Development of advanced genome-editing tools for basidiomycetous yeasts and engineering the biosynthetic pathway of mannosylerythritol lipids in *Moesziomyces antarcticus*

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Abstract

High demand of green and sustainable way for producing chemicals lead to usage of microorganisms for bio-based production of various types of products including biosurfactants. One of the most promising biosurfactants are mannosylerythritol lipids, which have been gaining a huge interest due to its high yield and mild production, various application. Non-conventional dimorphic yeast *Moesziomyces antarcticus* was used as a host organism for genetic manipulations and production of MELs. The MELs, excreted by *M. antarcticus* consist of three main forms of MEL-A, MEL-B and MEL-C and only very low amount of non-acetylated MEL-D is being produced. For homogenous production of MEL-D, knock-out of the *MAT1* gene, acetyltransferase responsible for acetylation of MEL-D was attempted. Different gene engineering techniques were applied for disruption of *MAT1* gene such as gene replacement with homologous recombination using a PCR amplified fragment with upstream and downstream flanking regions of *MAT1* embedding *NatMX4* selection cassette; split-marker approach using truncated fragments with homologous regions for recombination within the *NatMX4* cassette; RNP mediation for better gene targeting and increasement of homologous recombination by supplying with dDNA. Transformation with PCR amplified NatMX_MaMat1 fragment resulted 2-fold lower efficiency of *NatMX4* cassette insertion into the chromosome compared to split-marker with 70 bp overlap. RNP mediated transformation with 100x dilution of RNP obtained couple of mutants. However, *MAT1* gene was not successfully targeted by any of the cases resulting in random event integration and inability to disrupt the gene of interest. Construction of 231 bp overlap for a split-marker approach resulted in slight increase of transformation efficiency. Optimization of transformation was performed and pre-treatment of the cells before electroporation transformation increased the efficiency by 3-folds. Two transformation techniques were applied, where electroporation resulted in 26% higher transformation efficiency compared with PEG/LiAc/ssDNA mediated transformation and was used for further experiments with RNP.

Keywords: gene targeting, gene replacement, split-marker, homologous recombination, ribonucleoprotein, CRISPR/Cas9, *Moesziomyces antarcticus*, transformation.

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Abbreviations

BS	Biosurfactant
clonNAT	nourseothricin antibiotic
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
dDNA	donor DNA
DSB	double-strand break of DNA
gDNA	genomic DNA
HR or HDR	homologous recombination or homologous direct repair
LiAc	lithium acetate
MEL	mannosylerythirotl lipid
natMX4	selectable marker conferring nourseothricin resistance
NHEJ	non-homologous end joining
OD	optical density
PCR	polymerase chain reaction
PEG	polyethylene glycol
RNP	ribonucleoprotein which consists of CRISPR RNA-Cas9 protein complex
sgRNA	single guide RNA
ssDNA	single stranded DNA (salmon sperm DNA)
tracrRNA	trnas-activating crRNA

1. Introduction

Surfactants are one of the most essential and versatile products of the chemical industry. It has widespread applications in every industrial area fluctuating from household detergents to drilling muds and food items to pharmaceuticals (Kumar et al., 2013).

In general, surfactants can be described as compounds lowering the surface tension or interfacial tension between two liquids, a gas and a liquid or between liquid and solid. Structural composition of all surfactants can vary significantly but all of them have common amphipathic nature generated by hydrophobic and hydrophilic groups within the same particle. These two chemical groups make biosurfactants soluble in both aqueous and organic solvents. These features describe them as useful compounds in wide variety of applications for its solubility and ability to decrease surface tension (Rosen & Kunjappu, 2012).

As surfactants have a huge demand worldwide, it was estimated that the global market of surfactants reached USD 30.64 billion in 2016 and was predicted to grow until USD 39.86 billion by 2021. Due to a huge need of greener, more sustainable products, biosurfactants form a major share of the surfactant market with 344,068.40 tons in 2013 and is expected to reach 4,61,991.67 tons by 2020. Revenue of biosurfactants generated over USD 1.8 billion in 2016 and expected to reach USD 2.6 billion by 2023 while others estimated to be over USD 5.52 billion by 2022 (Singha et al., 2018). Large concern about surfactant toxicity has increased a worldwide alert followed by various regulations. The cycle of surfactant toxicity starts from its synthesis, disposal and subsequent exposure to the environment causing global warming, climate change, ozone layer depletion and greenhouse gas emission. Increasing awareness resulted in production of green surfactants from renewable sources and by microorganisms resulting so called environmentally and ecologically friendly biosurfactants as an alternative for petrochemical surfactants (Rebello et al., 2014). For more efficient production of biosurfactant, advanced gene engineering techniques can be applied resulting in production of desired product and higher yield.

1.1. Biosurfactants

Most of the currently produced surfactants are chemically obtained from petroleum and its synthesis requires toxic agents that are troublesome to break down by the organisms. This led to the research for more environmentally friendly surfactants which can be accomplished by microbial production of biosurfactants (BSs). These developments of naturally produced surfactants can be used as an alternative to existing products (Santos et al., 2016). Biosurfactants express higher preference over chemical surfactants due to lower toxicity, greater biodegradability, performance at extreme temperature or pH values, biocompatibility and digestibility (Shiomi, 2015).

Biosurfactants are defined as the surface-active biomolecules which are produced by microorganisms and used for various applications. Significant interest in biosurfactants was attracted due to its unique properties as specificity, low toxicity and relative ease of preparation with mild conditions. Due to the unique functional properties of biosurfactants, it was used in many industries including petroleum, petrochemical, mining, metallurgy (bioleaching), agrochemicals, fertilizers, foods, beverages, cosmetics, pharmaceuticals. Biosurfactants itself can be used as emulsifiers/demulsifiers, wetting, foaming, spreading agents, functional food ingredients and detergents (Vijayakumar & Saravanan, 2015).

Great biosurfactant producers are bacteria of the genera *Pseudomonas* and *Bacillus*, however, most of the biosurfactants produced by bacteria consider to be inadequate for usage in the food industry due to their possible pathogenic origin. The key of using yeast as a host producer such as *Yarrowia lipolitica*, *Saccharomyces cerevisiae*, *Kluyveromyces lactis and Moesziomyces antarcticus* is because of their GRAS (generally regarded as safe) status, meaning no risk of inducing toxicity or pathogenic reactions and ability to be used in the food and pharmaceutical industries. Yeast is also known for their higher biosurfactant productivity compared to bacteria (Shiomi, 2015; Santos et al., 2016).

Biosurfactants are amphiphilic compounds possessing both a polar (hydrophilic) moiety, which consists of mono-, oligo- or polysaccharides, peptides or proteins and non-polar (hydrophobic) group, build from saturated, unsaturated and hydroxylated fatty acids or fatty

alcohols. BSs are classified corresponding to their microbial origin and chemical structure, and can be divided into main classes (Pacwa-Płociniczak et al., 2011):

- **Glycolipids** (rhamnolipids, trehalolipids, sophorolipids, mannosylerythritol lipids)
- **Phospholipids**, **fatty acids and neutral lipids** (corynomycolic acid, spiculisporic acid, phosphatidylethanolamine)
- **Polymeric biosurfactants** (emulsan, alasan, biodispersan, liposan, mannoprotein)
- **Lipopeptides** (surfactin, lichenysin)

1.2. Mannosylerythritol lipids

Mannosylerythirotl lipids (MELs) belong to the group of biosurfactants, produced by living cells, for instance, microorganisms, plants, animal cells and others. MELs are amphilic structures which are possessing both lipolytic/hydrophobic (non-soluble) and hydrophilic (soluble) properties or in other words consist of polar and non-polar moieties. Biosurfactants include other well-known lipids such as rhamnolipids, surfactin, sophorolipids nonetheless mannosylerythirotl lipids have been getting progressively more attention due to its versatile forms, applications and interesting characteristics such as high biodegradability, low toxicity, effectiveness at extreme temperature or pH and mild production conditions compared with chemical surfactants (Andrade et al., 2017; Konishi et al., 2018; Goossens et al., 2016; Faria et al., 2014). MELs are usually produced by several smut fungi of the *Ustilaginaceae* family (*Ustilago*, *Pseudozyma* and *Moesziomyces*). Product diversity depends on different type of yeast and carbon source. It has been identified that first-known MEL producer *M. antarcticus* is able to yield mainly MEL-A along with smaller quantities of MEL-B and MEL-C as a major secondary metabolite with a high production while *Ustilago* spp. (*Ustilago maydis*) compose MELs as a minor together with cellobiose lipids (Morita et al.,2015). *M. antarcticus* secrets MELs when being grown on water-soluble or/and insoluble substrates such as glucose and rapeseed oil. Mild production conditions, multipurpose biochemical functions, environmental compatibility and structural biodiversity provides a huge interest in industrial production of mannosylerythirotl lipids.

MELs contain of 4-O-β-D mannopyranosyl-meso-erythritol as the part of hydrophilic group and a fatty acid or/and acetyl groups as the hydrophobic element and can be classified due to the degree of acetylation at C-4 and/or at C-6 position of the mannosyl moiety: C-4 and C-6 of the mannosyl unit can be di-acetylated (MEL-A), mono-acetylated (in C-4: MEL-C; in C-6: MEL-B) or non-acetylated (MEL-D)(Figure 1). The number of acetyl groups and the length of the acyl carbon chains produced during metabolism depend on the species of fungus and type of carbon source due to that there are about five more different structures (Morita et al., 2015). As it was mentioned above, *M. antarcticus* is able to produce a mix of three major MELs: mostly MEL-A and smaller quantities of MEL-B and MEL-C (Yu et al., 2015). Those three types of MELs can be separated from microbial metabolites, although, MEL-D could be synthesized via the lipase-catalyzed hydrolysis of MEL-B. Production of MELs is not growth associated, therefore it can be produced during the stationary phase of growth by using a batch/fed-batch reactor to achieve higher yields of the desired product.

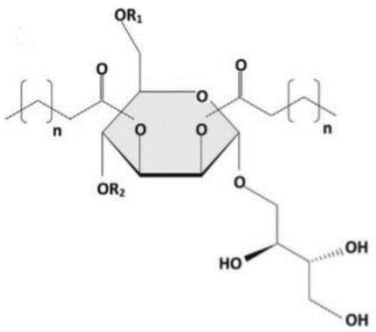

Figure 1: MEL structure (MEL-A: R1 = R2 = Ac; MEL-B: R1 = Ac, R2 = H; MEL-C: R1 = H, R2 = Ac: n = 4–16) (Faria et al. 2014)

1.2.1. Biosynthetic pathway of MELs

As it was described above, MELs can be produced from different type of feedstocks (sugars, various vegetable oils) by various fungi from *Ustilago* and *Pseudozyma* (*Moesziomyces*) genera. Combinations of selective substrates (soybean oil, olive oil, rapeseed oil) and nutrients (sodium nitrate) could serve as the mixture for a high production of MELs specific for selected species (Yu et al., 2015). All production of MELs lead to biosynthesis background of its formation. *U. maydis* yeast was used as a model organism for genetic studies and identification of gene cluster for MEL biosynthesis expressing under nitrogen starvation (Morita et al., 2014). By using the database of *U. maydis* as a reference, many different protein-coding genes were predicted and found to be homologous to proteins in *M. antarcticus* type strain. The five genes responsible for encoding erythritol/mannose transferase (*EMT1*), acyl transferase (*MAC1* and *MAC2*), putative transporter (*MMF1*) and acetyl transferase (*MAT1*), which are responsible for MEL biosynthesis, are located on scaffold 31 (Saika et al., 2014). Work of these five proteins in MEL biosynthetic pathway can

be divided into three steps processes. First, the production route of extracellular mannosylerythritol lipids is initiated by EMT1 enzyme which catalyzes the synthesis on mannosyl-D-erythritol by transfer of GDP-mannose. After, MAC1 and MAC2 are initiated to transfer short- and medium-chain fatty acids to positions C-2 and C-3 of mannose. The last step is acetylation of deacetylated MEL at position C-4 and C-6 catalyzed by a single enzyme MAT1 (Yu et al., 2015). It encodes an acetylCoA-dependent acetyl-transferase. *MAT1* gene performs independent regioselectivity and is able to acetylate mannosylerythritol at both the C-4 and C6 hydroxyl groups, as a result, possibility to produce at least three main monnosylerythritol lipids: MEL-A, MEL-B and MEL-C (Figure 2).

In this study, several different genetic engineering techniques are used for targeting *MAT1* gene in order to stop acetylation step of MELs and eliminate further production of MEL-A, MEL-B and MEL-C. Disruption of *MAT1* should result in accumulation of MEL-D lipid which cannot be naturally produced by any organism in a high amounts and requires synthesis from MEL-B by enzymatic reactions.

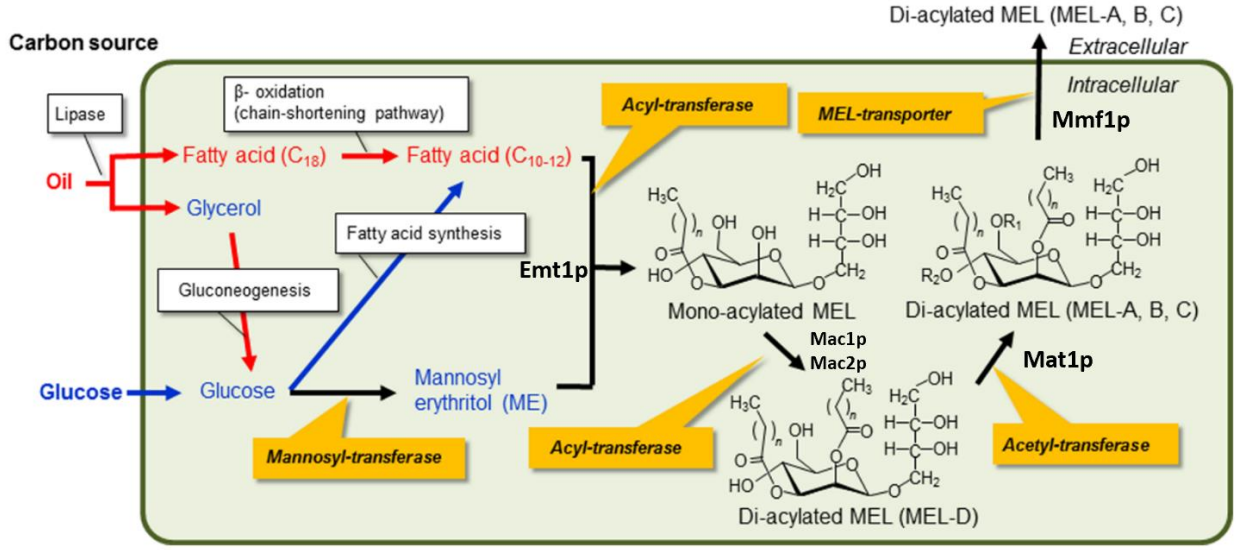

Figure 2: Biosynthetic pathway of mannosylerythritol lipids (MELs) production (Morita et al., 2014)

1.2.2. Applications of MELs

Mannosylerythritol lipids are functional compounds which gain significant attention due to its pharmaceutical and versatile biochemical activities including differentiation inducing

activities against human leukemia cells, rat pheochromocytoma cells and mouse melanoma cells. MELs demonstrate high binding affinity towards different immunoglobulins and lectins, as well as self-assembling properties lead to increase of interest due to its efficiency in gene transfection and drug delivery. Furthermore, gene transfection could be dramatically increased by one of the major components of MELs, MEL-A, mediated by cationic liposomes (Arutchelvi et al., 2008; Faria et al., 2014a; Morita et al., 2009). Some MELs exhibit anti-inflammatory characteristics as inhibition of the secretion of inflammatory mediators from mast cells (Morita et al., 2015).

Moreover, significant biochemical and physicochemical properties of MELs lead to lowering toxicity, increasing biodegradability, biocompatibility and surface activity. MEL-A and MEL-B present tremendous antibacterial activities against Gram-positive bacteria and low activities against Gram-negative bacteria leading to the smaller values of minimal inhibitory concentrations (MIC) towards Gram-positive bacteria rather than from other glycolipid biosurfactants such as sorbitan monolaurate (Span 20), rhamnolipids (RLs) and sucrose monocaprate (SE 10) (Nashida et al. 2018) . This antibacterial property leads to possibility of creating novel antibiotics with improved inhibitory abilities and could be used as a food preservative.

The aim in this study is engineering of MELs biosynthetic pathway which should lead to the production of MEL-D lipid. MEL-D, non-acetylated MEL, does not possess any acetyl group and instead retains hydrogen group which allows higher solubility, wettability and be applied as detergent (Morita et al., 2015). It is been reported that the only non-acetylated MEL homologue, MEL-D, and probably the only biosurfactant which is able to form reverse vesicles from a single component in wide variety of organic solvents without any assistance from co-solvents and co-surfactants due to its fine balance between hydrophobic and hydrophilic domains. The potential of reverse vesicles lies in their ability to encapsulate molecules and materials such as enzymes and inorganic ions, providing ideal micro and nano reaction centers for biological tests and synthesis of inorganic materials (Fukuoka et al., 2012).

Its mild production conditions, biodegradability, low toxicity and possibility of various functions brings MELs as a potential chemical in environmental field. MELs expose excellent interfacial properties, high enhancement in biodegradation and solubilization towards low solubility compounds such as petroleum (Yu et al., 2015).

Recently, studies revealed that *P. antarctica* strain, isolated from a plant surface, is able to produce extracellularly an outstanding biodegradable plastic-degrading enzyme, capable of degrading poly-butylane succinate and poly-butylane succinate-co-adipate (Kitamoto et al., 2011).

1.3. MEL producing strains

Organisms, producing mannosylerythritol lipids have been classified as Ustilagomycetous anamorphic yeasts which contains genus *Pseudozymyma* and *Ustilago*, including a smut fungus *Ustilago maydis.* In total, more than fifteen MEL-producing fungi, belonging to a variety of *Pseudozyma* yeast, were isolated from different types of vegetables and fruits. Taxonomy of MEL producing species can be strongly associated with their main products (Morita et al.,2015). For example, genus *Pseudozyma*, including *P. rugulosa*, *P. aphidis, P. Antarctica, P. parantarctica* belongs to a taxonomic branch of high producers of MEL-A as a main product, while *U. maydis* fungus is producing MELs as a minor product togethers with cellobiose lipids (CLs) (Morita et al., 2015; Faria et al., 2014a).

The first known MEL producer was identified and extracted over two decades ago from the bottom of a lake in Antarctica and named as *Candida antarctica.* It was discovered that this strain can produce industry-relevant extracellular lipases at a high yield (Morita et al., 2014; Morita et al., 2015). This study is focused on genetic engineering of *Moesziomyces antarcticus*, previously named as *Pseudozyma antarctica*, haploid anamorphic basidiomycetous yeast belonging to *Ustilaginaceae* family. It was reported as one of the most promising and efficient organisms for industrial MEL and recombinant protein production (Faria et al., 2014a; Morita et al., 2009; Morita et al., 2014), In addition, this strain is also the most efficient producer of MELs from sugar substrates (e.g. D-glucose and D-xylose) compared with other *Moesziomyces* species. Highest MEL production can be achieved by using vegetable oils as substrate, although it affects the total costs due to higher raw materials expenses, controversial usage towards food supply chain, difficulties in downstream processing reduce MELs sustainable production (Santos et al., 2018). Further screening for MEL production revealed an upregulated gene cluster in *M. antarcticus* with a strongly promoted gene expression in condition of nitrogen starvation, responsible for MEL biosynthesis. Previously, this gene cluster was first identified by genetic studies in *U. maydis* under

same limiting conditions. Comparative genomic and transcriptomic analysis between *M. antarcticus* and *U. maydis* have confirmed close similarity at genomic level, while gene expression pattern under the oil usage is tremendously different (Morita et al., 2014). Database from close related *U. maydis* strain was used as a reference strain for blasting and 6,845 protein-coding genes were predicted using AUGUSTUS program where 5,901 (86.2%) of genes were found to be homologous to the proteins in *M. antarcticus* T-34 strain (Figure 3). The five genes encoding erythritol/mannose transferase (*EMT1*), acyl transferase (*MAC1* and *MAC2*), putative transporter (*MMF1*) and acetyl transferase (*MAT1*), responsible for MEL biosynthesis, were clustered on scaffold 31 for the *M. antarcticus* type strain and found to be highly homologous to another *M. antrcticus* T-34 strain with 94.8%, 91.1%, 86.8%, 93.4%, and 92.4% uniqueness. As a result, it gives a possibility that plasmid, designed for *U. maydis* could be applied for usage in *M. antarcticus* strain.

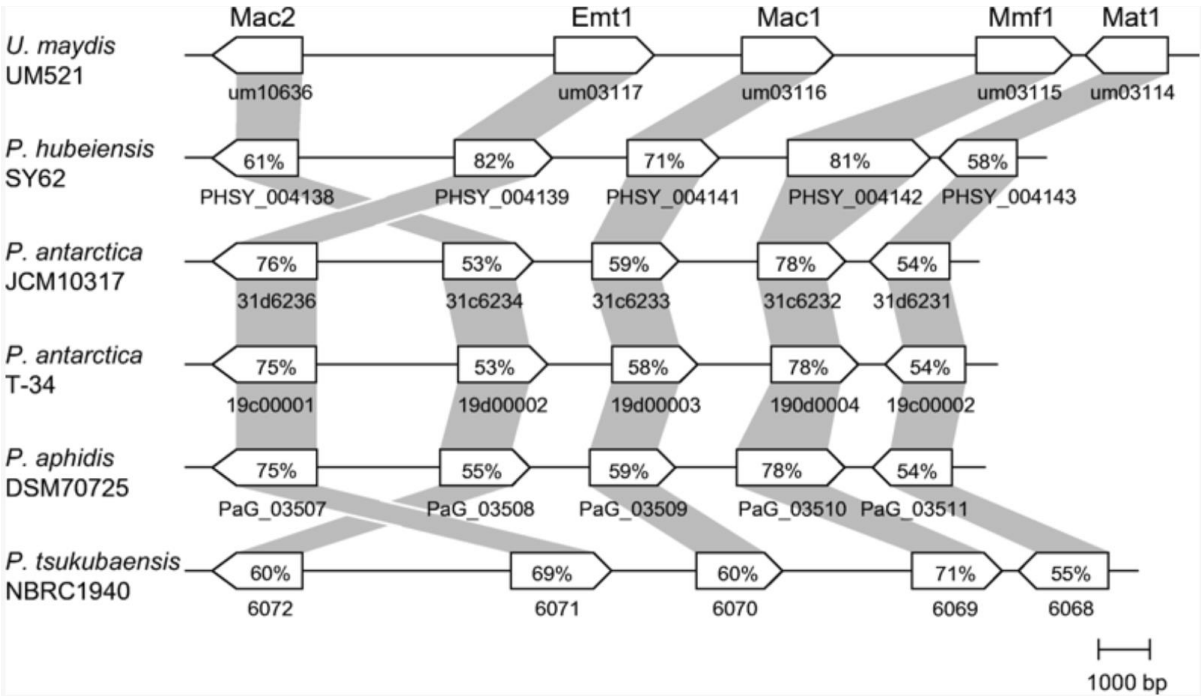

Figure 3: Similarity of the gene cluster for MEL biosynthesis in *Pseudozyma* strains compared with *U. maydis* together with a gene cluster for MEL biosynthesis represented in each strain's scaffolds (Saika et al., 2018)

1.4. Genetic engineering tools in *M. antarcticus*

Designing of suitable genetic engineering tools is crucial in modern molecular biology. Engineering of organism could be divided into three steps: choosing target genes, which would be inserted or/and mutated in a host organism; gene manipulation, where gene isolation and modification with different genetic elements are combined to assemble a construct for targeting; insertion of desired DNA construct into the host genome by various gene transfer tools such as protoplast transformation, electroporation and lithium acetate mediated transformation, which all have been established in *Pseudozyma* species (Saika et al.,2018). In many cases gene targeting tools are designed first *in silico* by artificial plasmid constructs and its compatibility, where afterwards it undergoes construction *in vitro*. Plasmid assembly is performed by digestion of DNA fragments by restriction enzymes at specific sites (restriction sites) and then assembling (ligating) them into final fragments. Gene engineering in *Pseudozyma* species was first described by constructing a plasmid containing a hygromycin B resistance carrying gene with *U. maydis* hsp70 promoter and terminator, which after was successfully integrated into *P. flocculosa*. Method was carried by polyethylene glycol and calcium chloride-mediated transformation of protoplast and obtained 4 transformants/µg of DNA per 10^8 of protoplasts. Though, preparation of protoplast is time consuming and complex due to its inability to store competent cells and high cost of suitable lytic enzymes for formation of protoplast in different organisms, leading to establishment of more novel transformation methods (Cheng et al., 2001; Marchand et al., 2007).

Another alternative method for *Moesziomyces* (*Pseudozyma*) *antarcticus* gene manipulation is electroporation due to its advantages in simplicity, speed and efficiency. Electroporation was successfully applied for several strains and proved to be working in *P. antarctica* CBS 516.83 (200 transformants per µg DNA per 10^8 cells) (Marchand et al., 2007), *P. antarctica* T-34 (48 transformants/µg of plasmid DNA) (Morita et al., 2007) and other *Pseudozyma* spp. including *P. antarctica* JCM10317, *P. aphidis* JCM10318, *P. ruglosa* JCM10323, *P. hubeiensis*. All above mentioned transformantions were performed with hygromycin B resistance marker for selection of mutants (Saika et al., 2018; Konishi et al., 2015). In addition, plasmid carrying nourseothericin resistance marker was applied in *M. antarcticus* GB-4(0) (Yarimizu et al., 2017). Efficiency of the transformation can vary depending on the target strain, expression of selection marker and vector system which could be episomal or integrative . Integrative vector lead DNA/RNA delivery for

parmanent incorporation into the host chromosome where cell undergoes mitosis together with vector DNA inside the daughter cell. Episomal vector integration lead for uniqueness due to precence outside of the chromosome and advantage of not disturbing essential host genes on chromosome which may cause mutations and changes in morphology and activity of the organism. It also could lead to a higher efficiency rate of transformation. Although electroporation could lead up to 50% of cell death rate which lead to one of the bottlenecks of this method (Berg et al., 2014 Saika et al., 2018).

In this study several gene engineering techniques are used for desired gene targeting in *M. antarcticus* by electroporation and polyethylene glycol/lithium acetate/single-stranded carrier DNA (PEG/LiAc/SS DNA) transformations. For targeting *MAT1* gene, gene replacement by homologous recombination, split-marker and ribonucleoprotein (RNP) gene targeting tools were used to observe its application ability.

1.4.1. Gene replacement by homologous recombination

Integrative gene transfer method is usually limited by availability of transgene expression in different organism and by the outcome of random insertional mutagenesis that complicates researches in gene expression and function studies and may lead to unfavorable occurrence in gene therapy (Lombardo et al., 2011). Integrative and conjugative elements (ICEs) and conjugative plasmids contains genes and sites which are required for processing their DNA for transfer and expression. Due to a randomness of gene transfer, most of the genes are not expressed during the integration in the chromosome (Johnson & Grossman, 2015). Site-specific integration and gene replacement could overcome these difficulties.

Gene replacement has been used as one of the most vital techniques for genetic modification of yeast strains. Although, the fidelity of gene targeting and input, required for modification of particular strain, diverge significantly depending on transformation method and design of DNA, needed for transformation, which includes the length of flanking regions and selected marker compared to the length of the target region in the host. Accurate modification of the *M. antarcticus* genome depends on homologous recombination (HR) between the inserted DNA for targeting and

yeast genome promoted by proteins, which are involved in the repair machinery of endogenous double-strand breaks (DSBs) in DNA. Gene replacement is achieved by transformation of linear DNA fragment containing dominant marker for selection, in this case is nourseothricin, flanked by the homologous to the target site sequences, and replaces the target gene in the host genome by crossing-over method. (Štafa et al., 2017).

Researches in other yeast species determined that low homology region is sufficient to initiate recombination of targeted gene and recombination efficiency can be increased exponentially by increasing the size of the flanking regions up to 1 kb (Kung et al., 2013; Morita et al., 2010). It was also reported that recombination efficiency may decrease depending on a size of non-homologous insert where no recombination occurred using 6 kb non-homologous DNA fragment flanked on each side by 1 kb homologous regions (Kung et al., 2013). In this research, flanking regions for homologous recombination were designed to be around 1 kb length, flanking *MAT1*, responsible for di-acetylation of MEL biosynthetic pathway, replacing it with nourseothricin containing cassette (*natMX4*) as a dominant marker for selection of mutant (Figure 4). It was decided also to use gene targeting fragment independently from plasmid, as a single-stranded DNA oligomer is much smaller and can be introduced into the cell with higher amount than target vector.

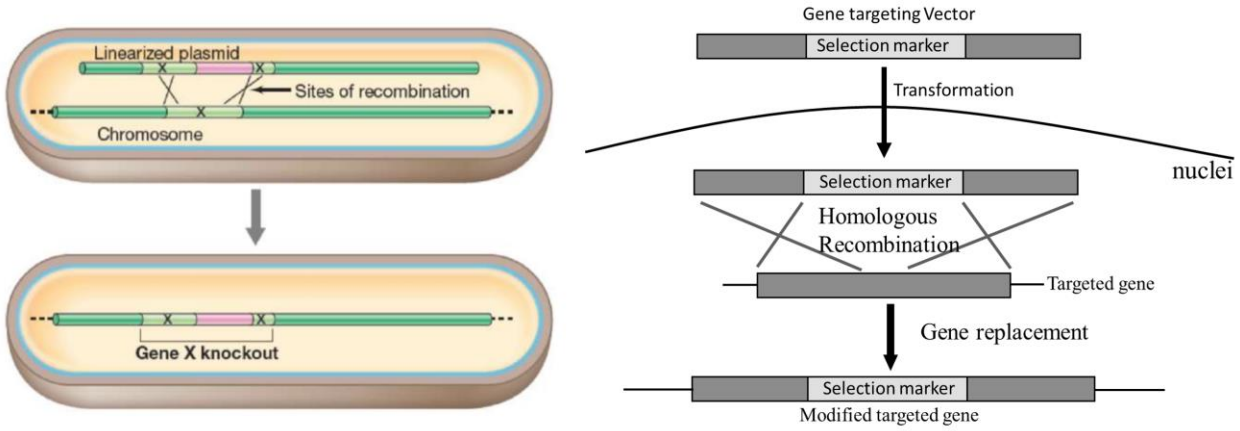

Figure 4: Gene-target replacement with knock-out fragment (selection cassette with donor DNA) mediated by homologous recombination. On the right it is more detailed representation of gene replacement by selection marker cassette flanked with upstream and downstream regions of target gene sequence: disruption fragment is inserted into the host organisms via transformation where double crossing-over is initiated at the homologous sites of the target gene leading to replacement with selection marker cassette and knockout of target gene (Madigan et al., 2015).

1.4.2. Split-Marker mediated gene replacement

Split-marker method is complementary to the simple gene replacement. Difference is in usage of the DNA fragments, overlapping within the sequence of dominant marker. Application of this approach facilitates in incretion of homologous integration and gene disruption. Due to selection marker abridge into smaller oligomers, the gene responsible for selection is not functional until homologous recombination occurs between overlapping oligomers for selection marker. One of the advantages of that method that it only requires two rounds of PCR for amplifying homologous regions of a target gene and dominant marker followed by fusing them into two fragments, each containing around 1 kb long homologous sites for target gene and overlapping regions of selection. As it noted in Figure 5, in total, three homologous events should occur within the host organism, one within each flanking region and one in the marker gene, leading to the replacement of the target gene (e.g. *MAT1* gene) with a fully functional marker gene for selection (*natMX4*) (Goswami, 2011; Chung & Lee, 2015).

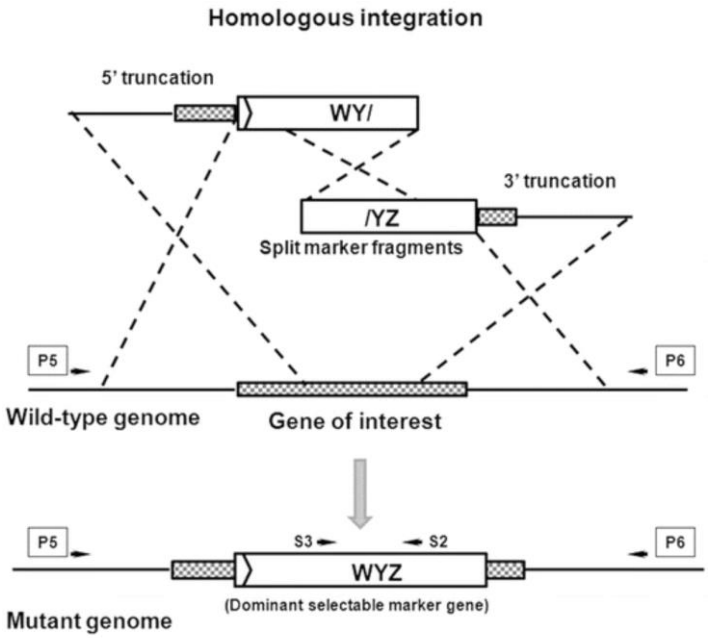

Figure 5: Schematic illustration of targeted gene disruption by split-marker-based transformation. Two truncated, overlapping marker gene fragments flanked with the truncation of a target gene are directly transformed into cells prepared from a wild-type yeast strain. Three cross-over events are required to generate functional marker gene and homologous integration. Only transformants containing a functional marker gene cassette will grow on a medium containing the selection agent. Employing split-marker-based gene disruption could enhance homologous recombination (Chung & Lee, 2015)

1.5. CRISPR-Cas9 system

When it comes to genetic modification of desired organism, a various gene manipulation tools could be applied such as PCR based method described above with integration of target sequences or newly applied CRISPR (clustered regularly interspaced short palindromic repeat) and CRISPR-associated (Cas) protein system, which originally serves as an adaptive immune system in bacteria and archaea. This system in bacteria can act against invasion of foreign DNA as plasmids or viruses by initiating immunity system mediated by three-stage processes of (1) adaptation of foreign DNA (spacers) into the host genome in CRISPR site, (2) expression of the guide CRISPR-RNA (crRNA) containing a spacer and guiding CRISPR/Cas complex to the cleavage locus and (3) interference of CRISPR/Cas system which invades foreign DNA for propagation of DSB in the target site (Makarova et al., 2011; Deltcheva, et al., 2011). CRISPR-Cas9 system is used as easy and efficient tool for genome editing by carrying specific mutations via non-homologous end joining (NHEJ) or homology-directed repair (HDR) with introduction of donor DNA for repair. It has potential advantages against other genome editing tools such as zinc-finger nuclease (ZFNs), transcription activator-like effector nuclease (TALENs) as it is easy to customize, higher target efficiency and ability to target multiple genes (Ran et al., 2013). CRISPR/Cas9 system rapidly developed as an efficient genome editing tool in various organisms such as *ascomycete* and several *basidiomycete* fungi. CRISPR-Cas9 system consists of Cas9 protein which serves as endonuclease, capable of catalyzing a DNA double-strand break (DSB) aimed by a single-guided RNA (sgRNA), containing a 20 nucleotide sequence which matches the sequence upstream of the protospacer-adjacent motif (PAM; 5'-NGG-3') site on the target site (Kunitake et al., 2019). Above mentioned sgRNA is a duplex consisting of a crRNA and a trans-activation crRNA (tracrRNA) which together guide Cas9 protein to the target locus for DSB propagation. The crRNA usually consists of 20 nt which are homologous to the target site and tracrRNA is universal sequence which creates stem loop structure for Cas9 protein to bind (Sternberg et al., 2014). The integrated DSB can be followed by deletion, insertion or substitution of the sequence using NHEJ. By introducing donor DNA (dNDA) for DSB repair, HR process takes place, which enables highly efficient site-specific gene manipulation (Kunitake et al., 2019) (Figure 6).

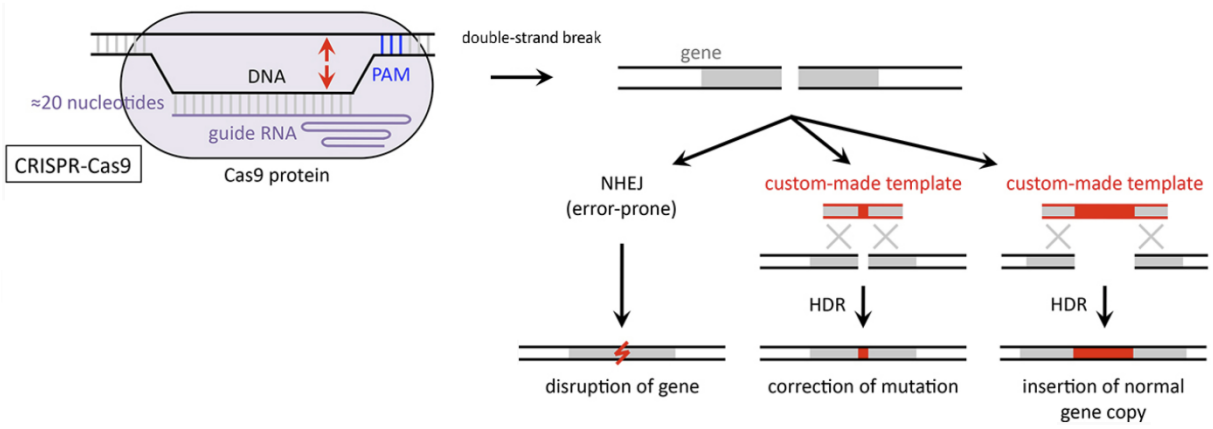

Figure 6: Schematic representation of CRISPR-Cas9 gene editing system. Cas9 protein is binded with gRNA complex which guides the protein to the homologous target sequence for introduction of double strand break (DSB). Subsequently, repair mechanisms of DNA is initiated for non-homologous end joining (NHEJ) or homologous direct repair by suplementing with donor DNA (Chadwick & Musunuru, 2018).

1.5.1. Plasmid-free CRISPR/Cas9 system

Some studies demonstrated the successful usage of CRISPR-Cas9 method in human cells by using purified Cas9 protein and CRISPR RNAs rather than using the Cas9-gRNA expressing construct (plasmid). Plasmid-free CRISPR-Cas9 gene editing was also used in plants, algae and filamentous fungus *Penicillium chrysogenum* mediated by protoplast transformation (Grahl et al., 2017). Ribonucleoprotein (RNP) complex is assembled *in vitro* and is able to be delivered to cells using standard electroporation or transfection methods. RNP is capable of cleaving genomic DNA target with similar efficiency as plasmid-based CRISPR-Cas9 system and could be used for most of the current CRISPR gene editing applications including generation of single or multi-gene knockouts, gene editing with dDNA implementation for HDR, which leads to generation of large genomic deletions/replacements. RNP system differs from plasmid CRISPR-Cas9 system by delivery pathway of components into the host cell and time of its absence within the cell. While plasmid system requires transcription/translation machinery for generation of functional gRNA-Cas9 complex and has high lag phase for Cas9 protein expression, RNPs are supplied as complete complexes, removable quickly after transfection and cleared by protein degradation within the cell. RNPs induce the rate of mutations for target genes compared with plasmid system and are supplied as functional complexes able to cleave a DNA target without the need of transcription/translation

(Figure 7). Moreover, quick removal of RNPs from the cell may increase the specificity by reducing the time for Cas9 cleavage to prevent off-targeting. This system can be used in the organisms which requires limited activity of Cas9 and higher specificity for a gene knockout or homologous recombination generation. Drawback of RNPs is temporary expression, which lead to plasmid-based system more desired for stable expression of CRISPR components if required (McDade, 2017). In this study CRISPR RNA-Cas9 protein complex (RNP) would be used together with a repair/target construct that contains the desired genome modification, in this case for *MAT1* targeting by replacing with *NatMX4* cassette. Introduction of RNP potentially minimizes off-target effects compared to CRISPR-Cas9 plasmid system as a purified Cas9 protein is able to immediately cut chromosomal DNA and is degraded rapidly (Kunitake et al., 2019).

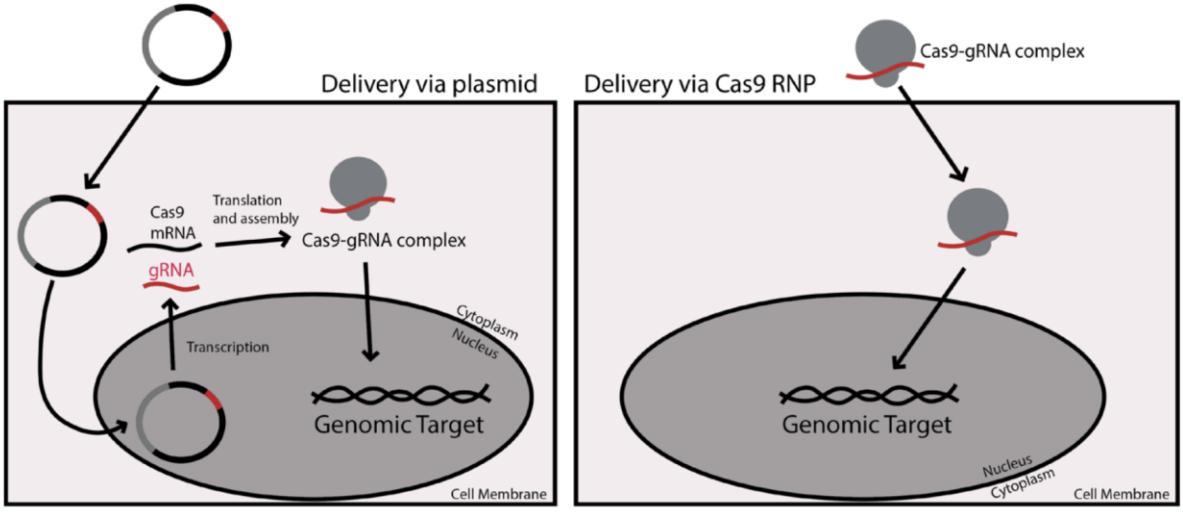

Figure 7: CRISPR component delivery through plasmid system (left) and Cas9 RNP system (right). Plasmid-based transport of CRISPR compounds requires transcription, translation and assembly of the gRNA-Cas9 complex in the host organism before targeting desire gene. Cas9 RNPs are delivered straight into the cells as pre-assembled complex of gRNA-Cas9 and are plasmid-free with no intermediate steps for interaction with target DNA (Addgene©)

1.6. Aim and approach

The aim of this project is to eliminate further steps in mannosylerythirotl lipid (MEL) biosynthetic pathway by knockdown of the *MAT1* gene, which is responsible for acetylation of mannosylerythritol at both the C-4 and C6 hydroxyl groups, resulting in production of only MEL-D (non-acetylated) as it is not naturally produced in a high amount.

First aim of this project is implementation of several different gene engineering techniques into *M. antarcticus* such as traditional homologous recombination by replacing gene of interest with disruption cassette, split-marker approach by using two truncated fragments from disruption cassette leading to homologous recombination and RNP mediation to observe plasmid-free gene targeting ability and homologous recombination efficiency.

The other aim of the project is to design a mutant with disrupted *MAT1* gene and perform MEL production in shake flasks by additional feeding steps with oil and carbon source. Comparison of mutant and wild type strain should be performed to have a clear vision of MEL production and its efficiency.

2. Materials and methods

2.1. Microorganisms and maintenance

In this study *M. antarcticus* PYCC 5048^T (CBS 5955) strain was used. It was provided by the Portuguese Yeast Culture Collection (PYCC), CREM, FCT/UNL, Caparica, Portugal. Strain was cultivated on Petri dish plate with YM agar medium (yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L, glucose 10 g/L, agar 20 g/L). Plate was incubated for 2-3 days at 30°C. Afterwards, cell culture was kept at 4°C and replated every 2 weeks for maintaining viability of the cells.

Escherichia coli (Top10 strain) was used as a host organism for propagation of modified plasmid. Organism was cultivated in LB medium (tryptone 10 g/L, yeast extract 5 g/L, sodium chloride 10 g/L, pH 7.0) with addition of ampicillin (100 μg/mL) for selection of modified cells. Incubation was performed at 37°C and 200 rpm for 16-18 h. Transformed cells were plated on LB agar plates containing the same concentration of ampicillin and incubated at 37°C.

2.2. Cultivation of *M. antarcticus*

Seed cultures of *M. antarcticus* were prepared in two steps by inoculating cells grown on agar plates into a growth medium. Three different media were used for cell growth: YM (yeast extract

3 g/L, malt extract 3 g/L, peptone 5 g/L, glucose 10 g/L); YPD (yeast extract 10 g/L, peptone 20 g/L, glucose 20 g/L); mineral (MEL) medium without sugars (yeast extract 1 g/L, NaNO3 3 g/L, KH2PO4 0.3 g/L, MgSO4x7H2O 0.3 g/L). First, yeast colonies were taken from YM agar plates and pre-cultivated in 50 mL falcon tubes containing 5 mL of YPD medium at 28°C and 180 rpm for 24 h. For better cell resuspension, pipetting of inoculated cells was performed. Later, 1mL from the top layer of pre-cultures was added to 500 mL Erlenmeyer flasks containing 50 mL of YPD medium (1/10 working volume) and cultivated at same conditions (28°C and 180rpm). Cotton plugs were used for suitable oxygen supply and prevention of contamination. To investigate cell growth, Optical Density (OD) was followed and measured at 640nm. Dilution were made to obtain absorbance in range of 0.1-0.5 for reliable estimation of cell growth.

Microscopy of cell broth was followed during the propagation of yeast-like cell growth and overall morphology of the organism.

Stock cultures were prepared from liquid cell broth and stored in 25% v/v glycerol aliquots at -80°C.

2.3. Plasmid construction for *MAT1* gene targeting

Plasmid (pUC57mini- NatMX_MaMat1) used in this study was synthesized and ordered from Genscript Corporation (NJ, USA). Plasmid contains 1 kb *MAT1* gene flanking regions: upstream region is 936 bp and downstream region of 1133 bp. For selection of plasmid, nourseothricin selection cassette (*natMX4*) was introduced in-between two flanking regions containing TEF promoter, nourseothricin gene and TEF terminator from *S. cerevisiae*. For plasmid engineering *in vitro*, ori (origin of replication) and AmpR (ampicillin resistance marker) were introduced. For more efficient plasmid engineering, EcoRV and PvuII restriction sites were designed at the end of each flanking regions.

2.4. Oligonucleotide primers

Primers used in this study were designed *in silico* via Benchling and Primer Blast (NCBI) softwares, following the parameters of length (17-24bp), G/C content of 40-60%, Tm difference between primers of <5°C.

Table 1: Primers used in this study for construction of different targeting cassetes and for screening of obtained transformants

Name	Sequence (5' → 3')
MaMat1-NatMX_FOR	CTCGATAGCACGCTCGGAAA
MaMat1-NatMX_REV	AGGCAGAGTGCAAAGGTTGA
natMX_Mat1-5'FOR	TGTCGCGAAGTCATCACCAT
natMX_Mat1-5'REV	CAGAGGGTGAACCCCATCC
natMX_Mat1-5'REV2	GTCGCGAGCCCCATCAAC
natMX_Mat1-3'FOR	TCTGGCTGGAGGTCACCAA
natMX_Mat1-3'REV	GTGGATCTGATATCGCCCATCT
natMX_Mat1-3'FOR2	GCCCCTGACCAAGGTGTT
Out_natMX_Mat1-5'FOR	TCCTCATCGCTCGGAAGAGA
Out_natMX_Mat1-3'REV	GATCGGACTTCTGGCGTTCT

2.5. Fragment construction

Fragment with *MAT1* gene flanking regions and *NatMX4* cassette for selection was constructed using PCR approach. It was amplified from plasmid pUC57mini-NatMX_MaMat1 by usage of two primers binding to each end of flanking regions (natMX_Mat1-5'FOR and natMX_Mat1-3'REV). The PCR was performed with Q5 high fidelity polymerase in 50 µL reaction (Appendix ii). PCR products were confirmed through 1.0% w/v agarose gel electrophoresis and purified by NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Quantification of purified fragment was made by Nano-drop 1000 (Thermo Scientific, USA).

2.6. Generation of Split-Marker fragments

Fragment for split-marker approach was constructed as well from plasmid pUC57mini-NatMX_MaMat1 as it already contained flanking regions for gene of interest and dominant selection marker (clonNAT). Four primers were used to amplify two truncated but overlapping fragments. Primers natMX_Mat1-5'FOR and natMX_Mat1-5'REV were used to amplify upstream flanking region with part of *NatMX4* selection cassette (74%) and natMX_Mat1-3'FOR together

natMX_Mat1-3'REV were used to amplify another half of split-marker fragments generating 70 bp overlap region within selection marker gene. For generating other set of split markers, natMX_Mat1-5'FOR and natMX_Mat1-5'REV2 primer set was used for upstream region amplification and natMX_Mat1-3'FOR2 with natMX_Mat1-3'REV were used for downstream region amplification resulting in 231 bp (2/3rd of the gene) overlap within clonNAT gene (Figure 8). The PCR was performed with Q5 high fidelity polymerase in 50 µL reaction (Appendix ii). PCR products were confirmed through 1.0% w/v agarose gel electrophoresis and purified by NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Quantification of purified fragment was made by Nano-drop 1000 (Thermo Scientific, USA).

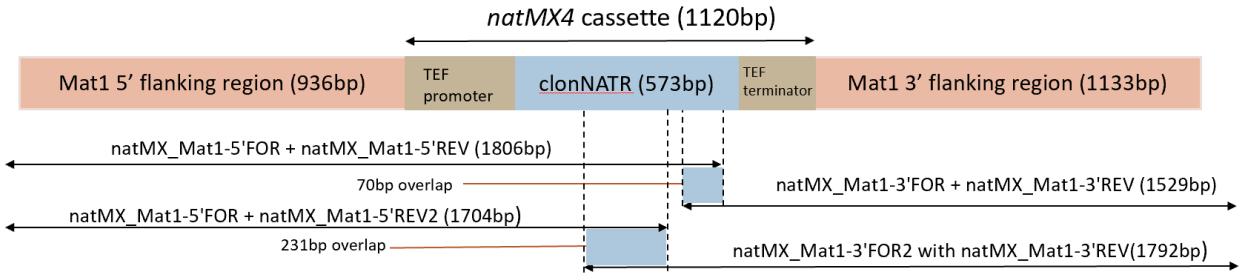

Figure 8: Creation of split-markers for gene knockout. Two sets of split-markers were created. First set results in 70 bp homology region (overlap) with upstream region (1806 bp) amplified by natMX_Mat1-5'FOR + natMX_Mat1-5'REV primers and downstream region (1529 bp) amplified with natMX_Mat1-3'FOR + natMX_Mat1-3'REV. Second set of split-markers results in 231 bp homology region (overlap) between two fragments (2/3rd of the clonNAT gene) where upstream region (1704 bp) was amplified by natMX_Mat1-5'FOR + natMX_Mat1-5'REV2 and downstream region (1792 bp) amplified with natMX_Mat1-3'FOR2 with natMX_Mat1-3'REV

2.7. Creation of backbone plasmid of pUC57

Original plasmid contains flanking regions for *MAT1* gene targeting (pUC57mini-NatMX_MaMat1), therefore, for targeting other genes, plasmid needs to undergo removal of these regions (Figure 9). As plasmid was designed with restriction sites at each end of flaking regions, restriction digest was performed for PvuII and EcoRV restriction sites. For removal of upstream flanking region, PvuII enzymatic digestion 50 µL reaction was performed with PvuII enzyme (New England BioLabs) with incubation time of 1h at 37ºC (Table 2). According to supplier's protocol, enzyme does not require any enzyme inactivation.

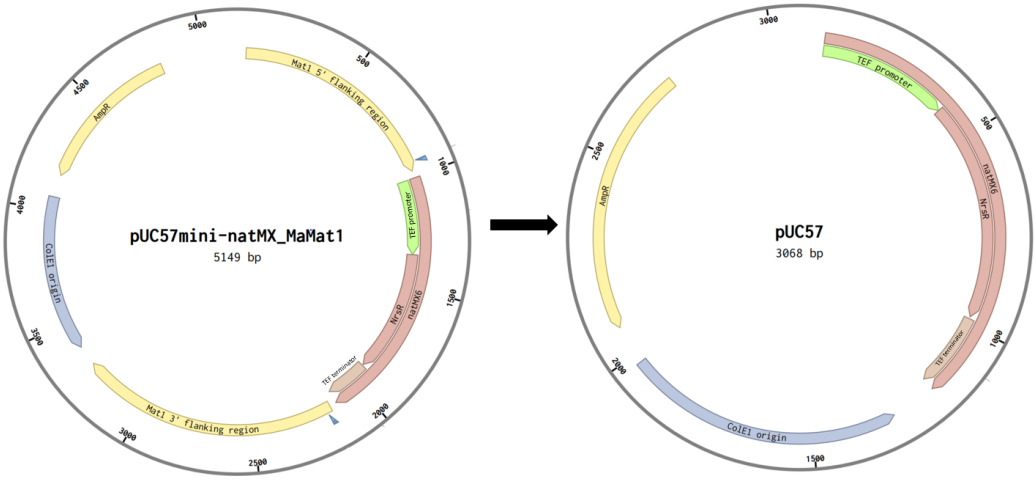

Figure 9*:* Creation of backbone pUC57 plasmid by restriction digestion of PvuII and EcoRV locus

Table 2: PvuII restriction digestion protocol for 50 µL reaction mix

Componets	**50 µl reaction**
Resctriction enzyme	1 µl
DNA	1 µg
10X NEB Buffer (NEB Buffer 3.1)	5 µl (1X)
Water	Up to 50 µl
Total Rxn Volume	**50 µl**

Self ligation of digested fragment was performed with T4 DNA ligase in a 50 µL reaction mixed which included 2.5 µL of 10X T4 DNA ligase buffer, 50 ng of DNA and 1 µL of T4 DNA ligase Weiss U enzyme. Reaction was mixed thoroughly, spun briefly and incubated for 1 h at 22ºC. Up to 5 µL of the mixture were used to transform 50 µL of the chemically competent cells.

For removal of downstream flanking region, EcoRV (New England BioLabs) enzymatic digestion was performed in 50 µL reaction mix (Table 3). Incubation was made at 37ºC for 1 h followed by inactivation of enzyme 80ºC for 20 min. Ligation, transformation was performed as described above.

Table 3*:* EcoRV restriction digest protocol for 50 µL reaction mix.

Components	**50 µl reaction**
Restriction enzyme	1 µl
DNA	1 µg
10X NEBuffer (NEBuffer 3.1)	5 µl
Water	Up to 50 µl
Total Rxn Volume	**50 µl**

2.7.1. Bacterial transformation

Aliquot of 50 µL of *E. coli* 5-alpha high efficiency chemical competent cells were pre-chilled on ice until cell broth melts. 2 µL of the ligated DNA was used to transform 50 µL chemically competent *E. coli* cells. DNA and cell mix was pipetted gently and kept on ice for 30 min and after incubated for 45 sec at 42ºC for heat shock transformation. Tubes were transferred on ice for 2 min to cool down. Subsequently, 300 µL of LB medium without antibiotics was added gently and incubated at 37ºC for 30 min. Cells were spread on LB agar plates containing 100 µg/ml ampicillin and incubated at 37ºC for approximately 12-24h.

Screening of assembled plasmid was followed by colony PCR approach and double restriction digestion.

2.8. Transformation of *M. antarcticus*

Two different methods were performed for fragment replacement and split-marker transformations which were electroporation method (Watanabe et al., 2016) and PEG/LiAc mediated transformation (Yarimizu et al., 2017) with some addition of changes in pre-treatment of the cells for electroporation to observe how lithium acetate pre-treatment can affect the cell wall and transformation efficiency.

Cultivation of organism prior transformation was performed as described in section 2.2.

2.8.1. Transformation by electroporation

Prior transformation, optical density measurements and microscopy of *M. antarcticus* cell broth was performed to observe cell morphology and single cell distribution. Cells with optical density value at 640 nm (OD_{640}) of approximately 2, 4 and 6 were used for transformation. Transformations were made with and without pre-treatment steps.

For preparation of competent cells, pre-treatment step was performed using lithium acetate (LiAc). Cultivated cell cultures were palleted and resuspended in 10 mL of transformation buffer containing 100 mM LiAc, 10 mM Tris-HCl and 1 mM EDTA. Samples were incubated at 28°C and 180 rpm for 1 h. Afterwards, 100 mM dithiothreitol (DTT) was added into the samples and incubated for additional 30 min at the same conditions.

Yeast cells were collected by centrifugation at 4400 x g at 4ºC for 10 minutes. Cells were washed twice with ice-cold sterilized water and once with ice-cold 1 M sorbitol before resuspension in 250 µL of ice-cold 1 M sorbitol. Fifty microliter aliquots of cell suspension were mixed with 1µg of DNA (1 µg fragment with flanking region or 0.5 µg of each slip-marker fragments) in a pre-chilled 0.2 cm electroporation cuvettes (Bio-Rad, USA). The cells were affected with electric pulse by Micro Pulser™ (Bio-Rad, USA) at 1500V, where time constant varied at 4.3-5.2 ms. Cells were resuspended in 1 mL 1 M ice-cold sorbitol immediately after the pulse and gently palleted at 3000 x g for 3 min. Subsequently, cell pallet was resuspended in 2 mL of YPD and incubated for 4 h at 180 rmp and 28ºC. Cell suspension (100 µL) was spread onto a YPD medium plate containing 100 µg/mL nourseothricin and 2% agar. Cell colonies appeared within 3-4 days at 30ºC incubation.

2.8.2. Transformation by PEG/LiAc/ssDNA treatment

Transformation by lithium acetate treatment was performed as described by Yarimizu, et al. (2017). Cell cultivation was performed as described in seciton 2.2. Cells were harvested at OD_{640} of 4 and centrifuged at 4400 x g, 4ºC for 10 minutes. The cell pallet was rinsed with 4 mL of transformation buffer (TFB: TFB: 40% polyethylene glycol 3350 (Sigma-Aldrich), 0.2 M lithium acetate, and 0.1 M dithiothreitol) and aftwerwards resuspended in 100 µL of TFB. Cell suspention was mixed with 10 µL of 10 mg/mL single-stranded salmon sperm DNA (ssDNA) and 1 µg of DNA fragment for transformation. ssDNA was pre-heated for 5 min before addition into the mix. Cell suspention was incubated at 37ºC for 1 h for a heat-shock transformation. 200 µL of YPD medium was added imidiatelly after the heat-shock. Cell were transfered into 2 mL of YPD medium and incubated for 4 h at 180 rmp and 28ºC for a cell recovery. Cell suspension was spread

of YPD medium plate containing 100 µg/mL nourseothricin and 2% agar and incubated at 30ºC for 3-4 days.

2.8.3. Transformation with RNPs

Transformation with RNP was performed by electroporation method as described above. RNP targeting *MAT1* gene was created using Alt-R CRISPR-Cas9 system from IDT (Integrated DNA Technologies, Inc.) and assembled during the final washing step to transform into competent cells of *M. antarcticus*. Stock of crRNA (gene specific for *MAT1* targeting) and tracrRNA (universal) were dissolved in RNase-free distilled water or TE buffer (final concentration 100 µM) and were stored at -20ºC. For creation of assembled guide RNA, equimolar concentrations (final of 4 µM and 7 µM) of the gene-specific crRNA and tracrRNA were mixed in nuclease-free duplex buffer, with a final volume of 3.6 µL (4 µM) and 1.2 µL (15.4 µM) per transformation required and afterwards incubated at 95°C for 5 min. Alt-R Cas9 nuclease 3NLS (61 µM stock provided by IDT)was diluted to 4 µM and 7 µM in Cas9 dilution buffer with the final volume of 3 µL (4 µM) and 1 µL (15.4 µM) used per transformation. Assembled gRNA (crRNA with tracrRNA) was cooled down at a room temperature and mixed with diluted Cas9 protein in a ratio of 1.2:1 respectively (3.6 µL of guide RNA (4 µM) to 3 µL of Cas9 protein (4 µM) or 1.2 µL of guide RNA (15.4 µM) to 1 µL of Cas9 protein (15.4 µM)). Mix was incubated at room temperature for at least 5 min to assemble the RNP complex. RNPs were used in two volumes of 6.6 µL (1.8 µM final concentration) and 2.2 µL (7 µM final concentration) per transformation with three dilution of x0, x10 and x100 for second test (2.2 µL (7 µM final concentration)). For RNP transformation, 1 µg of donor DNA (amplified fragment with *MAT1* flanking regions and *NatMX4* selection cassette) was used as RNP mix, targeting *MAT1*, has no selection. Moreover, RNP influence on homologous recombination could be observed.

2.8.4. Screening of *M. antarcticus* transformants

Colonies obtained after transformation were checked for insertion of *NatMX4* cassette and its targeting locus of *MAT1* gene. Integration of gene was determined by colony PCR or amplifying

from genomic DNA (Appendix iii) using DreamTaq DNA polymerase (Thermo Fisher)(Appendix i). Single colonies were resuspended in 20 mM NaOH, incubated at 98ºC for 15 min, and then used directly as a PCR template for screening. To identify clones containing *NatMX4* cassette, primers were designed to bind at the *NatMX4* cassette locus (MaMat1-NatMX_FOR and MaMat1-NatMX_REV; Table 1) and for checking correctly targeted locus of *MAT1*, primers were designed to bind outside of the target locus (Out_natMX_Mat1-5'FOR and Out_natMX_Mat1-3'REV; Table 1).

2.9. MEL production in batch culture

2.9.1. Fermentation of *M. antarcticus*

Pre-cultures of yeasts were made by inoculating cells from cryo-stocks of wild type *M. antarcticus* and potential transformant (to observe the difference in a cell growth and MEL formation) into Erlenmeyer flasks (1/10 working volume) containing sugar-free mineral medium (yeast extract 1 g/L, NaNO3 3 g/L, KH2PO4 0.3 g/L, MgSO4x7H2O 0.3 g/L) and 40 g/L glycerol. Cells were incubated for 72 h at 28°C and 180 rmp. Fermentation was launched in 500 mL Baffled flasks containing mineral medium and 40 g/L glycerol. 10 mL of each inoculum was transferred into the flasks to start fermentation at OD_{640} of 1 where final working volume increased until 60 mL. Fermentation was performed at 28°C and 180 rmp for 21 day. Feeding step was performed after 96 h where 40 g/L glucose and 40 g/L rapeseed oil were inoculated into the cell broth. Samples were taken for the first 5 days until feeding step (day 0, 1, 2, 3, 4) to check glycerol consumption and after samples were taken at day 5, 6, 11, 16 and 21 for MEL, glycerol and glucose quantification.

2.9.2. Sugar quantification by HPLC

For quantification of glycerol and glucose, 1 mL of cell culture samples were taken and centrifuged to extract supernatant containing sugars. Samples were diluted 2x with 4 mM sulphuric acid and filtered through 0.22 µm filter. Quantification was performed by high performance liquid

chromatography system (UltiMate 3000, Thermo Fisher Scientific, Massachusetts, United States) equipped with RI-101 detector (Shodex) and Aminex HPX-87H column (300 × 7.8 mm, BioRad, United States). 4 mM sulphuric acid was used as a mobile phase with 0.6 mL/min flow rate injecting 10 µL of sample.

2.9.3. MEL quantificaion by HPLC

All samples were extracted with equal volume of ethyl acetate (ratio 1:1) prior quantification. The mixture of cell broth and ethyl acetate was vigorously mixed on vortex and put onto thermo-shaker at 1000-1200 rmp and 30°C for 30 min. Samples were centrifuged at 8000 x g for a better phase separation where top layer of ethyl acetate phase was then used for quantification. Samples were quantified by UltiMate 3000 HPLC module (Thermo Fisher Scientific, Massachusetts, United States) equipped with Corona Veo Charged Aerosol Detector (Thermo Fisher Scientific, United States) and silica gel column (Inertsil SIL-100A, 5 µm, 4.6 × 250 mm, GL Science, Tokyo, Japan). A mobile phase containing chloroform-methanol was used with a linear gradient of flow of 1 mL/min from 100:0 to 0:100 for 35 min.

3. Results

3.1. Manipulation of yeast morphology

Cells of *M. antarcticus* were cultivated in two different media of YPD and YM. High concentrations of sugars induce the cell growth, on another hand, it induces the cell hyphae formation due to a fast growth as it was described in previous work (Bobkov, 2017). Simple inoculation of cells from agar plate resulted in formation of high number of clumps which negatively effects transformation efficiency due to predominantly filamentous morphology of cells and lower area of cell wall for available gene insertion (Figure 10 A, 10 D). To obtain more yeast-like form from selected high-sugar medium (YPD), cell morphology manipulation was performed. It was observed that cultivation conditions by constant re-inoculation of top layer of cell broth

containing mostly yeast-like form resulted in extraction of hyphae-free cell form which was later used for future transformations (Figure 10).

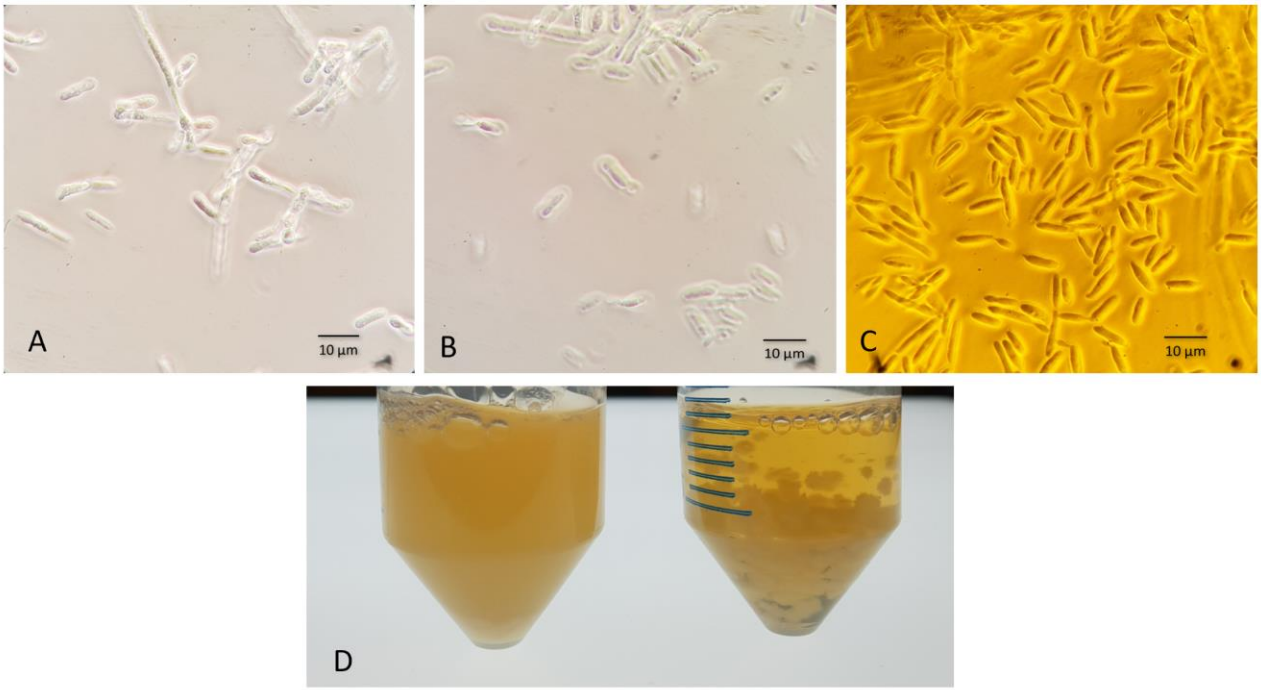

Figure 10: Microscopy (x1000 magnification) of *M. antarcticus* from 2 different cultivation methods. **A:** *M. antarcticus* cell morphology by inoculation of cells from YPD agar medium into YPD liquid medium **B:** *M. antarcticus* cell morphology by inoculation of cells from re-cultured cell broth (1 time) into YPD medium **C:** M. antarcticus cell morphology after re-culturing 2-3 times in YPD medium **D:** left tube is re-cultured *M. antarcticus* cell broth containing mostly yeast-like form and right tube shows inoculation of yeast from YPD agar plate into liquid YPD medium where cell clumps were observed.

3.2. pUC57 backbone plasmid construction

Bacterial colonies appeared after final bacterial transformation for pUC57 plasmid manipulation on LB agar medium containing selection agent (100 µg/mL ampicillin). Restriction digest of pUC57mini-NatMX_MaMat1 plasmid proved to be successful as PvuII and EcoRV resulted in single cutting site by each restriction site of pUC57 obtained from the final transformation. Two cutting sites were observed at 1174 bp for PvuII and 1894 bp for EcoRV for double enzymatic digestion while single restriction digests resulted in ~4000 bp length (Figure 11). Empty pUC57 plasmid later used for other disruption plasmid construction.

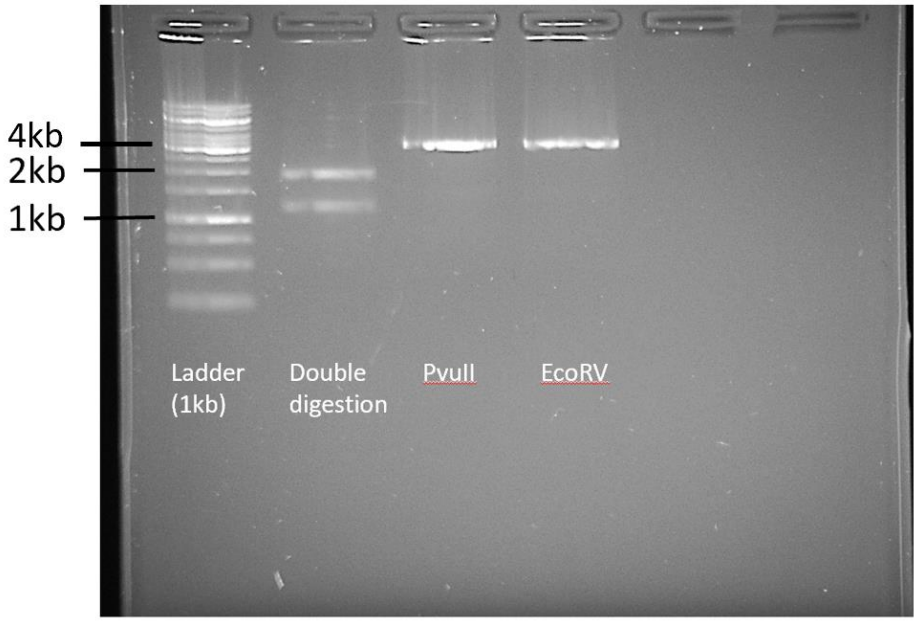

Figure 11: Double restriction digest with PvuII and EcoRV. Expected sizes for PvuII (~1200 bp) and EcoRV (~1900 bp) were obtained. Single restriction digests for PvuII and EcoRV have ~3000 bp bands.

3.3. Test of different conditions for *M. antarcticus* transformation with electroporation

Transformations were made by inserting NatMX_MaMat1 fragment containing *MAT1* gene flanking regions. Cells with OD_{640} of around 2, 4 and 6 were used for first transformations without a lithium acetate pre-treatment approach prior electroporation. Cell suspension was also diluted with 0x, 1x and 3x v/v sorbitol prior transformation to obtain 50 µL (original), 100 µL and 200 µL final volume to check how different dilutions affected pulse time variations. Selection of transformants was observed on YPD agar plates containing 100 µg/mL of clonNAT. Colonies started to appear within 3 days of growth at 30°C. Big colonies appeared after 3 days from all tested conditions where OD2 resulted in only 40.7 CFU/ µg, OD4 was 133.75 CFU/ µg DNA and OD6 showed much higher number of transformants of 431.25 CFU/ µg DNA (Table 4). There is a clear correlation between different OD resulting in increase of 3-folds with OD2, OD4 and OD6 respectively. Unfortunately plates with OD6 showed high number of small colonies which were a background growth without *NatMX4* cassette insertion and were stated as not suitable for further transformations. OD2 and OD4 showed low transformation efficiency but 20-40 randomly picked

colonies from each transformation showed *NatMX4* cassette insertion in almost all transformants confirmed by colony PCR and grew on regeneration plates. OD2 was used as standard conditions for further transformations. Usage of different volumes of aliquots in electroporation cuvette influenced the pulse time variations with 4.7-5.2 ms for 50 µL cell suspension, 2.9-3.1 ms for 100 µL and 2.4-2.7 ms for 200 µL where usage of 50 µL showed the highest pulsing time for DNA integration into the organism and was selected for further transformations. Moreover, usage of 100 µL and 200 µL cell suspention did not result in any colony formation after transformation.

Table 4: Different conditions tested for electroporation of *M. antarcticus*. Three different OD values were tested with different cell amount. Plating was performed in two ways of plating 1:20 dilution factor (100 µL of recovered cells from 2 mL total volume) and after with 1:4 dilution factor (100 µL of 5x concentrated cells) onto agar selection plates.

Optical density (OD_{640})	Transformation efficiency (CFU/ µg DNA)
2	40.7
4	133.75
6	431.25

Pre-treatment of the cells with transformation buffer (100 mM LiAc, 10 mM Tris-HCl, 1 mM EDTA) and 100 mM DTT before transformation was also tested and resulted in significant increase of transformation efficiency (Figure 12). Treatment increased transformation efficiency 3-folds compared with non-treated cells for fragment (52 ± 8 CFU/ µg DNA) and split-marker (30 ± 9 CFU/ µg DNA) approach (Table 5 shows transformation efficiency of pre-treated cell).

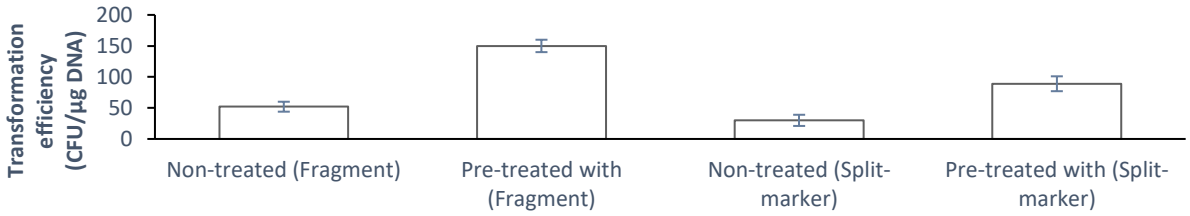

Figure 12: Transformation efficiency with and without pretreatment of cells prior transformation. Pre-treatment was performed for both fragment replacement and split-marker (70 bp) approach. Transformations were made with 1 µg of NatMX_MaMat1 fragment and truncated fragment for split-marker.

3.4. Transformation with fragment and split-marker approach

Transformations were made with both NatMX_MaMat1 fragment and split-marker (70 bp) approach containing two truncated fragments of NatMX_MaMat1 with 5' flanking region and 3' flanking region in each part. Transformants could occur only if two truncated split-marker fragments are assembled together at the selection marker site. Transformation efficiency resulting in 150 ± 10 CFU/µg DNA for NatMX_MaMat1 fragment replacement and 89 ± 12 CFU/µg DNA for split-marker approach where split-marker decreased the efficiency by almost two-fold (Table 5). No colonies were observed in the negative control and big colonies appeared on the selection medium plates within 3 days with fragment insertion and split-marker approach (Figure 13). Out of 20 randomly selected and re-plated colonies from each transformation, 12-15 (60-75%) grew on regeneration medium with selection pressure (clonNAT) and were evaluated as *natMX4* disruption cassette containing mutants which had to undergo screening with primers amplifying disruption cassette to prove its presents and other primers amplifying outside of the flanking regions to check if targeting was accurate.

Table 5: Transformation efficiency with NatMX_MaMat1 fragment insertion containing flanking regions and disruption cassette and Split-marker (70 bp) approach with two truncated fragments (pre-treated cells with LiAc prior transformation).

Transformation approach	Number of colonies (CFU/µg DNA)
Fragment insertion	150 ± 10
Split-marker insertion (70 bp)	89 ± 12

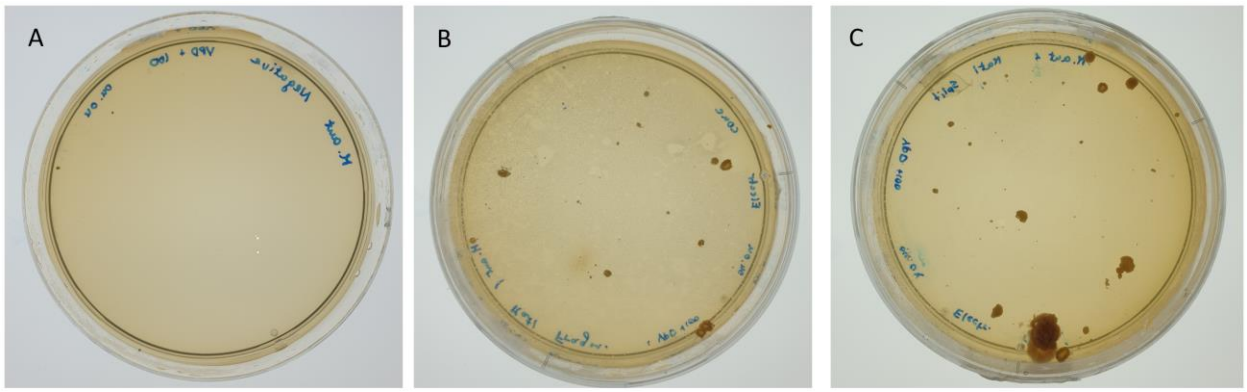

Figure 13: Transformation of *M. antarcticus* by electroporation with fragment containing *NatMX4* cassette and split-marker approach with two truncated fragments. A – negative control; B –transformants from fragment insertion containing *NatMX4* cassette; C – transformants from split-marker (70 bp) approach with two truncated fragment insertion.

3.5. Lithium acetate mediated transformation

Transformation of *M. antarcticus* with lithium acetate was performed to introduce NatMX_MaMat1 fragment into the host organism. Colonies were plated on YPD plates containing 100 µg/mL clonNAT for selection. Colonies started to appear after 3 days of incubation at 30ºC. Lithium acetate mediated transformation resulted in 119 ± 1 CFU/µg DNA. Mostly big colonies of transformants appeared on selection plate, while no colonies were observed on control plate (Figure 14). Transformation with NatMX_MaMat1 fragment and lithium acetate transformation resulted in 20.6% decrease of transformation efficiency compared to electroporation. Colonies were randomly selected and plated on regeneration medium plate with selection pressure. 50% of re-plated colonies grew with selection pressure.

Table 6: Transformation efficiency with Lithium acetate mediated transformation in *M. antarcticus*. Two dilution factors were used for plating and different number of colonies were observed.

Transformation approach	Number of colonies (CFU/µg DNA)
Fragment insertion	119 ± 1

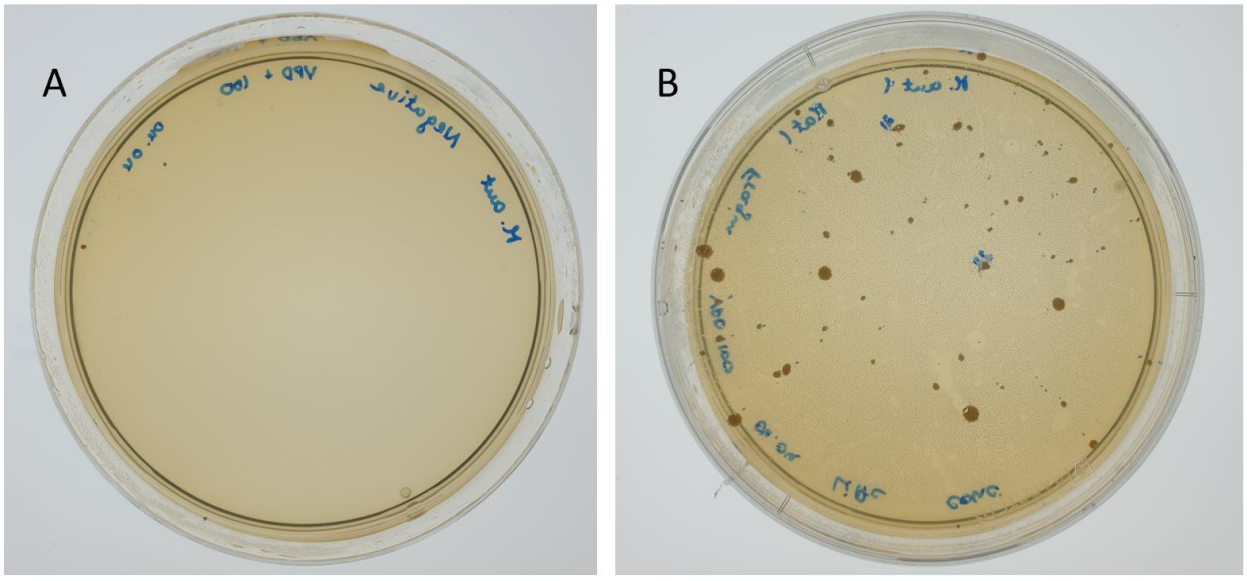

Figure 14: LiAc mediated transformation of M. antarcticus with fragment insertion. A – negative control; B – transformants with NatMX_MaMat1 fragment containing *NatMX4* cassette.

3.6. Transformation with RNP mediated approach

Transformation of *M. antarcticus* with NatMX_MaMat1 disruption cassette was performed with RNP mediation. Two different volumes of RNP mix with the same final concentration of 0.26 µM in transformation mix were tested. Test with 6.6 µL (4 µM) of RNP mix was unsuccessful, as during the electric pulse, cell suspension burst and pulse time decreased to 1.2 ms leading to 4 ± 1 CFU/µg DNA transformation efficiency. Obtained small colonies after re-plating on a new selection medium plate did not show any growth leading to no disruption cassette insertion. Reduction of RNP mix to 2.2 µL (7 µM) per transformation resulted in stable pulse time, which varied at 4.9-5.1 ms. Test of different RNP mix dilutions resulted in no transformant formation on plates with 0x and x10 dilution and 20 CFU/µg DNA with x100 RNP mix dilution. Colonies were re-plated on regeneration medium plates containing clonNAT and showed stable growth after 3 days of incubation.

3.7. Transformation of *M. antarcticus* with different length of split-markers mediated by RNP

Transformation of *M. antarcitus* was performed with pre-treatment step before transformation to increase transformation efficiency. Fragment NatMX_MaMat1 containing upstream and downstream flanking regions of *MAT1,* split-marker fragments with 70 bp overlap and split-marker fragments with 231 bp overlap were used for transformation with/without 0.07 µM RNP mix. Colonies appeared on the plate within 3 days of growth on selection medium at 30ºC. Different size of colonies was observed. From previous screenings it was observed that small colonies of mutants do not contain any *NatMX4* cassette insertion, therefore it was decided to count them as a background growth. All different transformation did not result any significant difference in transformation efficiency as it varied between 35-69 CFU/ µg DNA without RNP insertion and 44-80 with RNP. Transformation with split-marker fragments containing 231 bp overlap resulted in slightly higher transformation efficiency with 69 CFU/ µg DNA (without RNP) conpared to linear NatMX_MaMat1 fragment (40 CFU/ µg DNA) and split-marker fragments containing 70 bp overlap (35 CFU/ µg DNA).

Fragment and split-marker (70 bp overlap) showed increasement in transformation efficiency with RNP mediation resulting in 44 CFU/ µg DNA and 80 CFU/ µg DNA respectively while split-marker (231 bp overlap) had lower efficiency with RNP resulting in 60 CFU/ µg DNA (Table 7).

In general, transformation efficiencies did not vary significantly and it was possible to notice effect of RNP in slightly higher efficiency for two cases (Figure 15). However, to distinguish better targeting method, PCR screening needs to be performed for presence of *NatMX4* cassette and its integration locus.

Table 7: Transformation efficiency of *M. antarcticus* by electroporation. Three different approaches were tested without and with RNP mediation. Φ – diameter of a colonies (mm).

DNA fragment	CFU/ µg DNA	
	$\varphi > 0.5$	$\phi < 0.5$
Fragment	40	38
Split-marker (70 bp overhang)	35	33
Split-marker (231 bp overhang)	69	34
Fragment + RNP	44	23
Split-marker (70 bp overhang) + RNP	80	40
Split-marker (231 bp overhang) + RNP	60	22

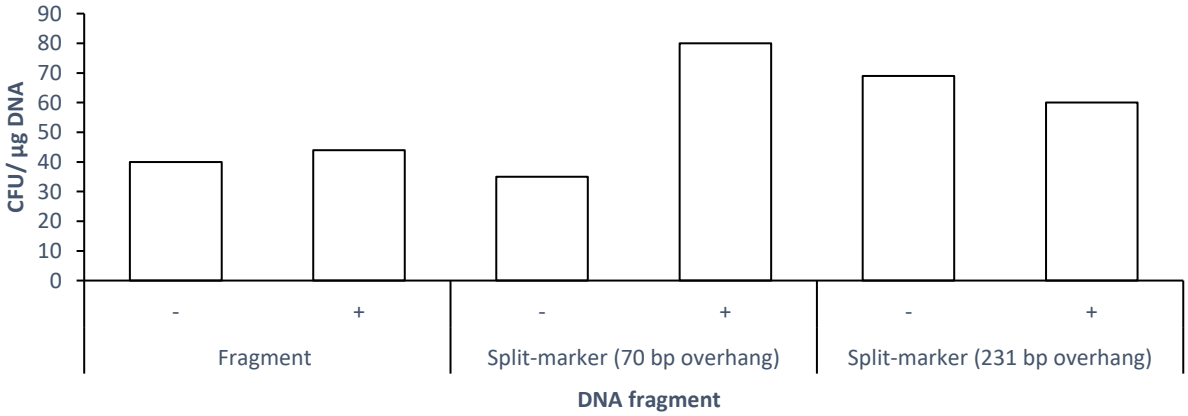

Figure 15: Visual representation of transformation efficiency of *M. antarcticus* by electroporation. Three different approaches were tested without and with RNP mediation. – without RNP, + with RNP mediation.

3.8. Screening of transformants

Screening of transformants was performed with two sets of primers. One set was with MaMat1-NatMX_FOR and MaMat1-NatMX_REV to see if *NatMX4* disruption cassette is inserted into the organism with expected size of 1200 bp. Another set of primers was Out_natMX_Mat1-5'FOR and Out_natMX_Mat1-3'REV to check if the insertion of the disruption cassette was in the right place as primers amplify outside of the target region with expected size of 3500 bp.

Transformation with fragment and split-marker (70 bp) approach

To confirm the presence of *NatMX4* cassette in obtained transformants, 14 colonies were picked from each fragment and split-marker transformations and analyzed by colony PCR. Screening of transformation with insertion of NatMX_MaMat1 fragment resulted in amplification of *NatMX4* cassette only in 35% of transformants with expected size of around 1200 bp (bands 1, 2, 4, 8, 12) while there was no *NatMX4* insertion or random insertion in the genome which leads to amplicons with double bands where *NatMX4* cassette is 1200 bp and target locus without replacement around 1500 bp (bands 6, 7, 9, 10, 11). Lane 14 was used as control with purified plasmid (Figure 16 A). Transformation with a split marker resulted in 71% correct amplicons which consist *NatMX4* cassette and is 2-fold more than insertion with linearized fragment (Figure 16 B). Transformant number 2 (named T2-3) was chosen as possible mutant and used for fermentation in later experiment to observe the difference in MEL formation against the wild type.

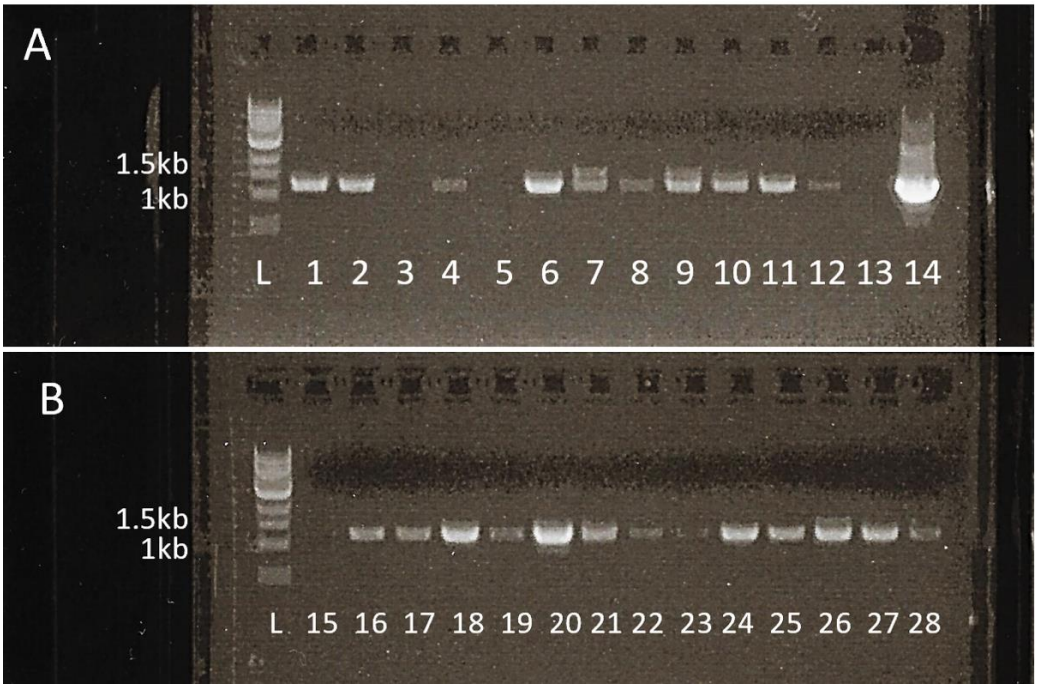

Figure 16: Colony PCR of *M. antarcticus* transformants. **A:** Transformants from NatMX_MaMat1 insertion. L – 1 kb DNA ladder; 1-13 – transformants obtained after fragments insertion where expected band with replacement is 1200 bp and without insertion is 1500 bp; line 14 represents purified plasmid as a control. **B:** Transformants obtained after split-marker (70 bp) approach (lane 15-28).

Amplification of outside the flanking site region was not successful with colony PCR and showed no band formation. Subsequently, gDNA was extracted from several different approaches where colonies grew on regeneration plates. For checking correct integration site, Out_natMX_Mat1-5'FOR and Out_natMX_Mat1-3'REV primer set was used for generation product of 3600 bp (with replacement) or 3900 bp (without replacement). All transformants were checked for *NatMX4* presence in the genome. Most of the transformants does not correspond to the expected size of 1200 bp for accurate *NatMX4* cassette insertion or have double bands, which leads to random integration events and growth on selection plate except mutant Nr4 which showed on-target mutation (Figure 17 A). Screening of transformants with Out_natMX_Mat1-5'FOR and Out_natMX_Mat1-3'REV primer set showed no integration as all bands corresponded to the same size as wild type organism (3900 bp) which was used as a control (Figure 17 B).

Two obtained colonies from RNP approach with 0.07 µM of gRNA:Cas9 mix also did not show the correct target as double band was observed from screening the presence of *NatMX4* cassette which lead to random event integration for dDNA (Figure 17 A). As a result, no changes

in size for outside of the flanking region screening was observed and no on-target transformants was obtained (Figure 17 B).

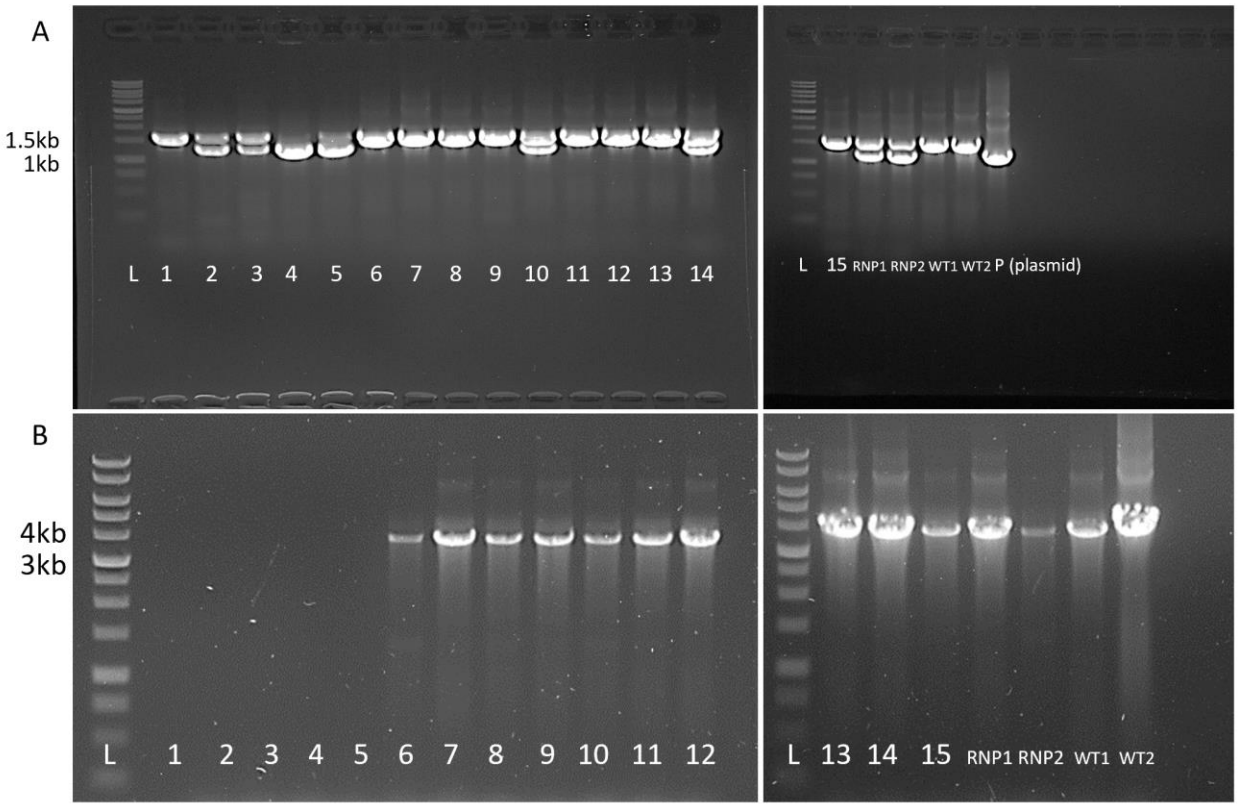

Figure 17: PCR amplification from gDNA of transformants. Picture **A** represents screening of *NatMX4* cassette with MaMat1-NatMX_FOR and MaMat1-NatMX_REV primer set which lead to amplification of 1200 bp fragment if *NatMX4* cassette insertion was on-target and 1500 bp for original MAT1 gene. L – 1 kb DNA ladder; bands 1-3 and 7-9 represent NatMX_MaMat1 fragment insertion by electroporation; bands 4-6 and 10-12 represent split-marker integration by electroporation; bands 13-15 represent NatMX_MaMat1 fragment insertion by LiAc mediated transformation; RNP1 and RNP2 represent two colonies obtained from NatMX_MaMat1 fragment insertion together with RNP complex (0.07 µM concentration); WT1 and WT2 represent amplicons from wild type strain of *M. antarcticus*; P – purified pUC57mini-NatMX_MaMat1 plasmid used as a control for the right amplicon of *NatMX4* cassette. Picture **B** shows the screening of outside of the flanking region which leads to 3600 bp amplicon if insertion was on-target and 3900 bp if there is no on-target insertion. Band number corresponds to the numbers from picture **A.**

3.9. Fermentation of MELs

Fermentation was performed in duplicates for both wild type and mutant T2-3 to check if there are any changes in MEL production for different MEL derivatives which are MEL-A, MEL-B, MEL-C and MEL-D. For optimal MEL production, fermentation was performed as described by Hlinický (2018) with 40 g/L of glycerol as a substrate for biomass formation at the first stage of

fermentation and 40 g/L glucose for synthesis of sugar moiety of MEL + 40 g/L rapseed oil for synthesis of fatty acid chains for MEL at day 4 as substrates for feeding.

Cell growth of *M. antarcticus* was observed by measuring optical density for both wild type and T2-3 mutant. Growth rate of both organisms was evaluated by calculating the slope in exponential phase of growth. It was determined that T2-3 mutant has slightly higher growth rate of 0.18 h^{-1} while wild type was estimated to be 0.136 h^{-1}. Stationary phase was reached at day 6 for both strains. (Figure 18).

Glycerol quantification was performed to observe its consumption and after 4 days of cell growth, it dropped until 18.3 g/L and 16.6 g/L with 0.24 h^{-1} and 0.257 h^{-1} glycerol consumption rate for wild type and T2-3 mutant respectively defining no cell starvation and relatively similar sugar consumption for formation of biomass.

Fermentation was performed in Baffled flask for better mixing and aeration of cells, but it should be noted that after day 4 cells were distributed on the walls of the flasks due to higher biomass formation and shaking, as a result, MEL production was performed with incomplete supernatant composition. Baffled flasks were considered as invalid for *M. antarcticus* batch fermentation.

During the fermentation, separation of oil phase was observed form the cell broth. Fermentation of the cultures was prolonged to 21 day as significant amount of cells was lost from the cell broth. After 21 day of fermentation, it was observed that red coloring oil phase appeared in one of the flasks from T2-3 mutant and precipitated on the bottom of the flask in the form of red beads. This analogy to hydrophobic substance considered to be extracellularly extracted MELs.

3.9.1 Quantification through HPLC

Test was performed to observe the difference in MEL formation from wild type and T2-3 mutant strains. Samples were prepared by extracting MELs with ethyl acetate (1:1 ratio) and run through HPLC equipped with silica gel column. Test quantifications of 21-day fermentation samples were run through HPLC to observe total MEL concentration and its products. Group of peaks was observed within 35 min rotation time. 7+ peaks appeared starting with elution at minute

1 corresponding to ethyl acetate and at minute 3.5 rapeseed oil and 4 peaks followed which corresponds to MEL-A, B, C, D (Appendix iv). No difference between wild type and mutant was observed which lead to conclusion of no disruption in *MAT1* gene site. Samples for MEL quantification from day 4 until day 21 were prepared but due to technical issues with the equipment, could not be proceeded, leading to unknown concentration of MELs throughout the fermentation and unable comparison in MEL formation between two strains.

For sugar quantification, HPLC machine equipped with silica gel column was used in order to develop a new method, able to determine MELs and sugars throughout the same run on the samples. Standards of glycerol, glucose and rapeseed oil were prepared for quantification by extraction with ethyl acetate. Original rotation time used for MEL quantification of 30 min lead to no peak formation of sugars. Time was increased until 35, 40 min with no successful detection. Mobile phases were switched to methanol-chloroform with the same linear gradient resulting in no sugar detection. It was assumed that sugars cannot be detected through the same method used for MEL quantification.

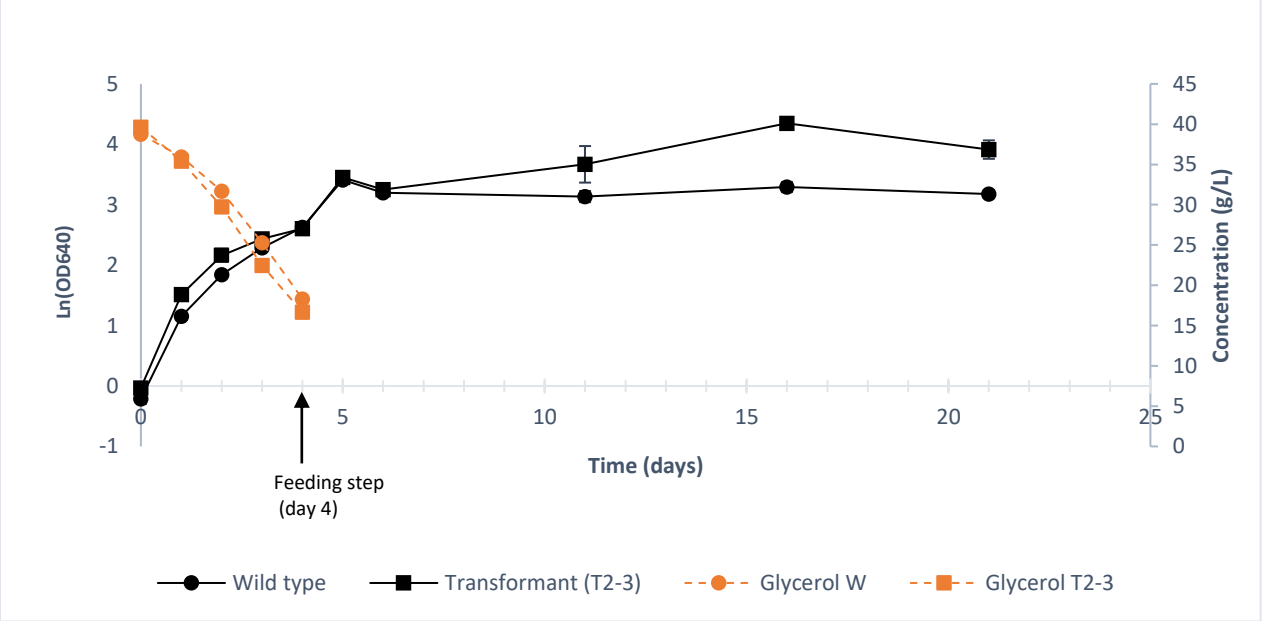

Figure 18: Fermentation experiment for wild and T2-3 mutant.

4. Discussion

4.1. Transformation of *M. antarcticus*

Other studies reveled using *P. Antarctica* GB-4(0) strain which demonstrates smooth yeast-like form under the same culture conditions which can lead to more stable transformation efficiency (Yarimizu et al., 2017; Kunitake, et al., 2019). For transformation of *M. antarcticus*, it is very crucial to have stable cell composition as type strain tend to form hyphae form in chains and branches and causing difficulties for separation and isolation of single cells. Adapted cell growth had to be performed in order to achieve fast growth from carbon-rich medium and yeast-like shape for further experiments. It is hypothesized that hyphae form of cells decreases the efficiency of transformation due to formation of clumps and branches leading to smaller cell wall surface available for the DNA insertion into the host.

Transformation by electroporation was carried with few modifications to check if pre-treatment of the cells with LiAc prior transformation can inhibit or increase transformation efficiency. It was revealed that LiAc treatment used in Grahl et al. (2017) for treatment of *Candida* species increased transformation efficiency 3-folds compared to non-treated cells where treated cells resulted in 150 ± 10 CFU/ µg DNA for fragment insertion and 89 ± 12 CFU/ µg DNA for split-marker approach. Fragment insertion with *NatMX4* selection cassette was used in Yarimizu, et al. (2017) and resulted in 36 CFU/ 6.9 µg DNA and 96 CFU/ 5 µg DNA of big colonies for different fragment insertions with the same selection pressure of clonNAT (100 µg/mL). It shows that treatment of the cells prior transformation can significantly increase transformation efficiency although higher selection pressure needs to be used for selection to avoid small colonies as in some cases only 35% of the mutants were carying *NatMX4* cassette for fragment insertion and 71% for split-marker approach.

Lithium acetate mediated transformation was also successful after several trials and yielded 119 ± 1 CFU/ µg DNA with NatMX_MaMat1 fragment insertion. Efficiency is sligtly lower than described by Yarimizu, et al. (2017) with 145 CFU/µg DNA. Eventhough transformation efficiency with LiAc was slightly lower for disruption fragment insertion, but it has an advante of

carrying the whole reaction in a single plastic Eppendorf tube and does not require expensive electroporation cuvettes which makes it more convenient for large number of transformations. However, selection of mutants and different gene engineering techniques may influence transformation efficiency (Saika et al., 2018).

On another hand, LiAc mediated transformation was not sufficient for further work with RNP experiments as it is degrading DNA, RNA proteins, in this case Cas9 protein (Norcum, 1991).

4.2. Disruption cassette for *MAT1* targeting

Since transformation efficiency for *M. antarcticus* is not significant and homologous recombination is inefficient, gene knockout strategy was used for allele disruption without the original pUC57 plasmid and only with PCR amplified disruption fragments. For targeting *MAT1*, plasdmi containing around 1 kb upstream and downstream regions of *MAT1* from each side of *natMX4* selection cassette was constructed. The *natMX4* gene was developed as a selection marker for *S. cerevisiae* containing three parts: TEF gene promoter, a dominant drug resistance ORF (nourseothricin) and transcriptional terminator from the TEF gene (Goldstein & McCusker, 1999). It was proved that *NatMX4* could be used as a selection marker in *P. antarctica* GB-4(0) where 85-100% transformants were selected as cassette containing mutants (Yarimizu et al., 2017). Previous approaches implementing the usage of selectable markers such as hydromycin B or neomycin/G418 in *P. antarctica* by integration into chromosome by electroporation resulted in low efficiency of gene targeting. In this study, insertion of *NatMX4* disruption cassette together with 1 kb flanking regions for *MAT1* targeting resulted only in 35% presence of *NatMX4* with fragment insertion and 71% with split-marker approach leading to lower efficiency of target than mentioned in other studies. Result from Yarimizu et al. (2017) revealed that gene targeting is relatively infrequent compared with random integration due to lower than 10% of on-target efficiency in *M. antarcticus* for *URA3* disruption which could lead to even lower efficiency for targeting *MAT1* as none of the screenings revealed possitive on-target gene replacement.

Moreover, only one study performed by Konishi & Makino (2018) was successful for *MAT1* gene disruption in close related specie *Pseudozyma hubeiensis*. Disruption efficiency was only 2% where only 1 out of 47 mutants was obtained with insertion of gene disruption fragment containing

5’ and 3’ flanking regions with hygromycin B resistant gene cassette. No studies were made for *MAT1* disruption in *M. antarcticus* leading to uknown disruption efficiency.

4.3. Homologous recombination

In this study, homologous recombination was a key repair mechanism for gene replacement technique. In most of the cases, 90% percent of screened mutants were showing double bands on agar gel which correspond to *NatMX4* fragment and *MAT1* gene with no mutation in a wild type strain. It was concluded that *NatMX4* fragment was randomly integrated into the chromosome by non-homologous end joining (NHEJ) in these mutants. Gene targeting of the desired site can outcome in off-targeting transformation occurrence as random integration of DNA by non-homologous end-joining (NHEJ), tandem repeat formation by DNA integration neighboring target site, heteroallelic transformants due to diploid strain where only one allele being transformed (Štafa et al., 2017). Moreover, low gene-targeting efficiency can be caused by predominant double-strand break (DSB) repair by NHEJ compared to HR, which is preferable in *M. antarcticus* (Kunitake et al., 2019).

It is also possible to improve the efficiency of gene targeting in different yeast and fungi by disruption of genes responsible for NHEJ such as orthologs of the *ku70*/*ku80* or *lig4*. By targeting these genes, it was observed significant increase in targeting efficiency of approximately 100% (Krappmann, 2007). On another hand, obtained mutants are sensitive to different mutagens (methyl methane sulfonate and ultraviolet) or antibiotics (phleomycin, bleomycin). Due to high risk of stress, mutants cannot be suitable for industrial applications. Selectable marker gene usage for disruption of essential genes in NHEJ in *M. antarcticus* is low efficient due to limited selectable marker gene availability in this species (Kunitake et al., 2019).

It should be noted, that knockout mutations in haploid organisms as *M. antarcticus* strain used in this research, yield viable cells only if the disrupted gene is non-essential (Madigan et al., 2015).

Efficient gene targeting in *M. antarcticus* is dependent on numerous amount of factors and part of them can remodeled for increasion of gene targeting fidelity. Some articles describes 40 bp homology region sufficient for gene replacement in *S. cerevisiae*, although usage of longer DNA

flanking regions usually results in increment of efficiency. Furthermore, higher GC content of the flanking regions rises the fidelity of the gene targeting (Štafa et al., 2017; Manthey et al., 2004; Gray & Honigberg, 2001). Researches in other yeast species determined that low homology region is sufficient to initiate recombination of targeted gene and recombination efficiency can be increased exponentially by increasing the size of the flanking regions up to 1kb (Kung et al., 2013; Morita et al., 2010). Due to minimal flanking region reported above, it was chosen to use 70 bp homology region for overlapping of split-markers with around 1 kb homology region upstream and downstream the target site. However, it is described that sufficient size for flanking regions is critical when applying slip-marker approach and should contain not less than 500 bp length, although the minimal size for efficient recombination of selective marker is unclear and can vary depending on a species. For fungal targeting, successful recombination achieved by DNA fragments overlapping at a range of 200-450 bp for selection gene or using 2/3rd size of a split markers for recombination (Chung & Lee, 2015; Goswami, 2011). Due to that, other split-markers were designed to have 231 bp overlap over nourseothricin gene. Transformation with bigger overlap between truncated fragments was successful, however transformants need to be screened for a clear picture of targeting.

4.4. RNP mediated transformation

RNP mediated transformation was performed with two different volumes of RNP mix: 6.6 µL and 2.1 µL with 1.8 µM and 7 µM of mix concentration relatively. Higher volume of RNP in transformation mix resulted in a burst of cell suspension which was due to too large volume of mix diluting the cell suspension and increasement of salts. It was decided to decrease RNP mix volume until 2.1 µL but to keep the same final concentration of RNPs (0.255 µM) and decrease the dilution of cell suspension. Electroporation procedure was performed successfully. However, first transformation resulted only in 2 transformants/ µg DNA which showed no on-target insertion. As it was described in Kunitake, et al. (2019), RNP mediated transformation in *P. antarctica* increases freaquency of target gene manipulation with 25 bp homologous regions and even higher frequencies by increasing the lengh of homology arms. In this research, homology arm length was around 1 kb, which is more specific for gene replacement. RNP mix used in this

experiment was prepared acording to Grahl et al. (2017) where they used RNP for knocking out known or putative catalase genes in diverse *Candida* species with molar ratio of RNP 1.2:1 (sgRNA:Cas9). After converting molarity to grams of product used, it was noticed that ration of Cas9 is 3-fold higher than sgRNA resulting in 1:3.4 ration sgRNA:Cas9 (0.739 µg sgRNA : 2.52 µg Cas9). It is hypotesized, that too large amount of Cas9 may inhibit formation of RNP complex or cause random cleavages within a cell. In Kunitake, et al. (2019) research, they used *P. antarctica* as a host organism for *URA3, PaADE2, PaCLE1* gene manipulation mediated by RNP complex with 1:2 ration of sgRNA:Cas9 in weight (0.3 µg of sgRNA and 0.6 µg of Cas9 protein with 0x (no RNP), x0.5 (0.3 µg of sgRNA and 0.6 µg of Cas9), x1 (0.6 µg of sgRNA and 1.2 µg of Cas9), and x2 (1.2 µg of sgRNA and 2.4 µg of Cas9 concentrations) which resulted in incresement of on-target mutations at all cases using RNP and significantly increased HR efficiency of knockout cassette insertion (3-4-folds depending on RNP concentration). More screening for obtained transformants from the last experimt with 0.07 µM RNP mix needs to performed although number of transformants do not vary depending on RNP usage but target efficiency may vary with fragment and two spilit-marker approaches (70 bp and 231bp overlaps). Moreover, Kunitake, et al. (2019) described an new methood for *P. antarcticus* transformation with polyethylene glycol-mediation by creating protoplast which fully degrades cell wall of the host and allows easier integration of DNA.

The problem of achieving low transformation efficiency with RNP could be because of the particular sgRNA on-target activity which was described to be 96%. The guide was designed through IDT Alt-R design tool where insertion of target locus is limited to 1 kb and *MAT1* gene contains 1,5 kb sequence. It had to be screened separately by introducing different regions of target gene and obtaining highest possible on-target score. Moreover, it does not contain the off-target score for designed guide as no analysis for score rate was performed in *M. antarcticus* to confirm the guide cleavage efficiency, however, test for *in vitro* DNA cleavage activity may be designed and performed through PCR reaction.

4.5. Fermentation of *M. antarcticus*

M. antarcticus is described as one of the most promising and excellent organisms for MEL production. Although the highest titer of MELs is recorded to be 165 g/L by close related specie *M. aphidis* in fed-batch bioreactor with continuous feeding of glucose and oil (Rau et al., 2005). It was reported that by usage of *n*-octadecane as a substrate for MEL production by *M. antarcticus T-34*, titer of MELs reached 140 g/L, while highest titer by non-sythetic substrate was 47 g/L (soybean oil) (Kitamoto et al., 2001; Kitamoto et al., 1992). Fermentation was performed according to feeding steps described in Hlinický (2018) where MEL titer was 47 g/L by adding glycerol for cell biomass growth and at day 4 feeding it with glucose and rapeseed oil for synthesis of MELs. This feeding methods was chosen as having higher productivity (3.4 g/L/day) compared to glycerol as a start substrate and rapeseed oil for feeding (2.4 g/L/day). Expected results would be lower than described above due to usage of Buffled flasks instead of Erlenmaeyr as cell biomass mass lost from the cell broth and spread across the flask walls. The shake flask fermentation in this study increased the time of fermentation until 21 day instead of 14 days as it was described in Hlinický (2018), which makes Buffled flasks not sutable for long cell cultivation. One more advantage of feeding step with both sugars along with oil is shortening time for MEL sythesis as glucose can be used directly for sythesis of sugar moyiety and rapeseed oil used for sythesis of faty acid chain, consequently increasing production rate compared to feeding only with oil where cell had to undergo sythesis of glucose prior production of MELs. Formation of red MEL beads was observed after 21 day only in one of the flasks which describes <40 g MEL/L other flasks. Moreover, formation of MEL beads indicates enhaced product formation which leads to increacement in critical micelle concentration (CMC) allowing MELs to parcipitate on the bottom of the bioreactor resulting in easier extraction and decreation of dowstream extraction processes (Rau et al., 2005).

Development of new method for sugar quantification simultaneously with MELs through silica gel column was unsuccessful as no peaks appeared corresponding to sugar signals. Sugar extraction by using ethyl acetate as a solvent could lead to no sugar solubilization, subsequently no product detected (Tsavas et al., 2002). More polar solvent had to be used for sugar detection.

5. Future perspectives

Transformation of *M. antarcticus* with *NatMX4* selection cassette resulted in comparably low transformation efficiency making it difficult to select the right mutant due to low on-target efficiency which may be lower than 2%. Due to that, more efficient transformation protocol needs to be applied such as protoplast transformation described by Kunitake, et al. (2019) for *P. antarctica* yeast and more mutants checked. Last transformation experiment was performed with split-marker approach containing 231bp together with RNP 0.07 µM and big colonies were found on the plate. Colonies need to undergo genomic DNA extraction procedure and PCR screening for presence of *NatMX4* cassette and it presence in the right integration locus to observe efficiency of split-marker with larger overlap between two truncated fragments. Together with this experiment, RNP was applied with fragment (NatMX_MaMat1 – downstream and upstream regions of target gene together with *NatMX4* selection cassette) and split-marker with 70 bp overhang. Both experiments yielded colonies on selection plate and needs to be checked for RNP on-target integration efficiency and observe better method for *MAT1* gene disruption.

Although, obtained colonies from the first RNP transformation did not show any on-target integration of disruption fragment, but just random *NatMX4* integration into the chromosome, sequencing of colonies needs to be performed. RNP contains sgDNA sequence for *MAT1* targeting and ability to cause DSB which later can lead to NHEJ with mismatch mutations and gene knockdown. Moreover, *in vitro* DNA cleavage assay may be performed to observe RNP ability to induce DSB in the right locus.

Plasmid system containing CRISPR-Cas9 can be tested with the same sgDNA as it was tested for RNP approach and observe the efficiency of plasmid integration and target efficiency against single RNP complex with dDNA implementation.

Empty plasmid pUC57 was created for usage in targeting other genes responsible in MELs biosynthetic pathway such as *EMT1*, *MAC1*, *MAC2* by amplifying a new flanking regions and construction of disruption fragments. It is relevant to observe how disruption of different genes responsible for acylation can change formation of MEL and may create a new, more efficient derivatives for certain applications depending on their physical and chemical characteristics.

6. Conclusion

It this study, it was demonstrated that *NatMX4* cassette can be used as a selection marker for basidiomycetous yeast *M. antarcticus* transformations. Treatment of cell prior transformation by electroporation is also crucial due to its ability to increase transformation efficiency dramatically. Gene engineering of *M. antarcticus* by targeting *MAT1* gene was unsuccessful as none of the methods resulted in positive gene disruption. It is possible to observe that split-marker approach with 70 bp overlap results in 2-fold higher selection cassette integration compared with PCR amplified disruption fragment with downstream and upstream flanking regions of target gene embedding selection marker cassette however in both cases homologous recombination efficiency was low and selection marker cassette got integrated randomly in the genome. Split-marker approach with 231 bp overlap at the selection cassette locus between two truncated fragments resulted in 2-fold higher transformation efficiency compared to previously described methods. RNP-mediated gene targeting resulted in obtaining colonies only at a low concentration of 0.07 µM of RNP mix where transformation efficiency increased slightly for fragment and split-marker (70 bp overlap) while no significant difference observed with other split-marker (231 bp overlap) approach. Screening of the split-marker (231 bp overlap) needs to be carried out in order to investigate its on-target efficiency. In summary, low homologous recombination efficiency in *M. antarcticus* for *MAT1* gene targeting leads to complications using only fragment replacement and requires more efficient techniques for targeting. Unscreened colonies had to be checked for RNP effect in targeting efficiency.

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Appendix

i. PCR using Dream Taq Green DNA Polymerase (Thermo Scientific)

Reaction was carried for colony PCR of *M. antarcticus*.

1. **Reaction setup:**

Components	**Volume (µL)**
10X Standard Taq Reaction Buffer	0.95
10 mM dNTPs	0.19
10 µM Forward Primer	0.19
10 µM Reverse Primer	0.19
Taq DNA Polymerase	0.0475
Template DNA	5
Nuclease-free water	3.4325

2. **Thermocycling conditions**

Steps	**Temperature**	**Time**
Initial Denaturation	95°C	3 minutes
25-40 cycles	95°C	30 seconds
	*55°C	30 seconds
	72°C	1 minute/kb
Final Extension	72°C	10 minutes
Hold	4°C	-

*temperature dependent on Tm value of primers. All primers used in this experiment had almost the same Tm value of 54-55°C.

ii. PCR using Q5 Hot Start High-fidelity DNA Polymerase

1. Reaction setup:

Components	**Volume (µL)**
5X Q5 Reaction Buffer	10
10 mM dNTPs	1
10 µM Forward Primer	2.5
10 µM Reverse Primer	2.5
Template DNA	< 1 µg
Q5 High-Fidelity DNA Polymerase	0.5
Nuclease-Free Water	to 50 µL
Total Volume	**50**

2. Thermocycling conditions

Steps	**Temperature**	**Time**
Initial Denaturation	98°C	30 seconds
25-35 cycles	98°C	10 seconds
	*67-68°C	30 seconds
	72°C	20–30 seconds/kb
Final Extension	72°C	2 minutes
Hold	4–10°C	

*temperature dependent on Tm value of primers. All primers used in this experiment had almost the same Tm value of 67-68°C.

iii. Genomic DNA extraction from yeast

Chemicals, solutions and materials:

- Lysis buffer
- Phenol:Chloroform:Isoamyl alcohol 25:24:1
- Isopropanol
- Ethanol 70% (v/v) (-20°C)
- TE buffer (pH 8)
- Sterile pure water
- Sterile 1.5 mL tubes
- Thermomixer (65°C)

Protocol:

1. Suspend one loop of cells in 100 µL of Lysis Buffer and mix thoroughly.
2. Incubate 30 min (at least) at 65°C.
3. Add 200 µL of phenol:chloroform:isoamyl alcohol 25:24:1 (in a fume hood)
4. Gently mix by inversion (approx. 20 times).
5. Centrifuge 15 min at 14000 rpm.
6. Transfer the aqueous phase (approx. 100 µL) to a new tube.
7. Add 1 volume (approx. 100 µL) of isopropanol.
8. Gently mix by inversion (approx. 20 times).
9. Incubate at room temperature for 15 min.
10. Centrifuge 10 min at 14000 rpm.
11. Remove the supernatant.
12. Add 200 µL of ethanol 70% (v/v) (20°C).
13. Gently mix by inversion (approx. 20 times).
14. Centrifuge 10 min at 14000 rpm.
15. Repeat steps 12-14.
16. Remove supernatant.
17. Dry the DNA at room temperature.
18. Dissolve the DNA in 50 µL TE.

Lysis buffer:	(for 500 mL)
50 mM Tris-HCl	25 mL of 1 M Tris-HCl (pH 7.5-8.0) – sterilize in autoclave
5 mM EDTA	5 mL of 500 mM EDTA – sterilize the water in autoclave (not the EDTA solution)
100 mM NaCl	50 mL of 1 M NaCl – sterilize in autoclave
1% (w/v) SDS	50 mL of 10% (w/v) SDS – sterilize the water in autoclave (not the SDS solution)

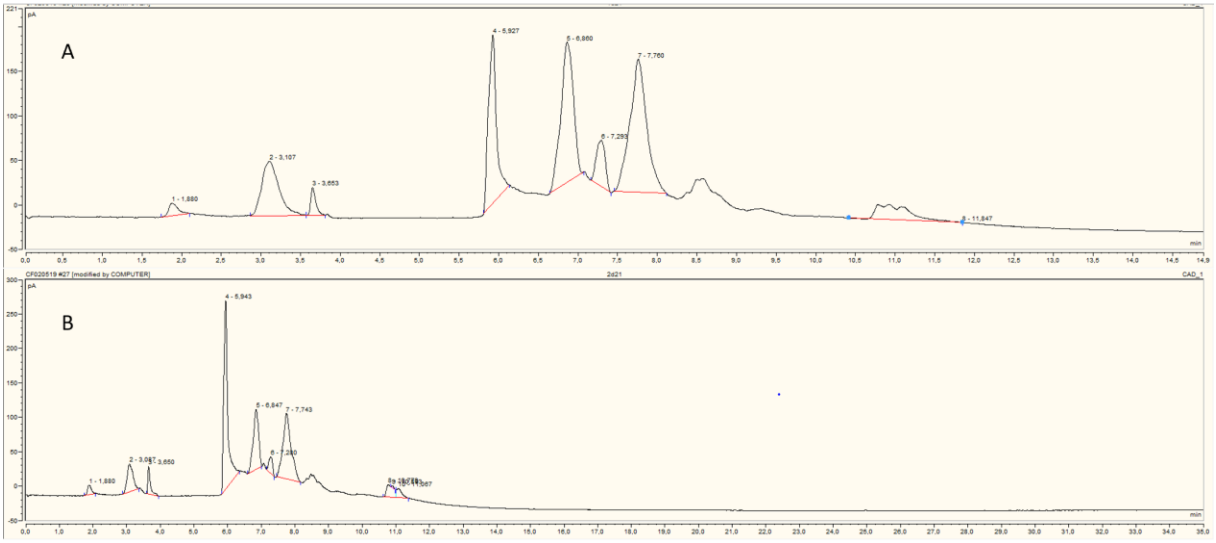
TE buffer:	(for 200 mL)
10 mM Tris-HCl	2 mL of 1 M Tris-HCl (pH 7.5-8.0) – sterilize in autoclave
1 mM EDTA	0.4 mL of 500 mM EDTA – sterilize the water in autoclave (not the EDTA solution)

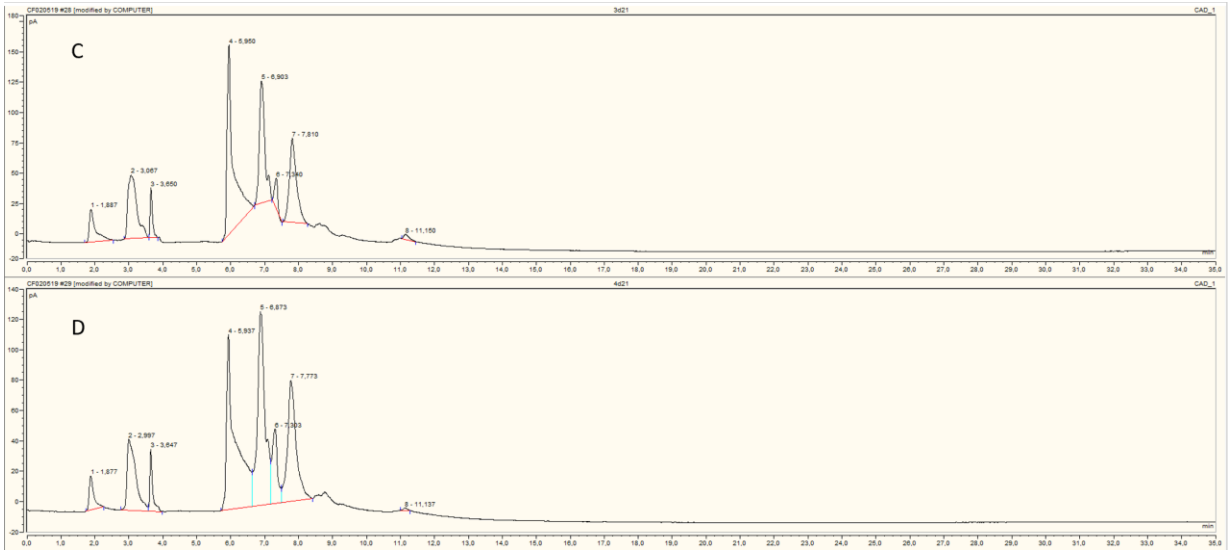
iv. HPLC test run of 21-day samples from wild type and T2-3 mutant

A – wild type strain C – T2-3 mutant

B – wild type strain D – T2-3 mutant

First 2 peaks represent ethyl acetate elution, peak Nr3 corresponds to rapeseed oil and 4 following peaks corresponds to 4 different derivatives of MELs. No difference between wild type and mutant observed.





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