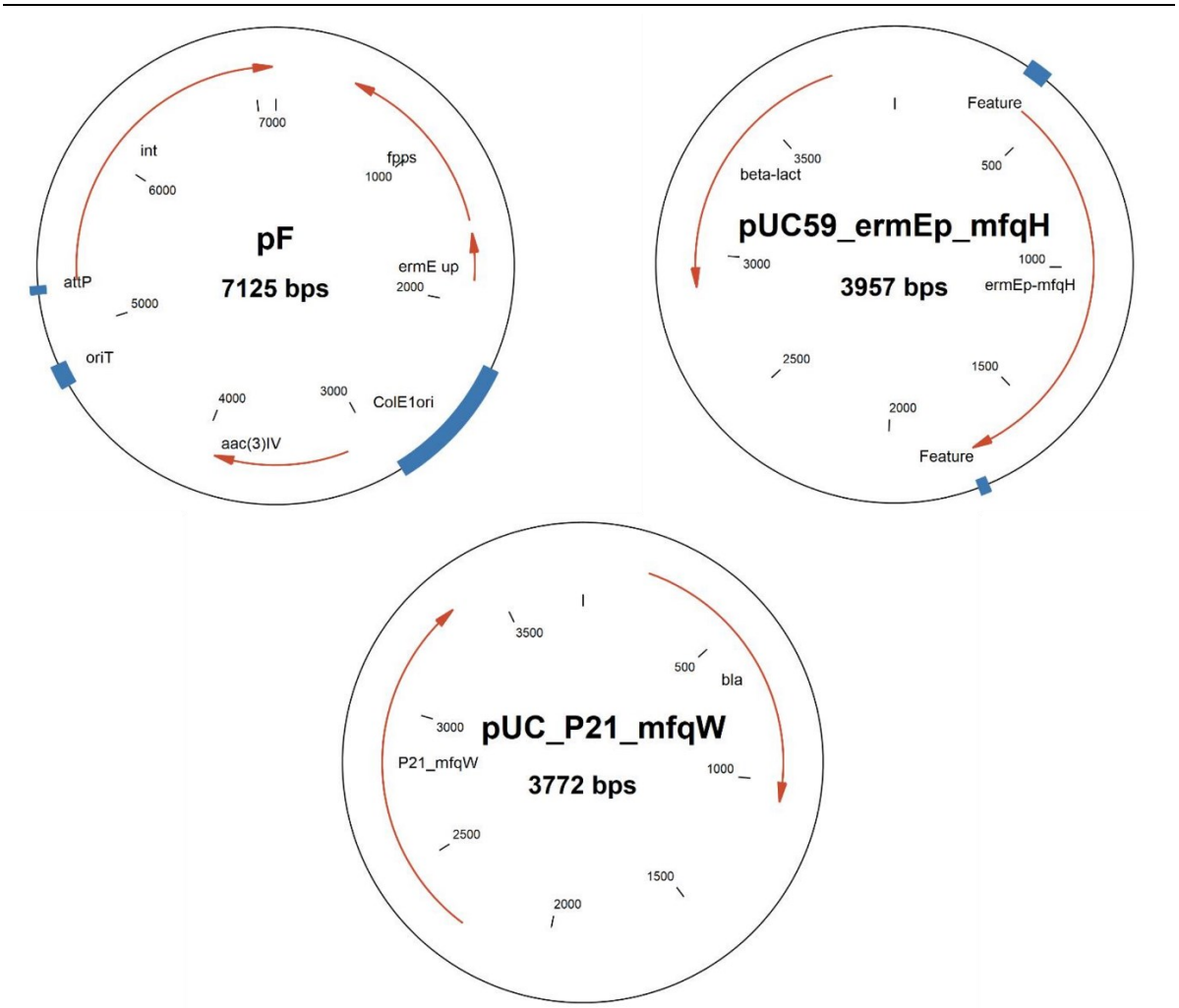

Figure 15: ***Intermediate vectors used for assembly of pF_mfqWH.*** *pF contained the gene fpps to express farnesyl pyrophosphate synthase (FPPS). pUC59_ermEp_mfqH: expresses O-methyl-transferase gene mfqH (ermEp-mfqH). pUC_P21_mfqW: contains gene mfqW for expressing an aromatic prenyltransferase (P21_mfqW). ErmE: a strong promotor. bla or beta-lact: beta lactamase. aac(3)IV: apramycin resistance. Int: integrase, ColE1ori: replication origin for Escherichia coli. AttP: attachment site.*

5.3 pG_mfqMH and its gene functions

The final plasmid vector pG_mfqMH contained the genes *gpps* (GPP-synthase) and *mfqH* (*O*-methyltransferase), which should induce the geranyl-bioproduction and methylation of the added MTP precursors (substrates S1 to S5). The vector also included *g*ene *mfqM,* which putatively encodes an aromatic prenyltransferase and thus induces prenylation of S1 to S5. The corresponding vectors are displayed in *Figure 16*.

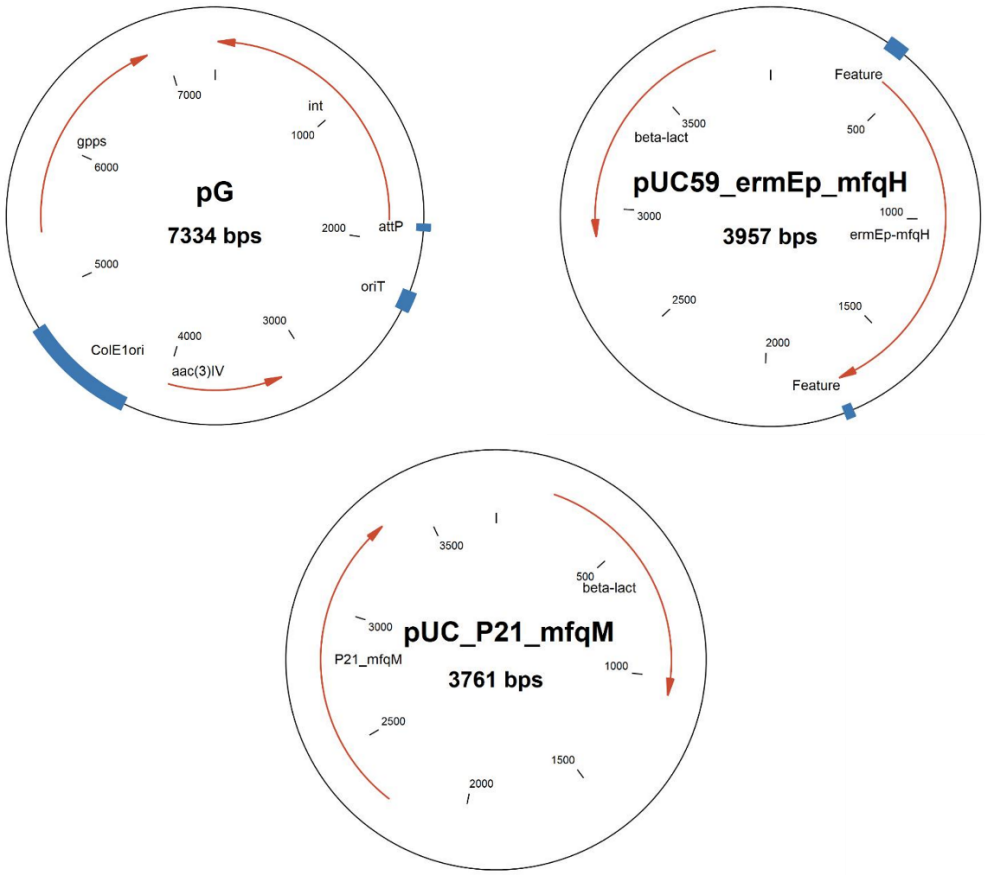

Figure 16: ***Intermediate vectors used for assembly of pG_mfqMH.*** *pG contained the gene gpps for expressing geranyl pyrophosphate synthase (GPPS). Vector pUC59_ermEp_mfqH expressed the O-methyltransferase gene mfqH (ermEp-mfqH), and vector pUC_P21_mfqM contained gene mfqM for the putative expression of an aromatic prenyltransferase (P21_mfqM). ErmE: a strong promotor. beta-lact: beta lactamase. aac(3)IV: apramycin resistance. Int: integrase, ColE1ori: replication origin for E. coli. AttP: attachment site.*

5.4 Vector construction in Escherichia coli sp.

All materials and methods used for these steps are explained in *Chapters 8 and 9*.

5.4.1 pF-harboring

The laboratory provided a purified DNA sample of pF, amplified in *E. coli* XL1-Blue MR cells. Purified DNA of pF was digested with *BamHI-HF®*/*KpnI®*, forming a linearized vector. According to the Clone Manager software, the vector had a size of 7116 bps. Gel electrophoresis confirmed that.

5.4.2 pUC59_ermEP_mfqH vector amplification and preparation

The laboratory provided stock DNA samples of pUC59_ermEP_mfqH. After its dilution, the DNA was transformed into *E. coli* XL1-Blue MR cells and amplified. The calculated restriction of pUC59_ermEP_mfqH/*BamHI-HF®*/*NotI®* led to two DNA fragments with a size of 1282 and 2675 bps. The fragment of interest, *ermEp_mfqH* had a size of 1282 bps; see *Figure 17.*

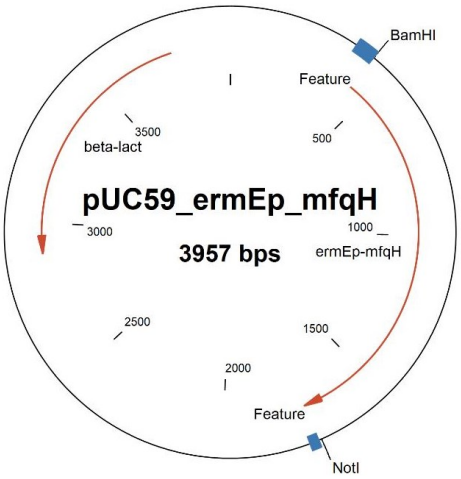

Figure 17: ***Vector map of pUC59_ermEp_mfqH/BamHI-HF®/NotI®.*** *The DNA fragment ermEp_mfqH had a size of 1282 bps. Beta-lact: beta lactamase. aac(3)IV: apramycin resistance. ermEp-mfqH: DNA fragment of interest. Int: integrase, Feature: ColE1ori, replication origin for E. coli.*

Gel electrophoresis confirmed a successful restriction of pUC59_ermEP_mfqH and its insert *ermEp_mfqH*; see *Figure 18*.

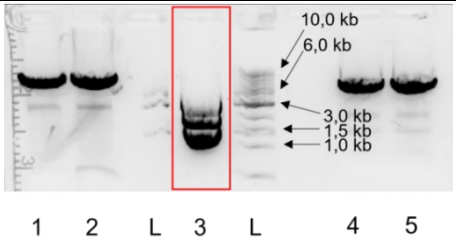

Figure 18: ***DNA fragments of pUC59_ermEP_mfqH/BamHI-HF®/NotI® (highlighted in red) and other samples.*** *L: 1kb DNA ladder. 1, 2: pF_mfqW/BamHI-HF®/NotI®. 3: pUC59_ermEP_mfqH/BamHI-HF®/NotI®. The DNA fragment ermEp_mfqH had a size of 1282 bps and correlated to the lowest gel band. 4, 5: pSET152/BamHI-HF®/XbaI®.*

Later on in this project, pUC59_ermEP_mfqH was reloaded on a gel, re-evaluating its DNA content. DNA fragments at 1282 and 2675 bps were calculated and validated by gel electrophoresis. The DNA bands at 1282 bps of three clones were gel extracted; see *Figure 19*.

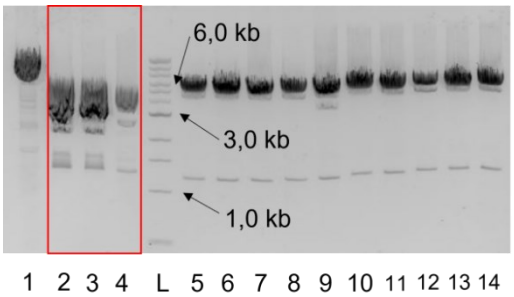

Figure 19: ***Gel image of pUC59_ermEP_mfqH (highlighted in red) and other samples.*** *L: 1kb DNA ladder. 1: pF_mfqW. 2, 3, 4: pUC59_ermEP_mfqH. Expected DNA bands at 1282 and 2675 bps were validated by the gel. Gel bands at 1282 bps were extracted, and PCR amplified. 5 to 14: pG/BamHI-HF®/XbaI®.*

For the construction of pG_mfqMH, pUC59_ermEP_mfqH was digested with *EcoRV-HF®*/*NotI-HF®*. The calculated fragments were expected to be 1285 and 2671 bps, which was validated by gel electrophoresis; see *Figure 20.*

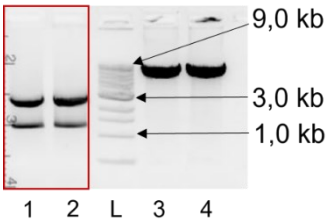

Figure 20: ***Gel image of pUC_ermEP_mfqH/EcoRV-HF®/NotI-HF® (highlighted in red) and other samples.*** *L: 1kb DNA ladder. 1, 2: pUC59_ermEP_mfqH/EcoRV-HF®/NotI-HF®. The gel confirmed the expected size of the calculated fragments at 1285 and 2671 bps. 3, 4 : pG_mfqM/EcoRV-HF®/NotI-HF®.*

5.4.3 pUC_P21_mfqW-harboring

pUC_P21_mfqW was diluted and amplified in *E. coli* XL1-Blue MR cells. Purified DNA of pUC_P21_mfqW was digested with *BamHI-HF®*/*KpnI®*, unveiling the DNA fragment *P21_mfqW* with a calculated size of 1093 bps; see *Figure 21*.

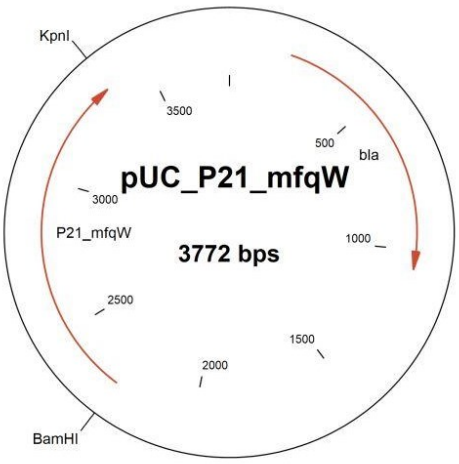

Figure 21: ***Vector map of pUC_P21_mfqW/BamHI-HF®/KpnI®.*** *The DNA fragment P21_mfqW had a size of 1093 bps. Bla: beta-lactamase.*

5.4.4 pF_mfqW-harboring

For the assembly of pF_mfqW, pF was ligated with the DNA fragment *P21_mfqW*. The ligation mixture was transformed into *E. coli* XL1-Blue MR cells and amplified. To validate the correct assembly of pF_mfqW, the constructed vector was digested with *ApaLI*® (*Figure 22*), leading to fragments of 1942 and 6259 bps.

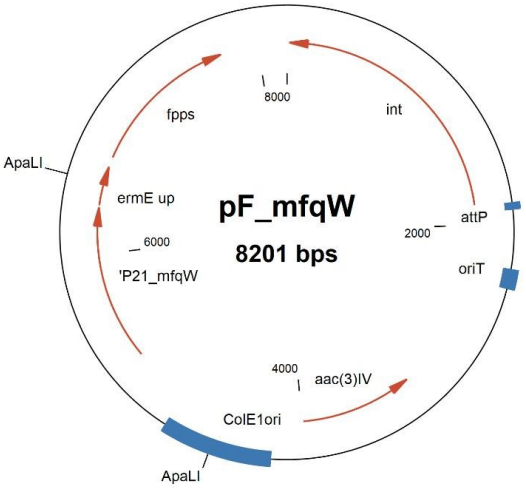

Figure 22: ***Vector map of pF_mfqW/ApaLI®.*** *The fragments had a calculated size of 1942 and 6259 bps. Int: integrase. attP: AttP: attachment site. aac(3)IV: apramycin resistance. ColE1ori: replication origin for E. coli. P21_mfqW: DNA fragment of interest, gene mfqW. ErmE up: a strong promoter. Fpps: DNA fragment of interest, gene fpps.*

Gel electrophoresis confirmed the correct construction of the vector; see *Figure 23*.

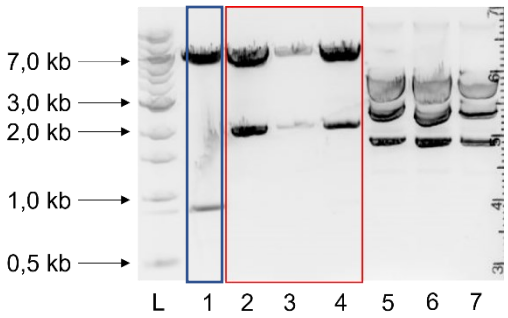

Figure 23: ***Gel image of the vector pF_mfqW/ApaLI® (highlighted in red), its negative control (highlighted in blue), and other samples.*** *L: 1kb DNA ladder. 1: negative control sample of pF_mfqW/ApaLI®. 2, 3, 4: pF_mfqW/ApaLI®. Bands were expected at 1942 and 6259 bps and confirmed by the gel. 5, 6, 7: pUC_gpps/BamHI-HF®/XbaI®.*

Later, pF_mfqW was digested with *BamHI-HF®*/*NotI®*, which linearized the vector and prepared it for further ligation.

5.4.5 pF_mfqWH vector construction

pF_mfqW was ligated with the DNA fragment *ermEP_mfqH*, transformed into *E. coli* XL1-Blue MR cells and amplified. The purified construct pF_mfqWH was digested with *BamHI-HF®*/*NotI®* to validate the correct assembly. Calculation by the Clone Manager indicated expected DNA fragments at 1282 and 8194 bps; see *Figure 24*.

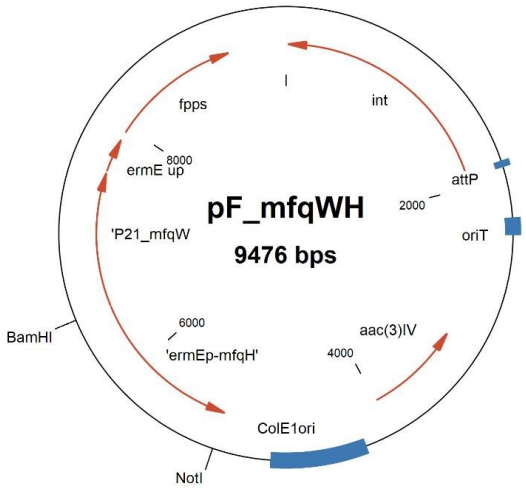

Figure 24: ***Vector map of pF_mfqWH/BamHI-HF®/NotI®.*** *The fragments had a calculated size of 1282 and 8194 bps. Int: integrase. attP: attachment site. aac(3)IV: apramycin resistance. ColE1ori: replication origin for E. coli. ermEp-mfqH: DNA fragment of interest, gene mfqH. P21-mfqW: DNA fragment of interest, gene mfqW. fpps: DNA fragment of interest, gene fpps. ErmE up: a strong promoter.*

Gel electrophoresis was performed to validate pF_mfqWH. Out of 7 clones, 3 clones contained the expected fragment *ermEP_mfqH*. The DNA bands at 1282 bps were gel extracted; see *Figure 25*.

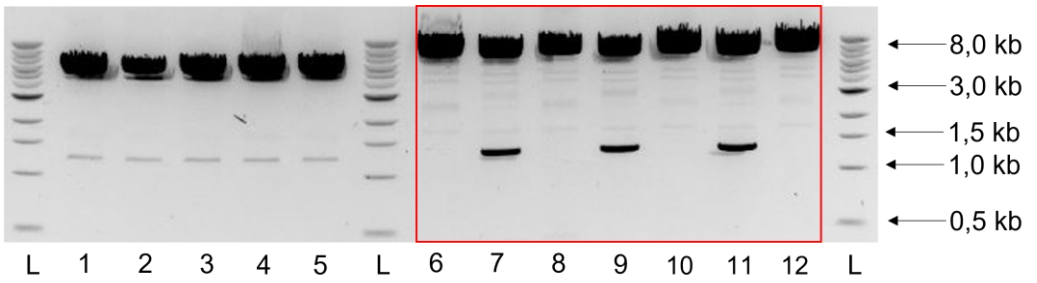

Figure 25: ***Gel image of the vector pF_mfqWH/BamHI-HF®/NotI® (highlighted in red) and other samples.*** *L: 1kb DNA ladder. 1 to 5: pG/BamHI-HF®/XbaI®. 6 to 12: pF_mfqWH/BamHI-HF®/NotI®. The gel confirmed calculated fragments at 1282 and 8194 bps.*

To validate the DNA sequence of the vector, sequencing was performed. The primers pSET_fwd1 and pSET_rev1 were used for sequencing by Eurofins Genomics. Sequencing confirmed the correct construct of pF_mfqWH.

5.4.6 pUC_P21_mfqM-harboring

The laboratory provided stock DNA samples of pUC_P21_mfqM. After its dilution, the DNA was transformed into *E. coli* XL1-Blue MR cells, amplified and later purified.

5.4.7 pG vector construction

pG was constructed by ligating pSET152 and pUC_gpps. A provided plasmid DNA of pSET152 was transformed into *E. coli* XL1-Blue MR cells and amplified. Purified pSET152 was cut with *BamHI-HF®/XbaI®*, which linearized the vector. Expected DNA bands at 5709 bps were validated by the gel and recovered.

To validate the DNA fragment *gpps*, a restriction of pUC_gpps with *BamHI-HF®/XbaI®* was performed. Gene *gpps* encodes a geranyl pyrophosphate synthase, producing C_{10} (geranyl) prenyl moieties. Calculated fragments had a size of 1625 and 2700 bps. Gel electrophoresis confirmed them; see *Figure 26*.

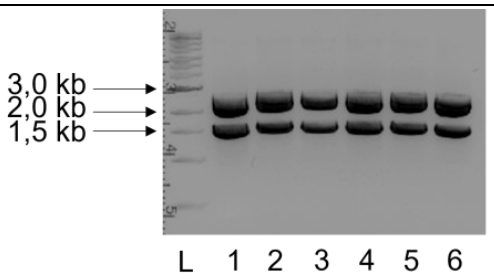

Figure 26: ***Gel image of pUC_gpps/BamHI-HF®/XbaI®.*** *L: 1kb DNA ladder. 1 to 6: Clones of gpps/BamHI-HF®/XbaI®. The DNA fragment of interest, gpps, had a calculated size of 1625 bps. The gel showed DNA bands at ~1500 bps, which were gel extracted.*

The isolated and purified DNA of *gpps* was ligated with pSET152/*BamHI-HF®*/*XbaI® and* transformed into *E. coli* XL1-Blue MR cells, plated and amplified. The purified vector pG was digested with the enzyme XhoI® and validated by gel electrophoresis to prove its correct assembly. Calculated by the Clone Manager, the restriction should result in two DNA fragments with a size of 2107 and 5227 bps; see *Figure 27*.

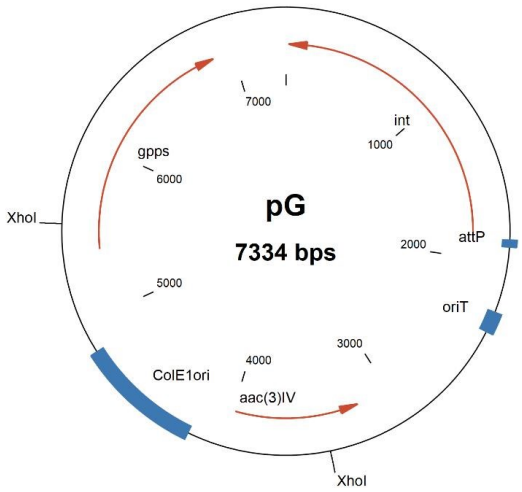

Figure 27: ***Vector map of pG/XhoI®.*** *The calculated size of the DNA fragments was 2107 and 5227 bps. Int: integrase. attP: attachment position. aac(3)IV: apramycin resistance. ColE1ori: replication origin for E. coli. Gpps: gene of interest, gpps.*

As a result, gel electrophoresis confirmed the construction of the vector pG; see *Figure 28*. Clones containing the DNA fragments at 2107 bps were further processed.

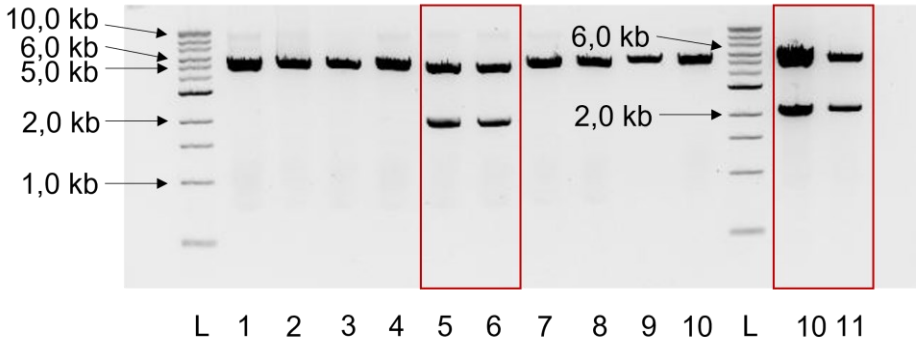

Figure 28: ***Gel image of pG/XhoI®.*** *L: 1kb DNA ladder. 1 to 12: pG/XhoI®. DNA bands for the vector pG were predicted at 2107 and 5227 bps, which were confirmed by the gel. Clones highlighted in red were further processed.*

5.4.8 pG_mfqM vector construction

The vector pG was digested with *BamHI-HF®*/*KpnI-HF®,* linearizing it with a calculated size of 7334 bps. Parallel to that, pUC_P21_mfqM was digested with *BamHI-HF®*/*KpnI-HF®*, leading to fragments at 1082 and 2675 bps.

The digested samples were loaded on agarose and separated; see *Figure 29*. Gel electrophoresis confirmed the success of the restriction; the DNA at 1082 bps, confirming the fragment *mfqM*, and the DNA at 7334 bps, confirming the vector pG, was gel extracted.

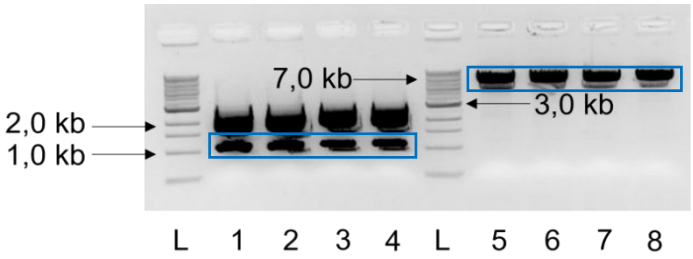

Figure 29: ***Gel image of pUC_P21_mfqM/BamHI-HF®/KpnI-HF® and pG/BamHI-HF®/KpnI-HF®.*** *L: 1kb DNA ladder. 1 to 4: pUC_P21_mfqM/BamHI-HF®/KpnI-HF®. The restriction should lead to fragments of 1082 and 2675 bps, which was confirmed by the gel. The bands highlighted in blue were gel extracted. 5 to 8: pG/BamHI-HF®/KpnI-HF. The restriction should result in 2 DNA bands with 16 and 7318 bps. The gel confirmed the DNA band at 7318 bps. The bands highlighted in blue were gel extracted.*

The extracted DNA of mfqM and pG*/BamHI-HF®/KpnI-HF®* was purified and ligated. The new vector construct pG_mfqM was transformed into *E. coli* XL1-Blue MR cells and amplified. Purified DNA of pG_mfqM was then digested with *BamHI-HF®*/*KpnI-HF®* and separated on agarose. According to the Clone Manager, DNA bands at 1082 and 7318 bps were expected; see *Figure 30*.

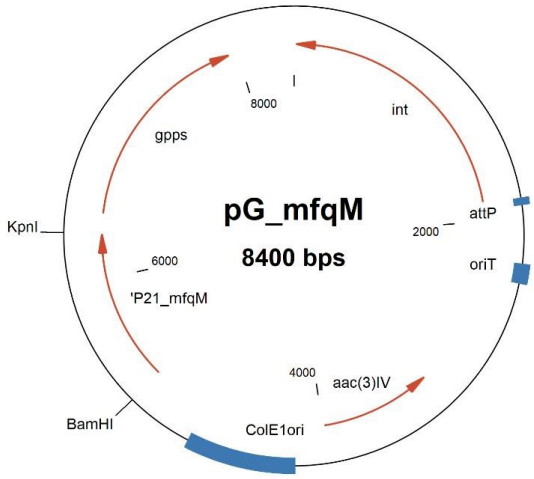

Figure 30: ***Vector map of pG_mfqM/BamHI-HF®/KpnI®.*** *The calculated size of the DNA fragments was 1082 and 7318 bps. Int: integrase. attP: attachment site. aac(3)IV: apramycin resistance. ColE1ori: replication origin for E. coli. P21_mfqM: DNA fragment of interest, gene mfqM. gpps: DNA fragment of interest, gene gpps.*

Consequent gel electrophoresis confirmed the DNA fragment *P21_mfqM*, which is 1082 bps; see *Figure 31.*

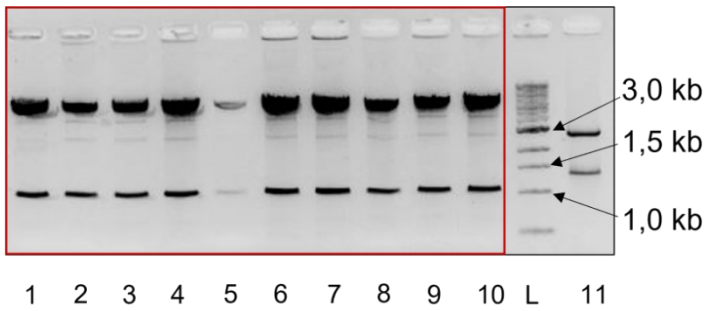

Figure 31: ***Gel image of pG_mfqM/BamHI-HF®/KpnI-HF® (highlighted in red) and other samples.*** *L: 1kb DNA ladder. 1 to 10: pG_mfqM/BamHI-HF®/KpnI-HF®. The restriction should lead to fragments of 1082 and 7318 bps, which was confirmed by the gel. 11: pUC_ermEP_mfqH/EcoRI-HF®/NotI-HF®.*

The constructed vector pG_mfqM was prepared for later ligation with the DNA fragment *ermEP_mfqH.* For that, the vector was linearized with *EcoRV-HF®*/*NotI-HF®*.

Subsequent gel electrophoresis confirmed pG_mfqM; see *Figure 32*. The DNA bands of pG_mfqM/*EcoRV-HF®*/*NotI-HF®* at 8391 bps were gel extracted and further processed.

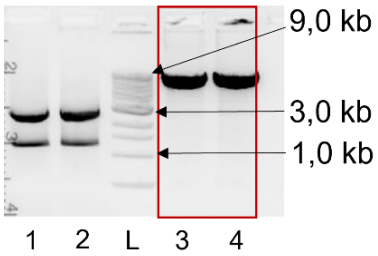

Figure 32: ***Gel image of pG_mfqM/EcoRV-HF®/NotI-HF® (highlighted in red) and other samples.*** *L: 1kb DNA ladder. 1, 2: pUC59_ermEP_mfqH/EcoRV-HF®/NotI-HF®. 3, 4: pG_mfqM/EcoRV-HF®/NotI-HF®. The restriction should lead to 9 and 8391 bps fragments, which the gel confirmed.*

5.4.9 pG_mfqMH vector construction

Two different clones of pG_mfqM were digested simultaneously in 2 steps. At first, pG_mfqM was digested with *EcoRV-HF®* and pG_mfqM with *NotI-HF®*. The restriction was validated by gel electrophoresis. The gel DNA pattern indicated the vector pG_mfqM at 8391 bps. The digestion was repeated with pG_mfqM/*EcoRV-HF®* and *NotI-HF®,* and pG_mfqM/*NotI-HF®* and *EcoRV-HF®,* respectively. The samples were loaded on agarose and separated.

The DNA band of pG_mfqM was gel extracted, purified, and ligated with the DNA fragment *ermEp_mfqH*. The ligation mixture was transformed into *E. coli* XL1-Blue MR and amplified. The purified DNA of pG_mfqMH was digested to check its correct construction. pG_mfqMH/*EcoRV-HF®*/*NotI-HF®* should lead to calculated fragments of 1286 and 8391 bps. Subsequent gel electrophoresis was performed, which confirmed the correct assembly of pG_mfqMH; see *Figure 33*.

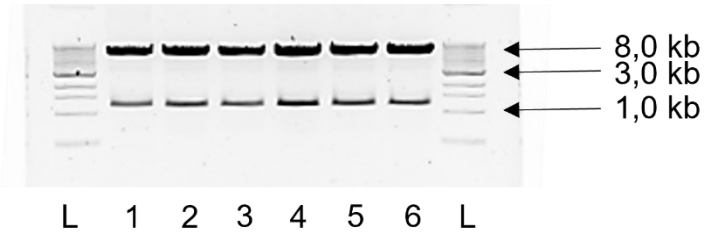

Figure 33: ***Gel separation pattern of pG_mfqMH/*EcoRV-HF®*/*NotI-HF®.*** *L: 1kb DNA ladder. 1 to 6: pG_mfqMH/*EcoRV-HF®*/*NotI-HF®*. The restriction should lead to fragments of 1286 and 8391 bps, which was confirmed by the gel.*

5.5 Conjugation in *S. venezuelae* T1-4D2

Both final vector constructs pF_mfqWH, pG_mfqMH and their intermediate vectors pF, pF_mfqW, pG, and pG_mfqM were successfully conjugated in *S. venezuelae* T1-4D2 as described in *Chapter 9*.

5.6 Determination of the MIC of substrates

All methods and materials are described in the *Chapters 8 & 9*.

Before fermentation, experiments determined the minimal inhibitory concentration (MIC) of the substrates S1 to S5. Different volume shares of the *Streptomyces T1-4D2* spore suspension, seeded in MYM and 0.25 M substrate S1 (juglone), were mixed and incubated, examining the substrates' inhibitory influence on mycelial growth. The experiment was repeated with different molar concentrations in various media for all the substrates. The defined MIC range for the project substrates is displayed in *Table 1*.

Table 1: ***MIC range of the project's substrates.***

Substrate	**Range MIC (mM/mL)**
S1 (juglone)	0.25 - 1.25
S2 (2-hydroxy-1,4-naphthoquinone)	1.00 - 5.00
S3 (2-methoxy-1,4-naphthoquinone)	0.10 - 0.50
S4 (1,4-naphthoquinone)	0.50 - 2.50
S5 (purpurin)	0.01 - 0.05

These concentrations were utilized for all fermentation experiments except those conducted in February and March 2023. New stock solutions of the substrates with standardized molarities for S1 to S4 (0.10 M) and S5 (0.01 M) were prepared and used for these particular experiments. This step should control potential variations in the experimental results that could arise from differences in substrate concentration, e.g., caused by the solvent DMSO. If the experimental run involved the fermentation of substrates, they were added at the end of the fermentation process. Depending on the mycelial growth, these mixtures were further incubated for 2 to 3 days.

Substrates, which were added after fermentation and extraction, and the sample run containing glycine contained different volumes of the substrates.

5.7 Fermentation

All methods and materials are described in the *Chapters 8 & 9*.

During the timeframe of this project, the fermentation medium MYM showed superior performance. PM4 was considered, at last, to perform solely for wild-type and pF-strains experiments.

The project also focused on setting the optimal fermentation parameters, such as the durance of the fermentation cycle and the number of precultures. Multiple fermentation steps were carried out to achieve optimal growth conditions and induce biotransformation. First, seeding cultures with different volumes of wild-type and recombinant *Streptomyces* strains were prepared and monitored for mycelial growth. Then, several volumes were transferred into a fresh medium and further incubated. The project considered one or two preculture cycles. Finally, two precultures cycles were chosen as best, which should further increase the yield and prevent contamination. *Figure 34* summarizes the specific fermentation parameters defined during this project.

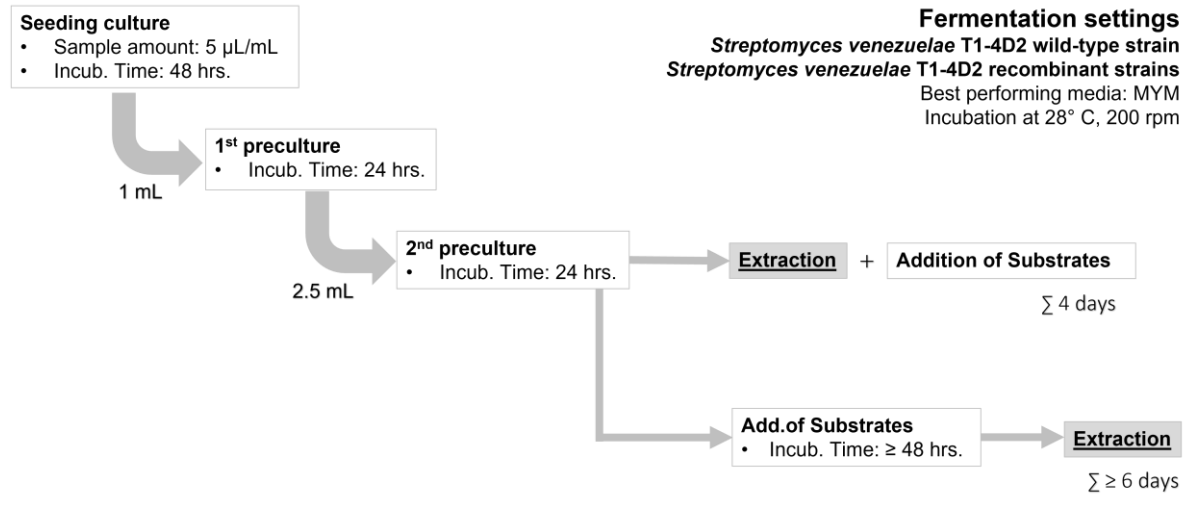

Figure 34: ***The projects' fermentation settings for S. venezuelae T1-4D2 wild-type and recombinant strains.***

Table 2 describes the strains of the recombinant *S. venezuelae* being fermented, the final concentration of the substrates, as well as the extraction method used. Since it was not clear if the substrates were going to be biotransformed intra- or extracellularly, the project also considered lysis experiments and the addition of the substrates at different time points of the analysis process. 8 experimental runs added the substrates into the 2nd preculture, and further incubated them. 3 experiments added the substrates at the end of the extraction process; they weren't fermented. Lysis experiments were performed twice with a 100 mM TRIS-HCl buffer, followed by MeOH extractions. HPLC analyzed all listed samples, and selected ones underwent further LC-MS analyses performed by Martin Zehl (MZ).

All fermentation, extraction, and analysis protocols are described in *Chapters 8* and 9.

*Table 2: **Fermentation experiments performed during this project and extraction methods.***

Date	**Samples *S. venezuelae* T1-4D2**	**Extraction method**	**HPLC sample preparation**	**LC-MS**
21.10. 22	**Wild-type (Wt)** **All pF-strains** Fermented in **MYM** S1 0.5 mM/mL S2 2 mM/mL S3 0.2 mM/mL S4 1 mM/mL S5 0.1 mM/mL	**Acetone (Ace) & Ethylacetate (EA)**	-	wt_Ace_S2 wt_Ace_S5 wt_EA_S4 pF_Ace_S2 pF_Ace_S5 pF_EA_S4 pF_mfqW_Ace_S2 pF_mfqW_Ace_S5 pF_mfqWH_Ace_S2 pF_mfqWH_EA_S4
08.11. 22	**All pG-strains** Fermented in **MYM** S1 0.5 mM/mL S2 2 mM/mL S3 0.2 mM/mL S4 1 mM/mL S5 0.1 mM/mL	**MeOH**	-	No LC-MS analysis
18.11. 22	**Wt** **All pF-strains** Fermented in **MYM** S1 0.5 mM/mL S2 2 mM/mL S3 0.2 mM/mL S4 1 mM/mL S5 0.1 mM/mL wt 0 mM substrates pF strains 0 mM substrates	**MeOH**	-	wt_0 wt all substrates pF_0 pF all substrates pF_mfqW_0 pF_mfqW all substrates pF_mfqWH_0 pF_mfqWH all substrates
30.11. 22	**Wt** **All pF-strains** Fermented in **TSB** wt 0 mM substrates pF strains 0 mM substrates	**100 mM TRIS-HCl lysis buffer** **MeOH**	Addition of: **S2** 1 M 0.5 µL, 1 µL, 5 µL, 10 µL **S5** 0,01 M 0.5 µL, 1 µL, 5 µL, 10 µL	Wt_S5_0.5 pF_S5_0.5 pF_mfqW_S5_0.5 pF_mfqWH_S5_0.5

Date	**Samples *S. venezuelae* T1-4D2**	**Extraction method**	**HPLC sample preparation**	**LC-MS**
16.12. 22	**Wt** **All pF strains** Fermented in **TSB** wt 0 mM substrates pF strains 0 mM substrates	**100 mM TRIS-HCl lysis buffer** **MeOH**	Addition of: **S1, S2, S3, S4** 0.1 M 1 µL, 2.5 µL **S5** 0.01 M 1 µL, 2.5 µL	wt_S3_2.5 pF_S2_2.5 pF_S3_1 pF_S5_2.5 pF_mfqW_S3_1 pF_mfqWH_S2_2.5 pF_mfqWH_S3_2.5 pF_mfqWH_S5_2.5
21.12. 22	**Wt** **All pG strains** Fermented in **2xYT**	**100 mM TRIS-HCl lysis buffer** **MeOH**	Addition of: **S1, S2, S3, S4** 0.1 M 1 µL, 2.5 µL **S5** 0.01 M 1 µL, 2.5 µL	pG_S1_1 pG_S2_2.5 pG_S3_1 pG_S3_2.5 pG_S4_1 pG_S4_2.5 pG_S5_1 pG_S5_2.5 pG_mfqM_S1_1 pG_mfqM_S2_1 pG_mfqM_S3_1 pG_mfqM_S3_2.5 pG_mfqM_S5_1 pG_mfqM_S5_2.5 pG_mfqMH_S1_1 pG_mfqMH_S2_2.5 pG_mfqMH_S3_1 pG_mfqMH_S3_2.5 pG_mfqMH_S4_2.5 pG_mfqMH_S5_1 pG_mfqMH_S5_2.5
12.01. 23	**Wt** **All pG strains** Fermented in **TSB** No substrates added	**MeOH**	-	Wt_0 pG_0 pG_mfqM_0 pG_mfqMH_0
16.02. 23	**Wt** **All pF strains** Fermented in **PM4** S1 0.2 mM/mL S2 0.2 mM/mL S3 0.2 mM/mL S4 0.2 mM/mL S5 0.02 mM/mL wt 0 mM substrates pF strains 0 mM substrates	**MeOH**	-	No LC-MS analysis

Date	**Samples *S. venezuelae* T1-4D2**	**Extraction method**	**HPLC sample preparation**	**LC-MS**
16.02. 23	**Wt** **All pF strains** Fermented in **MYM** S1 0.2 mM/mL S2 0.2 mM/mL S3 0.2 mM/mL S4 0.2 mM/mL S5 0.02 mM/mL wt 0 mM substrates pF strains 0 mM substrates **Wt** **pF** Fermented in **MYM**, **0.8 mM glycine** S2 0.2 mM S5 0.01 mM wt 0 mM substrates pF strains 0 mM substrates	**MeOH**	-	No LC-MS analysis
10.03. 23	**Wt** **All pF strains** Fermented in **PM4** S1 0.2 mM/mL S2 0.2 mM/mL S3 0.2 mM/mL S4 0.2 mM/mL S5 0.02 mM/mL wt 0 mM substrates pF strains 0 mM substrates all substrates, no cells solely PM4 medium	**MeOH**	-	wt_0 pF_0 pF_mfqW_0 pF_mfqWH_0 wt_S1 pF_S1 pF_mfqW_S1 pF_mfqWH_S1 wt_S2 pF_S2 pF_mfqW_S2 pF_mfqWH_S2 wt_S3 pF_S3 pF_mfqW_S3 pF_mfqWH_S3 wt_S4 pF_S4 pF_mfqW_S4 pF_mfqWH_S4 S1, S2, S3, S4

Date	Samples *S. venezuelae* T1-4D2	Extraction method	HPLC sample preparation	LC-MS
10.03.22	**Wt** **All pF strains** Fermented in **MYM** S1 0.2 mM/mL S2 0.2 mM/mL S3 0.2 mM/mL S4 0.2 mM/mL S5 0.02 mM/mL wt 0 mM substrates pF strains 0 mM substrates all substrates, no cells solely MYM medium	**MeOH**	-	wt_0 pF_0 pF_mfqW_0 pF_mfqWH_0 wt_S2 pF_S2 pF_mfqW_S2 pF_mfqWH_S2 S2

5.8 HPLC and LC-MS analyses of the extracts

HPLC analysis was performed in the laboratory, and Martin Zehl (MZ) and his team did LC-MS analysis.

HPLC analysis of the extracts of the wild-type and all recombinant pF-strains, fermented in MYM with S2 (2-Hydroxy-1,4-naphthoquinone), extracted with acetone (21.10.2022) yielded chromatographic peaks at ~21.25 min with different intensities, as *Figure 35* shows. This retention time was defined as the non-modified S2, which should lead to homologous intensities in all samples, according to the sample prep. protocol. However, wt and the recombinant pF showed the lowest intensities. The chromatogram also revealed small peaks at 27.50 and ~32.00 min for all the samples with comparable intensities. Since it was unclear whether all strains took up the substrate to the same extent or not, LC-MS analysis was performed. This run detected peaks at a deviating retention time of ~24.50 min with a $[M+H]^+$ of $C_{25}H_{20}O_6$ without appreciable differences in the intensities for all samples, including the wt; see *Figure 36*. Further analysis was performed, which classified these peaks as non-modified S2. The variances in the retention times could be attributed to the differences in settings between HPLC and LC-MS analysis, such as variations in the column, flow rate, and solvent employed. The HPLC peaks observed at 27.50 and approximately ~32.00 min were not verified by LC-MS analysis either.

Figure 35: ***HPLC spectrum of wt and all recombinant pF-strains (MYM, fermented with S2, Ace, 21.10.2022) at 254 nm.*** *Peaks at ~ 21.25 min, equivalent to S2, were detected in all samples, and wt showed the lowest intensity.*

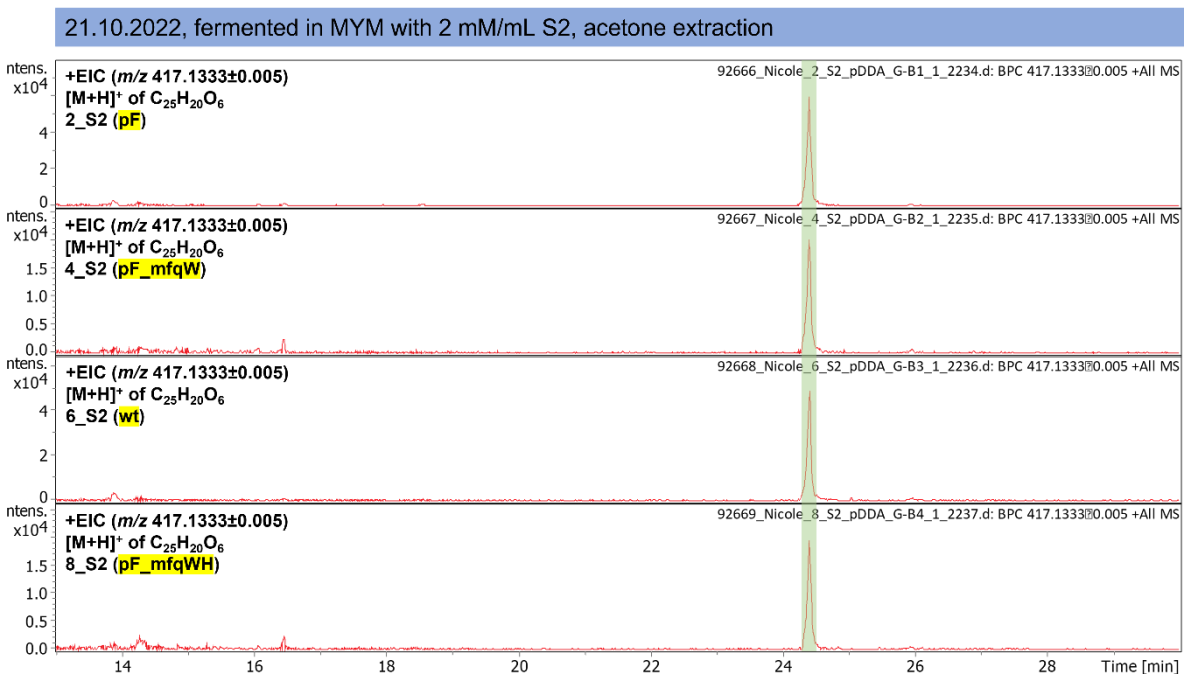

Figure 36: ***LC-MS analysis of wt and all recombinant pF-strains (MYM, S2, Ace, 21.10.2022)****. Peaks at ~24.50 min were detected and classified as non-biotransformational products with a calculated [M+H]⁺ of $C_{25}H_{20}O_6$., meaning S2, since it was detected in all samples without appreciable differences in the intensities. Figure provided by MZ, modified by NSCH.*

HPLC analysis of all recombinant pG-strains extracts, fermented in MYM with S1 to S5 and extracted with MeOH (08.11.2022), yielded no promising chromatographic peaks. Thus, no further LC-MS analysis was performed.

HPLC analysis of the extracts of wt and all recombinant pF-strains, fermented in MYM with S1 to S5 and extracted with MeOH (18.11.2022), revealed chromatographic peaks for S2 at 21.50 min, as *Figure 37* examines.

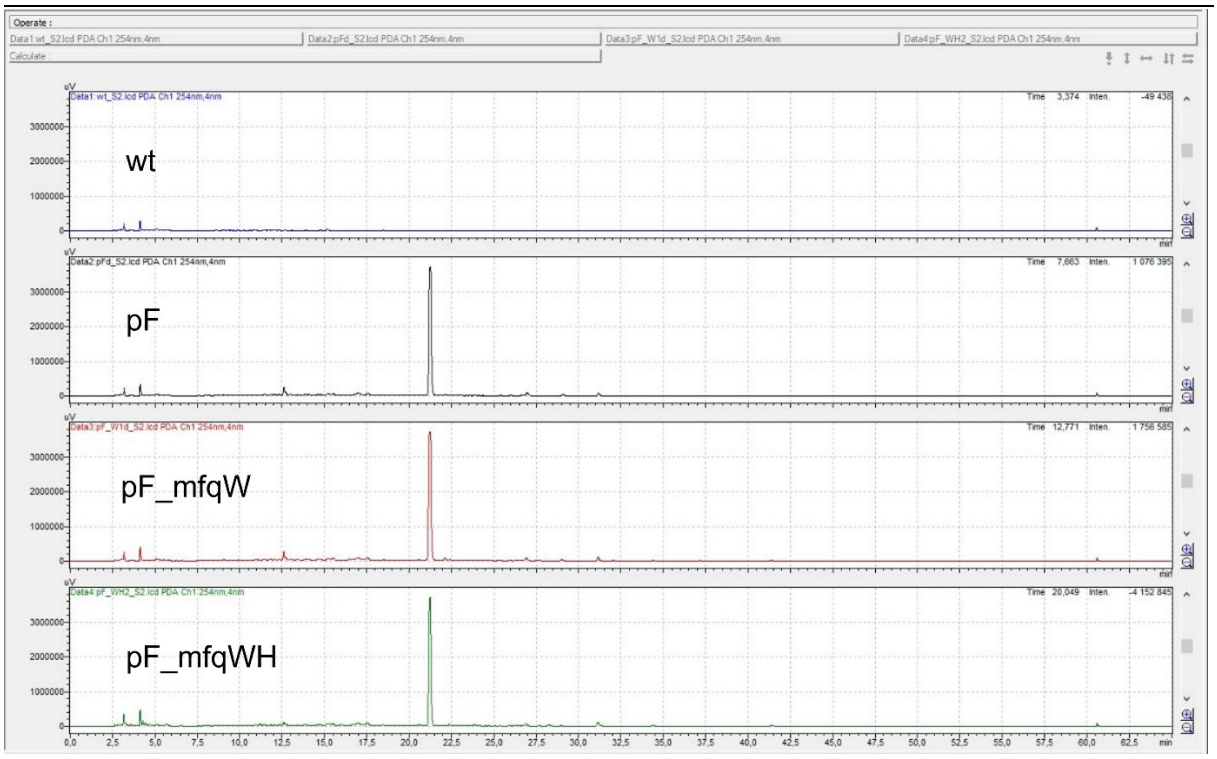

Figure 37: ***HPLC spectrum of wt and all recombinant pF-strains (MYM, fermented with S2, MeOH, 18.11.2022) at 254 nm.*** *Peaks at ~ 21.25 min (S2) were detected in all the recombinant samples but not in the wild-type.*

The wild-type didn't contain S2 since it showed no peak at 21.50 min, whereas the recombinant pF-strains yielded promising chromatographic peaks at this retention time with the same intensity. Small peaks at 27.50 and ~32.00 min were detected for all recombinant strains but not for wt. At this point, the wild-type strain appeared to not uptake substrate 2, while the recombinant strains did. Since the peaks at 27.50 and ~32.00 min were only detectable in the recombinant strains, a possible biotransformation of substrate 2 was presumed. To confirm this assumption, another LC-MS analysis was performed.

LC-MS analysis detected a relatively intense peak in sample pF_mfqWH_S2 at around 24.50 min with a $[M+H]^+$ of $C_{25}H_{20}O_6$. According to the previous analysis, this compound was classified as non-modified

S2. It was present in all samples of the series, including wt. Nonetheless, it was about 50 times more concentrated in pF_mfqWH; see *Figure 38*.

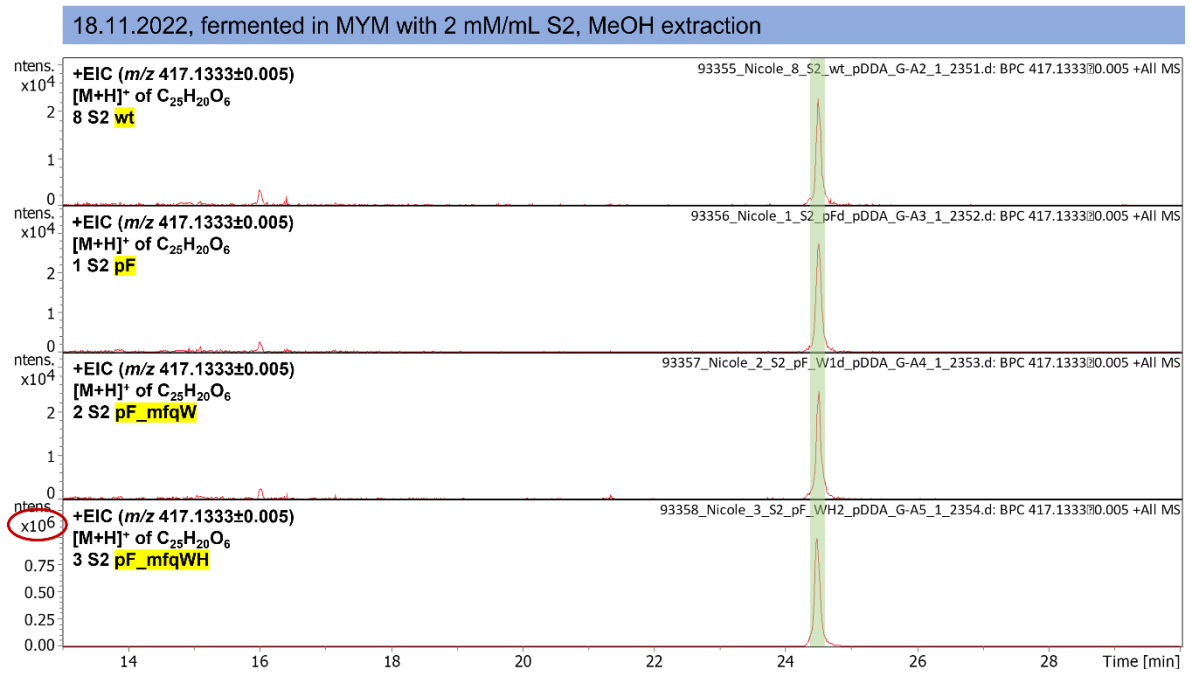

Figure 38: ***LC-MS analysis of wt and all recombinant pF-strains (MYM, S2, MeOH, 18.11.2022).*** *Peaks at ~ 24.50 min were detected in all the samples but with a 50x intensity in pF_mfqWH. Calculated $[M+H]^+$ predicted again $C_{25}H_{20}O_6$. Further analysis by MZ indicated S2 ($C_{10}H_6O_3$) with lapachol ($C_{15}H_{14}O_3$), a hydroxy-1,4-naphthoquinone with an attached prenyl group. Figure provided by MZ, modified by NSCH.*

Further analysis on $[M+H]^+$ of $C_{25}H_{20}O_6$ was performed by Martin Zehl. As a possible explanation, a biotransformation of S2 ($C_{10}H_6O_3$) into an unknown substance ($C_{15}H_{14}O_3$), presumably lapachol, was discussed. Lapachol is a hydroxy-1,4-naphthoquinone with an attached prenyl group, which shows structural similarities to S2; see *Figure 39* (Chemical Structure Search, 2022).

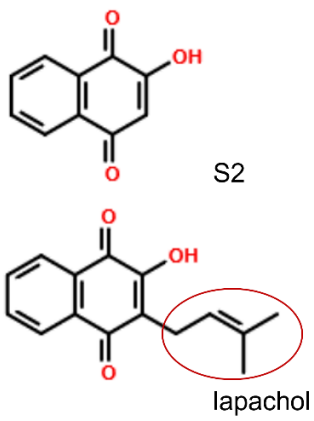

Figure 39: ***Structural similarities of S2, 2-Hydroxy-1,4-naphthoquinone, and lapachol ($C_{15}H_{14}O_3$).*** *The prenyl group is highlighted in red (Chemical Structure Search, 2022, modified by NSCH).*

It was assumed that the wild-type could also perform biotransformation. Thus, the experimental setting was repeated on 16.02.2023 and 10.03.2023, using MYM and PM4 as fermentation media with S1 to S5 and MeOH for extraction. The repetitions should confirm lapachol and, consequently, biotransformation. HPLC analysis detected promising peaks at ~22.00 min, in MYM, especially for substrate S2; see *Figure 40*.

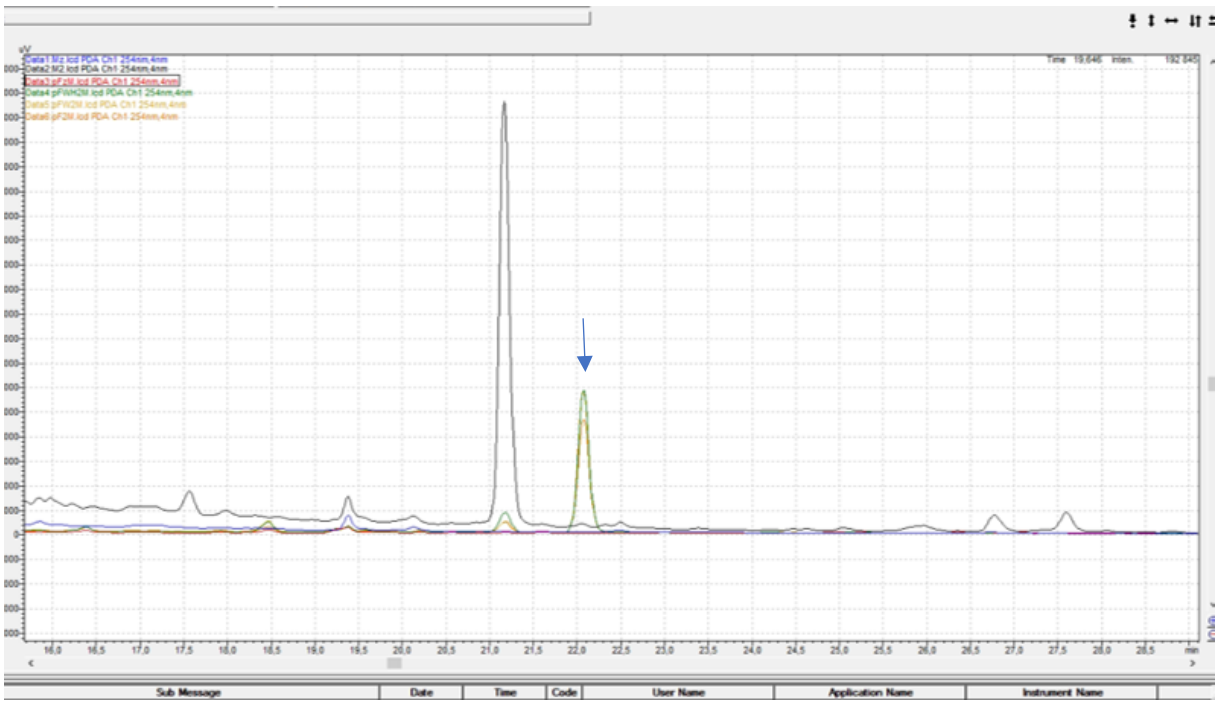

Figure 40: ***HPLC spectrum of solely MYM (blue), MYM S2 (black), pF 0 mM S2 (red), pF S2 (orange), pF_mfqW S2 (yellow), pF_mfqWH S2 (green) at 254 nm.*** *Peak comparison indicated a biotransformational product of S2 at 22.00 min for pF and pF_mfqWH.*

LC-MS further analyzed the samples of 10.03.2023. As a result, neither lapachol nor the supposed adduct of S2 with lapachol was detected. However, a biotransformational product of S2 (S2-CH_2O) was identified; see *Figure 41*. According to MZ, this compound was not correlating to the meroterpenoid BGC. In addition, it was detected in the negative control samples of wt and the recombinant strains.

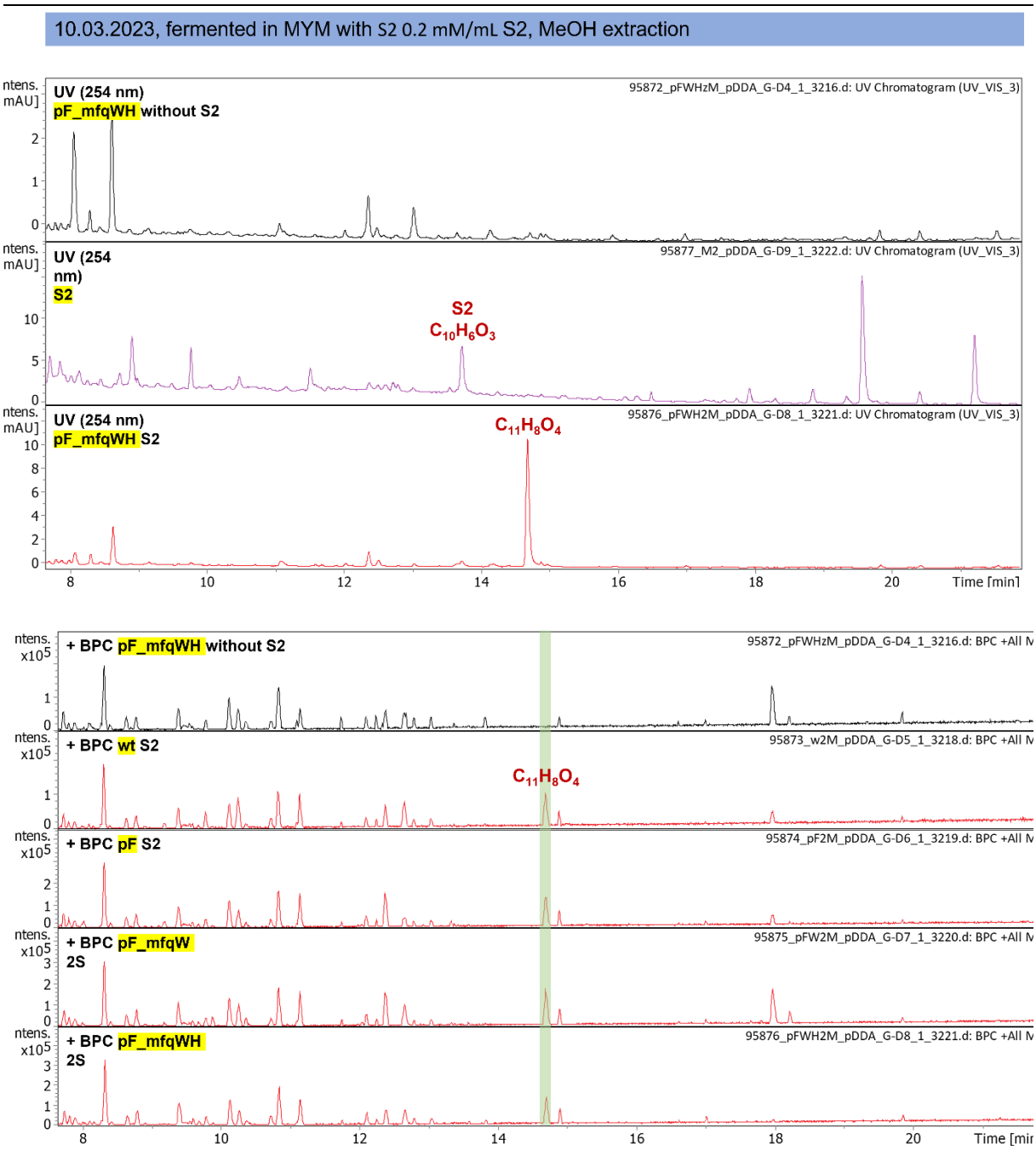

Figure 41: ***LC-MS analysis of S2, wt, and recombinant pF-strains, including negative control samples.*** *The peak for pF_mfqWH S2 indicated an adduct of S2 and CHO. However, the same peak occurred for wt too. Figure provided by MZ, modified by NSCH.*

From November 2022 onwards, the project also involved the lysis of the fermented project strains, as it had yet to be confirmed whether the substrates would be taken up by the wild-type and recombinant strains and whether the biotransformation occurred intra- or extracellularly. HPLC sample analysis of the extracts of wt and all recombinant pF-strains, fermented in TSB, lysed, and extracted with MeOH, HPLC sample prep. with adding S2 and S5 (30.11.2022) exposed unique peaks at ~28.00 min, which weren't correlating either to S2, or to S5. However, LC-MS analysis didn't confirm any reaction products of the substrates.

The HPLC analysis of the extracts of wt and all recombinant pF strains, fermented in TSB, lysed, and extracted with MeOH, HPLC sample prep. with adding S1 to S5 (16.12.2022) detected a promising peak at 21.00 min for pG_mfqWH_S2, which was not correlating to S2. The samples were analyzed by LC-MS, which didn't confirm any reaction products of the substrates.

The HPLC analysis of the extracts of wt and all recombinant pG-strains, fermented in 2xYT, lysed, and MeOH extracted, HPLC sample prep. with the addition of substrates S1 to S5 (21.12.2022) detected small but unique peaks for all the recombinant samples. Later performed LC-MS analysis didn't confirm any reaction product of the substrates. According to MZ, these peaks were classified as artifacts, lipids, and peptides but no reaction products of the substrates. Since this experimental run didn't involve negative control samples for wt and the recombinant pG-strains, it was again performed on 12.01.2023, using the promising MeOH extraction protocol performed on 18.11.2022. The HPLC analysis of the extracts of wt and all the recombinant pG-strains without substrates (12.01.2023) yielded promising chromatographic peaks at 14.75 min for all the recombinant samples. LC-MS analyzed these peaks at this appr. retention time, and identified chloramphenicol. The comparison to the samples containing substrates analyzed on 21.12., neither showed any results.

The project also examined the possible influence of nutritional supplements such as the amino acid glycine. Glycine can act as a carbon- and nitrogen source in nature, thus increasing the chances of the project's enzymatic activity and the formation and transfer of prenyl compounds on acceptor molecules. The experimental setting included extracts of wt and solely pF, fermented in MYM with 0.8 mM/mL glycine and the substrates S2 and S5. A MeOH extraction was performed afterward (16.02.2023). However, HPLC analysis didn't yield interesting chromatography peaks. Thus, no further LC-MS analysis was performed.

6 Discussion

Streptomyces sp. is acknowledged as a major source of bioactive and pharmacologically relevant secondary metabolites. *Streptomyces venezuelae* was first isolated from a soil sample out of Caracas, Venezuela, in the late 1950s. Since then, various *S. venezuelae* strains have been known for biosynthesizing chloramphenicol and other antibiotics, i.e., methymycin and pikromycin (Hill & Staunton, 2010, Ehrlich et al., 1947). Besides antibiotics, *Streptomyces* spp. yield prenylated meroterpenes (MTP), such as marfuraquinocins and prenylated phenazines. These secondary metabolites showed antibiotic and cytotoxic effects *in vivo*. Prenylation of chemical compounds is essential for their pharmacological activity due to their "anchor effect" on membranes or target proteins.

This project aimed at the prenylation of externally added aromatic compounds, i.e., naphthoquinones, by recombinant *Streptomyces venezuelae* strains expressing prenyltransferases, thus achieving biotransformation. The BGC for the biosynthesis of marfuraquinocin, identified in *Streptomyces* sp. S4.7 comprises various genes presumed to be involved in the biosynthesis of meroterpenoids and prenylated phenazines. Genes encoding farnesyl- and geranyl synthases, aromatic prenyltransferases, and *O*-methyltransferases, respectively, were cloned into vector plasmids in *E. coli* and then successfully transferred into *Streptomyces venezuelae* T1-4D2. This *Streptomyces* strain was genetically engineered by Phelan et al., deleting geosmin and 2-methylisoborneol (2-MIB) synthase genes, which may compete in the biosynthesis of FPP or terpenes (Phelan et al., 2015). Various fermentation and extraction methods, including cell lysis, were performed. An early LC-MS analysis of wt and recombinant pF-strains, expressing genes for farnesyl synthase, aromatic prenyltransferase, and *O*-methyltransferase fermented with S2 (2-hydroxy-1,4-naphthoquinone) detected the biotransformed substance lapachol. Lapachol is a derivative of 2-hydroxy-1,4-naphthoquinone with a C_5H_9-prenyl group attached at C-3. ABBA-prenyltransferases (encoded by genes *mfqW,* and *mfqM)* facilitate the transfer of the prenyl moiety through an electrophilic alkylation process, known explicitly as Friedel-Crafts alkylation, which occurs predominantly at positions C-1 or C-3 of the acceptor molecule's aromatic ring (Saleh et al., 2009). However, analogous experiment repetitions and final LC-MS analysis didn't prove biotransformation to lapachol. Instead, a compound of $C_{11}H_8O_4$ was detected, a putative adduct of S2 and an aldehyde. However, this compound was unrelated to the expressed prenyltransferases and consequently cannot be considered a biotransformation product.

The project plan focused on inducing biotransformation using the substrates S1 to S5, which served as targets for prenylation. These compounds are well known for their cytotoxicity and antibacterial activity (Majdi et al., 2023; Rahman et al., 2022; Singh et al., 2021; Chao et al., 2001). Although the used amounts were pre-tested (MIC of substrates), it needed to be clarified whether the applied concentration of substrates would be sufficient for biotransformation in *Streptomyces* spp. In general, the substrates' small, aromatic chemical structure and solubility may increase the chances of membrane permeability and, thus, intracellular biotransformation. Enzymes for prenylation, such as CloQ, have a substrate tolerance for various phenolic acceptors (Rudolf et al., 2021; Mori, 2020; Kuzuyama, 2017; Winkelblech et al., 2015; Zocher et al., 2012). However, it is noteworthy that modifications to functional groups, such as the conversion of a hydroxy group to a ketone group through oxidation, have the potential to impact enzymatic performance. The phenolic base frame of the substrates can transform quickly in contact with oxygen or UV light, especially juglone (S1) and purpurin (S5). In the case of juglone, UV light can lead to the generation of reactive oxygen species (ROS), such as singlet oxygen and superoxide radicals. These ROS can cause oxidative damage to biomolecules (Rahman et al., 2022; Mone et al., 2021). The substrates underwent multiple freezing and thawing cycles throughout the project, potentially impacting their chemical structure and, thus, their influence on biotransformation.

The project also considered potential extracellular biotransformation. For that, several experiments were performed using cell lysates. LC-MS analyses detected no potentially biotransformed compound and provided no additional data compared to the whole-cell experiments. The project's lysis protocol was based on a publication by Sun et al., 2020, which utilized several assays for testing enzyme activity in *Streptomyces roseosporus* spp. Besides using a 100 mM Tris-HCl buffer for lysis, the proposed protocol included 1 mM phenylmethylsulphonyl fluoride (PMSF) (Sun et al., 2020). This potent protease inhibitor may prevent enzymes that catalyze the breakdown or degradation of proteins. This master's project also considered PMSF; however, the provided sample was not soluble in DMSO or ddsH_2O. Due to concerns regarding the potential detrimental effects on *Streptomyces* lysate, recommended solvents such as isopropanol, ethanol, methanol, or 1,2-propanediol were not considered. As a consequence, PMSF was not used. Further investigations on the project samples may consider using a PMSF salt, which is water soluble.

Overall, this project didn't confirm the hypothesis that recombinant *Streptomyces venezuelae* strains induce prenylation of externally added aromatic compounds or precursors. The most likely cause of these results seems to be the inability of the substrates to efficiently enter the bacterial cells expressing prenyltransferases. Given the constraints of the project timeline, the most promising experimental fermentation and extraction method was solely performed with the recombinant pF-strains. Further experiments may repeat the protocol from November 18, 2022, incorporating the pG- and pF-strains and freshly prepared substrate solutions.

7 Conclusion and Outlook

The continual advancements in genetic manipulation, genome sequencing, analysis techniques, and innovative strategies like genome mining are pivotal in uncovering novel natural products (NP). These developments significantly enhance our understanding of the (dormant) biosynthetic gene clusters (BGC) within microorganisms and enable targeted genetic manipulation for their activation and production of unique NP.

Within this work, several important goals have been achieved. Recombinant vectors pF, pF_mfqW, pF_mfqWH, and pG, pG_mfqM, pG_mfqMH were assembled in *E. coli* sp. and successfully transformed into *S. venezuelae* T1-4D2. Before the biotransformation attempts, the MICs for the added aromatic compounds were determined. Numerous fermentation runs confirmed MYM and PM4 as the best-performing media regarding substrate tolerance. Several extraction methods, including cell lysis, were performed. Most promising results were obtained with MeOH extractions.

While biotransformation of externally added aromatic compounds was not demonstrated, the constructed vectors may be used in future experiments with the *Streptomyces* strains that naturally produce aromatic compounds. Since these compounds will be initially present inside the bacterial cells, the chances that they will be prenylated will be much higher, providing opportunities for producing novel bioactive prenylated aromatic compounds.

8 Materials

8.1 Microorganisms used in this work

Escherichia coli sp. and *Streptomyces* sp. were used in this work; see *Tables 3* & *4*. The methods for genetic engineering are explained in *Chapter 9.6*. The cultivation of these strains is further discussed in *Chapters 9.4 and 9.5*.

8.1.1 *Escherichia coli* spp.

The laboratory provided cryo aliquots of competent *E. coli* cells à 100 µL. The original supplier for all these cells was Agilent. This project also modified different *E. coli* strains for DNA plasmid amplification and conjugation. Due to their methylation deficiency, the conjugation of the recombinant vectors with *Streptomyces* was solely done with *E. coli* ET_{12567} cells.

Table 3: ***Escherichia coli spp. used in this project.***

Escherichia coli	**Phenotype**	**Additional info, carrying vector**	**Source**
XL1-Blue MR	Wild-type	Competent cells	Agilent
DH5α	Wild-type	Competent cells	Agilent
ET_{12567}	Wild-type	Competent cells	Agilent
XL1-Blue MR	Recombinant	pUC_gpps	AG Zotchev
ET_{12567}	Recombinant	pUC_gpps	AG Zotchev
DH5α	Recombinant	pSET152	AG Zotchev
spp.	Recombinant	pF (gene *fpps*)	AG Zotchev
spp.	Recombinant	pUC_P21_mfqW (gene *mfqW*)	AG Zotchev
spp.	Recombinant	pUC_gpps (gene *gpps*)	AG Zotchev
spp.	Recombinant	pSETgpps_trps13 (gene *gpps*)	AG Zotchev; discovered by Oberhofer et al., 2022
spp.	Recombinant	pUC_P21_mfqM (gene *mfqM*)	AG Zotchev
spp.	Recombinant	pUC59_ermEP_mfqH (gene *mfqH*)	AG Zotchev
XL1-Blue MR	Recombinant	pF	This work
XL1-Blue MR	Recombinant	pF_mfqW	This work
XL1-Blue MR	Recombinant	pF_mfqWH	This work
XL1-Blue MR	Recombinant	pSET152_gpps	This work
XL1-Blue MR	Recombinant	pG	This work
XL1-Blue MR	Recombinant	pG_mfqM	This work
XL1-Blue MR	Recombinant	pG_mfqMH	This work
ET_{12567}	Recombinant	pF	This work
ET_{12567}	Recombinant	pF_mfqW	This work
ET_{12567}	Recombinant	pF_mfqWH	This work
ET_{12567}	Recombinant	pG	This work
ET_{12567}	Recombinant	pG_mfqM	This work
ET_{12567}	Recombinant	pG_mfqMH	This work

8.1.2 *Streptomyces venezuelae* T1-4D2

For this project, *Streptomyces venezuelae* T1-4D2 wild-type was used, and 6 recombinant strains were created. Wild-type *S. venezuelae* T1-4D2 cells were provided by the laboratory.

Table 4: ***Streptomyces venezuelae T1-4D spp. used in this project.***

Phenotype	**Additional info**	**Source**
Recombinant	Deletion of geosmin and 2-methyl-isoborneol (MIB) synthase	AG Zotchev
Recombinant	pF	This work
Recombinant	pF_mfqW	This work
Recombinant	pF_mfqWH	This work
Recombinant	pG	This work
Recombinant	pG_mfqM	This work
Recombinant	pG_mfqMH	This work

8.1.3 Plasmid vectors

Additional information about the genetic engineering of the plasmid vectors used is specified in *Chapter 9.6.* All plasmid vectors shown in *Table 5* were provided by the laboratory.

Table 5: ***Plasmid vectors utilized within this project.***

Vector	**Features**	**Function**
pSET152	ColE1ori, aac(3)IV, oriT, attP, int	Vector used for cloning, as a backbone vector for gene *gpps*
pUC_gpps	beta-lact, *gpps*	Vector used for cloning and carrying of gene gpps
pUC57_1631[2]	*gpps*, P21 promotor, OroIR, trps13-1, ColE1ori, aac(3)IV, oriT, attP, int	Vector used for cloning and carrying of gene *gpps*
pUC_P21_mfqM	beta-lact, *P21_mfqM*	Vector used for cloning, including the gene *mfqM*
pUC59_ermEp_mfqH	*ermEp-mfqH*, beta-lact	Vector used for cloning, including the gene *mfqH*
pUC_P21_mfqW	bla, *P21_mfqW*	Vector used for cloning, including the gene *mfqW*

[2] This gene was discovered by the team of Sergey B. Zotchev (Oberhofer et al., 2022).

Table 6: ***Recombinant vectors within this project.***

Vector	Features	Functions
pG	Int, attP, oriT, aac(3)IV, ColE1ori, *gpps*	Recombinant vector, including gene *gpps*, for expression of GPP-synthase
pG_mfqM	int, attP, oriT, aac(3)IV, ColE1ori, P21_mfqM, *gpps*	Recombinant vector, including genes *gpps*, *mfqM*, for expression of GPP-synthase and putative expression of aromatic prenyltransferase
pG_mfqMH	Int, attP, oriT, aac(3)IV, ColE1ori, ermEp-mfqH, P21_mfqM, *gpps*	Recombinant vector, including genes *gpps*, *mfqM*, and *mfqH*, for expression of GPP synthase, putative aromatic prenyltransferase, and *O*-methyl-transferase
pF	ColE1ori, aac(3)IV, oriT, attP, int, *fpps*, ermE up	Vector used for cloning, including gene *fpps*, for expression of FPP synthase
pF_mfqW	Int, attP, oriT, aac(3)IV, ColE1ori, P21_mfqW, ermE up, *fpps*	Recombinant vector, including genes *fpps* and *mfqW*, for expression of FPP-synthase and aromatic prenyltransferase
pF_mfqWH	int, attP, oriT, aac(3)IV, ColE1ori, ermEp-mfqH, P21_mfqW, ermE up, *fpps*	Recombinant vector, including genes *fpps*, *mfqW*, and *mfqH*, for expression of FPP-synthase, aromatic prenyltransferase, and *O*-methyltransferase

8.2 Media

This chapter describes the preparation of media. The laboratory provided instructions for preparation.

8.2.1 Liquid cultivation media

All liquid cultivation media were stored at room temperature for appr. 12 months. Media supplemented with antibiotics were stored at 4° C for one month at the maximum.

Luria Bertani (LB)

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
ddsH_2O	add 1000 ml

MYM

Maltose	4 g
Yeast extract	4 g
Malt extract	10 g
Tap water	add 500 mL
ddsH_2O	add 500 mL

Adjusted to pH 7,3 with 1 M NaOH stock solution before autoclaving. Addition of 2 mL/L of R2 trace elements after autoclaving. The laboratory provided an R2 trace elements solution.

2xYT

Tryptone	16 g
Yeast extract	10 g
NaCl	5 g
ddsH_2O	add 1000 mL

Tryptone soya broth (TSB)

Tryptone soy broth powder	30 g
ddsH_2O	add 1000 ml

8.2.2 Solid cultivation media

L-Agar Miller (LA)

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15 g
ddsH_2O	add 1000 mL

Antibiotic LA plates were stored at 4° C. for appr. 6 months. LA plates with antibiotics were stored at 4° C. for one month at the maximum.

Soya flour mannitol (SFM)

D-Mannitol	20 g
Soy flour	20 g
Agar	20 g
Tap water	add 1000 mL

Add 1 mL/L of 1 M $MgCl_2$ stock solution after autoclaving.

SFM plates without antibiotics were stored at room temperature for appr. one month. SFM plates containing antibiotics were stored at 4° C. for 2 months at the maximum.

8.2.3 Fermentation media

Chapters 9.4 and *9.8* describe the methods of cultivation and fermentation of the project microorganisms, which success mainly depends on the right choice of media.

All liquid fermentation media were stored at room temperature for appr. 12 months. Media that contained antibiotics were stored at 4° C. for one month at the maximum.

MYM (recipe see *Chapter 8.2.1*)

Production medium 4 (PM4)

Glucose	15 g
Soy flour	15 g
Corn steep solids	5 g
$CaCO_3$	2 g
ddsH_2O	add 1000 mL

Addition of 6 mL/L of TMS 1 (trace elements) after autoclaving. The laboratory provided TMS 1 trace elements solution.

8.3 Solutions

8.3.1 Antibiotic stock solutions

The laboratory provided all antibiotic stock solutions with various concentrations (mg/mL) listed in *Table 7*, except nalidixic acid (NaI).

Table 7: ***Stock solutions and buffers used within this master thesis project.***

Antibiotics	**Stock concentration (mg/mL)**	**Used concentration *E. coli* (µL/mL)**	**Used concentration *Streptomyces* (µL/mL)**	**Solvent**
Ampicillin (Amp)	100	100	-	ddsH_2O
Apramycin (Am)	100	100	50	ddsH_2O
Chloramphenicol (Cml)	25	25	-	EtOH abs.
Kanamycin (Kana)	25	25	-	ddsH_2O
Nalidixic acid (NaI)	30	-	30	0.1 M NaOH

8.3.2 General stock solutions, buffers, and agarose-gel solution

Since each stock solution requires a different handling, short descriptions of their preparation are already mentioned in this chapter.

20% Glycerol

Glycerol (l)	20 mL
ddsH_2O	add 80 mL

Glycerol Rotipuran® solution with a purity of ≥ 99% by Roth was filled in a graduated cylinder and diluted with ddsH_2O up to 100 mL. The mix was homogenized, transferred into a laboratory bottle, and autoclaved. 20% glycerol solution was stored at room temperature until further usage but for one month at the maximum.

80% acetone

Acetone (l)	80 mL
ddsH_2O	add 20 mL

100% EMSURE® acetone by Merck Millipore was filled in a graduated cylinder and diluted with ddsH_2O up to 100 mL. The solution was stored at room temperature until further usage.

1 M NaOH

NaOH (s)	2 g
ddsH_2O freshly autoclaved	add 50 mL

Emsure® NaOH platelets with a purity of ≥ 99% by Merck-Millipore were weighed and carefully solved in water with constant stirring. The transparent solution was stored at room temperature until further usage.

1 M $MgCl_2$

$MgCl_2$ $6H_2O$ (s)	10.17 g
ddsH_2O	add 50 mL

Solid BioReagent® $MgCl_2$ hexahydrate with a purity of ≥ 99% by Sigma Aldrich was weighed and solved in water with constant stirring. The transparent solution was autoclaved and stored at room temperature until further usage.

100 mM TRIS-HCl lysis buffer, pH 7.5

TRIS (s)	4.84 g
ddsH_2O	add 400 mL

Adjust to pH 7.5 with 1 M HCl solution before autoclaving.

TRIS Pufferan® crystals with a purity of ≥ 99.9% by Roth were weighed, transferred into a conical flask, and solved with constant stirring. PH adjustment was enabled with a WTW Inolab pH electrode. For acidification, a provided 1 M HCl solution was used. The buffer was autoclaved and then stored at room temperature for one month at the maximum.

TRIS-boric acid-EDTA (TBE) buffer

89 mM TRIS base (s)	10.8 g
89 mM boric acid	5.5 g
2 mM EDTA-Na_2	0.7 g
ddsH_2O	add 400 mL

Adjusted to pH 8.0 with 1M HCl or 1M NaOH solution. The laboratory provided the TBE buffer used in this project.

0.8% Agarose gel, stained

Agarose (s)	1.6 g
TBE puffer	add 200 mL
GelRed® nucleic acid stain (10,000x)	add 10 µL

Solid agarose BioReagent® by Sigma Aldrich was mixed with TBE. For complete solvation, the mix was heated in a microwave at low watts for appr. 10 minutes. After cooling down to ~ 50° C, GelRed® nucleic acid stain (10,000x) by Biotium was added. The mix was then poured into a gel tray or stored at 50° Celsius for 7 days at the maximum.

8.3.3 Substrates S1 to S5

Substrates were crystals, and DMSO solution was used as a solvent. Sigma Aldrich provided the substrates as well as the solvent. The chemical structures of these compounds are displayed in *Chapter 4*.

The solid substrates were weighed and transferred into sterile 2.0 mL tubes. The required volumes of DMSO for a final concentration of 0.1 M or 0.01 M were added, and the tubes were further vortexed until transparent solutions were obtained. Substrate 1, juglone, had to be additionally protected from sunlight.

0.1 M Juglone (S1)

Juglone (s)	17.4 mg
Dimethyl sulfoxide (DMSO) (l)	add 1 mL

0.1 M 2-Hydroxy-1,4-naphthoquinone (S2)

2-Hydroxy-1,4-naphthoquinone (s)	17.4 mg
DMSO (l)	add 1 mL

0.1 M 2-Methoxy-1,4-naphthoquinone (S3)

2-Methoxy-1,4-naphthoquinone (s)	18.8 mg
DMSO (l)	add 1 mL

0.1 M 1,4-Naphthoquinone (S4)

1,4-Naphthoquinone (s)	15.8 mg
DMSO (l)	add 1 mL

0.01 M Purpurin (S5)

Purpurin (s)	2.56 mg
DMSO (l)	add 1 mL

8.4 Molecular Biology Kits

The laboratory provided kits for DNA preparation, extraction or purification, and DNA gel extraction. The protocol for plasmid DNA preparation and isolation is explained in *Chapter 9.6.2*, and the protocol for plasmid DNA gel extraction is discussed in *Chapter 9.7.1*.

Table 8: ***Molecular Biology kits used within this project.***

Functions	Name	Supplier
DNA preparation and isolation	Wizard® Plus SV Minipreps DNA Purification System	Promega
DNA gel extraction	Monarch® DNA Gel Extraction Kit	New England Biolabs Inc.

9 Methods

9.1 Preparation of substrate solutions S1 to S5

All substrates, as well as the solvent, were provided by Sigma Aldrich. A dimethyl sulfoxide (DMSO) solution was used as the solvent, see *Chapter 8.3.3*. All substrate solutions were immediately applied to fermentation cultures or aliquoted and stored at minus 20° Celsius for later usage.

9.1.1 Determination of the substrates' minimal inhibitory concentration (MIC)

The determination of the MIC was done under sterile conditions. Different volume shares of *Streptomyces* spore suspensions and 0.25 M substrate S1 (Juglone) were combined and incubated in 10 mL antibiotic-free MYM medium at 28° C and 200 rpm overnight. Negative control samples that validated the medium or showed the impact of DMSO on growth were included and incubated analogously.

The experiment was repeated with different molar concentrations for all of the substrates. The final MIC range is displayed in *Table 9*.

Table 9: ***MIC range of the project's substrates.***

Substrate	**Range MIC (mM/mL)**
S1 (Juglone)	0.25 - 1.25
S2 (2-Hydroxy-1,4-naphthoquinone)	1.00 - 5.00
S3 (2-Methoxy-1,4-naphthoquinone)	0.10 - 0.50
S4 (1,4-Naphthoquinone)	0.50 - 2.50
S5 (Purpurin)	0.01 - 0.05

9.2 Preparation of media

All media were prepared under S1 laboratory conditions. The laboratory provided the required instructions and recipes. The media recipes and storage proposals are discussed in *Chapter 8.2*.

All glassware was checked for residues before usage, and working instruments such as spatulas or stirring bars were cleaned with ddH_20 and EtOH 70%.

Half of the required solvent, mainly $ddsH_2O$, was poured into a conical flask containing a magnetic stirring bar. All solids were weighed precisely with an analytical balance and using spatulas and disposable weighing boats. Agar was considered separately. Solids were steadily added into the stirring water, agar at last.

Suspensions without agar, soy flour, or steep corn solids were stirred until clear solutions were obtained. Agar, SFM, or PM4 media were mixed until homogenization.

After this step, the other half of the solvent was mixed in for 3 to 4 minutes. The media were transferred into 1 L laboratory bottles to a maximum filling of 350 mL. The bottles were autoclaved at 121° C. for at least 20 minutes to ensure proper sterilization and stored afterward.

For media such as MYM, a defined pH was obligatory. PH adjustment was made before autoclaving, using the INOLAB WTW pH electrode and 1 M NaOH or 1 M HCl solutions.

Some media also requested additional salt- or puffer solutions added to the autoclaved and cooled-down bottle. These compounds were added under sterile conditions.

9.3 Preparation of plates

Solid media were prepared in Petri dishes under sterile conditions. The required media were prepared as described in *Chapter 8.2.2*. If the addition of antibiotics was required, the agar-containing media before supplementation with antibiotics were cooled down to approximately 50 °C.

Antibiotic-free plates were stored at room temperature, and antibiotics-containing plates were stored at 4° Celsius.

9.4 Cultivation of *Streptomyces venezuelae* T1-4D2 wild-type

Parallel to the liquid culture, SFM-$MgCl_2$ plates were inoculated with *Streptomyces* cells. À 100 µL of the *S. venezuelae* T1-4D2 lab sample were pipetted on two plates and spread evenly with a spatula. The plates were additionally dried for 20 to 40 minutes in the LAF. The dried plates were incubated at 28° C. and checked for growth daily. After 10 days, the surface of the plate was covered entirely with white-greyish *Streptomyces* mycelium and thus ready for further processing.

9.4.1 Preparation of cryo samples

After incubation and checked positively for growth, the amount of the liquid culture was divided equally into sterile 1.5 mL tubes in the LAF to a final volume of appr. 1 mL/tube. To separate the cells from other medium components, the tubes were centrifuged in a Minispin® centrifuge by Eppendorf at max. rpm (equals 13.400 rpm) for 5 minutes at room temperature. The supernatant was discarded entirely, and the pellet was carefully homogenized in 1 mL 20% glycerol solution. The cell-glycerol mix was transferred into cryotubes and stored until further usage at minus 80° Celsius.

9.4.2 Preparation of a *Streptomyces* spore suspension

Plates that showed a white-greyish surface indicated proper growth of *Streptomyces* and its mycelium. Such plates were further processed under sterile conditions in the LAF. Sterilized polystyrene syringes with cotton filters and cryotubes were prepared and held in the LAF.

5 mL of a 20% glycerol solution were carefully pipetted on the plate; the mycelium was scraped away with the pipette tip. The suspension was aspirated with the pipette, transferred into the prepared syringes, and filtered into cryotubes. The cryotubes were then stored at minus 80° C. until further usage.

9.5 Cultivation of recombinant *E. coli* on plates

Solid, agar, and antibiotics containing LA plates were coated under sterile conditions in the LAF. Plates, which were stored in the fridge, had to warm up to room temperature beforehand. For that, the Petri dishes were opened in the LAF and left there for appr. 30 minutes.

Depending on the coating volume, the liquid was spread with a spatula or an inoculation loop on the agar surface. Both techniques required a complete coverage of the plate surface for proper growth of the microorganisms. Before further incubation, the coated plates were dried additionally in the LAF for 20 to 40 minutes.

9.6 Genetic engineering of plasmid DNA

9.6.1 Transformation

Bacterial transformation is a process in which bacteria can take up DNA, mainly plasmid DNA. In this project, the transformation of plasmid DNA was realized with the heat shock method and later with conjugation.

The transformation of plasmid DNA with the heat shock method was performed with different *Escherichia coli* strains. The laboratory provided tubes containing à 100 µL of competent *E. coli* XL1-Blue MR, DH5α, or ET_{12567} cells and the transformation protocol.

The frozen tubes were thawed on wet ice before adding 1 µL of plasmid DNA. The mix was then kept on wet ice for 30 minutes and later transferred into a heating block at 42° C. for 45 seconds. After this heat shock, the mixture was quickly put into wet ice again and cooled down for 5 minutes. The chilled mixture was diluted with 900 µL of LB medium and incubated in a water bath by ARGOlab at 37° C. for 1 hour.

After incubation, prepared agar plates were coated with 100 to 150 µL of this sample and dried in the LAF. The plates were incubated at 37° C. overnight, followed by amplifying liquid cultivation and subsequent DNA preparation. The transformation method “conjugation” is described in *Chapter 9.7*.

9.6.2 Plasmid DNA preparation and isolation

DNA isolation and preparation, respectively, were processed with the Wizard® Plus SV Minipreps DNA purification kit by Promega, according to the manufacturer`s protocol.

9.6.3 Restriction

After amplification in liquid media and DNA preparation, the DNA plasmids had to be cut with restriction enzymes at priorly chosen restriction sites, creating compatible ligation fragments.

Table 10: ***Recipe for preparative restriction solution used in this project.***

Compound	**Volume [µL]**
DNA	25
Buffer, rCutSmart® (10x)	5
Enzyme 1	1
Enzyme 2	1
ddsH_2O, freshly prepared	18

The working volume was 50 µL in total. However, the recipe was adjusted within this project, especially if the DNA had to be higher concentrated or if the restriction was done analytically. For that, the volume of water was reduced, and the buffer was adjusted to the final working volume. While processing the restriction solution, all compounds had to be thawed and stored on wet ice.

Freshly prepared sterile water was transferred into a sterile 1.5 mL tube, followed by the rCutSmart® buffer provided by Biolabs. DNA was carefully pipetted into the mixture, the enzymes at last.

The enzymes were mainly supplied by Biolabs and labeled as high-fidelity (HF) restriction enzymes. In general, HF enzymes provide the same specificity as native enzymes but were initially engineered for significantly improved selectivity and performance in a single buffer.

The Biolabs vials were vortexed beforehand to ensure a complete homogenization of the provided enzyme solution. In addition, the enzymes were pipetted directly into the restriction mix and slowly pipetted up and down 2 - 3 times.

The final mix was vortexed and spun down shortly before incubation. The tubes were incubated in a 37° C. water bath for 1.5 hours. After this time, the samples were frozen and stored at minus 20° Celsius or processed promptly.

9.6.4 Ligation

To perform ligation, two DNA fragments were generated using restriction enzymes that cut the DNA at specific sites to create compatible, joined ends. In this project, the ends were sticky, which facilitated DNA cloning and improved the efficiency of DNA ligation.

T4 DNA Ligase and T4 Buffer by Biolabs were used as DNA ligase compounds.

Table 11: ***Ligation recipe used in this project.***

Compounds	**Volume [µL]**
Vector DNA (vDNA)0	1
Insert DNA (iDNA)	7
T4 Ligase Reaction Buffer	2
T4 DNA Ligase	1
ddsH_2O, freshly prepared	9

The working volume was 20 µL in total. All compounds for this solution had to be chilled on wet ice during the preparation and thawed before, respectively.

Freshly prepared sterile water was transferred into a sterile 1.5 mL tube, followed by the T4 buffer and the vector DNA- and insert DNA fragments (vDNA, iDNA). The tube was vortexed shortly before adding the T4 DNA Ligase.

The final mixture was again vortexed and incubated at room temperature for 1 hour. After that step, the ligated DNA was transformed into *E. coli* cells and plated.

9.7 Separation and analysis of plasmid DNA

Separation and analysis of previously performed restriction and ligation experiments were performed with a horizontal gel electrophoresis system. The laboratory provided all electrophoresis devices and their equipment. The agarose gel was handcrafted, as described in *Chapter 8.3.2*.

Before filling the gel tray, a gel comb had to be inserted. Enough conditions for forming a homogeneous porous gel membrane were achieved by leveling the gel caster and avoiding air bubbles while pouring the agarose solution into the tray. The hot agarose solution was slowly but consistently poured into the gel tray up to a height of ~ 5 mm. After cooling down for 35 to 45 minutes (depending on the size of the gel tray), the comb was removed, and the tray was transferred into the wet gel electrophoresis chamber. Before, all gel pockets needed to be filled with TBE buffer; thus, the filling level of the gel chamber had to be checked beforehand. Depending on the size of DNA fragments and the gel itself, gel electrophoresis was done at 80 to 100 Volts with a running time of at least 30 minutes.

Bio-Rad supplied the gel electrophoresis equipment used in this project. The gel loading process involved applying samples and a standard, such as 1 kb DNA ladder® by New England Biolabs Inc., onto a 0.8% agarose gel matrix. This matrix acted as a porous separating medium for the various-sized DNA fragments. The electrophoresis chamber was filled with TBE as a running buffer. An electric field was applied across the gel matrix to force the charged DNA fragments toward the oppositely charged electrode. The separation of the DNA fragments was visualized by adding the Gel Loading Dye Blue® (6x) to the sample. New England Biolabs Inc provided this stain.

The gel matrix was pictured with a Bio-Rad ChemiDoc® imaging system and discarded afterward. By illuminating the gel with ultraviolet (UV) light, the DNA bands stained by GelRed® could be observed and subsequently extracted.

9.7.1 Plasmid DNA gel extraction

DNA gel extraction was done with the Monarch® DNA Gel Extraction Kit, which also provided a protocol. After UV-imaging and cutting the desired bands out of the gel, the gel slices were transferred into 1.5 mL tubes and weighed.

The protocol involved dissolving the gel, washing, and eluting the DNA. As a DNA elution buffer, 15 µL of freshly prepared ddsH_2O were used. The samples were further processed or stored at minus 20° Celsius until further usage.

9.7.2 Sequencing

Sequencing was used to validate the construction of the plasmid vectors pF_mfqWH and pSET152_gpps and the correct DNA sequence of pUC_1631. This method was planned with the Clone Manager 11® software by Sci Ed.

Table 12: ***Recipe for sequencing plasmid DNA in this project.***

Compounds	**Volume [µL]**
DNA	12
Forward primer (fwd)	3
Reverse primer (rev)	3

The primers pSET_fwd1 and pSET_rev1 were provided by the laboratory and supplied by Eurofins Genomics. Due to their high concentration, they had to be diluted to 1:10 with freshly prepared ddsH_2O. À 3 µL of diluted primer was mixed with the DNA sample, labeled, and mailed to Eurofins Genomics, which did the sequencing.

9.7 Conjugation

Conjugation is another mechanism of bacterial transformation in which genetic material is transferred from one bacterial cell to another. This process is mediated by plasmids, small circular DNA molecules that can replicate independently of the bacterial chromosome.

Several plasmid modifications were performed in the laboratory and cloned into *E. coli* ET_{12567} and further conjugated with *Streptomyces venezuelae* T1-4D2. The laboratory also provided the conjugation protocol and most of the selection antibiotics.

NaI stock solution

NaI (s)	150 mg
0.1 M NaOH solution	add 5 mL

Adjustment/Solvation of precipitation with 1 M NaOH stock solution.

All antibiotics were sterile filtrated (0.2 µm sterile membrane filter), divided into aliquots, and stored at minus 20° C. until further usage.

The transformed ET_{12567} cells were incubated overnight on solid LA-AM plates at 37°C. New LA plates with three antibiotics, Kanamycin (Kana), Chloramphenicol (Cml), and Apramycin (AM), were prepared, and 4 sectors were drawn on their lids. Four colonies of E. coli ET_{12567} transformants were spread densely on each quarter and incubated at 37° C. overnight.

Grown cell colonies were then swiped from the plate with an inoculation loop and separately transferred into 1.5 mL tubes containing 500 µL of 2xYT medium. In addition, 50 µL of *Streptomyces* spores suspension was mixed with 350 µL of 2xYT medium and incubated at 50° Celsius for 7 minutes. 400 µL of the chilled spores medium was combined with 100 µL of the ET_{12567} cell mix. The mix was homogenized and spun down for 1 minute at 5000 rpm.

250 to 350 µL of supernatant were discarded, and the rest of the amount, including the pellet, were mixed and spread on antibiotic-free SFM $MgCl_2$ plates. The plates were incubated at 28° C. for 18 to 20 hours at the maximum.

Once signs of growth occurred (plate surface color changed to a slightly grey-white), each plate was coated with a mix containing 1 mL of freshly prepared ddsH_2O and 30 µL nalidixic acid (NaI) antibiotic. The mixture was evenly but carefully spread on the plate and dried thoroughly in the LAF for 40 minutes at the minimum.

After 3 -4 days of incubation at 28° C., grown colonies were obtained. They were scattered into 3 strokes with a sterile toothpick and further incubated at 28° Celsius. Colonies of each stroke were later picked up with an inoculation needle and separately plated on SFM AM/NaI plates. The plates were again incubated at 28° C. until notable growth was notable.

These colonies were then transferred on new SFM AM plates, incubated, and their grown mycelium was subsequently harvested as a spore suspension. The samples were stored at minus 80° Celsius.

9.8 Fermentation

Depending on the extraction protocol, the flasks were filled with various media to 10 mL or 25 mL and spiked with antibiotics. Several volumes of *Streptomyces* spore suspensions were added during this project and incubated. If growth-positive, 10% of these cultures were transferred into fresh liquid media and further incubated. Fermentation experiments mainly include the addition of substrates, which were incubated for 1 or 2 days, depending on the protocol and the growth. DMSO was implemented as a control sample. The flasks were incubated at 28° C., 200 rpm for 1 day at the minimum, and subsequently extracted.

9.9 Extraction methods

9.9.1 Ethyl acetate and acetone extraction

Ethyl acetate and acetone extraction methods were processed with a *Streptomyces venezuelae* T1-4D2 wild-type spore suspension, which was fermented in antibiotic-free MYM medium containing à 20 µL of 0.25 M substrate S1, 0.10 M S3, 0.50 M S4, and 100 µL of 0.01 M S5. Glycerol stocks of the transformants pF, pF_mfqW, and pF_mfqWH were prepared analogously.

The incubated substrate-cell suspensions were then poured into Falcon® tubes and centrifuged at room temperature, 4000 x G, for 30 minutes. 5 mL of the supernatant were transferred into new tubes. The rest was stored at minus 20° C until further use. The supernatant aliquot was diluted with 5 mL of EMSURE® ethyl acetate provided by Merck Millipore and shaken manually.

The pellet was eluted in 5 mL 80% acetone and vortexed till complete solvation. The samples were centrifugated at room temperature, 4000 x G, for minutes. The supernatant was saved into new Falcon® tubes; the pellet was discarded.

Acetone and ethyl acetate extracts were stored at 4° C. until further processing.

1 mL of each acetone extract and 1 mL of the ethyl acetate phase were transferred into 1.5 mL tubes. The tubes were evaporated in the Concentrator plus speed-vac® by Eppendorf at 45° Celsius. Ethyl acetate samples were dried after 35 minutes, and 80% acetone samples after more than 3 hours due to their water content.

All pellets were resolved in 100 µL of MeOH, vortexed, or sonicated till complete solvation. The tubes were centrifugated at max. rpm for 10 minutes, 60 µL of the supernatant were transferred into HPLC vials.

9.9.2 MeOH extractions with substrates S1 to S5

MeOH extraction methods were processed with a *Streptomyces venezuelae* T1-4D2 wild-type spore suspension, which was fermented in antibiotic-free MYM medium containing 20 µL of the substrates S1, S2, S3, S4 (0.10 M) and 100 µL of S5 (0.01 M). pF-transformants were processed analogously, using AM (50 µL/mL) containing MYM medium.

Liquid cultures were poured into Falcon® tubes, snap-frozen in liquid nitrogen, and stored at minus 80° Celsius. The frozen tubes were lyophilized with the VaCO 5-II® freeze dryer by Mitsubishi.

The dried pellets were then solved in à 15 mL MeOH. The tubes were placed in the shaker at 28° C., 200 rpm for 1 hour, filtered in round bottom flasks, and evaporated till dryness. Evaporation was done with a hei-VAP® rotary evaporator by Heidolph. Dried samples were eluted in 1 mL MeOH and transferred into 1.5 mL tubes. They were centrifugated at room temperature at max. rpm for 8 minutes before HPLC analysis.

9.9.3 MeOH extraction without substrates

As a control, MeOH extractions were also performed without substrates. For that, samples of *Streptomyces venezuelae* T1-4D2 and pG-transformants were fermented in TSB media containing Apramycin (AM, 50 µL/mL). After incubation, the samples were in a chilled EtOH bath before lyophilization. Dried pellets were eluted in 25 mL MeOH and shaken at room temperature, 150 rpm, for 3 hours. The extracts were filtered and rotavaped till complete dryness. Samples were then eluted with 2.5 mL MeOH each and sonicated shortly. 2 mL were transferred into tubes and centrifugated at room temperature, max. rpm for 10 minutes before HPLC analysis.

9.9.4 100 mM TRIS-HCl lysis

Streptomyces venezuelae T1-4D2 spore suspension was used and fermented in an antibiotic-free TSB medium for the TRIS-HCl lysis method. The liquid cultures were poured into Falcon® tubes and centrifugated at 4° C., 4000 x G for 30 minutes. The supernatant was discarded, and the pellet was stored at minus 20 °Celsius until further usage.

The Lysis method was also performed with pG-transformant spore suspensions, which were fermented in TSB and 2xYT media containing AM (50 µL/mL). After incubation and validation of the growth, the TSB samples were discarded. The 2xYT samples were poured into flacon tubes and centrifugated at 4° C., 4000 x G for 30 minutes. Supernatants were discarded, and pellets were stored at minus 20° Celsius until further use.

All pellets were processed analogously. They were thawed on wet ice and solved in à 5 mL of 100 mM Tris-HCl buffer at a pH of 7.5. To complete cell lysis, the suspensions were sonicated at 4° C., high mode, at 40% amplitude for 10 cycles of 30 sec. on and 30 sec. off with a Bioruptor Plus® sonicator by Diogenode.

Cell debris was pelleted by centrifugation at 4° C., max. rpm for 30 minutes. 400 µL lysate were transferred into 1.5 mL tubes, and volumes of à 0.5 µL, 1 µL, 5 µL and 10 µL of the substrate S2 0.10 M and S5 0.01 M were added.

The samples were vortexed shortly and incubated in an AccuBlock® by Labnet at 30° C. for 1 hour. After this step, the samples were snap-frozen and subsequently freeze-dried. The dried pellets were eluted in 200 µL MeOH each, vortexed shortly, and centrifugated at room temperature at max. rpm for 8 minutes. The final samples were frozen at minus 20° Celsius.

Glycerol stocks of pF-transformants were inoculated onto SMF plates containing AM (50 µL/mL). The plates were incubated at 28° C. for 4 days. Cell swipes of colonies were then transferred into TSB containing AM and fermented. Liquid cultures were processed as described above. The freeze-dried pellets were eluted in 200 µL MeOH each, vortexed shortly, and centrifugated at room temperature at max. rpm for 8 minutes before further HPLC analysis.

9.10 Analytical analysis of metabolites

9.10.1 Analytical HPLC

Analytical High-Performance Liquid Chromatography (HPLC) separates, identifies, and quantifies specific metabolites in a complex liquid mixture. The fermentation extracts were analyzed by a Shimadzu HPLC unit. Depending on the extraction protocol, the columns Luna® C18 5 µm (250 x 4.6 mm) by Phenomenex and Acclaim® 120 C18 3 µm (2.1 x 150 mm) by Thermo Scientific were used as lipophilic stationary phases. The mobile phase was set at a 1 mL/min flow rate and consisted of two solvents. Solvent A was a solution of 0.1% formic acid in ddH_2O and solvent B, 100% acetonitrile (ACN).

Regardless of the extraction protocol, the project samples were diluted in MeOH and transferred into HPLC glass vials to a maximum volume of 160 µL. The vials were capped and placed in an autosampler, including a pure MeOH sample, for washing and finishing the sample run *Table 13.*

Table 13: ***HPLC methods, columns, and gradients used in this project****.*

Methods	**Column**	**Elution gradients**	**Used for**
min flow_short_40min	Acclaim® 120 C18 3 µm (2,1 x 150 mm), Thermo Scientific	0.00 – 31.00 minutes 5% sol. A / 95% sol. B 31.01 – 40.00 minutes 95% sol. A / 5% sol. B	TRIS HCl-lysis MeOH extractions
95_65min_Flow 1min	Luna® C18 5 µm (250 x 4,6 mm), Phenomenex	0.00 – 55.00 minutes 5% sol. A / 95% sol. B 55.01 – 65.00 minutes 95% sol. A / 5% sol. B	Ethyl acetate & acetone extractions MeOH extractions MIC of substrates

9.10.2 Analyzed samples

The methods used are mentioned in *Table 13*. Selected samples were analyzed with LC-mass spectrometry (LC-MS). Martin Zehl and his team from the Department of Analytical Chemistry, Faculty of Chemistry at the University of Vienna, did the LC-MS analysis.

10 References

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