Metabolic Engineering for Enhancement of Itaconic Acid Production in Ustilago including Introduction of the QDR3 gene from Saccharomyces cerevisiae

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Abstract: *U. maydis* is a natural itaconic acid producer and a promising candidate for industrial production of this acid due to its yeast-like morphology that represents a great advantage to the current status: fermentation with filamentous fungi *A. terreus*. Previous studies in *U. maydis* already improved IA titers. However, the yields are still below the ones obtained with *A. terreus*. Aiming to enhance itaconate production of the best-producing *U. maydis* strain obtained so far, the XXX gene was deleted by homologous recombination. Moreover, mitochondrial transporters from natural itaconate producers (*At_mttA, Um_mtt1* and *UcN_mtt1*) were overexpressed. The combined effect of XXX deletion and *At_mttA* overexpression led to a 56% increase in the final itaconate concentration and 37% increase in yield when compared to the best-producing strain at the starting point of this thesis. Moreover, the QDR3 gene, which encodes for an MFS transporter responsible for MDR in *S. cerevisiae* was overexpressed in various *U. maydis* strains. The results showed that QDR3 does not complement *ipt1* function in *U. maydis*.

Keywords: Itaconic acid, XXX, mitochondrial transporters, QDR3

1. Introduction

Itaconic acid (IA) (C₅H₆O₄) has been gaining increasing interest since it was identified as one of the most promising and flexible building blocks derived from biomass (Werpy & Petersen, 2004) that can replace petrochemical-based monomers and therefore contribute to the sustainable development of the chemical industry (Willke & Vorlop, 2001).

The commercial uses of this organic acid include plastics, adhesives, elastomers and coatings that result from the polymerisation of methyl, ethyl or vinyl esters of IA (Willke & Vorlop, 2001). In terms of size, IA market surpassed 75 million US$ in 2015 (Global Market Insights, 2016) and is expected to grow, exceeding 216 million US$ by 2020 (Global Industry Analysts Inc., 2016). Despite the wide range of applications and market growth opportunities, the high cost of production of IA is still limiting its applications and remains an obstacle to be tackled in face of the availability of cheap substitutes like acetone cyanohydrins (Global Industry Analysts Inc., 2016).

Among the natural IA producing organisms there are *Aspergillus terreus* and *Ustilago maydis* (Guevarra & Tabuchi, 1990; Klement & Büchs, 2012). The current status in commercial production of IA is submerged fermentation with *A. terreus* that yields up to 86 g L⁻¹ when the process is optimized (Kuenz, Gallenmüller, Willke, & Vorlop, 2012). Nevertheless, *A. terreus* is a filamentous fungi which brings several challenges during fermentation such as high viscosity of the broth, clogging and a difficult balance between enough agitation power input for sufficient oxygen transfer and the mechanical stress caused by stirring that can break the mycelia (Geiser et al., 2015; Klement & Büchs, 2012).

Alternatively, *U. maydis* provides a major advantage in submerged fermentations due to its yeast-like morphology. Furthermore, it is a model organism for mating, pathogenicity, signal transduction and genomics having therefore its genome well-characterized (Martinez-Espinoza, García-Pedrajas, & Gold, 2002). The rising biotechnological interest in *U. maydis* is also a result of the natural production of several secondary metabolites such as organic acids (Guevarra & Tabuchi, 1990), glycolipids, siderophores and tryptophan-derived compounds (Böker, Basse, & Schirawski, 2008). Moreover, *U. maydis* a promising organism for production of value-added chemicals like IA using lignocellulosic biomass as raw material (Geiser, Wierckx, Zimmermann, & Blank, 2013).
Recently, all genes involved in the itaconate pathway in *U. maydis* have been identified as a cluster (Geiser et al., 2016). This pathway is compartmentalized between the mitochondria and the cytosol and starts with the transport of cis-aconitate to the cytoplasm by the mitochondrial tricarboxylate transporter (Mtt1) where it is isomerized to the unusual intermediate trans-aconitate by the aconitate-Δ-isomerase (Adi1). Finally, itaconate is formed by decarboxylation of trans-aconitate by the critical enzyme trans-aconitate decarboxylase (Tad1) (Geiser et al., 2015). Once itaconate is in the cytoplasm, it can be either directly exported by Itp1, the itaconate transport protein, or further converted to 2-hydroxyparaconate by the P450 monoxygenase (Cyp3) and then exported by the same transporter (Geiser et al. 2016). Another important gene in the cluster is ria1, which codes for a transcription factor that regulates all the genes in the cluster (Geiser et al., 2016). Iaconate yield improved more than twofold when cluster genes mt1 and ria1 were overexpressed. Since ria1 is probably upregulating all cluster genes including mt1 this results suggest that the mitochondrial transporter Mt1 is likely a bottleneck for itaconate production in *U. maydis* (Geiser et al., 2015).

The identification and characterization of itaconate pathway genes and enzymes layed ground for further metabolic engineering. Simultaneous deletion of the cyp3 gene and overexpression of ria1 increased itaconate production by almost 4-fold (compared to the wildtype) and ceased the production of 2-hydroxyparaconate (Geiser et al., 2016). Nevertheless, itaconate degradation was still verified which indicates the existence of other pathways of itaconate degradation in *U. maydis* (Geiser et al., 2016) that could be similar to the one recently reported in *A. terreus* (Chen, Huang, Zhong, Li, & Lu, 2016). Moreover, the malate titer of the engineered strain decreased 42% when compared to the wild-type. This reduction was associated with the upregulation of Mt1 since this transporter is likely to antiport malate with cis-aconitate into the mitochondria (Geiser et al., 2016). This function has already been identified in the *A. terreus* homolog MttA (Jaklitsch, Kubicek, & Scrutton, 1991).

As stated before, the pathway towards IA is compartmentalized between the mitochondria and the cytosol, which gives foremost importance to the transport reactions (Steiger, Punt, Ram, Mattanovich, & Sauer, 2016). In *A. terreus* this transport is done by the mitochondrial carrier protein MttA and in *U. maydis* by its homolog Mtt1 with 35% sequence similarity on protein level (Steiger et al., 2016). Previous unpublished studies by Tehrani showed a 50% and 35% increase in itaconate production when mt1 and AT_mttA, respectively, are overexpressed in *U. maydis*.

The other transporter involved in itaconate biosynthesis is the itaconate transport protein Itp1 that is responsible for the export of itaconate to the extracellular space and is annotated as a transporter of the major facilitator superfamily (MFS) (Geiser et al., 2015). The MFS includes a family of drug-H+ antiporters that confer multi-drug resistance (MDR) and several of this transporters have been associated to yeast response to weak acid toxicity by exporting the counter ions RCOO⁻ (Sá-Correia, dos Santos, Teixeira, Cabrito, & Mira, 2009a). The ODR3 gene in *S. cerevisiae* codes for a drug:H⁺ antiporter that belongs to the cluster I of the DHA12 drug efflux family. Expression of ODR3 is required for increased tolerance of *S. cerevisiae* to a broad range of cytotoxic compounds, structurally and functionally unrelated (Tenreiro, Vargas, Teixeira, Magnani, & Sá-Correia, 2005). Previous studies showed that overexpression of this transporter leads to a substantial reduction in IA accumulation being therefore crucial for the survival of the cells in the presence of (Nicole Martins Rodrigues, 2014). A possible explanation for these results is that Qdr3 directly exports the counter-ion (CsH₂O₄²⁻) to the cytosol. Alternatively, there is a possibility that Qdr3 translocates natural substrates whose homeostasis influences the accumulation of itaconate inside the cells (Sá-Correia, dos Santos, Teixeira, Cabrito, & Mira, 2009b).

### 2. Experimental Procedures

#### 2.1. Genetic Modification of Strains and Transformant Selection

All *U. maydis* strains used in this work are listed in Table I. *U. maydis* KO-cyp3 + KO-XXX + P_eto strain was constructed using FLP/FRT recombination system. For this, plasmid pJET1.2_Um_FRT- KO-XXX was constructed by Gibson assembly using Gibson Assembly® Cloning Kit (New England BioLabs, according to the manual) of four DNA fragments: the backbone plasmid pJET1.2 from CloneJET PCR Cloning Kit Mix (ThermoFischer Scientific), a hygromycin cassette and two FRT sites, flanking region 1 and flaking region 2 of the XXX gene. The Hyg cassette and the FRT sites make up the KO construct that was integrated in the genome of *U. maydis* strain 4175 (Table I) by homologous recombination.
The remaining *U. maydis* strains created during this work were constructed by integration of the overexpression constructs (etef promoter, Tnos and the gene to be overexpressed) in the ip-locus.

### Table I – List of *U. maydis* strains used and created during this work.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ustilago maydis</em></td>
<td>MB215 2229</td>
<td>a2b13</td>
<td>Prof. Dr. Michael Böcker, University Marburg (3048)</td>
</tr>
</tbody>
</table>
| *Ustilago maydis*         | MB215 KO-cyp3 + P

  etef

#6 | MB215 ∆UMAG_05074 + properties of integrated P

  etef

<table>
<thead>
<tr>
<th>MB215 KO-ipt1</th>
<th>MB215 ∆UMAG_11777 #23</th>
<th>Strain collection IAMB, RWTH Aachen (2329)</th>
</tr>
</thead>
</table>
| MB215 KO-cyp3 + KO-XXX + P

  etef

#6 | MB215 ∆UMAG_05074 + properties of integrated P

  etef

| MB215 + P

  etef

QDR3 #1, #3, #4 and #7 | WT + properties of integrated P

  etef

QDR3 | This thesis |
| MB215 KO-ipt1 + P

  etef

QDR3 #4, #5, #9 and #11 | MB215 ∆UMAG_11777 + properties of integrated P

  etef

QDR3 | This thesis |
| MB215 KO-cyp3 + Petef#6 + P

  etef

QDR3 #1-4 | MB215 ∆UMAG_05074 + properties of integrated P

  etef

QDR3 | This thesis |
| MB215 KO-cyp3 KO-XXX + P

  etef

#1, #2, #8 and #9 | MB215 ∆UMAG_05074 + properties of integrated P

  etef

QDR3 | This thesis |
| MB215 KO-cyp3 KO-XXX + P

  etef

+ P

etef

cbx_A_ter_mtt #4, #14 and #15 | MB215 ∆UMAG_05074 + properties of integrated P

  etef

cbx_A_ter_mtt | This thesis |
| MB215 KO-cyp3 KO-XXX + P

  etef

+ P

etef

cbx_05079 #2-5 | MB215 ∆UMAG_05074 + properties of integrated P

  etef

cbx_05079 | This thesis |
| MB215 KO-cyp3 KO-XXX + P

  etef

+ P

etef

cbx_05079 #6 #1, #5, #7 and #9 | MB215 ∆UMAG_05074 + properties of integrated P

  etef

cbx_05079#6 | This thesis |

Amplification of DNA fragments was performed by PCR. The polymerases used were either OneTag® 2X Master Mix with Standard Buffer, Q5® High-Fidelity DNA Polymerase (New England Biolabs) or Phire Plant Direct PCR Master Mix (ThermoFischer Scientific) according to the manual. For PCR product purification GenepHlow Gel/PCR Kit (GeneAid) was used according to the manual. The concentration and purity of genomic DNA was determined at 260/280 nm using NanoDrop One UV Vis Spectrophotometer (ThermoFischer Scientific). For separation and visualization of DNA fragments, 1 % (w/v) agarose gels stained with 50 µL L⁻¹ Roti®-Safe GelStain in 1x TAE-buffer (40 mM Tris, 2 mM EDTA, pH 8) were used.

Transformation of *U. maydis* strains was done via protoplasts: Aliquots of 50 µL containing the protoplasts to be transformed were thawed on ice for ten minutes before adding 500 ng of DNA. Afterwards ten minutes of incubation on ice took place. After this 500 µL of STC-PEG solution (40 % (w/v) PEG 4000 in STC) was added followed by ten more minutes of incubation on ice. After incubation, the whole suspension was spread on a freshly prepared REG-agar (1 M sorbitol, 1% (w/v) peptone, 1% (w/v) yeast extract, 1% (w/v) sucrose and 1.5% (w/v) agar) plate with the selection antibiotic (10 mL bottom REG-agar containing two-fold antibiotic concentration and 10 mL top REG-agar antibiotic-free added after solidification of bottom agar and not long before inoculation). Colonies were obtained after two to three days of incubation at 30°C.

To produce the protoplasts, an overnight culture of *U. maydis* was inoculated in 50 mL YEPS-medium to an OD₆₀₀ of 0.2. After an OD₆₀₀ of 0.8 was reached the culture was centrifuged for ten minutes at 4000 rpm and 4 °C. The resulting pellet was then resuspended in 25 mL SCS (1M sorbitol, 20mM sodium citrate, pH 5.8) and afterwards centrifuged for ten minutes at 4000 rpm and 4 °C. The resulting pellet was resuspended in 2 mL SCS containing 20 mg mL⁻¹ Lysing Enzyme (from *Trichoderma harzianum*; Sigma Aldrich Chemie GmbH) and left at room temperature while the protoplastation process was followed on the microscope until 80% of the cells were in state of protoplasts. Cells were then washed three times with 10 mL SCS (10 minutes at 2300 rpm and 4 °C) to stop the reaction. After
the final washing step the pellet was resuspended in 500 µl STC (100 mM calcium chloride, 1M sorbitol in 10 mM Tris-HCl, pH 7.5) and distributed in aliquots of 50 µl that were stored at -80°C.

To verify the success of the transformation a colony PCR was performed: cell material from agar plates was added to 1.5 mL microcentrifuge tubes containing 100 µl Chelex (5% (w/v)) and glass beads. The tubes were then vortexed for ten minutes and incubated at 100°C for six minutes. Finally, the tubes were centrifuged at maximum rpm for one minute. Finally, 1 µl of the supernatant was used as template for PCR using Phire Plant Direct PCR Master Mix according to the supplied manual. For storing the tubes were kept at 4°C.

For transformation of chemically competent E. coli cells DH5α™ (New England BioLabs), an aliquot was thawed on ice and afterwards 50 µL of chemical competent cells were transferred to a 1.5 ml micro-centrifuge tube after which up to 5 µL of assembled product or plasmid were added. The mixture was gently mixed using a pipet before being placed on ice for 30 minutes. Subsequently the mixture was subjected to a heat shock at 42°C for 30 seconds and then placed back on ice for 2 more minutes. Afterwards, 950 µL of LB media at room temperature was added to each tube. The cells were then incubated at 37°C and 250 rpm (shaking diameter 25 mm) for 60 minutes. After this time, 50 µl and 100 µl of the suspension was plated on LB medium with ampicillin and incubated overnight at 37°C. To verify the success of the transformation Colony PCR was performed (Chomczynski & Rymaszewski, 2006) using OneTaq® 2X Master Mix with Standard Buffer according to the supplied manual.

For plasmid isolation from E. coli the Monarch Plasmid Miniprep Kit (New England BioLabs) was used following its instruction manual.

2.2. Culture Conditions

E. coli strains were grown in LB medium (1% (w/v) peptone, 1% (w/v) sodium chloride, 0.5% (w/v) yeast extract) at 37°C and 250 rpm. U. maydis strains were grown in YEPS medium (1% (w/v) sucrose, 1% (w/v) peptone, 1% (w/v) yeast extract) at 30°C and 200 rpm. Shaking cultures of U. maydis mutants were performed in System Duetz® plates (24 well plates) with a filling volume of 1.5 mL at 30°C and 300 rpm. Screening medium contained either 50 or 100 g L⁻¹ glucose, 0.8 g L⁻¹ NH₄Cl, 1 mL L⁻¹ vitamin solution, 1 mL L⁻¹ trace element solution, 0.2 g L⁻¹ MgSO₄ • 7 H₂O, 0.01 g L⁻¹ FeSO₄ • 7 H₂O and 0.5 g L⁻¹ KH₂PO₄. As a buffer, either 30 mM MES, 100 mM MES or 99 g L⁻¹ CaCO₃ buffer (all pH 6.5) were used. Samples were taken at different time points during incubation. To ensure constant conditions in oxygenation, for each sample point a complete plate was taken.

For culture observation microscope Leica DM750 was used (Leica Microsystems) with 10x, 40x, 63x and 100x oil immersion objectives.

For long-term storage of cryocultures, 500 µL of glycerol (80% (v/v)) was added to 500 µL of overnight culture, the mixture was then vortexed and kept in aliquots at -80°C.

2.3. Analytical Methods

Optical density of cells was measured as absorbance at 600 nm (OD₆₀₀) with an Ultrospec 10 Cell Density Meter (Amersham Biosciences). To measure pH the pH electrode InLab® semi-micro (Mettler Toledo) was used connected to HI 2211 pH/ORP Meter (HANNA instruments).

Glucose and organic acid concentrations in the supernatants were analysed in a Beckmann Coulter System Gold High Performance Liquid Chromatography (Beckmann Coulter GmbH) with an Organic Acid Resin 300 x 8 mm column (CS-Chromatography) and a differential refractometer LCD 201 (MELZ, Germany). As eluent, 5 mM H₂SO₄, with a flow rate of 0.6 mL min⁻¹ and a temperature of 40°C was used. All samples were filtered with Rotilabo® syringe filters (CA, 0.2 µm, Ø 15 mm) and afterwards diluted 1:4 with bidistilled water.

3. Results

3.1. XXX deletion using FLP/FRT system and cultivation studies in U. maydis

For deletion of XXX in U. maydis the plasmid pJET1.2_Um_FRT-KO-XXX was constructed, transformed in E. coli and subsequently isolated. This plasmid contains the KO construct for XXX deletion which was amplified by PCR and purified prior to transformation in U. maydis KO-cyp3 + P₆₆₆₆ protoplasts. Success of the KO construct integrations was verified by colony PCR and transformant U. maydis KO-cyp3 + KO-XXX + P₆₆₆₆ #12 was chosen for cultivation and further studies.

The cultivation was performed in triplicates in SystemDuetz® plates in screening medium with 50 g L⁻¹ glucose over 96h using U. maydis KO-cyp3 + P₆₆₆₆ #6 as control. Samples were taken every 24h for HPLC analysis and OD and pH measurements. Three different buffers...
were used: 30 mM MES, 100 mM MES and 99 g L⁻¹ CaCO₃.

As expected (oral speech, H. Tehrani), measurement of OD values for the control strain (U. maydis KO-cyp3+P₆₇ #6) has high standard deviations not captured by the method. However, U. maydis KO-cyp3 + KO-XXX + P₆₇ cultures OD results show a typical profile (Figure 1). Therefore, it is hard to compare the two strains.

Furthermore, pictures of both strains amplified 10x, 40x, 63x and 100x were taken under the microscope every 24h in all three media (confidential data not shown). The results regarding itaconate production can be analyzed in Figure 2. The highest final itaconate concentrations were achieved in the medium buffered with CaCO₃: 20.4 g L⁻¹ for the control strain and 19.1 g L⁻¹ for the KO-cyp3 + KO-XXX + P₆₇ strain although itaconate degradation was registered in the last 24h of cultivation of the XXX knockout strain in the medium buffered with CaCO₃. Moreover, in the media buffered with CaCO₃ there is a striking difference between the itaconate production rate of the control strain and the XXX KO in the first 48h of cultivation: at this time point, the itaconate concentration are of 9.89 g L⁻¹ and 13.36 g L⁻¹ respectively.

In CaCO₃ buffered media, the XXX KO consumed all glucose after only 48h while the control strain consumed only 65% of the glucose available (data not shown). The yield obtained after 96h follows the same pattern as the itaconate concentration. In CaCO₃ buffered medium the control strain reached 0.36 g itaconate gglucose⁻¹ which was slightly higher than the XXX KO with 0.33 g itaconate gglucose⁻¹ (Figure 3).

3.2. Overexpression of Mitochondrial Transporters in U. maydis KO-cyp3 + KO-XXX + P₆₇

In this study, mitochondrial transporters from A. terreus (AT_MttA), U. cynodontis (UcN_Mtt1) and from U. maydis itself (Um_mtt1) were overexpressed in U. maydis KO-cyp3 + KO-XXX + P₆₇. For this, plasmids containing the mitochondrial transporters were restricted overnight with Sspl-HF® (New England BioLabs). Its size was verified in agarose gel followed by transformation in U. maydis KO-cyp3 + KO-XXX + P₆₇ protoplasts. The success of the transformation was evaluated by colony PCR. Three to four transformants of each transporter were cultivated in screening medium with 50 and
100 g L\(^{-1}\) glucose buffered with CaCO\(_3\) for 72h (n=3) in order to choose the best-producing transformants. *U. maydis* KO-cyp3 + KO-XXX + *P. eletro*_mttA #14, *U. maydis* KO-cyp3 + KO-XXX + *P. eletro* and *Um_mtt1* #3 and *U. maydis* KO-cyp3 + KO-XXX + *P. eletro* _UcN_mtt1_ #1 were chosen for further itaconate production study.

A new screening was performed in screening medium buffered with CaCO\(_3\) and 50 g L\(^{-1}\) glucose for 96h. The cultivation was performed in triplicates and three control strains were used: the WT (*U. maydis* MB215), *U. maydis* KO-cyp3 + *P. eletro* #6 and *U. maydis* KO-cyp3 + KO-XXX + *P. eletro*. Aiming to further evaluate the itaconate production profile, especially in the first 48h of cultivation a new sampling frequency was established and samples were collected every 6h. A new sampling method was also established since a previous similar cultivation led to formation of a white precipitate, presumably a salt of itaconate. Therefore, HCl (37% (50%(v/v)) was added to a duplicate of the cultures previously to any measurement and samples were stored at two different temperatures: 4°C and -20°C. Precipitation was only observable in the samples without HCl stored at -20°C, especially in the ones corresponding to the final hours of cultivation.

![Figure 4 - Itaconate concentration measured in samples without HCl stored at 4°C (full lines) and with HCl stored at -20°C (dotted lines). Cultivation was performed in SystemDuetz® plates in 99 g L\(^{-1}\) CaCO\(_3\) buffered media with 50 g L\(^{-1}\) glucose: Three control strains were used (WT in orange, *U. maydis* KO-cyp3 + *P. eletro* #6 in red and *U. maydis* KO-cyp3 + KO-XXX + *P. eletro* in blue). The transformants screened are the *mttA* transformant in light blue, *Um_mtt1* transformant in black and *UcN_mtt1* transformant in green. Error bars indicate deviation from the mean (n=3).](image)

Figure 4 shows that all the transformants achieve higher itaconate concentrations than any of the control strains throughout the 96h of cultivation and higher itaconate production rates in the first 60h. The transformant where *AT_mttA* is overexpressed reached a final itaconate concentration of 26.1 g L\(^{-1}\) after 96h followed by *Um_mtt1* overexpression (25.6 g L\(^{-1}\)) and the transformant expressing the mitochondrial transporter from *U. cynodontis* with 22.8 g L\(^{-1}\). These values correspond to an increase of 38%, 35% and 21% when compared to *U. maydis* KO-cyp3 + KO-XXX+ *P. eletro* (19.0 g L\(^{-1}\)), respectively.

All values indicated before correspond to the samples stored at 4°C and where no HCl was added since the data also showed that the addition of HCl influences negatively the quantification of itaconate. In fact, the higher the amount of itaconate in the media, the more significant the difference between samples with or without HCl.

![Figure 5 - Malate concentration measured in samples without HCl stored at 4°C. Cultivation was performed in SystemDuetz® plates in 99 g L\(^{-1}\) CaCO\(_3\) buffered media with 50 g L\(^{-1}\) glucose: Three control strains were used (WT in orange, *U. maydis* KO-cyp3 + *P. eletro* #6 in red and *U. maydis* KO-cyp3 + KO-XXX + *P. eletro* in blue). The transformants screened are the *mttA* transformant in light blue, *Um_mtt1* transformant in black and *UcN_mtt1* transformant in green. Error bars indicate deviation from the mean (n=3).](image)

Data in Figure 5 shows that malate is produced by the wild-type during the first 62h of cultivation, time at which it reaches a maximum of 13.3 g L\(^{-1}\). After this the concentration drops to 12.7 g L\(^{-1}\) at 96h. All three transformants showed lower final malate concentration than the *U. maydis* KO-cyp3 + KO-XXX + *P. eletro*. Strain that reached 2.37 g L\(^{-1}\). Comparing the transformants, the one overexpressing the *AT_mttA* gene had the lowest final concentration (0.94 g L\(^{-1}\)).
The yield data shows that overexpression of the mitochondrial transporters resulted in a final yield increase for all three transformants that were screened. The most significant increase was due to AT_mttA overexpression, reaching a final yield of 0.48 g itaconate g glucose\(^{-1}\) which corresponds to a 37% increase when compared to the XXX KO strain (U. maydis KO-cyp3 + KO-XXX + P\(_\text{etf}\)). The transformants overexpressing Um_mtt1 and UcN_mtt1 increased the itaconate yield in 34% and 20%, respectively, comparing to the same control strain.

### 3.3. Heterologous expression of MDR transporter Qdr3 from S. cerevisiae in U. maydis

To study the function of QDR3 in U. maydis four strains of this species were transformed with this gene: U. maydis MB215 (WT), U. maydis KO-ltp1, U. maydis KO-cyp3 + P\(_\text{etf}\) #6 and U. maydis KO-cyp3 + KO-XXX + P\(_\text{etf}\). Transformation was performed via protoplasts and confirmed by colony PCR. Four colonies of each strain were selected for a tolerance screening in MES 100 mM medium with 50 g L\(^{-1}\) glucose. The cultivation was performed in triplicates and lasted 72h, after which OD and pH were measured and glucose and itaconate quantified by HPLC. Strains U. maydis MB215 (WT), U. maydis KO-ltp1, U. maydis KO-cyp3 + P\(_\text{etf}\) and U. maydis KO-cyp3 + KO-XXX + P\(_\text{etf}\) were used as control. Moreover, pictures of every strain were taken under the microscope with four different amplifications (10x, 40x, 63x and 100x).

The analytic results show that there was no improvement in itaconate production by the transformants. In fact, the itaconate concentration even decreased in all transformants of the strain U. maydis KO-cyp3 + P\(_\text{etf}\) QDR3 when compared to the control (U. maydis KO-cyp3 + P\(_\text{etf}\)). Moreover, the transformants of the KO strain for the ltp1 transporter show residual levels of itaconate just like the control strain U. maydis KO-ltp1 which indicates that Qdr3 does not complement ltp1 function. Regarding glucose consumption, expression of qdr3 in the WT resulted in lower final glucose concentration for all three transformants when compared to the control (WT). On the other hand, it had the opposite effect on the U. maydis KO-cyp3 + P\(_\text{etf}\) strain, in which the control had 5.85 g L\(^{-1}\) glucose after 72h while the transformants registered higher glucose concentration values: between 14.6 g L\(^{-1}\) and 17.5 g L\(^{-1}\). Moreover, the observation of the cultures under the microscope did not show any difference between the transformants and respective control strains (data not shown).

### 4. Discussion

The aims of this thesis were the enhancement of IA production in U. maydis using metabolic engineering strategies and to characterize the function of the QDR3 gene from S. cerevisiae in U. maydis.

Regarding the first aim, two strategies were used: deleting XXX gene and improving itaconate production by overexpressing mitochondrial transporters of itaconate producing organisms: AT_mttA from A. terreus,
Um_mtt1 from U. maydis and UcN_mtt1 from U. cynodontis.

To create the XXX KO strain, FLP/FRT system was used allowing the creation of marker-free strains which is important since there is a very limited number of selection markers available in U. maydis (Khrunyk, Münch, Schipper, Lupas, & Kahmann, 2010).

In terms of IA production, there was a striking difference in the itaconate production rate in the CaCO3 buffered media over the first 48h with the XXX KO strain achieving as much as 13.96 g L⁻¹ while the KO-cyp3+P_eto #6 strain only produced 9.89 g L⁻¹. Such high production rate had also a great impact on glucose consumption that was depleted in the first 48h. The absence of glucose can be a plausible explanation for the activation of possible itaconate degradation pathways that caused the decrease in itaconate concentration in the last 24h of cultivation. The same behaviour had already been acknowledged in U. cynodontis (Guevarra & Tabuchi, 1990).

Strain U. maydis KO-cyp3 + KO-XXX + P_eto was selected for overexpression of AT_mttA, Um_mtt1 and UcN_mtt1 under the control of the constitutive etef promoter.

A new screening with the best transformants was performed aiming to study the itaconate production profile in more detail. A new sampling method was adopted: HCl was added to the samples to solubilize itaconate that precipitates as a salt of calcium (CaC₂H₄O₄) after storage at -20°C for HPLC analysis. It was also found that the addition of HCl negatively influences the itaconate quantification by HPLC. Up to this date, no explanation was found for this discrepancy. For analysis of the results and discussion purposes, only the HPLC measurements of the samples without HCl stored at 4°C were used.

All transformants showed very high production rates in the first 48h, slowing down after but maintaining high rates until 61h of cultivation. During the last 24h, the itaconate concentration of some cultures drops, namely for the AT_mttA transformant and KO-cyp3+P_eto #6 control strain, probably due to unknown itaconate degradation pathways in U. maydis. As expected, the overexpression of the transporter led to higher final itaconate concentrations than the ones achieved by the XXX KO strain, namely 38%, 35% and 21% increase when AT_mttA, Um_mtt1 and UcN_mtt1 are overexpressed, respectively. These results proved that mtt1 was in fact a bottleneck for IA production in U. maydis.

The yield is an important parameter for evaluating process efficiency and profitability especially because of the high cost of the substrate (Klement & Büchs, 2012). The maximum yield obtained after 96h of cultivation was of 0.48 gitaconate gglucose⁻¹ for the AT_mttA transformant. Assuming zero growth, the maximum theoretical product to substrate yield is 0.72 gitaconate gglucose⁻¹. Therefore, the best-producing strain obtained in this study (U. maydis MB215 KO-cyp3 + KO-XXX + P_eto + P_eto_cbx_A_ter_mtt #14) reached 67% of the theoretical maximum. Since it is impossible to reach 100% because part of the glucose must be used for cell growth there is not much room for further metabolic engineering. Furthermore, the best yield obtained in this study in DuetzPlates® (0.48 gitaconate gglucose⁻¹) is already more than 2-fold higher when compared to the last published results obtained in a fed-batch reactor (Geiser et al. 2016) and corresponds to 84% of the industrial yields obtained in IA fermentation with A. terreus (up to 0.57 gitaconate gglucose⁻¹) (Klement and Büchs 2012).

Regarding malate production there seems to be an opposite relation between the amount of itaconate produced and the amount of malate in the media. This supports the theory that mttA from A. terreus is probably a cis-aconitate/malate transporter (Jaklitsch et al., 1991) but also indicates that the homolog transporters from U. maydis (Um_mtt1) and U. cynodontis (UcN_mtt1) share the same function therefore reverting the malate flux from the cytoplasm to the interior of the mitochondria, as suggested by Geiser at al. (Geiser et al., 2016).

The second aim of this thesis was to characterize the function of the QDR3 gene from S. cerevisiae in U. maydis. For this purpose, a codon-optimized version of the gene under the control of the constitutive etef promoter was used. Unlike the previous screenings that aimed to study itaconate production this one was designed to study tolerance to itaconate. Therefore, MES 100 mM buffer, that has a lower buffer effect, was chosen.

Since QDR3 and ipt1 are both MFS transporters (Geiser et al., 2015; Tenreiro et al., 2005), it was expected that the first would complement the function of the latter. However, itaconate concentration of U. maydis KO-ipt1 + P_eto qdr3 transformants was residual, just like the respective control strain (U. maydis KO-ipt1) which means that QDR3 does not complement ipt1 function being unable to transport itaconate from the cytoplasm to the extracellular space.
Interestingly, the overexpression of QDR3 in the KO-cyp3P_0 deficiencies #6 strain led to lower itaconate concentrations: from 14.96 g L⁻¹ in the control to between 11.11 g L⁻¹ and 12.29 when QDR3 is overexpressed. Moreover, it also affected negatively the glucose uptake. However, for the WT the opposite impact on glucose uptake was observed. A possibility could be the fact that QDR3 is transporting itaconate into the cytoplasm and not the opposite with the transformants therefore achieving lower itaconate concentrations. Another possibility could be that itaconate was used as carbon source. In any case, further research is needed to understand the effects of QDR3 expression in U. maydis.

5. Conclusions and Future Work

In this thesis it was possible to confirm that Mtt1 was in fact a bottleneck in itaconate production and that overexpression of Um_mtt1 or mitochondrial transporters from A. terreus (AT_mttA) or U. cynodontis (UcN_mtt1) result in higher itaconate production rates, final concentration and product to substrate yield. The combined effect of XXX deletion and AT_mttA overexpression increased itaconate final concentration to 26 g L⁻¹ and yield 0.48 g itaconate gglucose⁻¹ which is 67% of the theoretical maximum yield (assuming zero growth). Therefore, and taking into account that part of the glucose has to be used for cell growth, there might be little room for further metabolic engineering improvements. A possible strategy to further improve the yield can be avoiding itaconate degradation either by using a glucose fed-batch or by investigating and deleting genes associated with this degradation pathway.

It was also proved that Qdr3 does not complement ltp1 function of itaconate export probably due to low homology (27% on protein level). However, it could be interesting to test the influence of QDR3 in pH and itaconate tolerance in U. maydis. For this, a new screening where media buffered with higher and lower buffer effect and itaconate concentration should be performed. Moreover, to test if Qdr3 is transporting itaconate into the cytoplasm and if itaconate is being used as carbon source, a new screening with glucose-free media and to which itaconate is added could be performed in order to evaluate itaconate concentration profile over time.

6. References


