5 APPLIED MICROBIOLOGY

Volume 5

The production process of biodiesel results in a huge waste stream of crude glycerol, reducing the economic and ecological advantage. Thus, the overall aim of this thesis was to establish a production process for organic acids from glycerol with Ustilaginaceae. In a screening of 126 Ustilaginaceae, two candidates for organic acid production were found - *Ustilago trichophora* for malic acid and *U. vetiveriae* for itaconic acid. Glycerol uptake and growth rate of both strains were improved by adaptive laboratory evolution. Selection of the best growing colony for each strain and medium and process optimization drastically improved the production.

The itaconic acid titer of *U. vetiveriae* TZ1 was increased to 35 g L^{-1} (rate: 0.09 g L^{-1} h^{-1}). Simultaneously about 60 g L^{-1} malic acid were formed. First metabolic engineering approaches overexpressing the mitochondrial transporter Mtt1 and the regulator of the itaconic acid gene cluster Ria1, both from *U. maydis*, increased the itaconic acid titer by 1.5- and 2.0-fold, respectively. Simultaneously, the malic acid titer was reduced by 96 % and 61 %, respectively.

For *U. trichophora* TZ1 the malic acid titer was improved to 200 g L^{-1} produced within 264 h reaching a maximal production rate of 1.53 g L^{-1} h^{-1}. Potential native target genes for metabolic engineering were identified after de novo genome sequencing. To enable the improvement of malic acid production with *U. trichophora* TZ1 by metabolic engineering, existing tools were investigated and adapted to be suitable for creation of overexpression mutants. Using these tools, overexpression mutants for two different malate dehydrogenases (Mdh1 and Mdh2), pyruvate-carboxylase (Pyc) and two different malic acid transporters (Ssu1 and Ssu2) were generated. While overexpression of Pyc did not improve malic acid production, transformants overexpressing Mdh1, Mdh2, Ssu1 and Ssu2 showed an up to 38 % increased malic acid production rate and an up to 54 % increased yield in shake flasks compared to *U. trichophora* TZ1. In bioreactor cultivations with the mutant overexpressing Ssu2, an increased production rate could not be observed. Due to a drastically lowered optical density, however, this strain had a 29 % higher specific production rate. Additionally, the product yield was improved by 1.4-fold.

These results clearly strengthen the applicability of Ustilaginaceae as production organisms. By valorization of crude glycerol, the overall biodiesel bio refinery concept is improved.

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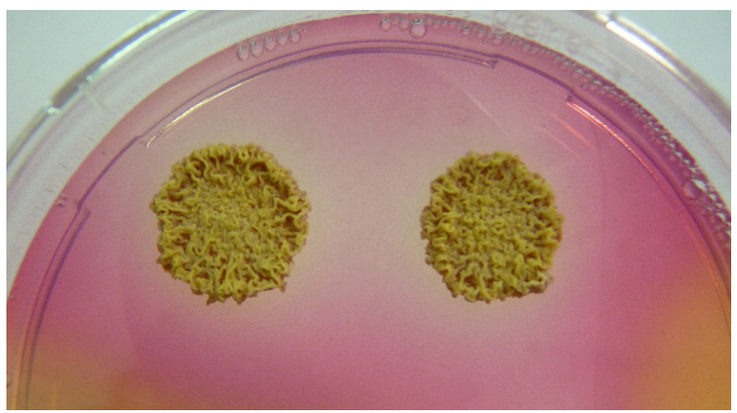

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Summary

The rapidly growing need for energy and fuel combined with an increased awareness of the detrimental influence of fossil resources has evoked the investigation of renewable alternatives, such as biodiesel. The production process of biodiesel, however, results in a huge waste stream of crude glycerol, reducing the economic and ecological advantage.

The overall aim of this thesis was to establish a production process for organic acids from biodiesel derived glycerol with Ustilaginaceae. The biodiversity within this family is well-known and in a screening of 126 Ustilaginaceae, two promising candidates for organic acid production were found - *Ustilago trichophora* for malic acid production and *U. vetiveriae* for itaconic acid production. Glycerol uptake and growth rate of both strains were improved by adaptive laboratory evolution. Selection of the best growing single colony for each strain and medium and process optimization drastically improved the production values.

The itaconic acid titer of *U. vetiveriae* TZ1 was increased to 35 g L^{-1} produced at a production rate of 0.09 g L^{-1} h^{-1}. Simultaneously about 60 g L^{-1} malic acid were formed. In first metabolic engineering approaches overexpressing the mitochondrial transporter Mtt1 and the regulator of the itaconic acid gene cluster Ria1, both from *U. maydis*, the production values could be shifted in favor of itaconic acid increasing it by 1.5- and 2.0-fold, respectively. Simultaneously, the malic acid titer was reduced by 96 % and 61 %, respectively.

For *U. trichophora* TZ1 the malic acid titer was improved to nearly 200 g L^{-1} produced within 264 h reaching a maximal production rate of 1.53 g L^{-1} h^{-1}. Since the knowledge on this obscure *U. trichophora* was scarce, potential native target genes for metabolic engineering were identified after de-novo genome sequencing. To enable the improvement of malic acid production with *U. trichophora* TZ1 by metabolic engineering, existing tools, such as antibiotic markers and promoters, were investigated and adapted to be suitable for creation of overexpression mutants. Using these tools, overexpression mutants for two different malate dehydrogenases (Mdh1 and Mdh2), pyruvate-carboxylase (Pyc) and two different malic acid transporters (Ssu1 and Ssu2) were generated. While overexpression of Pyc did not improve malic acid production, transformants overexpressing Mdh1 and Mdh2 and malic acid transporters Ssu1 and Ssu2 showed an up to 38 % increased malic acid production rate and an up to 54 % increased yield in shake flasks compared to *U. trichophora* TZ1. In bioreactor cultivations with the mutant overexpressing Ssu2, an increased production rate could not be observed. Due to a drastically lowered optical density, however, this strain had a 29 % higher specific production rate. Additionally, the product yield was improved by 1.4-fold.

These results clearly strengthen the applicability of Ustilaginaceae as industrially valuable production organisms. By this valorization of biodiesel derived crude glycerol, the overall biodiesel bio-refinery concept is improved on an economic as well as ecological level.

Zusammenfassung

Der schnell wachsende Bedarf an Energie und Treibstoffen hat zusammen mit dem steigenden Bewusstsein für die schädlichen Auswirkungen fossiler Brennstoffe die Forschung nach erneuerbaren Alternativen, wie Biodiesel, hervorgerufen. Der Produktionprozess von Biodiesel resultiert jedoch in einem enormen Abfallstrom an Rohglycerin, wodurch der ökonomische und ökologische Vorteil geschmälert wird.
Das übergeordnete Ziel dieser Arbeit war es, einen Produktionsprozess für organische Säuren aus Glyzerin mit Ustilaginaceen zu etablieren. Die Biodiversität innerhalb dieser Familie ist gut bekannt und ein Screening von 126 Ustilaginaceen lieferte zwei vielversprechende Kandidaten für die organische Säureproduktion – *Ustilago trichophora* für Malat und *U. vetiveriae* für Itakonat. Durch adaptive Laborevolution wurden sowohl die Glyzerinaufnahme als auch die Wachstumsrate beider Stämme verbessert. Anschließend konnte durch Selektion der bestwachsenden Einzelkolonien für jeden Stamm und Medium- und Prozessoptimierung die Produktionswerte drastisch verbessert werden.
Der Itakonsäuretiter von *U. vetiveriae* TZ1 wurde auf 35 g L^{-1} erhöht, welche mit einer Rate von 0.09 g L^{-1} h^{-1} produziert wurden. Gleichzeitig wurden 60 g L^{-1} Malat gebildet. In ersten Ansätzen des „metabolic engineering" durch Überexpression des mitochondrialen Transporters Mtt1 und des Regulators für das Itakonsäurecluster Ria1 aus *U. maydis*, konnte die Produktion zu Gunsten von Itakonat verschoben werden, wobei der Titer auf das 1,5-fache bzw. 2,0-fache erhöht wurde. Gleichzeitig wurde der Malattiter drastisch um 96 %, bzw. 61 % gesenkt.
Für *U. trichophora* TZ1 wurde der Malattiter auf fast 200 g L^{-1} erhöht, welche innerhalb von 264 h produziert wurden, wobei eine maximale Produktionsrate von 1.53 g L^{-1} h^{-1} erreicht wurde. Da bisher wenig über diesen obskuren *U. trichophora* Stamm bekannt war, wurden potentielle, native Zielgene für das anschließende „metabolic engineering“ durch de-novo Genomsequenzierung identifiziert. Zur Verbesserung der Malatproduktion mit *U. trichophora* TZ1 wurden bereits existierende *U. maydis* Werkzeuge, wie Antibiotikamarker und Promotoren untersucht und angepasst. Durch Anwendung dieser Werkzeuge wurden Überexpressionsmutanten für zwei verschiedene Malatdehydrogenasen (Mdh1 und Mdh2), die Pyruvatcarboxylase (Pyc) und zwei unterschiedliche Malattransporter (Ssu1 und Ssu2) erstellt. Während die Überexpression der Pyc die Malatproduktion nicht verbesserte, zeigten die Überexpressionen von Mdh1 und Mdh2 und von den beiden Malattransportern Ssu1 und Ssu2 im Vergleich zur Referenz *U. trichophora* TZ1 eine bis zu 38 % erhöhte Malatproduktionsrate und eine bis zu 54 % erhöhte Ausbeute in Schüttelkolben. In Bioreaktoren konnte keine erhöhte Produktionsrate für die Überexpressionsmutante von Ssu2 beobachtet werden. Jedoch zeigte dieser Stamm aufgrund einer drastisch reduzierten optischen Dichte eine 29 % erhöhte spezifische Produktionsrate. Zusätzlich wurde die Produktausbeute um das 1,4-fache erhöht. Diese Ergebnisse stärken deutlich die Anwendbarkeit von Ustilaginaceen als industriell wertvolle Produktionsorganismen. Durch diese Valorisierung von Rohglyzerin wird das gesamte Biodiesel-Bioraffinerie-Konzept sowohl ökonomisch als auch ökologisch verbessert.

List of Abbreviations

List of Organisms	
A. flavus	*Aspergillus flavus*
A. itaconicus	*Aspergillus itaconicus*
A. niger	*Aspergillus niger*
A. oryzae	*Aspergillus oryzae*
A. terreus	*Aspergillus terreus*
C. krusei	*Candida krusei*
E. coli	*Escherichia coli*
L. lactis	*Lactococcus lactis*
P. antarctica	*Pseudozyma antarctica*
P. tsukubaensis	*Pseudozyma tsukubaensis*
R. oryzae	*Rhizopus oryzae*
S. reilianum	*Sporisorium reilianum*
S. cerevisiae	*Saccharomyces cerevisiae*
U. avenae	*Ustilago avenae*
U. cynodontis	*Ustilago cynodontis*
U. hordei	*Ustilago hordei*
U. trichophora	*Ustilago trichophora*
U. vetiveriae	*Ustilago vetiveriae*
U. xerochloae	*Ustilago xerochloae*
U. zeae	*Ustilago zeae*
U. maydis	*Ustilago maydis*
Y. lipolytica	*Yarrowia lipolytica*

List of enzymes/proteins/promotors/genes	
ampR	ampicillin resistance cassette
bleR	phleomycin resistance cassette
Cas	CRISPR associated protein
cbx	carboxin resistance gene
CoA	coenzyme-A
ColE1	origin of replication in *E. coli*
crg1	carbon source-regulated gene
cyp1	cytochrome P450 family gene
emt1	elongator methionine-accepting tRNA gene
FLP	flippase
FRT	flippase recognition target
Gfp/*gfp*	green fluorescent protein/gene
GS1	glutamine synthetase
hph	hygromycin resistance cassette
hyg	hygromycin resistance gene
ip	iron sulphur protein subunit of succinate dehydrogenase
ip^R	ron sulphur protein subunit of succinate dehydrogenase (resistant)
ip^S	iron sulphur protein subunit of succinate dehydrogenase (sensitive)
mae1	malic acid transport protein gene
Mat1	mating type
Mdh/*mdh* 1,2,3	malate dehydrogenase/gene
mig1	multicopy inhibitor of GAL gene expression gene
Mtt1/*mtt1*	mitochdonrial *trans*-aconitate transporter/gene
nar1	nitrate reductase gene
natR	nourseothricin resistance cassette
Ndh-2	NADH dehydrogenase
Nox	NADH oxidase
Poma	oma promotor
Potef	otef promotor
prf1	perforin gene
Psc	Promoter *S. cerevisiae*
Pyc/*pyc 2*	pyruvate carboxylase/gene
Ria1/*ria1*	regulator of the itaconic acid cluster/gene
sdh2	succinate dehydrogenase gene
Ssu/*ssu* 1,2	plasma membrane sulfite pump/gene
tad1	*trans*-aconitate decarboxylase gene
UARS	*Ustilago* autonomous replication site

List of chemicals/elements

A	adenine
ATP	adenosin triphosphate
C	carbon
C	cytosine
Ca	calcium
$Ca(OH)_2$	calcium hydroxide
$CaCl_2$	calcium chloride
$CaCO_3$	calcium carbonate
cbx	carboxin
CM	carboxy methyl
CO_2	carbon dioxide
CO_3	carbon trioxide
DMSO	dimethyl sulfoxide
DNP	2,4-dinitrophenol
FAME	fatty acid methyl ester
$FeSO_4$	ferrous sulfate
G	guanine
GAL	galactose
gly	glycerol
H	hydrogen
H_2O	water
HCl	hydrogen chloride
HCO_3	hydrogen carbonate
hyg	hygromycin
KH_2PO_4	potassium dihydrogen phosphate
mal	malate
MEL	mannosylerythritol lipid
MES	2-(N-morpholino) ethanesulfonic acid
$MgSO_4$	magnesium sulfate
N	nitrogen
NAD	dicotinamide adenine dinucleotide (reduced)
NADH	nicotinamide adenine dinucleotide (oxidized)
NaOH	sodium hydroxide
nat	nourseothricin
NH_4Cl	ammonium chloride
O	oxygen
O_2	molecular oxygen
PCR	polymerase chain reaction
phl	phleomycin
T	thymine
UA	ustilagic acid

List of Units

%	percentage
°C	degree Celsius
µL	microliter
µm	micrometer
bp	basepairs
g	gram
g	gravitational-force
h	hour
ha	hectar
kb	kilo basepairs
kDa	kilo Dalton
kg	kilogram
kt	kilotons
kV	kilovolt
L	liter
M	molar
Mbp	mega basepairs
Mg	megagram
min	minute
mL	milliliter
mm	millimeter
mM	millimolar
mol	molar
ms	milliseconds
nm	nanometer
rpm	revolutions per minute
t	ton
v/v	volume per volume
vvm	volume flow per unit of liquid volume per minute
w/v	weight per volume

General Abbreviations	
ALE	adaptive laboratory evolution
approx.	approximately
ATCC	American Type Culture Collection
BLASR	Basic Local Assignment with Successive Refinement
BLAST	Basic Local Alignment Search Tool
BMBF	Bundesministerium für Bildung und Forschung
BRAIN AG	Biotechnology Research And Information Network Aktiengesellschaft
bzw.	beziehungsweise
(c)	cytoplasmatic
CAS	Chemical Abstracts Service
CBS	Centraalbureau voor Schimmelcultures
CLR	continuous long read
CRISPR-Cas	clustered regularly interspaced short palindromic repeats – CRISPR associated
CS	Chromatographie Service
CurTiPot	Potentiometric Titration Curves
D-	dextro
DDBJ	DNA Data Bank of Japan
DNA	deoxyribonucleic acid
DO	dissolved oxygen
DoE	Department of Energy
DOT	dissolved oxygen tension
DSM	Deutsche Sammlung von Mikroorganismen
e.g.	exempli gratia
EN	European Standard
ENA	European Nucleotide Archive
et al.	*et alii* or *et aliae*
EU	European Union
fwd	forward
GRAS	generally regarded as safe
HPLC	high-performance liquid chromatography
iAMB	Institute of Applied Microbiology
L-	levo
(m)	mitochondrial
MB	Michael Bölker
Mio	million
mRNA	messenger ribonucleic acid
MTM	modified Tabuchi medium
N	number of samples
n.a.	not available
no.	number
OD	optical density

General Abbreviations	
ORF	open reading frame
PCR	polymerase chain reaction
pH	negative logarithm of the concentration of hydronium ions
pK_a	symbol for the acid dissociation constant at logarithmic scale
prod.	production
rev	reverse
RI	refractive index
RNA	ribonucleic acid
rTCA-cycle	reductive tricarboxylic acid cycle
RWTH	Rheinisch-Westfälische Technische Hochschule
SMRT	single molecule real time
SSAKE	Short Sequence Assembly by progressive *k*-mer search and 3′ read Extension
SSF	simultaneous saccharification and fermentation
ssp.	species
SSPACE	SSAKE-based Scaffolding of Pre-Assembled Contigs after Extension
TCA-cycle	tricarboxylic acid cycle
TMFB	Tailor-Made Fuels from Biomass
TZ	Thiemo Zambanini
U.S.	United States
UK	United Kingdom
USA	United States of America
UV	ultraviolet
VWR	Van Waters & Rogers
WT	wildtype
YEP	yeast extract peptone
ZeroCarbFP	ZeroCarbonFootprint

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Chapter 1

General introduction

1. General introduction

1.1. The inevitable switch from fossil fuels to renewable resources

1.1.1. Fossil resources and their consequences

Within the last 200 years the global population has increased from around 1 billion to more than 7 billion people [1, 2], with a peaking increase between 1900 (1.5 billion) and 2000 (6.1 billion). This rapidly increasing population was accompanied by an even stronger increasing need for food, energy, chemicals and other materials, to improve living standards. Even though the growth rate for the global population is already declining and is expected to become negative before the end of this century [3], the global energy, food and chemical demand will further increase. For instance, the demand for energy is predicted to grow by more than 50 % until 2025 [4], while the global food demand is expected to double until 2050 [5]. For transportation fuels an even stronger increase is expected [6]. The main reason for this is the development of the third world and emerging markets. Combined with a decreasing availability of fossil resources, such as crude oil, coal and natural gas, this process will on the one hand lead to a future shortage in fossil resource supply resulting in a strong dependency on oil-exporting countries. On the other hand the consequences of the broadly discussed drawbacks of fossil fuels will further increase.

The discussion about advantages and disadvantages of fossil resources is ongoing as long as mankind is using them. Even though, the list of potential drawbacks and resulting problems, including global warming and pollution of the air and the water, is already known for a long time, the world oil production increased about 10-fold within 60 years between 1950 and 2010 [7]. This huge increase results to a great extent from the larger traffic volume, which consumes the major amount of crude oil. Specifically, 55 % of the total petroleum output is used for transportation fuels [8, 9], while only 5 % goes into the production of chemicals [10] and 40 % into energy supply. Since the needed supply in all of these areas is expected to increase further without any change in its distribution [4], it becomes obvious, that with diminishing fossil fuels, the switch to renewable, environment friendly and sustainable alternatives is inevitable.

1.1.2. Sustainability through bio-based, renewable alternatives

In the beginning of the 20th century, many industrial production processes for relevant materials still mainly relied on bio-based raw materials, such as agricultural crops. However, by the middle of the 20th century, many of these were replaced by petro-chemicals [11, 12]. Even though the interest in a bio-based industry was regained after the oil crisis in the 1970s, stagnating and even decreasing oil prices over the following decades mitigated this effect. These decreasing prices even resulted in a tripling of the global consumption of liquid petroleum within following years. To stop this process, governmental regulations were made and laws were passed for a strictly reduced use of petroleum-based resources, while the use of alternative technologies was elevated. With “the Biofuels Directive”, the European Union intended 1.4 % and 5.75 % of all transportation fuels to be biomass-based by the end of 2005 and 2010, respectively. Even though these goals were not reached, the directive already showed a switch even on an institutional level increasing the acceptance for biofuels more than ever before. The U.S. Department of Energy, for instance, decided that by 2025, the use of liquid petroleum transportation fuels should be reduced by 30 % and the usage of industrial organic acid chemicals by 25 %. To compensate for the lowered

petroleum-based production, bio-based processes should be established [13].

Even though nowadays the term "bio-based" is omnipresent, the exact definition often stays unclear. So what is actually a bio-based resource or chemical? In the business dictionary "bio-based" is defined as a "material or product derived from biological or renewable resources" [14] and in the European Standard EN 16575:2014 'Bio-based products - Vocabulary' the term "bio-based" is defined as "derived from biomass; can have undergone physical, chemical or biological treatment(s)", while the term "bio-based product" is defined as "product wholly or partly derived from biomass, which is normally characterized by the bio-based carbon content or the bio-based content. Product can be an intermediate, material, semifinished or final product" [15]. In contrast to fossil resources with $10^6 – 10^8$ years, bio-based resources have a carbon turn-over of only about 10^2 years. Combined with nearly carbon neutral production processes, this sustainability is their main advantage [16]. Another advantage is the positive effect on local economies, since bio-based resources can grow in most areas of the world, however with differences in the yield for different crops. The average yield is about 10 dry Mg ha^{-1} $year^{-1}$ ranging from 8 dry Mg ha^{-1} $year^{-1}$ for willow in Sweden to 22 dry Mg ha^{-1} $year^{-1}$ for short rotation woddy crops in the United States [17, 18].

In first attempts to exploit bio-based, renewable substrates, mainly the kernels and seeds of crops, such as wheat, corn and sugar cane or beet were used. These first generation feedstocks, however, quickly ignited the "food vs. fuel" discussion, since the new industries competed with the traditional use for the feedstock, namely as food and feed [19]. As cropland and thus the harvest are limited, an increased production cannot be coped by expanding farmland [20]. Consequently, alternatives had to be investigated. Generally, an increase in biomass yield per hectare is considered a good starting point to increase the produced biomass, without a need to simultaneously increase the agricultural land. In Tabaco plants, the biomass yield could be improved by overexpression of GS1 [21] and for poplar, the tree height could be increased by 1.4-fold via overexpression of glutamine synthase [22]. Additionally, the low efficiency in light capturing by photosynthesis, which is presently stated to be around 2 %, was tackled to improve the yield. Photosynthesis has already been shown to be accessible for genomic manipulation in Tabaco plants, improving the overall efficiency of light capturing [23]. Another possibility to improve the yield is to use not only the kernels or seed, but also or even exclusively other parts of the plants, such as hemicellulose or lignin. Using these parts, which in the first processes were disposed of or burned and consequently lost energy, drastically improves the ratio of energy resulting from the produced biofuel to energy required to produce the biofuel. These processes using so called second generation feedstock, thus both circumvent the mentioned "food vs. fuel" dilemma and simultaneously help to lower the price of biofuels improving the competitiveness with fossil fuels [24]. The advantages of biofuels are further increased by their high similarity to conventional fuels. Therefore, no major effort is needed for incorporation in terms of use, storage and distribution. In this context, the cluster of excellence at RWTH Aachen University, "Tailor-Made Fuels from Biomass" (TMFB), is driving the investigation for novel biofuels. By a rather new reverse approach, the desired product, the "tailor-made" fuel, is designed in the first step and the production pathway is generated afterwards. By this they are able to exactly customize the resulting biofuels to the needs of the engines, resulting in the production of better fuels [25]. Potential target molecules as biofuels, chemicals, solvents or materials are, 3-methyl-γ-butyrolactone, γ-valerolacton, 2-methyl-butanediol, 2-methyl-tetrahydrofuran, 3-methyl-tetrahydrofuran, and 1,4-pentanediol.

1.2. Biofuels

The most important biofuels are biodiesel (mainly EU), which is a fuel made exclusively from plant oils, and bioethanol (mainly United States and Brazil), which is ethanol derived from fermentation of plant starches or sugar cane. Compared to petroleum-based production processes, which have been investigated intensively for decades, these production processes are still in their infancy, and have a huge potential for improvement. Even though the process of biodiesel production was already established in 1853 [26, 27], it was not before the 1990s, that the use of biodiesel as transportation fuel became popular. With increasing interest since the 2000s a production of 6.0 million tons was reached in 2013 [28]. For bioethanol, the development is rather similar. The first car using exclusively bioethanol was introduced in 1978 in Brazil. However, it took several more decades for bioethanol to gain more and more importance as a biofuel. Especially in countries apart from Brazil and the United States, bioethanol production and the use of bioethanol as addition to conventional fuel did only increase considerably since the 2000s reaching a production of 66.8 million tons in 2011 [29]. The production of bioethanol from first generation feedstock relied mainly on wheat starch in Germany, corn in the United States and sugar cane in Brazil [4]. Since these raw materials sincerely inflamed the already mentioned "food vs. fuel" debate, contemporary research mainly focuses on other non-food bio-based materials, such as low-cost municipal or agricultural waste materials, trees or corn stover [30, 31].

Hill *et al*. postulated the following five criteria a biofuel needs to fulfill in order to efficiently replace fossil fuels: 1) must generate a net energy gain; 2) must have environmental benefits; 3) must be economically competitive; 4) must be producible in large quantities; and 5) must not reduce food supplies [32]. Based on these criteria, they evaluated the production of bioethanol and biodiesel. The energy output of bioethanol produced from corn grain is 25 % higher than the invested amount of energy, while the greenhouse gas emission is reduced by 12 % compared to fossil fuels. For biodiesel produced from soybean the corresponding values are 91 % and 41 % [32]. These values clearly demonstrate a net energy gain and environmental benefits, which are additionally increased by a minimal release of other pollutants, especially for biodiesel. The economical competitiveness of biofuels drastically correlates to fluctuating oil prices, resulting in non-competitiveness in 2005 without subsidies. However, taking not only direct costs but also social and environmental costs into account, both biodiesel and bioethanol are competitive compared to fossil fuels [32, 33]. The two aspects, which most often result in difficulties when comparing fossil fuels and biofuels, are the producibility in large quantities without reducing food supplies. Hill *et al*. stated, that 12 % (gasoline) and 6 % (diesel) of contemporary demand could be met, if all U.S. corn and soybean would be used for biofuel production [32] and the U.S. Department of Energy stated in 2011, that by 2030 30 % of contemporary fossil fuel demand could be replaced by biofuels [9]. This could be drastically improved, if other substrates, such as waste biomass or other industrial waste streams would be used.

1.2.1. The process of biodiesel production

Biodiesel is the most prominent example for a biofuel, especially in the EU. The term biodiesel refers to animal fat- or vegetable oil-based diesel fuels, which consist of long-chain alkyl-esters. The production process of biodiesel starts from vegetable oils or animal fats, which via transesterification and esterification with a short-chain alcohol, such as methanol or ethanol, are processed to the biofuel. The most common one is fatty acid methyl ester (FAME), which results from the reaction with methanol (Figure 1).

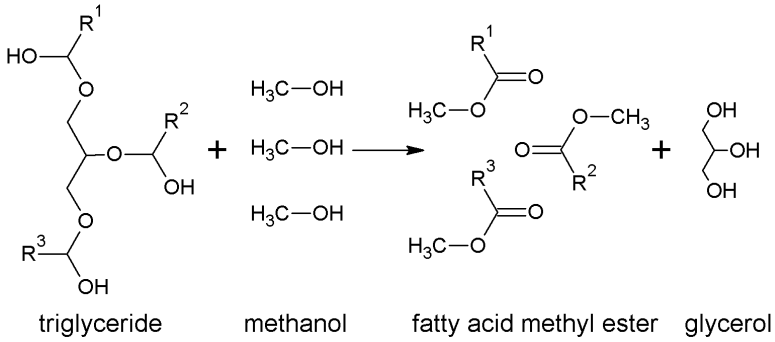

Figure 1: Reaction equation for the production of FAME.

As substrate, a broad range of oils can be used, such as virgin oil feedstock, waste vegetable oil, tallow, and yellow grease, which greatly contributes, to overcome the problems arising from producibility in large quantities, while not reducing food supplies. The typical production process can be divided into the following four steps: 1) pretreatment of the feedstock; 2) determination and treatment of the free fatty acids; 3) transesterification and esterification of the fats and oils; 4) purification of the product. In the first step, the feedstock is cleared from impurities, which mainly result from cooking, and storage. The removal of water is of special importance, since it would result in the formation of soap instead of biodiesel in the transesterification step. In the second step, the amount of free fatty acids is determined by titration and afterwards they are either esterified into biodiesel or removed. The third step is the actual production step. The most common mechanism for this step is the transesterification of the lipids with an alcohol (commonly methanol) under presence of an acid or base, to produce biodiesel. The base helps to deprotonate the alcohol, resulting in a strong nucleophile, which can attack the ester. Other possibilities for the production process either rely on mixing of the immiscible phases using ultra-shear reactors, which can reduce the droplet size, ultrasonic reactors, which cause a good mixture by ultrasonic waves, or the use of alternative catalysts, such as lipases. Another possibility is the use of a supercritical process, in which the catalyst is replaced by high temperature and pressure. These conditions result in the formation of a single phase, which drastically improves the reaction speed. Further, this method simultaneously improves the fourth step of biodiesel production, the product purification, due to the missing catalyst. In this last step, the by-products are removed from the biodiesel. These by-products are soap, resulted from water residues in the substrate, excess alcohol, catalyst depending on the method, water and the main by-product crude glycerol.

1.2.2. The by-product glycerol

The simple polyol (sugar alcohol) glycerol is a colorless, non-toxic, viscous liquid, with a molecular mass of 92.09 g mol^{-1}. It is used as sweetener or solvent in the food industry, in pharmaceutical formulations, personal care and cosmetic applications, such as toothpaste, skin or hair care products, soaps, and syrups or elixirs to improve smoothness or as anti-freezing agent, as a result of its strong hydrogen bonds formed with water molecules (Figure 2).

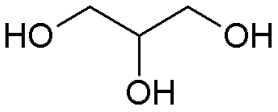

Figure 2: Chemical structure of glycerol.

Due to the different applications, it was still considered one of the top twelve building block chemicals to be produced from biomass in 2004 [34]. A drastically increasing biodiesel production, however, resulted in a huge stream of crude glycerol flooding the markets in the following years, since the process of biodiesel production is accompanied by the generation of 10 % (w/v) crude glycerol. Thus, the price for glycerol dropped, resulting in most companies producing glycerol chemically to shut down [35]. Depending on the biodiesel production process, the crude glycerol contains impurities, such as methanol, ash, soap, salts, non-glycerol organic matter, and water [36, 37]. These impurities pose severe problems to the use of the crude glycerol making it a burden rather than a blessing for the biodiesel industry. The disposal of crude glycerol results in pollution of the environment and is consequently quite expensive. However, the purification to pharma-grade glycerol is also not profitable, especially, since the need for pharma-grade glycerol is smaller than the amount producible from the crude glycerol stream. Besides the use of cheaper substrates, substrate costs make up about 75 - 95 % of the final costs for biodiesel production [38], the exploitation of the crude glycerol as feedstock for microbial production processes is frequently discussed as possibility to improve the biodiesel production process [37, 39, 40]. By this, the crude glycerol could add value to the production chain of biodiesel, making the overall production process more competitive against fossil fuels. Even though, many microbes struggle with the contained impurities, different production processes starting from unpurified crude glycerol are known. These comprise among others, production of 1,3-propanediol using *Klebsiella pneumoniae* [41, 42], glycolipids with *Ustilago maydis* [43], citric acid [44, 45], erythritol [46], and α-ketoglutarate [47] with *Yarrowia lipolytica*, succinic acid with *Bacillus succiniciproducens* [48], triacylglycerol with *Pseudozyma* [49], and hydrogen production by different organisms [50]. One of the main advantages of glycerol as substrate for microbial conversion, besides its abundance as structural component in many lipids in nature, is the higher degree of reduction than sugars, which especially benefits the production of reduced chemicals, such as organic acids. On top, glycerol as liquid can be directly used for feed, without dilution of the culture broth by addition of extra water.

1.3. Eye on platform chemicals (organic acids)

In order to succeed in a complete transition from a fossil- to a bio-based economy, not only the previously discussed transportation fuels, but the whole range of petroleum-derived products needs to be replaced by bio-based alternatives [9]. This also includes the production of platform chemicals. The term platform chemical derives from the vision, that simple, small molecules can be utilized as building blocks for the production of higher-value chemicals and materials [51]. In 2004, the U.S. Department of Energy investigated more than 300 potential candidates as most important building blocks to be produced from biomass [34]. The evaluation was organized in different steps. In the first step, the 50 most interesting molecules were selected according to the following criteria: raw material, estimated processing costs, estimated selling price, the technical complexity associated with the best available processing pathway, the market potential for each of the candidate building blocks, direct product replacement, novel products and building block intermediates. In the next step the selected compounds were organized into different groups using the criteria direct replacement, novel properties, and potential utility as a building block intermediate. These groups were analyzed regarding chemical functionality and potential use and in a next step, the single compounds were classified with regard to their current utility to serve as a simple intermediate in traditional chemical processing, as a reagent molecule for adding functionality to hydrocarbons, or as byproducts from petrochemical syntheses. A first list of 30 building block candidates met the following criteria: exhibits multiple functionalities suitable for further conversion as derivatives or molecular families, can be produced from both lignocellulosics and starch, are C1 - C6-monomers, are not aromatics derived from lignin, and are not already super-commodity chemicals. Further narrowing down, the list identified the top 15 sugar derived building blocks (Table 1) [34].

Table 1: Top 15 platform chemicals to be produced from biomass assigned in 2004. [34]

platform chemical
1,4 succinic, fumaric and malic acid
2,5 furan dicarboxylic acid
3 hydroxy propionic acid
aspartic acid
glucaric acid
glutamic acid
itaconic acid
levulinic acid
3-hydroxybutyrolactone
glycerol
sorbitol
xylitol/arabinitol

Since the state of the research, with new techniques, methodologies and technologies drastically contributes to the viability of a production process, this list was revisited six years later in 2010 using the following 9 criteria: 1) The compound or technology has received significant attention in literature, 2) The compound illustrates a broad technology applicable to multiple products, 3) The technology provides direct substitutes for existing petrochemicals, 4) The technology is applicable to high volume products, 5) A compound exhibits strong potential as a platform chemical,

6) Scaleup of the product or a technology to pilot, demo, or full scale is underway, 7) The bio-based compound is an existing commercial product, prepared at intermediate or commodity levels, 8) The compound may serve as a primary building block of a biorefinery, 9) Commercial production of the compound from renewable carbon is well established [52]. The evaluation resulted in a new list of the top 10 sugar derived building blocks (Table 2) [52].

Table 2: Top 10 platform chemicals to be produced from biomass assigned in 2010. [52]

platform chemical
ethanol
furans
glycerol and derivatives
biohydrocarbons
lactic acid
succinic acid
hydroxypropionic acid/aldehyde
levulinic acid
sorbitol
xylitol

Already in 2010, the authors stated, that this list might differ drastically, depending on the intention, since they just tend to offer a starting point for the development of production processes for biorefineries, which, however, does not exclude other possibilities [52]. This is clearly shown by the exclusion of 8 of the original potential platform chemicals from the 2004 list. Additionally, another six years have past since the revisited list has been published. In these six years, technology has further advanced and changed, consequently once again resulting in changed interest in molecules as platform chemicals. The production of glycerol as platform chemical, as just one example, which has already been mentioned, has lost relevance, due to the increased biodiesel production. Which, however, did not change is the demand of the market for low-cost monomers able to compete with and substitute for inexpensive petroleum-derived carbon backbone molecules [12]. One group of chemicals, which always were in focus as promising platform chemicals are the organic acids. In the 2004 list of the top sugar derived building block chemicals, nine chemicals belong to the group of organic acids [34] and also in the revisited list, many organic acids are still present [52]. This clearly demonstrates the potential of this group, which is also consistently discussed in literature [40, 53-56].
The main reason for interest in organic acids is their broad range of applications as polymers or commodity chemicals per se, which to a major part result from their functional groups [56, 57]. Another aspect which drastically increases the potential of organic acids is that most of them are natural products of microbes. Thus, new production processes can start from a natural producer, which facilitates the development as complicated techniques can be avoided at least in the first production steps [57]. Even though the contemporary market volume for many organic acids seems small, there is a huge potential in emerging markets and with new technologies, to replace different existing petrochemical-based production processes. The group of 1,4-dicarboxylic acids (succinic, fumaric, and malic acid), for instance, could replace maleic anhydride, which would increase their annual market potential by more than 200,000 t [57]. Thus, the projected market volume for many organic acids is far higher than the contemporary production capacity (Table 3).

Table 3: Microbial organic acid production potential (adapted from Sauer et al.*, 2008).* [57]

organic acid	annual production [t]	annual microbial production [t]	projected marked volume [t]
acetic acid	7,000,000	190,000	-
3-hydroxypropionic acid	n.a.	-	up to 3,600,000
lactic acid	150,000	150,000	-
fumaric acid	12,000	-	more than 200,000
malic acid	40,000 [54]	-	more than 200,000
succinic acid	40,000 [58]	-	more than 270,000
itaconic acid	80,000	80,000	more than 410,000 [59]
levulinic acid	450	-	high
citric acid	1,600,000	1,600,000	-
glucaric acid	n.a.	-	high
gluconic acid	87,000	87,000	-

The differences between contemporary annual production and projected marked volume clearly demonstrate the potential for further research in the field of microbial organic acid production. In the following paragraphs, more detailed information on citrate, itaconate, malate, and succinate is given. Citrate was chosen, since it is the microbial organic acid production process with by far the highest output and itaconate, malate and succinate were chosen, since they have an immense expected market volume, if the price could be lowered by introduction of novel production processes. In Figure 3 the microbial production pathways for these four organic acids are depicted (Figure 3).

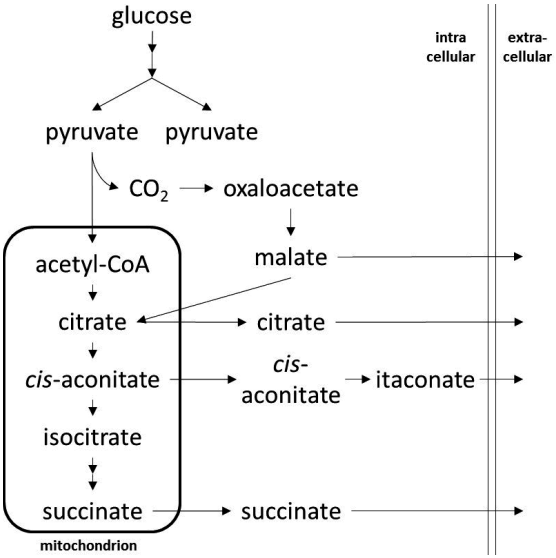

Figure 3: Schematic pathways for the microbial production of malate, citrate, itaconate and succinate.

1.3.1. Citric acid

Citric acid (3-carboxy-3-hydroxypentane-1,5-dioic acid, 3-carboxy-3-hydroxypentanedioic acid, 2-hydroxy-1,2,3-propanetricarboxylic acid) (CAS 77-92-9) is a saturated, non-toxic, organic, C6-tricarboxylic acid with a molecular mass of 192.12 g mol^{-1} (Figure 4). Its solubility in water is 147.76 g L^{-1} at 20 °C with pK_a-values of 3.13 (pK_{a1}), 4.76 (pK_{a2}), and 6.39 (pK_{a3}), while the solubility in ethanol, ether, ethyl acetate, and DMSO is lower.

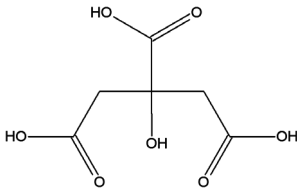

Figure 4: Chemical structure of citric acid.

The main use for citric acid is with about 70 - 75 % in the food and beverage industry as acidifier, flavoring, and chelating agent. The rest is used for metal cleaning, pharmaceutical formulations, and in the detergent market [53, 60]. It was discovered by Carl Scheele in 1784 and originally the production was covered by extraction from citrus fruits, since the concentration can reach up to 8 % of the dry weight of lemons and limes. Increasing population with consequently higher needs, however, resulted in the investigation of other production processes. As it is a natural product of all organisms, microbial production of citric acid is the oldest fermentative production process for an organic acid at high volume. Microbial citric acid accumulation was first observed in 1893 and in 1913, the first patent was filed for production of citric acid with *Aspergillus niger* [61-63]. Four years later, in 1917 the first fermentation process with *A. niger* was reported [64]. Since then, the process with *A. niger* has been investigated thoroughly improving it drastically reaching yields of about 200 g L^{-1} from 240 g L^{-1} glucose, which is about 90 % of the theoretical value [65-68]. For many years the industrial production of citric acid has been exclusively covered by fermentation of carbohydrates using *A. niger* [60]. Recently, the production from different carbon sources, especially industrial waste streams, such as molasses or glycerol, and with different organisms, such as *Y. lipolytica* or *Candida* species has been investigated [44, 69, 70]. For the microbial production of citric acid, different processes are known, such as the surface method, the submerged process, continuous and immobilized processes and the koji process. Already in 1998, the market volume for citric acid was 879,000 tons [71]. Until 2007, the annual citric acid production has nearly doubled to about 1.6 mio tons and has thus become the largest microbial production process, making up 80 - 90 % of microbial organic acid production [55, 72]. The microbial production of citrate starts from pyruvate, which can be generated for instance from glucose by glycolysis. One molecule of pyruvate is decarboxylated to acetyl-CoA by pyruvate decarboxylase in the mitochondrion, while the other is carboxylated to oxaloacetate by pyruvate carboxylase in the cytosol. The oxaloacetate is converted to malate, which is transported to the mitochondrion. Together with the acetyl-CoA, it is converted to citrate, which is transported out of the mitochondrion and out of the cell [73, 74]. This pathway allows for the already mentioned high yields, since all six carbon atoms are preserved during the microbial conversion steps.

1.3.2. Itaconic acid

Itaconic acid (2-methylidenebutanedioic acid, 1-propene-2-3-dicarboxylic acid, methylenesuccinic acid) (CAS 97-65-4) is an unsaturated, non toxic, organic, C5-dicarboxylic acid with a molecular mass of 130.1 g mol^{-1} (Figure 5). Its solubility in water is 83 g L^{-1} at 20 °C with pK_a-values of 3.84 (pK_{a1}) and 5.55 (pK_{a2}) [75], while solubility in methanol, 2-propanol, acetone, and ethanol is lower.

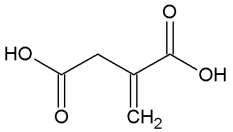

Figure 5: Chemical structure of itaconic acid.

In contrast to citric acid, it is exclusively used in non-food applications, such as anti-scaling polymers in water treatment, carbon fiber technology, cement additives, chelant dispersants for minerals in coatings, co-builders in phosphate-free detergents, dye intermediates, hair treatment, lubricant additives, pH-responsive hydrogels, plastics, coatings, superabsorbance, rubber, surface active agents, adhesives, thickeners or binders, and synthetic latex [53, 76-79]. Itaconic acid production as chemical synthesis from citric acid monohydrate was discovered in 1836 [80]. In the following decades, several other methods were investigated, starting from different precursors or using different techniques [81-83]. Even though given the broad range of different production methods, no chemical itaconate production is practiced commercially, since they all depend on expensive substrates lowering the product margin, if economically feasible at all [34, 76, 77]. Microbial itaconate production processes can circumvent these drawbacks, due to the use of cheap and abundant substrates. The first microorganism discovered to produce itaconic acid was *A. itaconicus* in 1931 [84] and only 8 years later, *A. terreus*, contemporary exclusive industrial production organism, was discovered [85]. The production of itaconic acid with *A. terreus* has been investigated intensively over the years and the pathway has been clarified [83, 86, 87]. It starts from *cis*-aconitate in the TCA-cycle, which is exported to the cytosol. In the cytosol it is decarboxylated to itaconate by *cis*-aconitate decarboxylase and the resulting itaconate is exported out of the cell (Figure 3) [88-92]. The production process mainly relies on submerged fermentation, in which a low pH-value (<4), a low phosphate concentration as growth limiting nutrient to induce production, a high magnesium sulfate and glucose concentration, and a high DOT are essential [55, 77, 86, 93-96]. Since *A. terreus* is able to grow on a broad variety of different carbon sources, itaconic acid production starting from different feedstock has been investigated, however with huge differences in the obtained yields reaching up to 70 % on sucrose [86, 97, 98]. The obtained titers are normally in a range of 80 - 100 g L^{-1} [56, 99, 100]. However, a titer of 146 g L^{-1} was reached by cultivation of a strain overexpressing the *cis*-aconitate decarboxylase and a transport protein under pH-optimized conditions [56]. Since the discovery of *A. terreus*, several other organisms producing itaconic acid have been reported, such as *P. antarctica* [101], *U. zeae* [102], *U. maydis* and *U. cynodontis* [103, 104] and different *Candida* [105] and *Rhodotorula* [106] species. Another interesting point for microbial itaconic acid production is that the production process is possible in the same production facilities as citric acid, since even the same recovery process is possible [53].

1.3.3. Malic acid

Malic acid (hydroxybutanedioic acid or 2-hydroxysuccinic acid) (CAS 6915-15-7), which is named after the latin word "malum" meaning apple, is the only organic acid comprising an asymmetric C-atom and thus occurs as L- (CAS 97-67-6) and as D-isomer (CAS 636-61-3). It is a saturated, non-toxic, organic, C4-dicarboxylic acid with a molecular mass of 134.09 g mol^{-1} (Figure 6). Its solubility in water is high with 558 g L^{-1} at 20 °C for DL-malic acid and 363.5 g L^{-1} for D- or L-malic acid. It is also soluble in ethanol and aceton and moderately soluble in diethyl ether. The pK_a-values are 3.46 (pK_{a1}) and 5.10 (pK_{a2}). The racemic mixture DL-malic acid is classified as "generally recognized as safe" GRAS by the U.S. Food and Drug Administration [54].

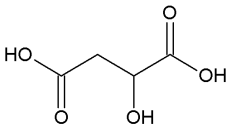

Figure 6: Chemical structure of malic acid.

Most of the 40.000 tons produced annually are used in the food, beverage and candy industry as acidulant [54]. However, malic acid is also used in pharmaceuticals, infusions, metal cleaning and finishing, paints and dyes, electroless plating, textile finishing. Additionally it has great potential as building-block chemical as precursor for maleic anhydride, substitute for tetrahydrofuran derivatives or for the production of the novel bio-degradable polymer polymalic acid, which increases the projected market volume drastically [34, 54, 92, 107]. Malic acid was first isolated from apples in 1785 [108] and for many decades, extraction from apple juice was the method of choice to produce malic acid. Contemporary commercial production exclusively relies on chemical synthesis via hydration of maleic or fumaric acid resulting in a racemic mixture or the biotransformation of fumaric acid with immobilized cells hyper-expressing the enzyme fumarase. This process is stereospecific and yields enatiomerically pure L-malic acid at a yield of 70 % of the theoretical one using *Brevibacterium ammoniagenes* [54, 55, 109-111]. This yield was further improved to nearly 100 % using an *Saccharomyces cerevisiae* strain overexpressing fumarase in a highly optimized bioreactor process [54, 112, 113]. The L-form of malic acid is produced by all organisms as part of the TCA-cycle. Especially filamentous fungi, such as *Rhizopus* ssp. and *Aspergillus* ssp., are known to secrete malic acid into the cultivation medium, naturally, reaching high titers [54, 109].
In 1962, *A. flavus* was found to produce malic acid and the production process was patented [114]. However, this production process was not applicable in the food industry due to aflatoxin production. Consequently other organisms were investigated and *S. cerevisiae*, other *Aspergillus* species and even bacteria have been reported to secrete malic acid [115-118]. In general, malic acid is not accumulated under normal conditions but under stress, often nitrogen or phosphate starvation, as it is also known for other organic acids [54, 119]. There are four different known pathways for microbial malic acid production; the TCA-cycle, the cytosolic rTCA-cycle, the cyclic glyoxylate pathway, and the non-cyclic glyoxylate pathway [115]. These do not only differ in the enzymes involved, but also in their compartmentation. However, the cytosolic rTCA-cycle comprising the reaction of pyruvate to malic acid via oxaloacetate with the help of pyruvate carboxylase and malate dehydrogenase has been reported to be the predominant pathway for extracellular malic acid

accumulation in many different organisms [54, 115, 117, 120-122]. One of the main disadvantages of microbial malic acid production is the unspecificity of the production process yielding nearly always succinate and fumarate as by-products. The mixture of end products makes the down-stream processing difficult [109]. However, this close correlation of the production of different organic acids in microorganisms can generally be an advantage, as it results in the possibility to easily change the flux in favor of another organic acid. It has been reported that a decreased activity of the cytosolic fumarate hydratase in a fumarate producer or the succinate dehydrogenase in a succinate producer, for instance, results in an efficient malic acid producer [54]. In contrast to this, the overexpression of the succinate dehydrogenase in a malic acid producer helps to increase succinate production and an increased activity of the itaconate pathway in a malic acid producer results in improved itaconate production [123].

1.3.4. Succinic acid

Succinic acid (butanedioic acid, ethane-1,2-dicarboxylic acid) (CAS 110-15-6) is a saturated, non-toxic, organic, C4-dicarboxylic acid with a molecular mass of 118.09 g mol^{-1} (Figure 7). Its solubility in water is 58 g L^{-1} at 20 °C with pK_a-values of 4.2 (pK_{a1}), 5.6 (pK_{a2}), while the solubility in ethanol, acetone, ether, and glycerol is lower.

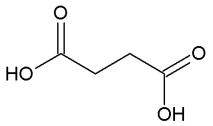

Figure 7: Chemical structure of succinic acid.

Succinic acid can be used as surfactant, detergent, foaming agent, ion chelator, acidulant, flavoring agent, antimicrobial agent, in pharmaceuticals and antibiotics, and as precursor for the production of different polymers, resins and solvents [56, 57, 124]. Even though, contemporary annual production of about 40,000 tons is rather small, the possibility to replace petroleum derived maleic anhydride, opens up a new market resulting in a potential market volume of more than 270,000 tons per year, if further research can lower production costs [57]. Succinic acid was first isolated in 1546 from amber by Georgius Agricola [125]. After a period of fermentative production for application in agriculture, food and pharmaceutics in the beginning and middle of the 20th century, contemporary commercial production mainly relies on petroleum-based chemical synthesis [124, 125]. As intermediate of the TCA-cycle, also succinic acid, just as malic and citric acid, is present in all organisms. Originally, most production processes were based on bacteria, however, by-product formation and the need for expensive, complex nutrients resulted in the search for other production organisms [124-126]. Among the investigated organisms are different *Fusarium* [127] and *Aspergillus* [122] species, *Penicillium simplicissimum* [128], *Basfia succiniciproducens* [129, 130], and yeast, such as *S. cerevisiae* and *C. krusei* [131]. In general, three different pathways for microbial succinic acid production are known, the already mentioned oxidative and reductive TCA-cycle and the glyoxylate bypass [53]. One of the main benefits in microbial succinic acid production, just as in malic acid production, is the CO_2-fixation resulting in a negative carbon balance, which greatly contributes to reduce environmental pollution [57]. Since succinic acid is generally a by-product in microbial malic acid

production [53], the production process has been investigated and improved in different steps concerning the elimination of by-product formation [132-135]. Additionally, the production and product tolerance were increased by adaptive laboratory evolution [136-138].

1.4. The development of novel biocatalysts - Ustilaginaceae

Given the broad range of intensively investigated production organisms for organic acids, it is surprising, that apart from some exceptions for most of them, the biggest amount, if not all, is still produced chemically from petroleum (Table 3). The main reason is that many bio-based processes are not yet economically competitive in direct comparison to petroleum-based processes [57]. High costs often result from low production values in microbial production processes. Low yields entail a great need of substrate, drastically lowering the profit margin. This factor can at least to some extend be circumvented by the use of low-cost or even waste-stream substrates, such as biodiesel derived crude glycerol, which is available in abundance (see chapter 1.2.2). Immense downstream processing costs due to low titers or by-product formation can be seen as most relevant cost driver for many microbial production processes. Consequently, the investigation and improvement of existing and the development of novel production processes is inevitable. One possible approach in rational whole-cell biocatalyst and bioprocess design (Figure 8) has been proposed in 2010 by Kuhn *et al*. [139].

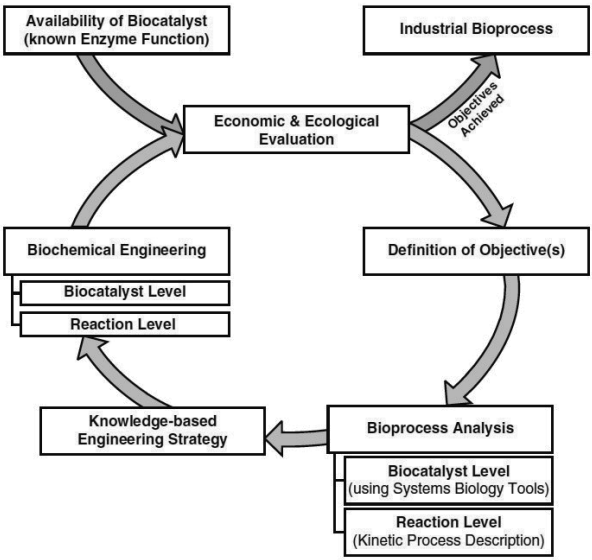

Figure 8: Systems biotechnology-based bioprocess development. [139]

In this iterative process a biocatalyst is evaluated on an economic and ecological level. Based on the evaluation, objectives for improvement are defined, the bioprocess is analyzed in consecutive steps and the biocatalyst is engineered on a knowledge-based level. The resulting, manipulated

biocatalyst is then re-evaluated. Depending on the outcome, an industrially applicable bioprocess can be launched, if the objectives were achieved. Otherwise, the biocatalyst undergoes further rounds of optimization, until the desired attributes are acquired.

Since detailed strain characterization for improvement of existing processes, especially for already intensively investigated organisms, is costly and time consuming [140], the use of novel production organisms quite often is a promising approach. The family of Ustilaginaceae is well-known for the natural production of a versatile range of industrially interesting compounds, such as organic acids, polyols, extracellular and intracellular lipids, tryptophan derivatives and siderophores [103, 104, 141-145]. Since most of the members are plant pathogens, even though there are some exceptions, such as *P. antarctica* or *P. tsukubaensis*, Ustilaginaceae were mainly investigated in terms of phylogeny and pathogenicity for many years. They are known to infect crops, mainly belonging to the family of Graminaceae, such as corn, barley, wheat, oats, sorghum, sugar cane, and forage grasses [146]. By the formation of tumors and phyllody in the inflorescences, they drastically reduce the harvest, leading to severe losses in the agricultural industry. Their biotechnological potential, however, apart from the possibility of producing a broad range of products, has been of minor focus. Yet, with 607 described species, Ustilaginaceae pose a significant biodiversity, which can be investigated and exploited by simple screening experiments, as already shown in 2014 by Geiser *et al*. [145]. The best known genera within the family of Ustilaginaceae are *Ustilago* and *Sporisorium* and the best known species within these genera are *U. maydis*, *U. hordei*, and *S. reilianum* [142, 144]. *U. maydis* causes corn smut disease, which is responsible for severe losses in agricultural economy. Besides being a model organism for biotrophic plant pathogen interactions [144, 147-149], *U. maydis* has become a fungal model organism in many other research areas, such as cell biology, DNA repair, mRNA transport, and molecular techniques [150-153]. Especially, the fully annotated genome sequence, which was published in 2006, allowed for further research regarding molecular biology and the development of novel tools [154, 155]. For instance, the application of the FLP/FRT system [156], Golden Gate Cloning [157] and the CRISPR-Cas system [158] have been demonstrated in *U. maydis*. Combined with a distinct set of available constitutive (Potef, Poma) [159-161] and inducible (*crg1*, *nar1*, *mig*, *prf1*) [162-164] promoters and functional antibiotics with corresponding resistance cassettes (carboxin, hygromycin, nourseothricin, phleomycin) [155, 165-169] these tools pose a solid basis, for metabolic optimization of value-added chemical production. Apart from the broad range of useful tools, the main advantage of *Ustilago* as production organism for industrially interesting compounds is the possibility to grow in haploid form in liquid culture medium. This phenotype results in different beneficial character traits. The duplication time of about two hours is rather short, and the sensitivity against osmotic pressure, hydro mechanical stress from shear forces, and stress from impurities in the medium and high product concentrations are low compared to filamentous fungi, such as *Aspergilli* [76, 119, 144]. Besides, natural utilization of many different carbon sources, such as glucose, glycerol, xylose, xylan, CM-cellulose, and homogenized plant tissue, has been reported [119, 144, 170-173].

All these aspects clearly demonstrate the potential of the family of Ustilaginaceae to find promising strains for the production of value-added chemicals from biodiesel-derived crude glycerol. Especially the possibility to grow on many different carbon sources, combined with the insensitivity towards impurities in the medium are of great advantage in the valorization of biodiesel-derived crude glycerol without prior cost intensive purification. Such a process would greatly benefit the overall process of biodiesel production, lowering the production costs and simultaneously posing the possibility to produce biofuels from the waste stream itself.

1.5. Scope and outline of this thesis

The overall aim of this thesis was the generation of a microbial production process for the valorization of crude glycerol from biodiesel production yielding organic acids with Ustilaginaceae as production organisms.

The general introduction in this chapter 1 is supposed to give the needed background knowledge and to elaborate on the underlying concepts and basics. These include the inevitable need to switch from fossil to renewable, bio-based resources for the generation of transportation fuels and chemicals, possible substitutes and their characteristics with a special focus on organic acids and both contemporary and future production processes, focusing on the microbial production with Ustilaginaceae. Additionally, the workflow of a systems biotechnology-based bioprocess development is introduced.

Following this workflow, the biodiversity of Ustilaginaceae was screened for organic acid production from glycerol in chapter 2.1, since these fungal production organisms are well-known for their ability to naturally produce a broad range of value-added chemicals. Titer, yield, and rate of the best malate producing strain, *U. trichophora,* were improved by adaptive laboratory evolution and medium optimization in shake flasks.

In chapter 2.2 the production process was taken to bioreactors. By optimizing the medium composition for bioreactors the reached production values could be further improved. Additionally, the process was characterized with regard to elevated and lowered process temperature, changing pH-values and differing buffer systems.

Since the new production strain, *U. trichophora* TZ1, is a not well characterized organism, the draft genome sequence is presented in chapter 2.3.

Using this genome sequence, potential target genes for overexpression were identified. However, to allow metabolic engineering, available tools had to be investigated and adapted for applicability in *U. trichophora* TZ1. The process of tool development and the overexpressions themselves including the resulting production values are described in chapter 2.4.

In chapter 2.5 the complete workflow was repeated in order to generate a production organism for itaconic acid from glycerol. In a screening of 126 Ustilaginaceae, *U. vetiveriae* was identified as promising organism. Adaptive laboratory evolution, medium optimization, and process-investigation in scaled-up bioreactors resulted in improved production values. Applying the previously investigated tools, especially the yield and product specificity could be drastically improved by metabolic engineering.

Finally, chapter 3 intends to elaborate on the possibility of further improvements, to make both, the production process for itaconic acid and malic acid industrially applicable. In this, special focus is spend on both, the microorganisms themselves and the production process, with possible challenges in up-scaling, downstream processing and the specific combination of production parameters.

In general this thesis strengthens the potential of Ustilaginaceae as industrial production organisms for different relevant platform chemicals and precursors, which can substitute petroleum-derived chemicals and fuels. A further improvement of the described production processes would strongly contribute to the needed switch from petroleum derived chemicals to sustainable, economically and environmentally feasible bio-based production processes.

Chapter 2

Results

Efficient malic acid production from glycerol with *Ustilago trichophora* TZ1

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Contributions:

Eda Sarikaya, Wiebke Kleineberg and Thiemo Zambanini performed shake flask experiments. Thiemo Zambanini and Nick Wierckx wrote the manuscript with the help of Lars M. Blank and Joerg M. Buescher. Thiemo Zambanini, Nick Wierckx, Joerg M. Buescher and Lars M. Blank designed experiments and analyzed results. Lars M. Blank, Nick Wierckx and Guido Meurer conceived and designed the project. We thank Elena Geiser for technical assistance and valuable discussion.

2. Results

2.1. Efficient malic acid production from glycerol with *Ustilago trichophora* TZ1

2.1.1. Summary

The large surplus of crude glycerol, as main low-value waste stream in biodiesel production, has led to the investigation of new possibilities for the production of value-added chemicals from this feedstock. New and efficient (bio-)catalysts are needed that are able to convert glycerol to versatile chemical building blocks. This would contribute to further develop away from a mainly petroleum based, to a sustainable, bio-based industry. One promising group of discussed building block chemicals are dicarbonic acids.

Here we report the efficient synthesis of malate from glycerol using *Ustilago trichophora* RK089, which was identified in a screening of 74 Ustilaginaceae. For economically feasible production that can compete with existing processes, a high productivity is required. By adaptive laboratory evolution, the growth and production rate were increased by 2.5-fold and 6.6-fold, respectively. Further medium optimization increased the final titer, yield, and overall production rate to 196 g L^{-1}, 0.82 g_{mal} g_{gly}^{-1}, and 0.39 g L^{-1} h^{-1}, respectively.

This titer is the highest reported for microbial malate production, making *U. trichophora* TZ1 a promising microbial production host for malate from crude glycerol, especially since it is not genetically engineered. Since this production process starts from an industrial waste stream as substrate and yields an interesting platform chemical, which can be used to replace petro-chemicals, it greatly contributes to a sustainable bio-economy.

2.1.2. Introduction

In recent years it has become apparent that a switch from our mainly petrochemical based industry towards a bio-based, carbon neutral economy is inevitable. This switch requires new precursors for many different chemicals in a broad range of sectors, such as the mobility, polymer, food additives, and pharmaceutical industries, which still rely mainly on fossil resources.

One chemical of interest is the C4-dicarbonic acid malic acid, which has been used as acidulant in foods and beverages for decades [109]. Malate has great potential as building-block chemical, for instance as a bio-based precursor for maleic anhydride, or for substituted tetrahydrofuran derivatives [34, 57]. It can also be used for the production of bio-degradable polymers [54]. In 2004 Werpy and Petersen considered 1,4-diacids (malate, succinate, fumarate) one of the twelve most promising chemicals to produce from biomass [34]. Since it is, as intermediate of the tricarboxylic acid cycle, a natural product of many microbes, microbial production is considered to be promising [54].

Indeed, the possibility of microbial production of malate has already been known and investigated for a long time. In 1962, Abe *et al.* selected *Aspergillus flavus* as production strain and patented the production process reaching a final titer of 58 g L^{-1} at a rate of 0.27 g L^{-1} h^{-1} and with a yield of 0.78 mol malate per mol glucose [114]. However, *A. flavus* is known to produce aflatoxins excluding it as industrially applicable production strain, especially for food grade malate [53]. To circumvent such problems, well established model organisms, such as *Saccharomyces cerevisiae*, *A. niger*, and *Escherichia coli*, were engineered for microbial malate production [115, 174-176]. These efforts resulted in production values comparable to the ones with *A. flavus*. In 2013 Brown *et al.* reported a production process for malate with *A. oryzae* producing 154 g L^{-1} malate with a rate of 0.94 g L^{-1} h^{-1} and a yield of 1.38 mol mol^{-1} on glucose [117]. *A. oryzae* is a close relative to *A. flavus* which produces no aflatoxins and is generally regarded as safe (GRAS).

However, production processes with *Aspergillus* species have certain drawbacks, such as the filamentous growth, which results in difficulties with the oxygen supply during large-scale fermentation [76]. Therefore a new, unicellular production strain would be favorable. In 2014, Geiser *et al*. screened 68 Ustilaginaceae for the production of organic acids [145]. They found many strains from this family to produce malate naturally, besides other organic acids, such as succinate or itaconate. Ustilaginaceae are a family of plant pathogenic fungi, of which the haploid form grows unicellularly. Further, they show tolerance to high concentrations of organic acids and they do not produce toxins, which makes them industrially applicable, even for the food industry.

Thus far, most malate production studies have focused on glucose as a substrate. Recently, glycerol has been heralded as new substrate for the production of chemicals [36]. The rising production of biodiesel, 123 million tons per year predicted by 2016 [177], is accompanied by the production of around 19 million tons per year of crude glycerol as main waste stream (10 % (w/v)). Although this glycerol itself has been considered one of the most important building blocks to be produced from biomass [52], the large volume of crude glycerol side streams has become a burden rather than a blessing. The overall process of biodiesel production would become economically more favorable, if new applications for the resulting crude glycerol were found. One application discussed frequently over the last years is the microbial conversion of the crude glycerol to value-added chemicals [36, 37]. Different production processes using glycerol as precursor have been reported, such as the production of 1,3-propanediol [42, 178], polyhydroxyalkanoates [179], lipids [180], succinate [48],

citrate [45], and erythritol [46]. The possibility of malate production from glycerol, however, has thus far only been proposed, but not investigated [37]. One advantage of the microbial conversion of glycerol to C4-dicarboxylic acids, such as malate or succinate is the possibility of CO_2 fixation through the action of pyruvate carboxylase. By this reaction, the three-carbon pyruvate and CO_2 are converted to the four-carbon oxaloacetate, theoretically enabling a process with a net carbon fixation [37].
In this study, we present the yeast-like growing smut fungus *U. trichophora* TZ1 as new production host for malate from glycerol, combining high productivity with little by-product formation and avoidance of consumer opinion and regulatory restrictions, due to production with a genetically not modified organism.

2.1.3. Material and Methods

2.1.3.1. Strains and culture conditions

The 68 strains belonging to the family Ustilaginaceae screened by Geiser *et al.* in 2014 [145], except for *U. avenae* CBS 131466 (2216), plus *U. maydis* DSM 3121 (1949), *U. maydis* DSM 4500 (1950), *U. maydis* DSM 14603 (1951), *U. maydis* Nr. 483 ATCC 22902 (2170), *U. maydis* Nr. 495 ATCC 22914 (2179), *U. trichophora* CBS 131473 (2219), *U. hordei* Uh4875-4 Mat1 [181] were screened in this study. The numbers in parenthesis indicate in-house strain numbers.
As standard medium, MTM was used according to Geiser *et al.* [145] with 0.2 g L^{-1} $MgSO_4$ 7 H_2O, 10 mg L^{-1} $FeSO_4$ 7 H_2O, 0.5 g L^{-1} KH_2PO_4, 1 mL L^{-1} vitamin solution, 1 mL L^{-1} trace element solution and 0.8 g L^{-1} NH_4Cl and 50 g L^{-1} glycerol, unless stated otherwise. As buffer, either 100 mM MES or differing concentrations of $CaCO_3$ were used. When using solid $CaCO_3$ buffer, the concentration of medium components is always based on the total volume of liquid and solid.
For solid medium screening, plates with MTM containing 20 mM MES pH 6.5, 2 % (w/v) Agar-Agar and 0.02 g L^{-1} methyl red were used. 10 µL of an overnight culture, grown in MTM with 10 g L^{-1} glucose and 100 mM MES was spotted on the plates in duplicates and the plates were incubated at 30 °C for 9 days.
For adaptive laboratory evolution, *U. trichophora* was grown in MTM with 100 mM MES in 100 mL Erlenmeyer flasks with 10 % (v/v) filling volume. OD_{600} was measured daily until an OD_{600} of >16 was reached, after which a new culture was inoculated to an OD_{600} of 0.5. This procedure was repeated sequentially for 57 days. Growth rates of evolved and original strains were assessed in separate cultures in MTM with $CaCO_3$ as buffer.
Medium optimization was performed in 24-deep well plates (Enzyscreen, System Duetz®) with 1.5 mL MTM containing either MES or $CaCO_3$ and differing concentrations of $FeSO_4$ and KH_2PO_4 incubated at 30 °C (relative air humidity = 80 %) shaking at 300 rpm (shaking diameter = 50 mm).
Shake flask production experiments were conducted in 500 mL Erlenmeyer flasks with 10 % (v/v) filling volume. All cultures were incubated at 30 °C (relative air humidity = 80 %) shaking at 200 rpm (shaking diameter = 25 mm). As preculture, MTM with 100 mM MES was inoculated from an overnight YEP culture and grown over night. All shake flask cultures were inoculated to a starting OD_{600} of 0.5. All yields were calculated based on the actual amount of glycerol consumed.

2.1.3.2. Analytical methods

All experiments were performed in duplicates. Shown is the arithmetic mean of the duplicates. Error bars and ± values indicate deviation from the mean.

When using $CaCO_3$ as buffer, 1 mL of culture broth was taken for OD_{600} determination and HPLC-analysis. The $CaCO_3$ was dissolved with HCl prior to further measurements. OD_{600} was determined in an Ultrospec 10 cell density meter (Amersham Biosciences, UK). Samples were diluted to an OD_{600} between 0.1 and 0.8.

For HPLC analysis centrifuged samples (13,000 g, 5 min) were filtered through cellulose acetate filters (diameter 0.2 µm, VWR, Germany) and subsequently diluted 1:10 with distilled water. Glycerol and organic acids were analyzed on a Dionex Ultimate 3000 HPLC (Dionex, USA) with an Organic Acid Resin column (CS Chromatographie, Germany) kept at 75 °C, with a constant flow rate of 0.8 mL min^{-1} of 5 mM sulfuric acid as eluent. For detection, a Shodex RI 101 detector at 35 °C and a variable wavelength UV detector (Dionex, USA) at 210 nm were used.

Ammonium concentration was determined by a colorimetric assay according to Willis [182].

2.1.4. Results and Discussion

2.1.4.1. Selection and evolution of *U. trichophora* as best producer of malate from glycerol

Ustilaginaceae are known to produce a broad variety of secondary metabolites and other products from glucose naturally, such as itaconate, malate, and succinate [145]. In order to identify strains that produce acids from glycerol efficiently, 74 Ustilaginaceae were initially screened on solid glycerol medium with methyl red as pH-indicator. The seven best strains were chosen for further characterization based on growth rate (colony size) and acid production (pink halo) (Figure 9A). Subsequently, these seven strains were assessed in more detail in liquid cultures in 2-(N-morpholino)ethanesulfonic acid (MES)-buffered modified Tabuchi medium (MTM) containing 50 g L^{-1} glycerol and 0.8 g L^{-1} NH_4Cl. *U. trichophora* (CBS 131473) was selected as the best growing strain with a growth rate of 0.11 ± 0.00 h^{-1}, producing 2.3 ± 0.1 g L^{-1} malate in 216 h at an overall rate of 0.01 ± 0.00 g L^{-1} h^{-1} (Figure 9E). Although *U. trichophora* was the best growing and producing strain on glycerol out of the 74 screened strains, these values are low compared to growth and malate production of this strain on glucose under the same conditions (0.45 ± 0.02 h^{-1} and 0.08 ± 0.00 g L^{-1} h^{-1}, respectively), indicating that its metabolic capacity has room for improvement on glycerol.

Adaptive laboratory evolution (ALE) is a method frequently used to improve different characteristics of microbes by adapting them to environmental conditions, such as sub-optimal pH-values or temperatures, different stress factors or the ability to utilize non-preferred carbon sources [183, 184]. Especially the potential to improve the growth rate on non-preferred carbon sources has been shown in many studies. For instance, Sonderegger *et al.* (2003) and Kuyper *et al.* (2005) could improve the growth rate of engineered *S. cerevisiae* strains on xylose and Ibarra *et al.* (2002) were able to double the growth rate of *E. coli* on glycerol at 30 °C by ALE [185-187]. Using a simple re-inoculation scheme with two parallel shake-flask cultures, as described in the materials and methods section, the growth rate of *U. trichophora* was improved after 58 days with 27 re-inoculations, corresponding to approx. 140 generations (Figure 9B).

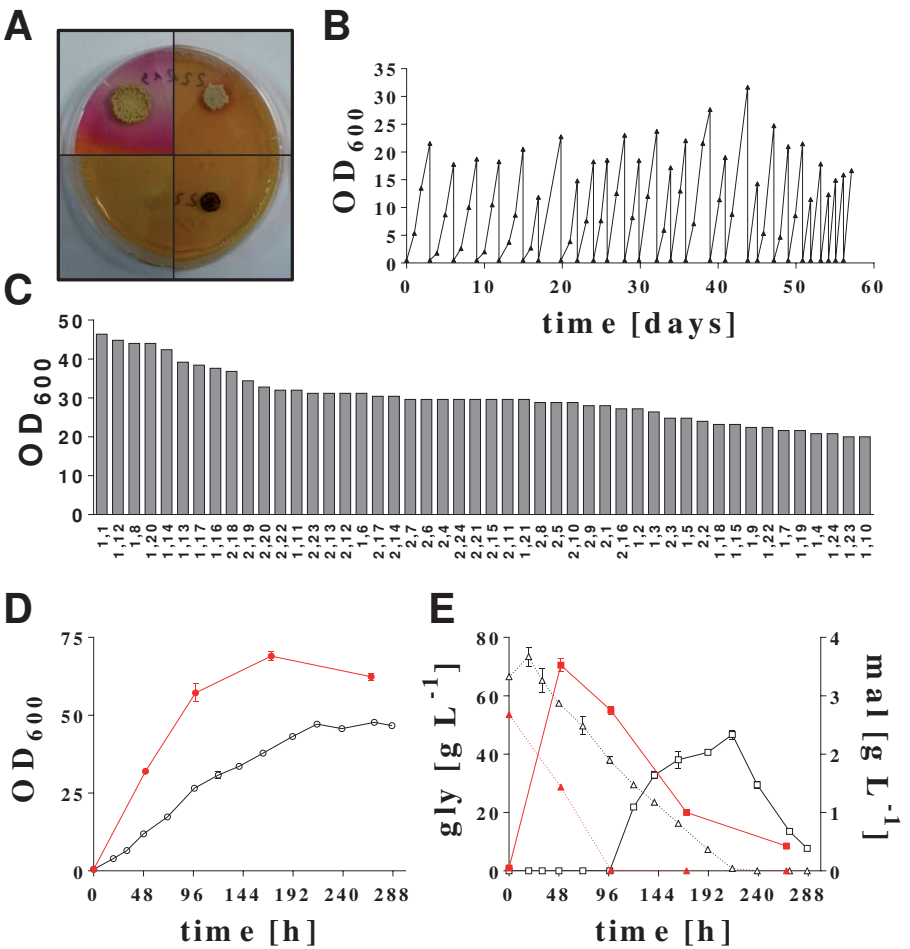

Figure 9: Screening and ALE for the production of organic acids from glycerol. A: four Ustilaginaceae in solid medium screening with glycerol as sole carbon source and methyl red as pH-indicator, pink halos indicate acid production, B: ALE of U. trichophora *on glycerol as sole carbon source. A single representative culture is shown, C: rank ordered OD_{600} after 24 h of 48 single colonies from the two parallel* U. trichophora *TZ1 cultures, the first numbers on the X-axis indicate from which parallel line the clone is derived. D: Comparison of growth for* U. trichophora *TZ1 (closed symbols, red) and wild type (open symbols, black) on glycerol, E: comparison of acid production (squares, solid lines) and glycerol consumption (triangles, dotted lines) for* U. trichophora *TZ1 (closed symbols, red) and wild type (open symbols, black).*

Screening 48 single colonies of the evolved cultures (Figure 9C) resulted in a clonal culture with increased growth rate of 0.26 ± 0.03 h^{-1} for the best colony, *U. trichophora* TZ1, which constitutes a 2.4-fold improvement over the wild type. It should be noted that, as known for Ustilaginaceae [145], the OD_{600} of *U. trichophora* generally continues to increase after nitrogen limitation (Figure 9D), likely as a result of intracellular lipid accumulation and associated morphological changes [170]. The indicated growth rates were therefore assessed separately in cultures with a higher sample resolution in the initial growth phase (data not shown). The malate production reached 3.5 ± 0.1 g L^{-1} within 50 h, corresponding to a malate production rate of 0.07 ± 0.00 g L^{-1} h^{-1} (Figure 9E).

2.1.4.2. Medium optimization increases malate production with *U. trichophora* TZ1

To further improve the malate production of *U. trichophora* TZ1, the influence of different medium components (MES and $CaCO_3$ buffer; 10, 50, 100 mg L^{-1} $FeSO_4$; 0.125, 0.25, 0.5, 1 g L^{-1} KH_2PO_4) was investigated in 24-deep well plates. Different $FeSO_4$ concentrations were tested, since $FeSO_4$ is known to influence organic acid production in *A. terreus* [87]. Influences of MES and $CaCO_3$ buffer on organic acid formation in Ustilaginaceae were already shown by Geiser *et al.* (2014) and the impact of KH_2PO_4 concentration on organic acid production has been shown by Jeon *et al.* (2013) [145, 188]. Changing the $FeSO_4$ and KH_2PO_4 concentrations did not influence growth or malate production for *U. trichophora* TZ1 (data not shown). A change from MES buffer to $CaCO_3$ buffer (33 g L^{-1}), however, resulted in a higher titer of 5.3 ± 0.3 g L^{-1} malate after 98 h of cultivation upon glycerol depletion. In MES-buffered cultures pH-values decreased during cultivation, while pH-values in cultures with $CaCO_3$ stayed constant. Given the higher buffer capacity of $CaCO_3$, it is likely that in MES-buffered cultures pH decreases to below the minimum for malate production of *U. trichophora* TZ1. A similar phenomenon was also observed for certain itaconate producing *Ustilago* strains [145]. Another advantage of $CaCO_3$ as buffer could be the additional supply of CO_2, since the microbial production of malate via pyruvate likely relies on CO_2 as co-substrate. Upon reaction of malic acid with $CaCO_3$ HCO_3^- is formed, which is in equilibrium with dissolved CO_2 dependent on the pH. This can provide an additional HCO_3^- supply for pyruvate carboxylase to form oxaloacetate from pyruvate. Indeed, feeding of additional CO_2 to an engineered malate producing *S. cerevisiae* strain significantly improved malate production [189]. By this, the malate production can theoretically be enhanced to yield 1 mole malate per mole of glycerol [115].
Since the malate production rate did not decrease until glycerol depletion, the initial glycerol concentration was increased to 200 g L^{-1}. In these cultures the malate concentration reached 129 ± 5 g L^{-1} upon glycerol depletion (Figure 10). This concentration was only observed upon dissolution of solid medium components with HCl prior to filtering for HPLC analysis. If HCl addition was omitted the concentration reached 28 ± 2 g L^{-1} after 96 h of cultivation, after which it dropped to 14 ± 1 g L^{-1} and stayed constant throughout cultivation. These results clearly show that the produced malate in combination with $CaCO_3$ forms Ca-malate, which precipitates after a brief super-saturation to its solubility limit. This solubility is somewhat higher than reported values (approximately 10 g L^{-1}) [190], which is likely due to differences in temperature and the presence of cells and other buffering agents.

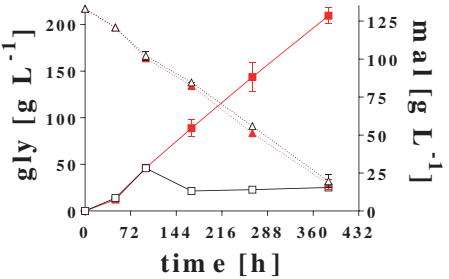

Figure 10: Shake flask cultivation of U. trichophora *TZ1 in MTM with 200 g L^{-1} glycerol. Cultures contained 100 g L^{-1} $CaCO_3$. Comparison of acid production (squares, solid lines) and glycerol consumption (triangles, dotted lines) for samples dissolved with HCl (closed symbols, red) and supernatant (open symbols, black). Error bars indicate deviation from the mean (n=2).*

2.1.4.3. Higher initial glycerol concentration further increases malate production

To further investigate the influence of starting glycerol concentrations on malate formation, the initial glycerol concentration was varied between 150 and 400 g L^{-1} in 50 g L^{-1} increments. Growth decreased with increasing initial glycerol concentrations (Figure 11A), leading to complete growth inhibition at concentrations exceeding 300 g L^{-1} (data not shown). An initial glycerol concentration of 150 g L^{-1} led to the highest overall volumetric production rate of 0.50 ± 0.08 g L^{-1} h^{-1}. Furthermore, malate production rates and glycerol uptake rates remained constant until depletion (Figure 11B). Due to handling issues (i.e. shaking of viscous liquid), samples for 300 g L^{-1} could not be taken after 672 h. Hence, the maximal malic acid titer of 196 ± 5 g L^{-1} was reached with 250 g L^{-1} glycerol as starting concentration after 504 h, corresponding to an overall production rate of 0.39 ± 0.01 g L^{-1} h^{-1} (Figure 11C). This culture also had the highest yield of 0.82 ± 0.02 g_{mal} g_{gly}^{-1} (= 0.57 ± 0.01 mol_{mal} mol_{gly}^{-1}) although in general the cultures did not show large differences in yield and no clear trend could be observed (average for all cultures: 0.74 ± 0.9 g_{mal} g_{gly}^{-1}, which equals 0.51 ± 0.06 mol_{mal} mol_{gly}^{-1}). Interestingly, the rate in the culture with 250 g L^{-1} glycerol as starting concentration is only 22 % lower than the rate achieved with 150 g L^{-1} glycerol, even though the overall OD_{600} of the former culture is less than half of the latter. It seems that with high glycerol concentrations the specific production rate per gram biomass is higher, although quantification is difficult due to possible differences in intracellular lipid formation, which significantly influences the biomass composition of *Ustilago* under these conditions [119, 170].

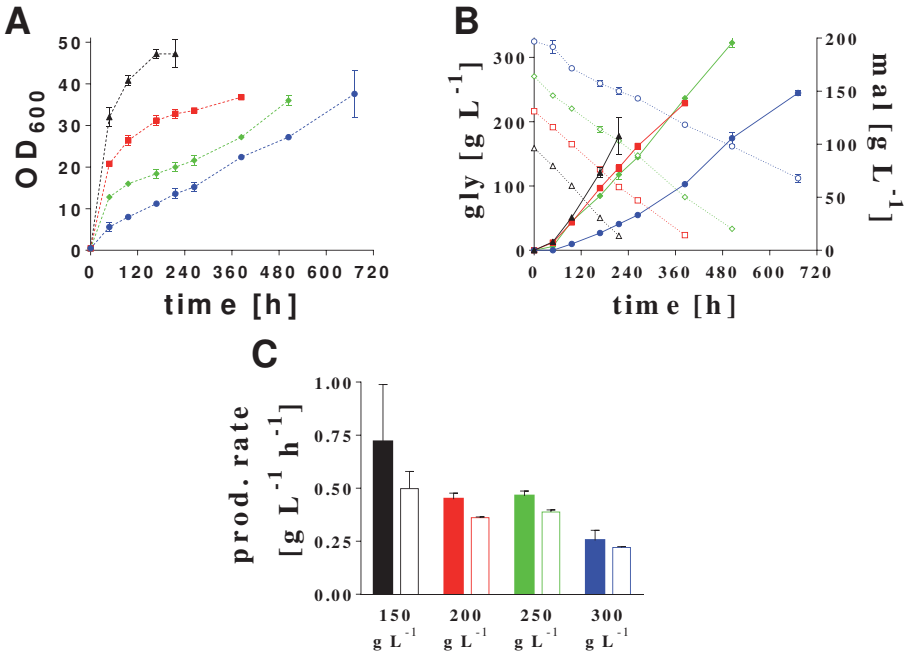

Figure 11: Shake flask cultivation of U. trichophora *TZ1 in MTM with differing glycerol concentrations. Cultures contained 100 g L^{-1} $CaCO_3$. A: comparison of growth, B: comparison of acid production (solid lines, closed symbols) and glycerol consumption (dotted lines, open symbols). 150 g L^{-1} (triangles, black), 200 g L^{-1} (squares, red), 250 g L^{-1} (diamonds, green), 300 g L^{-1} (circles, blue). C: maximal (filled bars) and overall (open bars) production rate per glycerol concentration. Error bars indicate deviation from the mean (n=2). Adapted from [191, 192].*

2.1.4.4. Separation of growth and production

Ustilaginaceae and other fungi generally only initiate organic acid production upon depletion of an essential nutrient [55, 119], which poses an inherent trade-off between biomass and product formation. In order to investigate this trade-off, as well as to establish the minimal set of compounds needed during the malic acid production phase, cells grown for 24 hours in 50 mL MTM containing 0.8 g L^{-1} NH_4Cl and 50 g L^{-1} glycerol were centrifuged, washed twice with demineralized water, and transferred to 50 mL of an aqueous solution of 100 or 200 g L^{-1} glycerol. The resting cell conversion was either buffered with 100 g L^{-1} $CaCO_3$ (200 g L^{-1} glycerol), 100 mM MES-buffer (Figure 12) or not buffered at all (100 g L^{-1} glycerol) (data not shown).

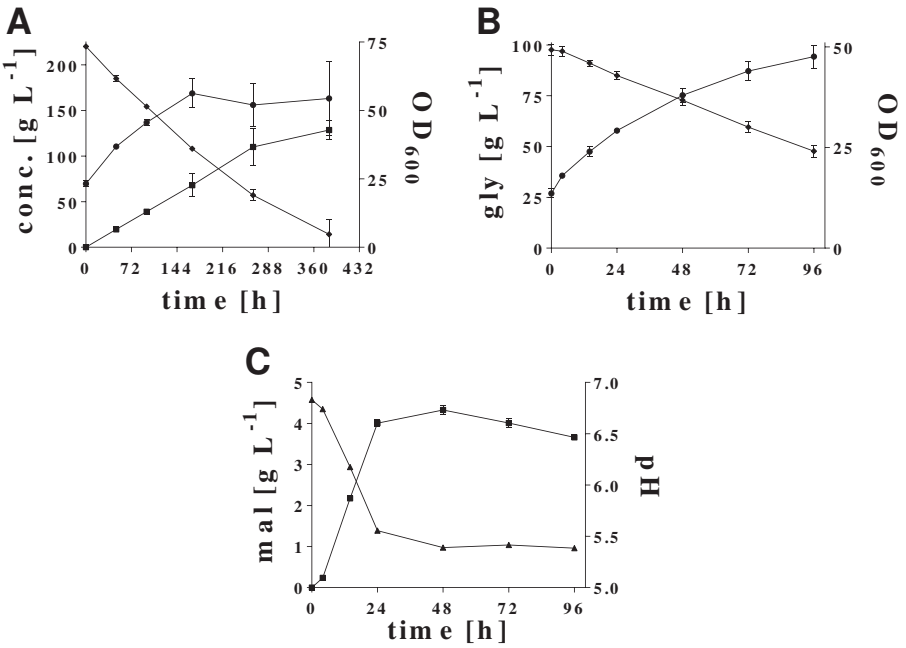

Figure 12: Malic acid production of U. trichophora *TZ1 in shake flasks with aqueous glycerol solutions. Cultures contained either 200 g L^{-1} glycerol buffered with 100 g L^{-1} $CaCO_3$ (A), or 100 g L^{-1} glycerol buffered with 100 mM MES-buffer (B, C). OD_{600} (circles), malic acid concentration (squares), glycerol concentration (diamonds) and pH-value (triangles). Error bars indicate deviation from the mean (n=2).*

In the $CaCO_3$-buffered conversion, a concentration of 129 ± 11 g L^{-1} malic acid was reached after 384 h corresponding to a production rate of 0.34 ± 0.03 g L^{-1} h^{-1} (Figure 12A), which equals the production rate of normal cultivations with 200 g L^{-1} initial glycerol (compare Figure 10). In the MES-buffered system, however, the resulting titer was significantly lower, reaching 4.01 ± 0.08 g L^{-1} (Figure 12C), while the unbuffered control produced only 20 ± 1 mg L^{-1}. Both in the MES-buffered and in the unbuffered system, the pH dropped below 5.5 and 4.5, respectively, in contrast to the $CaCO_3$ buffered system, which stayed above pH 6.0 throughout the production. This data indicates that during the production phase no supplements in the medium are needed. The pH, however, is a critical parameter. In 2014, Geiser *et al*. were already able to show the significant influence of buffer capacity on acid production with Ustilaginaceae [145]. They cultivated Ustilaginaceae in differing concentrations of MES buffer. Comparable to our data, a drop in pH inhibited further acid production. Apparently, a pH greater than 5.4 is needed for efficient malate production with *U. trichophora* TZ1. Interestingly though, even without further malate production in the MES-buffered system the glycerol consumption rate stays constant for 72 h. This suggests the activity of an overflow metabolism, possibly switching to alternative products, such as extracellular glycolipids, polyols or CO_2. Additionally, with both buffers OD_{600} increases more than 2.5-fold,

even though no source of nitrogen, phosphate or trace elements was present. This increase in OD_{600} can for a large part be attributed to the production of intracellular lipids [170]. A significant increase in dry cell weight after nitrogen limitation has also already been reported by Klement *et al.* in 2012 [119]. They could show, that the cell number still increased by about 30 % after limitation. However, the carbon-to-nitrogen weight ratio in the biomass increased from 5.9 in cells during unlimited growth to about 20 in cells during stationary phase, clearly showing that the increasing OD_{600} is a result of nitrogen "dilution". Consequently, one additional division cycle is possible after nitrogen depletion, which probably does not positively affect the bio-catalytic potential, since the total amount of proteins remains the same [119]. In addition, a significant amount of glycerol is used for lipid formation, which mainly occurs after nitrogen depletion. Although this generally detracts from the overall efficiency of malate production, the lipids themselves are a useful secondary product for food, cosmetic or biofuel applications, and their inclusion in the cells makes them relatively easy to separate. These phenomena may partly explain the yield of 0.43 ± 0.00 mol_{mal} mol_{gly}^{-1}, which is lower than the yield in cultures with complete mineral media containing 200 g L^{-1} glycerol (0.49 ± 0.00) even though no glycerol is needed for biomass production. In addition, the lack of micronutrients likely serves as an additional stress factor which reduces the cells' productivity and tolerance to malate.

In all, these results indicate the high potential of *U. trichophora* TZ1 as production organism for malic acid. Though the overall production rate of 0.50 ± 0.08 g L^{-1} h^{-1} is lower than reported rates for other organisms [117], a titer of almost 200 g L^{-1} is reached with a strain that is not genetically modified. To our knowledge this titer is the highest reported value for microbial malic acid production. In the future, this process can be taken to bioreactors for further improvement, making full use of *U. trichophora*'s unicellular growth, focusing on increasing the production rate and final titer while circumventing handling and downstream processing issues associated with $CaCO_3$ cultures. These issues include problems of oxygenation by shaking due to high viscosity and the general drawback of a huge gypsum waste stream for industrial scale processes resulting from production processes involving $CaCO_3$ as buffering or downstream processing agent.

By this, the overall production process for malic acid with *U. trichophora* could be further improved, making *U. trichophora* a promising industrially applicable production organism for malic acid.

2.1.5. Conclusion

The microbial conversion of glycerol to value added chemicals has been the focus of research for many years. The identification and optimization of *U. trichophora* TZ1 as efficient malate producer opens up novel opportunities for glycerol valorization, potentially adding to the overall feasibility of a biodiesel bio-refinery. The reached titer of almost 200 g L^{-1} is the highest titer reported for any microbial malic acid production, and further improvements in the production rate and yield can be expected from process optimization and metabolic engineering. Especially the generation of a closed carbon-balance would shed light on possible targets, since it would clarify the amount of glycerol used for by-product formation and respiration. Abovementioned facts reveal the potential for further research and improvement of *U. trichophora* TZ1 as promising, industrially applicable production organism for malic acid, or as a gene donor of interest for heterologous malate producers. This confirms in general the potential of the Ustilaginaceae for bio-catalysis.

Enhanced malic acid production from glycerol with high-cell density *Ustilago trichophora* TZ1 cultivations

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Contributions:

Thiemo Zambanini performed shake flask experiments. Wiebke Kleineberg, Eda Sarikaya and Thiemo Zambanini performed bioreactor experiments with pharma-grade glycerol. Joerg M. Buescher performed bioreactor experiments with crude glycerol. Thiemo Zambanini and Nick Wierckx wrote the manuscript with the help of Lars M. Blank and Joerg M. Buescher. Thiemo Zambanini, Nick Wierckx, Joerg M. Buescher and Lars M. Blank designed experiments and analyzed results. Lars M. Blank, Nick Wierckx and Guido Meurer conceived and designed the project. We thank Elena Geiser for technical assistance and valuable discussion.

2.2. Enhanced malic acid production from glycerol with high-cell density *Ustilago trichophora* TZ1 cultivations

2.2.1. Summary

In order to establish a cost-efficient biodiesel bio-refinery, valorization of its main by-product, crude glycerol, is imperative. Recently, *Ustilago trichophora* TZ1 was found to efficiently produce malic acid from glycerol. By adaptive laboratory evolution and medium optimization, titer and rate could be improved significantly.

Here we report on the investigation of this strain in fed-batch bioreactors. With pH controlled at 6.5 (automatic NaOH addition) a titer of 142 ± 1 g L^{-1} produced at an overall rate of 0.54 ± 0.00 g L^{-1} h^{-1} was reached by optimizing the initial concentrations of ammonium and glycerol. Combining the potential of bioreactors and $CaCO_3$ as buffer system, we were able to increase the overall production rate to 0.74 ± 0.06 g L^{-1} h^{-1} with a maximum production rate of 1.94 ± 0.32 g L^{-1} reaching a titer of 195 ± 15 g L^{-1}. The initial purification strategy resulted in 90 % pure calcium malate as solid component. Notably, the fermentation is not influenced by an increased temperature of up to 37 °C, which reduces the energy required for cooling. However, direct acid production is not favored as at a lowered pH-value of pH 4.5 the malic acid titer decreased to only 9 ± 1 g L^{-1}. When using crude glycerol as substrate, only the product to substrate yield is decreased. The results are discussed in the context of valorizing glycerol with Ustilaginaceae.

Combining these results reveals the potential of *U. trichophora* TZ1 to become an industrially applicable production host for malic acid from biodiesel derived glycerol, thus making the overall biodiesel production process economically and ecologically more feasible.

2.2.2. Introduction

The production of biodiesel, as one possible supplement for petroleum derived fuels, is a great opportunity to drive the needed switch to a bio-based economy. This is also reflected in the ever increasing amount of produced biodiesel, which is predicted to be 123 million tons per year in 2016 [177]. However, this process results in a 10 % (w/v) crude glycerol waste stream, decreasing the profit margin and ecological feasibility. Microbial conversion to valorize this large low-value side stream is considered a promising strategy to add value to the overall biodiesel bio-refinery concept. Microbial production processes starting from glycerol as substrate have been investigated and reviewed intensively over the last years resulting in processes for many different products [36, 37, 193].

The C4-dicarboxylic acid malic acid is widely used as acidulant and flavor enhancer in the food industry and has also received great interest in non-food applications, such as metal cleaning, textile finishing, and pharmaceuticals production [54]. Even though the annual world production in 2006 was only about 40,000 tons, future use of malic acid is predicted to be above 200,000 tons per year as raw material of a novel biodegradable polymer - polymalic acid [54, 57]. In 2004 malic acid has been identified by the Department of Energy (DoE) as one of the top twelve building block chemicals to be produced from renewable biomass at bulk scale [34]. Traditionally, malic acid was obtained by the extraction from apple juice at low yields [109]. Today malic acid can be produced both chemically and biotechnologically. In current industrial production processes it is mainly manufactured by chemical synthesis via hydration of maleic or fumaric acid producing a racemic mixture of D- and L-isomers [194]. Alternatively, enzymatic hydration of fumarate by immobilized bacterial cells of *Brevibacterium ammoniagenes* or *Bacillus flavum* containing a highly active fumarase yields enantiomerically pure L-malic acid [121]. However, these production methods are costly and substrates for the synthesis of malic acid are derived from non-sustainable petrochemical feedstocks [54]. Thus, as TCA-cycle intermediate, bio-based microbiological production processes based on renewable substrates for malic acid have become the focus of research. The first patented microorganism producing malic acid was *Aspergillus flavus* [114]. The fermentation process was improved by medium optimization resulting in a final titer of 113 g L^{-1} from 120 g L^{-1} glucose as substrate [109]. However, this organism is not applicable for industrial production, especially for food applications, due to the production of aflatoxins [53]. Besides *Saccharomyces cerevisiae* [115] and *Escherichia coli* [175, 176], an *A. oryzae* strain has been investigated as production organism. This strain, overexpressing a malate dehydrogenase, pyruvate carboxylase, and C4-dicarboxylate transporter produced a final titer of 154 g L^{-1} malic acid from glucose at a rate of 0.94 g L^{-1} h^{-1} [117].

Recently we reported that *U. trichophora* TZ1, a member of the family of Ustilaginaceae which is known to produce organic acids naturally [145], is able to produce malic acid from glycerol [195]. This strain has been adapted to glycerol by laboratory evolution, increasing glycerol uptake rates. After medium optimization the final malic acid titer reached 196 g L^{-1} produced from 250 g L^{-1} glycerol at an average rate of 0.4 g L^{-1} h^{-1} in shake flasks. The limiting factor in these shake flask cultivations was either glycerol depletion or problems concerning oxygen transfer, which result from viscous culture broth.

Here we report on malic acid production with *U. trichophora* TZ1 in bioreactors to overcome abovementioned problems. Further, the production process was investigated at differing temperature profiles and pH-values to determine the boundary conditions of an eventual industrial process, and the effects of using high concentrations of crude glycerol as a substrate were evaluated.

2.2.3. Material and Methods

2.2.3.1. Strains and culture conditions

U. trichophora TZ1 was used throughout this study [195].

As standard medium, modified Tabuchi medium (MTM) according to Geiser *et al.* containing 0.2 g L^{-1} $MgSO_4$ 7 H_2O, 10 mg L^{-1} $FeSO_4$ 7 H_2O, 0.5 g L^{-1} KH_2PO_4, 1 mL L^{-1} vitamin solution, 1 mL L^{-1} trace element solution [145] and differing concentrations of NH_4Cl and (crude) glycerol was used. For additional glycerol feeds 200 mL of an 800 g L^{-1} glycerol solution were added to the cultures. Additional 150 g $CaCO_3$ was fed to the cultures as solids, when the pH-value dropped below 5.5. Pharma grade glycerol was used for all cultures except for those where the use of crude glycerol is explicitly stated. Crude glycerol was used as 80 % (w/v) aqueous solution and autoclaved without prior purification. After addition of all medium components, the pH-value was adjusted to 6.5.

All batch cultivations were performed in New Brunswick BioFlo® 110 bioreactors (Eppendorf, Germany) with a total volume of 2.5 L and a working volume of 1.25 L. Temperature was maintained at 30 °C and the pH-value was either set to 6.5 and controlled automatically with 10 M NaOH or different amounts of $CaCO_3$ were added as buffer. To prevent foam formation, antifoam 204 (Sigma Life Science, USA) was added automatically using level sensor control. The aeration rate was set to 1.25 L min^{-1} (1 vvm) and the dissolved oxygen tension (DOT) was kept at 80 % saturation by automatically adjusting the stirring rate. As preculture, 50 mL MTM containing 0.8 g L^{-1} NH_4Cl, 50 g L^{-1} glycerol, and 100 mM MES in 500 mL shake flasks was inoculated from an overnight YEP culture to an OD_{600} of 0.5. This culture was grown over night, washed twice by dissolving the pelleted cells (5,000 rpm, 5 min, 30 °C) in 10 mL distilled water and used for inoculation of the bioreactor to an initial OD_{600} of 0.5. All shake flask cultures were incubated at 30 °C (relative air humidity = 80 %) shaking at 200 rpm (shaking diameter = 25 mm).

2.2.3.2. Analytical methods

All experiments were performed in duplicates. Shown is the arithmetic mean of the duplicates. Error bars and ± values indicate deviation from the mean.

From bioreactors 5 mL of culture broth was taken for OD_{600} and HPLC-analysis. When using $CaCO_3$ as buffer, the $CaCO_3$ in 1 mL culture broth was dissolved with HCl prior to further measurements. OD_{600} was determined in an Ultrospec 10 cell density meter (Amersham Biosciences, UK). Samples were diluted to an OD_{600} between 0.1 and 0.8.

For HPLC analysis centrifuged samples (13,000 g, 5 min) were filtered through cellulose acetate filters (diameter 0.2 µm, VWR, Germany) prior to diluting 1:10 with distilled water. For analysis of glycerol and organic acids, a Dionex Ultimate 3000 HPLC (Dionex, USA) was used with an Organic Acid Resin column (CS-Chromatographie, Germany) at 75 °C, with a constant flow rate of 0.8 mL min^{-1} 5 mM sulfuric acid as eluent. For detection, a Shodex RI 101 detector at 35 °C and a variable wavelength UV detector (Dionex, USA) at 210 nm were used.

Ammonium concentration was determined by a colorimetric assay according to Willis [182].

Calculation of the molar fraction of undissociated and dissociated species for malate was performed using CurTiPot [196].

2.2.4. Results and Discussion

2.2.4.1. Bioreactors enable higher cell density resulting in higher volumetric production rates

The potential of Ustilaginaceae as production organisms of different industrially relevant compounds, such as organic acids, lipids or polyols, has been discussed and demonstrated consistently over the last years [76, 104, 144, 145, 170, 197-199]. Recently, *U. trichophora* was found to produce malic acid naturally from glycerol at high titers. By adaptive laboratory evolution and medium optimization, the production rate of this strain in shake flask could be improved to around 0.4 g L^{-1} h^{-1} reaching titers near 200 g L^{-1} [195]. All cultivations ended either upon glycerol depletion, or by oxygen limitations due to the viscosity of the cultures. These viscosity issues resulted mainly from the buffering agent, $CaCO_3$, reacting with produced malate, forming insoluble calcium malate. Although this precipitation might be beneficial for alleviation of product inhibition, it greatly hinders oxygenation of the culture broth in shaking flasks [200].

To overcome handling issues with insoluble components and to avoid glycerol depletion, here we investigate the production process with *U. trichophora* TZ1 in bioreactors, in which the pH was kept constant by titration with NaOH. By this, effects of insoluble buffer components on production can be minimized. Further, by feeding additional glycerol prior to depletion, malate titers might be further increased. Additionally, better oxygenation through sparging and stirring, which has a strong influence on microbial organic acid production processes [95], also enables higher cell densities.

Initially, *U. trichophora* TZ1 was cultured in pH controlled bioreactors (pH 6.5, NaOH titration) in MTM containing 0.8 g L^{-1} NH_4Cl and 200 g L^{-1} initial glycerol. An additional 160 g glycerol were fed when the concentration dropped below 50 g L^{-1}. This results in a slight drop in the measured malate concentrations due to the dilution of the culture broth. The resulting titer (119.9 ± 0.9 g L^{-1}) and rate (0.13 ± 0.00 g L^{-1} h^{-1}) (Figure 13B) were significantly lower than those reached in shake flasks with $CaCO_3$ [195]. Likely, these reductions can be attributed to product inhibition caused by the drastically increased dissolved malate concentration in NaOH-titrated cultures. To improve the production rate, the cell density was increased by using higher concentrations of the growth-limiting nutrient NH_4Cl (1.6 g L^{-1}, 3.2 g L^{-1}, and 6.4 g L^{-1}). Dependent on the initial NH_4Cl concentration, a delay in the onset of malate production could be observed, which can be attributed to a longer growth phase. Maximal OD_{600} however could be increased from 42 ± 2 with 0.8 g L^{-1} NH_4Cl to 80 ± 0 and 115 ± 1 using 1.6 and 3.2 g L^{-1} NH_4Cl, respectively (Figure 13A). As expected, also the overall volumetric malic acid production rate (from beginning of cultivation until the end) increased to 0.46 ± 0.02 and 0.54 ± 0.07 g L^{-1} h^{-1} with 1.6 and 3.2 g L^{-1} NH_4Cl, respectively (Figure 13B). 6.4 g L^{-1} NH_4Cl, however, did not lead to increased biomass and subsequently production, but had the opposite effect (data not shown). In these cultures NH_4Cl was no longer depleted during the fermentation. A similar effect was observed for itaconate producing *U. maydis* MB215 in MTM with NH_4Cl concentrations above 4 g L^{-1} [170]. This likely explains the reduced productivity, since nitrogen limitation is the most efficient trigger for organic acid production with Ustilaginaceae [119]. To compensate for this effect all medium components except for glycerol were doubled in combination with 6.4 g L^{-1} NH_4Cl in a subsequent fermentation, resulting in an overall volumetric production rate of 0.54 ± 0.00 g L^{-1} h^{-1}, with a maximal production rate of 1.99 ± 0.04 g L^{-1} h^{-1} between 45 and 69 hours (Figure 13B).

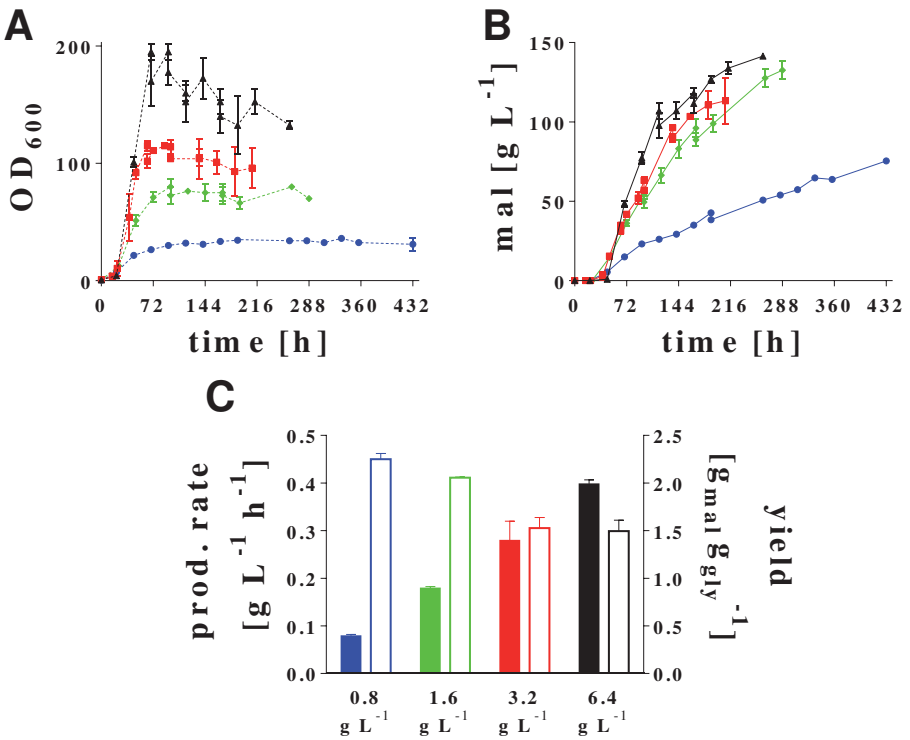

Figure 13: Fermentation of U. trichophora *TZ1 with different NH_4Cl concentrations. A: OD_{600}, B: malate production, C: maximum malate production rate (solid bars) and yield (open bars) for controlled batch fermentations in MTM containing 200 g L^{-1} initial glycerol at 30 °C and pH 6.5 with DO kept at 80 %. Colors indicate different initial NH_4Cl concentrations: 0.8 g L^{-1} (circles, blue), 1.6 g L^{-1} (diamonds, green), 3.2 g L^{-1} (squares, red), and 6.4 g L^{-1} with doubled concentrations of all medium components except glycerol (triangles, black). Values for 0.8 g L^{-1} are only shown until 432 h, however a further increase in concentration to a final titer of 120 ± 1 g L^{-1} could be observed until 908 h of cultivation. Error bars indicate deviation from the mean (n=2). Adapted from [191, 201, 202].*

As expected, an increase in the growth-limiting nutrient led to more biomass formation and consequently to a higher volumetric production rate. There is a good correlation between the maximum malate production rate and the initial NH_4Cl concentration, indicating that the production rate could be further increased as long as secondary limitations are excluded. However, further increases will strongly impact the product yield, since more glycerol is used for biomass formation. Assuming no CO_2 co-consumption, the maximum theoretical yield would be 0.75 mole malate per mole glycerol. However, the glycerol needed for biomass production reduces this maximum, and this reduction is proportional to the initial ammonium concentration. Based on the glycerol consumption during the growth phase (Figure 13A) approximately 11.5 grams of glycerol are

needed for biomass formation per gram NH_4Cl. Thus, taking into account the total amount of glycerol consumed by each culture, biomass formation reduces the maximum theoretical yield to 0.73, 0.71, 0.68, and 0.62 mol mol^{-1}, for 0.8, 1.6, 3.2, and 6.4 g L^{-1} NH_4Cl, respectively. This in part explains the reduction of the observed yields in the cultures with higher NH_4Cl concentrations, although in general the yields are only 30 - 55 % of these theoretical maxima, suggesting that the impact of biomass formation is at the moment relatively low. Improvement of the product yield should be the main focus of future optimization, possibly by the reduction of by-product formation through the disruption of competing pathways. The improvement of specificity for the production of one organic acid is generally considered a promising approach to improve microbial organic acid production. For *U. trichophora* TZ1, however, besides 5 - 10 g L^{-1} succinate, no significant amounts of other organic acids were found in HPLC analysis. Additionally, CO_2 and extra- and intracellular lipids are most likely the main by-products. The formation of lipids under organic acid production conditions and their effect on the cells have been described extensively [119, 203]. These by-products can be reduced by knock-out of single genes in the responsive gene clusters [123, 141, 204].

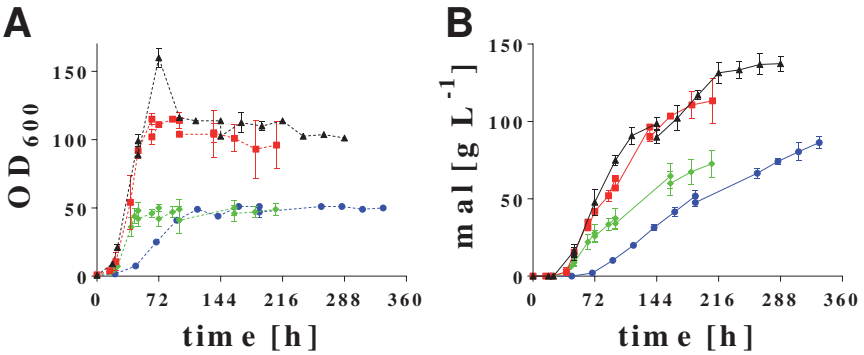

Figure 14: Fermentation of U. trichophora *TZ1 with different initial glycerol concentrations. A: OD_{600}, B: malate production for fermentations in MTM containing 3.2 g L^{-1} NH_4Cl at 30 °C and pH 6.5 with DO kept at 80 %. Colors indicate different initial glycerol concentrations: 300 g L^{-1} (circles, blue), 250 g L^{-1} (diamonds, green), 200 g L^{-1} (squares, red), 150 g L^{-1} (triangles, black). Additional 160 g glycerol were added when the concentration dropped below 50 g L^{-1}. Error bars indicate deviation from the mean (n=2). Adapted from [191, 202].*

Since a significant influence of the starting glycerol concentration on the malic acid production rate has been observed in shake flasks [195], this relation was also studied in bioreactors. Concentration steps of 50 g L^{-1} between 150 g L^{-1} and 300 g L^{-1} were investigated in MTM containing 3.2 g L^{-1} NH_4Cl. Additional 160 g glycerol were fed to the cultures one time (300 g L^{-1} initial glycerol), two times (150 and 200 g L^{-1} initial glycerol) and four times (250 g L^{-1} initial glycerol), when the concentration became lower than 50-100 g L^{-1} (150 and 200 g L^{-1} initial glycerol) or 200 g L^{-1} (250 and 300 g L^{-1} initial glycerol). Thus, after the consumption of the initial glycerol, its concentrations generally ranged between 50 and 150 g L^{-1} (150 and 200 g L^{-1} initial glycerol) and 100 and 250 g L^{-1} (250 and 300 g L^{-1} initial glycerol). Just as in shake flasks, increasing initial glycerol concentrations between 150 g L^{-1} and 300 g L^{-1} decreased growth rates, final OD_{600} and

malic acid production rates (Figure 14). Possibly, higher glycerol concentrations impose a stress upon the cells. This is also known in other organisms, such as *S. cerevisiae*, even though lower glycerol concentrations are generally known to contribute to osmotolerance in different yeast, such as *Zygosaccharomyces rouxii* and *S. cerevisiae* [205, 206].

2.2.4.2. *U. trichophora* TZ1 accepts a broad temperature range for production

In 1990, Guevarra and Tabuchi investigated the influence of temperature on itaconic acid production and growth of *U. cynodontis* [103]. They could show that the highest tested temperature (35 °C) was best for cell growth. However, the lowest tested temperature (25 °C) resulted in the highest organic acid titers. To investigate influences of temperature on acid production by *U. trichophora* TZ1, cells were grown at 30 °C and the temperature was changed after the growth phase to 25 °C and 35 °C. In a third approach heating was disabled and cooling was only activated at temperatures exceeding 37 °C (Figure 15). In this case the temperature remained at this maximum after 30 hours, indicating the considerable heat generated by these high-density cultures. As seen in Figure 15B, malic acid production was not influenced by temperatures exceeding 30 °C. However, 25 °C resulted in a lower malic acid production rate yet reaching the same final titer of approximately 120 g L^{-1}.

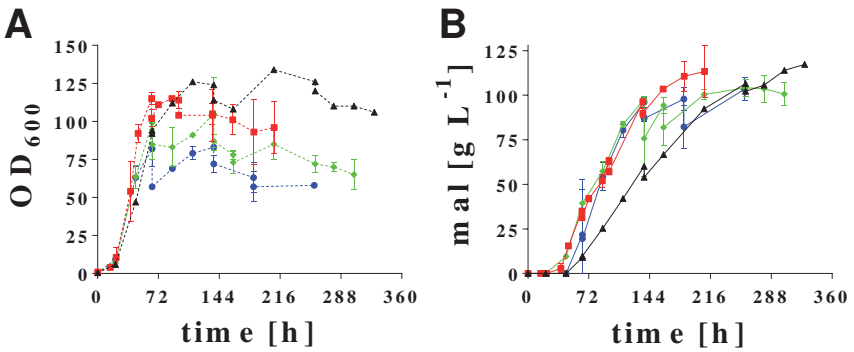

Figure 15: Fermentation of U. trichophora *TZ1 at different temperatures. A: OD_{600}, B: malate concentration for fermentations in MTM containing 200 g L^{-1} initial glycerol and 3.2 g L^{-1} NH_4Cl at 30 °C and pH 6.5 with DO kept at 80 %. Colors indicate different temperatures: 25 °C (triangles, black), 30 °C (squares, red), 35 °C (circles, blue) and 37 °C (diamonds, green). Error bars indicate deviation from the mean (n=2). Adapted from* [191].

Since malic acid production with *U. trichophora* TZ1 was not influenced by elevated temperatures and reduced use of heating and cooling systems could reduce operating costs, preliminary experiments without a heating and cooling system were conducted. These experiments indicated that uncontrolled temperatures above 37 °C negatively influence the malic acid production process. This was also observed in 2008 by Kuenz for itaconic acid production with *A. terreus* [207]. A temperature increase from 27 °C to 30 °C resulted in a 60 % increased production rate. Further increasing the temperature to 33 °C and 37 °C resulted in a 20 to 40 % increase compared to 30 °C. However, a process temperature of 40 °C reduced itaconic acid production drastically [207].

2.2.4.3. Decreasing pH-values drastically lower malic acid production

In a next step, the fermentation was investigated in respect to growth medium pH. Malic acid production with *U. trichophora* TZ1 was investigated in bioreactors at pH 4.5, 5.5, and 6.5. The tested pH-range neither influenced growth rate (Figure 16A), nor morphology (data not shown). However maximal OD_{600} was higher at lower pH. Malic acid production was clearly lowered by decreasing pH reaching 113 ± 15 g L^{-1} (pH 6.5), 64 ± 6 g L^{-1} (pH 5.5), and 9 ± 1 g L^{-1} (pH 4.5). In fungi such as *Aspergillus*, *Saccharomyces* and *Yarrowia*, organic acids such as succinic acid, itaconic acid, and malic acid are produced best at low pH, with some exceptions [95, 100, 208-211]. For Ustilaginaceae, however, mainly near neutral pH-values are best for organic acid production [170], although exceptions such as *U. cynodontis* have been reported [145].

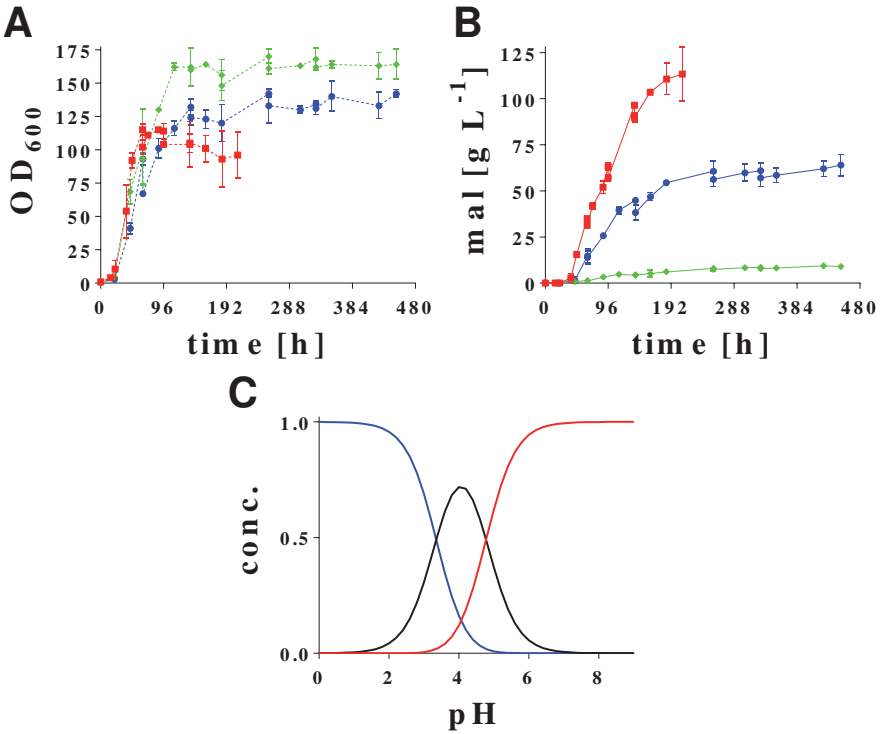

Figure 16: Fermentation of U. trichophora *TZ1 at different pH-values. A: OD_{600}, B: malate concentration for fermentations in MTM containing 200 g L^{-1} initial glycerol and 3.2 g L^{-1} NH_4Cl at 30 °C and pH 6.5 with DO kept at 80 %. Additional 160 g glycerol were added when the concentration dropped below 50 g L^{-1}. Colors indicate different pH-values: pH 6.5 (red), 5.5 (blue), and 4.5 (green). Error bars indicate deviation from the mean (n=2). C: Distribution of molar fractions of dissociated and (partly) undissociated malate species. Shown is the relative distribution of fully dissociated (blue), partially dissociated (black) and fully undissociated (red) malate dependent on the pH-value. Data was generated using CurTiPot [196]. Adapted from [191].*

Both, production at high and at low pH-value has different opportunities and disadvantages on microbial organic acid production and down-stream processing. A low pH can help to lower the risk of contamination in industrial-scale fermentations. Further, the production of environmentally unfriendly by-products can be reduced, since during the production process less titration agents, such as $CaCO_3$ or $Ca(OH)_2$, are needed, which in the later process have to be disposed of. However, the same by-product, namely gypsum, is also produced in the down-stream process of microbial citric acid production, resulting from the reaction of sulfuric acid with calcium-citrate [60]. However, more advanced down-stream technologies, such as simulated moving bed [212], are becoming ever more established and could enable a calcium-free process, provided that it does not negatively impact the overall process efficiency. Another advantage of producing acids at low pH is the easier down-stream processing itself, since methods such as cooling, evaporation-crystallization or salting are possible [76, 213]. Besides the positive effects of production at a low pH, there are many advantages for production at near neutral pH. One of those beneficial effects for Ustilaginaceae is the lowered burden, normally resulting from undissociated acids or low pH itself. Other advantages are the avoidance of thermodynamic constraints on acid export or the possibility of advanced process strategies, such as simultaneous saccharification and fermentation (SSF) in which the pH-optimum of the applied enzymes is essential [57, 119, 214].

pH-values near the lower pK_a value of malate (pK_{a1} 3.46, pK_{a2} 5.10) [115] result in undissociated malic acid. Although the molar fraction of this undissociated species is relatively low (approximately 0.002 % at pH 6.5, 0.1 % at pH 5.5 and 4.8 % at pH 4.5; Figure 16C), its protonophoric effect likely disrupts cellular pH-homeostasis. This possibly coupled to an increased intracellular malic acid concentration, likely leads to the observed reduction of malate production. The weak acid uncoupling effect caused by uptake of the protonated form via diffusion with a simultaneous import of a proton and needed active transport of the dissociated form out of the cell leads to a loss of energy [214, 215]. A further loss of energy can result from the export mechanism itself. It was reported, that the most likely mechanism for export of dicarboxylic acids at low pH is an antiport with protons [216]. This would lead to additional H^+ ions pumped against the proton motive force, which consequently increases ATP consumption [217]. The observation, that glycerol uptake is not decreased in cultures with lower pH, would strengthen this hypothesis, since its consumption could help to cope with the energy loss.

2.2.4.4. $CaCO_3$ as buffering agent helps to overcome product inhibition

Independent from final OD_{600}, malic acid production, glycerol consumption, growth rate, and temperature, a clear drop in production rate at malate concentrations above 100 g L^{-1} is visible and the maximal titer of around 140 g L^{-1} was not exceeded. In shake flask cultivations containing $CaCO_3$ as buffer agent, however, this titer had been exceeded with constant production rates until glycerol depletion [195]. In these cultures the $CaCO_3$ reacts with the produced malic acid forming calcium malate, which precipitates at a concentration above 14 g L^{-1}. As a consequence, additionally produced malate is no longer dissolved in the medium, thus alleviating product inhibition and toxicity. These results strongly suggest a negative effect of product inhibition at concentrations above 100 g L^{-1}.

To overcome the assumed product inhibition in fed-batch bioreactors, cultivations with MTM containing 3.2 g L^{-1} NH_4Cl, 200 g L^{-1} initial glycerol and 100 g L^{-1} $CaCO_3$ as buffer were

performed (Figure 17). An additional 150 g L^{-1} $CaCO_3$ was added when the pH dropped below 5.5 and additional 160 g glycerol were fed when the concentration fell below 50 g L^{-1}. This fermentation resulted in the production of 195 ± 15 g L^{-1} of malic acid within 264 h of cultivation, corresponding to an overall production rate of 0.74 ± 0.06 g L^{-1} h^{-1}. The process reached a yield of 0.43 ± 0.05 g_{mal} g_{gly}^{-1} and a maximal production rate of 1.94 ± 0.32 g L^{-1} between 47 and 71 hours (Figure 17A). Both glycerol consumption and malic acid production decreased over time. The yield during production phase, however, stayed constant in a range of 0.39 to 0.49 g_{mal} g_{gly}^{-1}, indicating that the decreasing production rate is rather an effect of dilution due to glycerol feed than an actual decrease in the specific productivity.

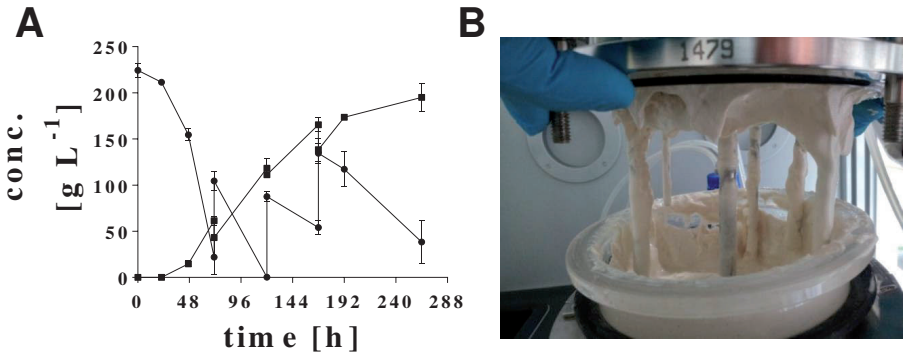

Figure 17: Fermentation of U. trichophora *TZ1 with $CaCO_3$. A: malate concentration (squares) and glycerol concentration (circles), B: fermentation broth after 264 h of fermentation in MTM containing 200 g L^{-1} glycerol, 3.2 g L^{-1} NH_4Cl and 100 g L^{-1} initial $CaCO_3$ at 30 °C with DO kept at 80 %.*

The yield achieved with $CaCO_3$ as buffer is 1.5-fold higher than with NaOH. This increase may either be due to an increase in CO_2 co-fixation through the action of pyruvate carboxylase or to a reduction of product inhibition by in-situ crystallization of calcium malate. Based on the current yield, and assuming that all remaining glycerol is converted to CO_2, 85 % of the total produced CO_2 originates from glycerol. The remaining 15 % originates from $CaCO_3$ (12 %) and aeration (3 %). Given this relatively low contribution of $CaCO_3$ to the overall CO_2 balance, a positive effect of additional CO_2 co-metabolism from $CaCO_3$ is unlikely. This suggests that the higher yield observed with $CaCO_3$ is mainly due to reduction in product inhibition.

At 264 h the fermentation had to be stopped due to bad mixing caused by high medium viscosity (Figure 17B) as had already been experienced for shake flask cultivations using $CaCO_3$ as buffering agent [195]. This increased viscosity, likely caused by calcium malate, results in poor and inhomogeneous oxygenation. Further, even though the formed calcium malate can easily be recovered for downstream processing, it is linked to a large stream of gypsum waste, which results from the reaction with sulfuric acid within the downstream process as already mentioned above [60]. This gypsum needs to be disposed of as environmentally unfriendly left-over of this process. However, the prior limit of 140 g L^{-1} malic acid in bioreactors could be exceeded, further sustaining the hypothesis of product inhibition at concentrations above 140 g L^{-1}. Additionally, the malic acid

production rate could be kept near constant for a longer time. These advantages have to be weighed against the abovementioned drawbacks in order to determine the beneficial effect of $CaCO_3$ as buffering agent.

As already mentioned the formation of solid calcium malate in bioreactors containing $CaCO_3$ as buffering agent enables efficient initial purification. To isolate the product from the fermentations, all solid components (settled for 48 h) resulting from an autoclaved fermentation with $CaCO_3$ (Figure 17B) were dried at 120 °C for 24 h. 0.2 g of this mixture was dissolved in 1 mL of HCl (37 %) and adjusted to 2 mL with water in triplicates. The mixture was filtered to remove cells and the malate concentration was determined via HPLC to be 68.1 ± 0.1 g L^{-1}. Assuming that all product is recovered in the form of calcium malate, this is nearly 90 % of the theoretical malic acid concentration (78 g L^{-1}), indicating that the solids recovered from the bioreactor are 90 % pure calcium malate. The remaining 10 % can be assumed to be biomass and remaining $CaCO_3$.

2.2.4.5. *U. trichophora* TZ1 can cope with impurities in crude glycerol

Biodiesel derived crude glycerol contains, depending on the biodiesel production process, impurities such as methanol, ash, soap, salts, non-glycerol organic matter, and water [36, 193]. Even though different microbial conversions of crude glycerol to value-added chemicals have been reported [40], many organisms struggle with the contained impurities, especially in fed-batch cultures with high substrate loads. The purification to pharma-grade glycerol, however, is a costly process often prohibiting the possible application of glycerol in microbial chemical production. To test whether *U. trichophora* TZ1 is able to cope with the contained impurities, we investigated malic acid production with *U. trichophora* TZ1 in MTM containing 100 g L^{-1} and 200 g L^{-1} crude glycerol in shake flasks. The used crude glycerol contained 1.5 % ashes and 1.9 % free fatty acids, with a pH-value between 6 and 8. Neither growth rate, nor maximal optical density, nor glycerol uptake were influenced by 100 and 200 g L^{-1} crude glycerol compared to the same amount of pharma-grade glycerol. Malic acid production, however, was lowered by 63 % (100 g L^{-1}) and 41 % (200 g L^{-1}) (data not shown). This indicates that the organism itself is capable of coping with the contained impurities, although at a cost resulting in a lower malic acid titer. This may in shake flasks be due to lower oxygen input as a result of increased salt concentrations, which can be up to 12 % in crude glycerol [193]. Increased osmotic pressure in media containing high concentrations of salts results in a lower maximum oxygen transfer rate in shake flasks [218]. The effect of this on growth and organic acid production was investigated in several organisms. For *U. maydis*, increased osmotic stress due to higher salt concentrations resulted in a prolonged lag-phase and lower growth rates. Interestingly, itaconic acid production slightly increased with higher salt concentrations [119], possibly due to high redox energy surplus generated with this product compared to malate. The same effect was observed in *Candida oleophila* with increased citric acid production with higher osmolarity of the medium [219]. Since the redox-potential of the different production pathways for malic acid, succinic acid and itaconic acid is completely different, the effect of reduced oxygen transfer rates will likely differ.

To rule out this effect we evaluated *U. trichophora* TZ1 in more industrially relevant conditions. To this end, it was cultivated in a bioreactor with MTM containing 200 g L^{-1} crude glycerol and 3.2 g L^{-1} NH_4Cl. The pH was kept stable at 6.5 by automatic addition of NaOH. Additional crude glycerol was fed upon glycerol depletion (Figure 18).

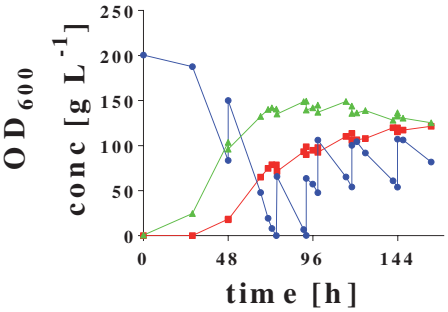

Figure 18: Fermentation of U. trichophora *TZ1 with crude glycerol. Malate concentration (red, squares), glycerol concentration (blue, circles) and OD_{600} (green, triangles) in MTM containing 200 g L^{-1} crude glycerol, 3.2 g L^{-1} NH_4Cl at 28 °C (37 °C during production phase, 48 h) with DO kept at 30 %. pH was kept at 6.5 by automatic addition of NaOH. Shown is one exemplary fermentation run.*

This fermentation resulted in OD_{600} values and growth rates comparable to the ones in bioreactors with pharma-grade glycerol. Also the glycerol uptake rate (2.90 g L^{-1} h^{-1}) and the malic acid production rate (0.75 g L^{-1}) were comparable to the ones with pharma-grade glycerol. Only the yield was lowered to 0.26 g g^{-1}. A slight negative impact of crude glycerol compared to pharma-grade glycerol on organic acid production has already been shown for *Y. lipolytica* in citric acid production [46]. Interestingly, for *U. trichophora* TZ1 the accumulation of impurities by glycerol feed adding up to 476 g glycerol did not result in lowered production properties, which hints at an effect which is perhaps limited to the initial growth phase. A possibility to overcome this issue would be a second adaptive laboratory evolution on crude glycerol. For this, however, it has to be taken into consideration that depending on the origin of the crude glycerol, the composition of contained impurities differs in a broad range, not only in concentration, but also in components themselves [220]. In addition, to the already high tolerance to impurities in crude glycerol by *U. trichophora* TZ1 and thus only slight negative effect, the contained salts might also have a beneficial effect. For *Actinobacillus succinogenes* it could be shown, that synthetic seawater can act as mineral supplement [221].

2.2.5. Conclusion

The strain *U. trichophora* TZ1, which recently has been reported as promising production organism for malate from glycerol, is capable of producing 200 g L^{-1} malic acid at an overall rate of 0.74 g L^{-1} h^{-1} reaching a maximal production rate of 1.94 g L^{-1} h^{-1} and a yield of 0.31 mol mol^{-1} (31 % of the theoretical maximum assuming CO_2 co-fixation or 41 % assuming no CO_2 co-fixation) in bioreactors. These values, which are some of the highest reported for microbial malic acid production, allow *U. trichophora* TZ1, though only having undergone adaptive laboratory evolution and medium and fermentation optimization, to compete with highly engineered strains overexpressing major parts of the malate production pathway. Thus, further optimization of *U. trichophora* TZ1 could focus on metabolic engineering, which would not only harbor considerable potential to increase the production rate but also allow for strain optimization in terms of product to substrate yield by targeted disruption of by-product formation pathways. A subsequent systems biology comparison between the wild-type and the evolved strain could not only shed light on the adaptational mutations that enhanced the growth and production rate of *U. trichophora* TZ1 on glycerol but might also provide insights into why the strain utilizes glycerol faster than other Ustilaginaceae. In addition, it could clarify the glycerol uptake and degradation pathway and expand the general knowledge base of this relatively obscure *Ustilago* strain. This would clearly help to develop it into a platform for the production of not only malate but also other industrially relevant chemicals, to be produced from biodiesel-derived crude glycerol.

Draft genome sequence of *Ustilago trichophora* RK089 – a promising malic acid producer

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Contributions:
Genomic DNA was isolated by Thiemo Zambanini. Thiemo Zambanini and Nick Wierckx wrote the manuscript with the help of Lars M. Blank and Joerg M. Buescher. Thiemo Zambanini, Nick Wierckx, Joerg M. Buescher and Lars M. Blank analyzed results. Lars M. Blank, Nick Wierckx and Guido Meurer conceived and designed the project. We thank Elena Geiser for technical assistance and valuable discussion.

2.3. Draft genome sequence of *Ustilago trichophora* RK089 – a promising malic acid producer

2.3.1. Summary

The basidiomycetous smut fungus *Ustilago trichophora* RK089 produces malate from glycerol. De-novo genome sequencing revealed a 20.7 Mbp (301 gapclosed contigs, 246 scaffolds). Comparison to the genome of *U. maydis* 521 revealed all essential genes for malate production from glycerol contributing to metabolic engineering for improving malate production.

2.3.2. Draft genome sequence

The members of the family Ustilaginaceae, belonging to the phylum basidiomycota, are known to produce many different, industrially interesting compounds, such as organic acids, lipids, and polyols naturally [76, 119, 144, 145, 170, 197]. In a screening of 74 strains belonging to 13 species, *U. trichophora* RK089 (CBS 131473) showed the highest malic acid production from glycerol [195]. After adaptive laboratory evolution and medium and process optimization, this strain was capable of producing more than 200 g L^{-1} malic acid at a maximum production rate of nearly 2 g L^{-1} h^{-1} demonstrating its potential as production organism [222]. *U. trichophora* was first isolated from *Echinochloa colonum* in Egypt by Kunze in 1830 [223]. Since then, this organism attracted little focus of research, apart from the description as plant pathogen belonging to the Ustilaginaceae including its phylogeny [224-227]. Hence, no prior knowledge exists about molecular techniques for genetic manipulation and the genome sequence is unknown. For further optimization of malate production by *U. trichophora*, however, metabolic engineering is required.

Here we present a draft genome sequence of *U. trichophora* RK089. Sequencing and *de-novo* assembly was done by BaseClear BV (Leiden, Netherlands) using Illumina Nextera (paired-end library) and PacBio RSII (10 kb library) with 1 SMRT cell for sequencing. CLC Genomics Workbench version 7 was used for draft assembly of the reads using the "De novo assembly" option. The optimal k-mer size was automatically determined using KmerGenie [228]. Alignment of the PacBIO CLR reads was performed with BLASR [229]. Analysis of the orientation, order and distance between resulting contigs was performed using SSPACE-LongRead scaffolder version 1.0 [230] and automated gap closure was performed using GapFiller version 1.10 [231]. The resulting sequence of 246 gap-closed scaffolds contains 20,691,595 bp consisting of 1,399 large contigs (>300 bp in size) and 77 smaller contigs with a GC content of 53.06 % and 124 gaps. The average sequence size is 84,112 bp with a maximum of 637,988 bp.

The sequence consists of 6424 open reading frames (ORFs). By comparison of the sequence with the closely related *U. maydis*, several genes expected to be involved in the conversion of glycerol to malic acid were identified that have 88 to 90 % homology on DNA level and 94 to 97 % homology on protein level. None of the genes from the itaconic acid cluster, recently discovered for *U. maydis* [232], are present in *U. trichophora* RK089, a strain that indeed does not produce itaconic acid. We were able to identify all genes from *U. maydis* glycolipid clusters, i.e., coding for ustilagic acid (UA) [204] and mannosylerythritol-lipids (MEL) [141] synthesis.

Nucleotide sequence accession number

This Whole Genome Shotgun project has been deposited in DDBJ/ENA/GenBank under the accession LVYE00000000. The version described in this paper is version LVYE01000000.

Metabolic engineering for improved malic acid production by *Ustilago trichophora* TZ1

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Contributions:

Hamed Hosseinpour Tehrani, Elena Geiser, Christiane K. Sonntag and Thiemo Zambanini constructed strains and performed screening experiments. Hamed Tehrani, Elena Geiser and Thiemo Zambanini performed bioreactor cultivations. Thiemo Zambanini, Nick Wierckx, Joerg M. Buescher and Lars M. Blank analyzed results. Thiemo Zambanini and Nick Wierckx wrote the manuscript with the help of Lars M. Blank. Lars M. Blank, Nick Wierckx and Guido Meurer conceived and designed the project. We grategully acknowledge BioEton for providing the glycerol.

2.4. Metabolic engineering for improved malic acid production by *Ustilago trichophora* TZ1

2.4.1. Summary

The organism *Ustilago trichophora* has been found recently as good natural malic acid producer from glycerol. Even though this organism has only underdone adaptive laboratory evolution, medium optimization and investigation of process parameters, the reached titer, yield and rate are already able to compete with those of organisms overexpressing major parts of the underlying metabolic pathways. However tools for metabolic engineering of this relatively obscure strain are not established, yet.

Here we adapt existing molecular tools for *U. maydis* for use in *U. trichophora*. We determined the needed concentrations for the antibiotics carboxin, hygromycin, nourseothricin, and phleomycin to be 10 mg L^{-1}, 300 mg L^{-1}, 100 mg L^{-1}, and 100 mg L^{-1}, respectively. The corresponding resistance cassettes from *U. maydis* are applicable in this organism to confer resistance. However, site-specific integration via homologous recombination into the *ip*^{S}-locus only yielded ectopic integration. Hence, a plasmid was constructed containing the *ip*-locus of *U. trichophora*, resulting in site-specific integration into the genome. With these tools in hand, overexpression of pyruvate carboxylase, two different malate dehydrogenases, and two different malate transporters was possible in *U. trichophora* TZ1. Overexpression of pyruvate carboxylase did not result in improved malic acid production. The overexpression of the genes encoding malate dehydrogenases (Mdh1 and Mdh2) and the two malate transporters (Ssu1 and Ssu2), however, resulted in an up to 54 % increased yield in shake flasks compared to *U. trichophora* TZ1. Interestingly, bioreactor cultivations of the mutants overexpressing *ssu2* and *mdh2* did not yield a higher production rate or malic acid titer. However, a drastically lowered biomass formation and glycerol uptake resulted in a 34 % higher specific production rate for both strains and a 30 % increased yield for the transporter mutant (Ssu1) compared to the reference strain *U. trichophora* TZ1.

These values demonstrate the potential of metabolic engineering to further improve malic acid production from glycerol in *U. trichophora* TZ1. Modern methods such as next-generation sequencing enabled the rapid adaptation of the considerable suite of genetic tools available for *U. maydis*. This opens up a wide range of possibilities for further optimization, especially combinatorial metabolic engineering to increase the flux from pyruvate to malic acid and to reduce by-product formation.

2.4.2. Introduction

The biotechnological production of chemicals has gained great interest in the last decades. Strongly fluctuating oil prices, concerns about pollution of the environment, and the resulting climate change, have driven the development of new sustainable microbial production processes [54]. One focused group of chemicals is organic acids, such as succinic, fumaric, citric, itaconic and malic acid. As natural metabolites produced by many organisms, it is not astonishing that production with a broad range of microbes has been investigated, including different *Candida* species [233], *Yarrowia lipolytica* [234], and *Aspergillus niger* [235] for citric acid, *A. terreus* [76, 78, 92] and different *Ustilago* species [103, 104, 119, 145] for itaconic acid, *Rhizopus oryzae* [236] and *Torulopsis glabrata* [237] for fumaric acid, *Y. lipolytica* [211], *Lactobacillus* species [238] and *Actinobacillus succinogenes* [239] for succinic acid, and *Aspergillus* species [174, 240] and *Saccharomyces cerevisiae* [115] for malic acid. Many of these organisms underwent considerable metabolic engineering in order to establish efficient production of the desired chemical.

The potential of metabolic engineering to improve malic acid production, for instance, has been demonstrated in different organisms. In *S. cerevisiae* combined overexpression of the native pyruvate carboxylase gene *pyc2*, an allele of the peroxisomal malate dehydrogenase gene *mdh3*, which has been retargeted to the cytosol by deletion of the C-terminal targeting sequence, and expression of the *Schizosaccharomyces pombe* malate transporter gene *mae1* resulted in a malic acid titer of 59 g L^{-1} produced with a yield of 0.42 mol_{mal} mol_{glu}^{-1} [115]. By overexpression of a native C4-dicarboxylate transporter, Brown *et al*. were able to improve the malic acid production rate of *A. oryzae* by more than two-fold [117]. Combined overexpression with cytosolic pyruvate carboxylase and malate dehydrogenase, increased the rate by additional 27 %. The final strain overexpressing all conversion and transport steps from pyruvate to extracellular malic acid reached a 2.6-fold increased titer of 154 g L^{-1} produced at a rate of 0.94 g L^{-1} reaching a yield of 1.38 mol mol^{-1} on glucose [117]. This clearly demonstrates the importance of the reductive tricarboxylic acid (rTCA) pathway among the four identified possible microbial production pathways for malic acid. The pathway starts with carboxylation of pyruvate to oxaloacetate by pyruvate carboxylase, which is followed by reduction to malate by malate dehydrogenase [115, 117]. Also in other organisms, such as different *Aspergillus* species and *R. oryzae*, this pathway has been shown to be essential in efficient malic acid production [54, 120-122].

In 2016, we reported *U. trichophora* as promising production organism for malic acid from glycerol. By adaptive laboratory evolution and medium optimization a titer of nearly 200 g L^{-1} was reached produced at a rate of 0.39 g L^{-1} h^{-1} [195]. In bioreactors the production rate was further improved to a maximum of nearly 2 g L^{-1} h^{-1}, while the reached yield was still low with 31 % of the theoretical maximum [222]. Combining the already high production capability of this genetically unmodified strain, with the possible positive effects of overexpressing rTCA pathway genes, seems to be a promising approach to further improve malic acid production by *U. trichophora*. However, molecular tools and methods, such as vectors, promoters and terminators for overexpression, applicable antibiotics with corresponding resistance cassettes, and transformation and screening protocols, are not available for this relatively obscure organism. Yet, these tools are known for the model organism *U. maydis* [156-158, 171]. Additionally, the genome of *U. trichophora* has recently been sequenced [241], providing a key enabling factor for genetic and metabolic engineering.

Here we report on the adaptation and development of molecular tools and methods for

U. trichophora and the use of these to overexpress native genes for a pyruvate carboxylase, two malate dehydrogenases (mitochondrial and cytoplasmic) and two malate transporters. The resulting strains were screened in liquid cultures both in small volumes in shaking flasks and larger volumes in bioreactors.

2.4.3. Material and Methods

2.4.3.1. Strains and culture conditions

All strains used and generated in this study are listed in Table 4.

Table 4: Strains used in this study.

Strain name	Description	Reference
U. trichophora RK089	wildtype strain	[242]
U. trichophora TZ1	*U. trichophora* RK089 evolved on glycerol	[195]
U. trichophora RK089 pSMUT	*U. trichophora* RK089 with genomic integration of pSMUT; hygromycin resistant	This study
U. trichophora RK089 pNEBUC	*U. trichophora* RK089 episomally expressing pNEBUC; carboxin resistant	This study
U. trichophora RK089 pNEBUN	*U. trichophora* RK089 episomally expressing pNEBUN; nourseothricin resistant	This study
U. trichophora RK089 pNEBUP	*U. trichophora* RK089 episomally expressing pNEBUP; phleomycin resistant	This study
U. trichophora TZ1 pUTr01	*U. trichophora* TZ1 with genomic integration of pUTr01; carboxin resistant	This study
U. trichophora TZ1 pUTr01-Mdh1	*U. trichophora* TZ1 with genomic integration of pUTr01-Mdh1; carboxin resistant	This study
U. trichophora TZ1 pUTr01-Mdh2 (m)	*U. trichophora* TZ1 with genomic integration of pUTr01-Mdh2 (m); carboxin resistant	This study
U. trichophora TZ1 pUTr01-Mdh2 (c)	*U. trichophora* TZ1 with genomic integration of pUTr01-Mdh2 (c); carboxin resistant	This study
U. trichophora TZ1 pUTr01-Pyc	*U. trichophora* TZ1 with genomic integration of pUTr01-Pyc; carboxin resistant	This study
U. trichophora TZ1 pUTr01-Ssu1	*U. trichophora* TZ1 with genomic integration of pUTr01-Ssu1; carboxin resistant	This study
U. trichophora TZ1 pUTr01-Ssu2	*U. trichophora* TZ1 with genomic integration of pUTr01-Ssu2; carboxin resistant	This study

As standard medium, MTM was used according to Zambanini *et al.* [195]. As buffer, either 100 mM 2-(N-morpholino)ethanesulfonic acid (MES) or 100 g L^{-1} $CaCO_3$ were used.

For drop tests, 5 µL of a YEP-grown overnight culture diluted to a starting OD_{600} of 1 were pipetted onto a YEP-plate containing different concentrations of carboxin, hygromycin, nourseothricin or phleomycin in different dilutions (1, 10^{-1}, 10^{-2}, 10^{-3}) and incubated (7 days, 30 °C).

Screening experiments were performed in 24 deep well plates (Enzyscreen, System Duetz®) with 1.5 mL MTM containing 100 g L^{-1} $CaCO_3$ and 0.8 g L^{-1} glycerol incubated at 30 °C (relative air humidity = 80 %) shaking at 300 rpm (shaking diameter = 50 mm).

Shake flask production experiments were conducted in MTM containing with 200 g L^{-1} glycerol and 0.8 g L^{-1} NH_4Cl as described previously [195].

Controlled batch cultivations were performed as described previously [222]. The pH was set to 6.5 and controlled automatically by 10 M NaOH. As medium MTM containing 200 g L^{-1} glycerol and either 3.2 g L^{-1} NH_4Cl or 6.4 g L^{-1} with doubled concentration of all other medium components was used.

2.4.3.2. Analytical methods

All shake flask experiments were performed in triplicates. Bioreactor cultivations were performed in duplicates. Shown is the arithmetic mean of the replicates. Error bars and ± values indicate deviation from the mean.

OD_{600} determination and HPLC analysis were performed as described previously [195].

Fluorescence was measured in black FLUOTRACK 96-well microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany) with a Synergy MxF Fluorescence Microplate Reader (BioTek Instruments Inc., Winooski, USA). For excitation and emission wavelengths of 485 nm and 530 nm were chosen and the gain was set to 80.

Fluorescence microscopy was performed on a Leica DM6000 B fluorescence microscope (Wetzlar, Germany) using the fluo green filter at a magnification of 630 with an oil-immersion object. As excitation and emission wavelengths 499 nm and 520 nm were chosen. Exposure time was set to 200 ms, gain to 10, and intensity to 4.3.

2.4.3.3. Cloning procedures

Standard cloning-related techniques were performed according to Sambrook *et al*. [243].

The genome sequence of *U. trichophora* RK089 (accession number: LVYE01000000) was used as reference [241].

Genomic DNA from *U. trichophora* was isolated according to Hoffman and Winston [244].

All vectors used and generated in this study are listed in Table 5.

Table 5: Vectors used in this study with description of contained elements.

Plasmid name	Description	Reference
pSMUT	Ori ColE1; ampR; Psc; *hph*	[245]
pNEBUC	ip^R-locus; ori ColE1; UARS; ampR	[246]
pNEBUN	natR; ori ColE1; UARS; ampR	[246]
pNEBUP	bleR; ori ColE1; UARS; ampR	[246]
pUMa43 Otef-gfp-nos cbx	Potef; *gfp*; Tnos; ori ColE1; ampR; *U. maydis* ip^R-locus	[247]
pUTr01	pUMa43 Otef-gfp-nos cbx with the *U. maydis* cbx cassette exchanged for the cbx cassette from *U. trichophora* RK089	This study
pUTr01-Mdh1	pUTr01 with *gfp* exchanged for UT11161	This study
pUTr01-Mdh2 (m)	pUTr01 with *gfp* exchanged for UT00403 (m)	This study
pUTr01-Mdh2 (c)	pUTr01 with *gfp* exchanged for UT00403 (c)	This study
pUTr01-Pyc	pUTr01 with *gfp* exchanged for UT01054	This study
pUTr01-Ssu1	pUTr01 with *gfp* exchanged for UT05271	This study
pUTr01-Ssu2	pUTr01 with *gfp* exchanged for UT05764	This study

For overexpression all genes were cloned into pUTr01 by exchanging the Gfp-gene. For this, the backbone pUTr01 was amplified via PCR using the primer pair pUMa otef-cbx-fw/pUMa otef-cbx-rv. The resulting fragment was digested using MluI and DpnI. All inserts were amplified via PCR using the primer pairs listed in Table 6.

Table 6: Primers used within this study with corresponding sequences and description.

Primer	**Sequence/description**
pUMa43-dCBX-fwd	TTGGCGCGCCAATTAGGCCGGCCTTACCCATTATTGAAGC Amplification of *ip*-locus from *U. trichophora*
pUMa43-dCBX-rev	CCTTGGCGCGCCAACCTTAATTAAGGTTGAAAAAGGAAGAG Amplification of *ip*-locus from *U. trichophora*
pUMa otef-cbx-fw	CGACGCGTCGATTTGCGGCCGCTTTACCGGCTGCAGATCGTTC Amplification of the backbone pUMa otef-gfp-nos cbx without Gfp
pUMa otef-cbx-rv	CGACGCGTCGGACTAGTCGATCGAATTCCTGCAGCC Amplification of the backbone pUMa otef-gfp-nos cbx without Gfp
UT11161+sig_fwd	GCAGGAATTCGATCGACTAGTATGGTCAAGGCTACTGTTATC Amplification of *mdh1* from *U. trichophora*
UT11161+sig_rev	TGCAGCCGGTAAAGCGGCCGCTTAAAGGTTGGCAGTGAAC Amplification of *mdh1* from *U. trichophora*
UT00403+sig_fwd	GCAGGAATTCGATCGACTAGTATGTTCGCTCGTCAGGCTC Amplification of *mdh2* (m) and *mdh2* (c) from *U. trichophora*
UT00403+sig_rev	TGCAGCCGGTAAAGCGGCCGCTCAAGGGTTGGCGGCGAC Amplification of *mdh2* (m) from *U. trichophora*
UT00403-sig_fwd	GCAGGAATTCGATCGACTAGTATGGCTTCGGGCGGTATTG Amplification of *mdh2* (c) from *U. trichophora*
UT_05271_fwd	CGATCGACTAGTCCGACGCGTATGGGCTTTGGTATCACC Amplification of *ssu1* from *U. trichophora*
UT_05271_rev	GCGGCCGCAAATCGACGCGTTTATCTAGAAGGTGAAGCC Amplification of *ssu1* from *U. trichophora*
UT_05764_fwd_II	CGATCGACTAGTCCGACGCGTATGTCACCCAACCCCTCG Amplification of *ssu2* from *U. trichophora*
UT_05764_rev_II	GCGGCCGCAAATCGACGCGTTTAGGTGAGGGTCGTCATTC Amplification of *ssu2* from *U. trichophora*
UT01054_fwd	TGCAGGAATTCGATCCCATGGATGCCCGTCGAGCCCGAG Amplification of *pyc* from *U. trichophora*
UT01054_rev	GATCTGCAGCCGGGCGGCCGCTTAGTGCTCAATTTCGCAGAGCAAGTC Amplification of *pyc* from *U. trichophora*
fwd-ampII	TCTGACGCTCAGTGGAAC Colony-PCR to test for integration into the *U. trichophora* genome
rev-ampII	TGGTGTCGACGTGAATGC Colony-PCR to test for integration into the *U. trichophora* genome

The targeting sequences for *mdh1* and *mdh2* were analyzed using a combination of Signal-3L and TargetP 1.1 [248].

All plasmids were assembled in *Escherichia coli* and correctness was confirmed by PCR, restriction digest and sequencing via Eurofins Scientific (Ebersberg, Germany).

For transformation of *U. trichophora*, protoplasts were prepared as described by Schulz *et al.* [249] and Tsukuda *et al.* [168] or whole cell transformation was performed according to Maassen *et al.* [250].

The plasmid conferring site-specific integration and resistance to carboxin in *U. trichophora,* pUTr01, was constructed by exchanging the cbx-resistant ip^R-locus on the plasmid pUMa43 Otef-gfp-nos cbx with the *ip*-locus from the genome of *U. trichophora* RK089. For this, the backbone pUMa43 Otef-gfp-nos cbx was amplified via PCR with the primer pair pUMa43-dCBX-fwd/pUMa43-dCBX-rev and the resulting fragment was self-circularized after digestion with AscI to give plasmid pUMa43 Otef-gfp-nos. The *U. trichophora* specific *ip*-locus was identified based on comparison to the *sdh2* gene from *U. maydis* 521. The sequence was point mutated to confer carboxin resistance (position 761-762: AC changed to TT [165 166] and ordered as 'string', linear synthetic DNA from Thermo Scientific (Waltham, USA). Additionally, an MfeI/MunI restriction site was added (position 437, 438: TG changed to GT). Backbone and insert were assembled using the restriction enzymes AscI and PacI giving plasmid pUTr01 (Figure 19C).

2.4.4. Results and Discussion

2.4.4.1. Establishing tools and methods for genetic engineering of *U. trichophora*

Production rate and titer for the recently discovered natural malic acid producer *U. trichophora* RK089 have been improved drastically by adaptive laboratory evolution, medium optimization and process investigation, while the product yield was still low with only 31 % of the theoretical maximum [195, 222]. In order to metabolically engineer the resulting strain *U. trichophora* TZ1, existing tools from the closely related *U. maydis* had to be investigated and adapted for applicability. Since antibiotics and the corresponding resistance cassettes are the basis of classic metabolic engineering, we performed a drop test on YEP plates containing different concentrations of carboxin (cbx), hygromycin (hyg), nourseothricin (nat) and phleomycin (phl).
For *U. maydis* used concentrations for these antibiotics are 1-4 mg L^{-1} for cbx [165, 167, 251], 200-400 mg L^{-1} for hyg [155, 165, 167, 168, 251], 50-300 mg L^{-1} for nat [167, 169, 251], and 50 mg L^{-1} for phl [169]. We tested concentrations in the range of 1-15 (cbx), 100-500 (hyg), 1-300 (nat), and 1-150 mg L^{-1} (phl). Plates were assessed for growth every 24 h by visual inspection. The results for carboxin after 48 h of incubation are shown exemplarily in Figure 19A. For *U. trichophora* no growth was observed after 48 h exceeding concentrations of 10 (cbx), 300 (hyg), 100 (nat), and 100 mg L^{-1} (phl). In contrast to *U. maydis*, however, prolonged incubation (>72 h) resulted in growth of *U. trichophora* even at the highest tested concentrations for cbx, hyg, and phl. Only for nat no growth could be observed at concentrations exceeding 200 mg L^{-1}. Thus, after transformation colonies should be picked after approximately 48 h of cultivation. To test, whether transformation of *U. trichophora* is possible and the corresponding selection markers are functional, protoplasts were transformed with the episomally replicating plasmids pNEBUC (cbx resistance cassette), pNEBUN (nat resistance cassette), pNEBUP (phl resistance cassette), and genome-integrated pSMUT (hyg resistance cassette). Resulting colonies on selective medium plates with the respective antibiotics were screened for different incubation times (Figure 19B). Plasmid-isolation from protoplasts and re-transformation into *E. coli* followed by re-isolation resulted in the correct plasmid for pNEBUN, pNEBUP, and pSMUT. It should be noted, that for all three plasmid transformations the number of background colonies increased after 72 h of incubation, as it has already been observed earlier for *U. maydis* with nat [169]. This also correlates with the observations from the drop test screening. In contrast to the other plasmids, for pNEBUC mainly negative clones could be found for cbx concentrations below 5 mg L^{-1} and no colonies for concentrations above

5 mg L^{-1}. Thus, whole cell transformation was performed, using 5, 10 and 15 mg L^{-1} cbx for selection. This transformation resulted in positive results for all concentrations (Figure 19B). The discrepancy between transformation via protoplasts and whole cells is likely to result from sensitivity of protoplasts, which has been described previously for different organisms. Some antibiotics act as effective growth inhibitors on yeast protoplasts already in low concentrations [252]. Also for *Corynebacterium glutamicum* it was shown, that an increasing concentration of penicillin reduces the regeneration frequency of protoplasts after transformation [253].

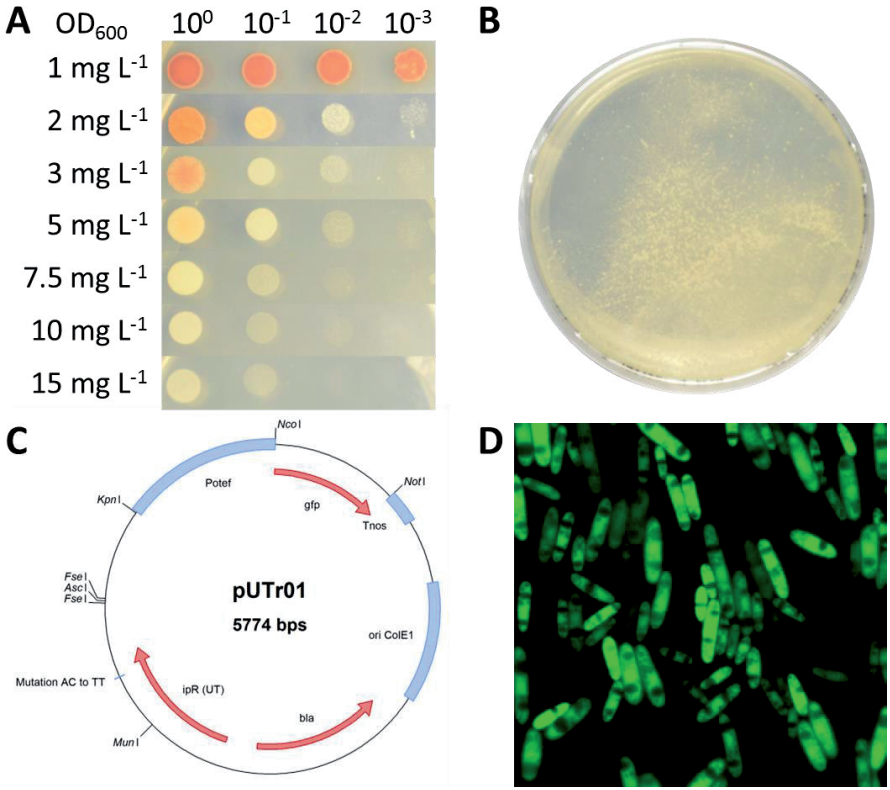

Figure 19: Genetic tool development for U. trichophora*. A: drop test of 5 µL* U. trichophora *RK089 culture with different dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}) on YEP plates containing different concentrations of carboxin (1, 2, 3, 5, 7.5, 10, 15 mg L^{-1}) B: YEP plate containing 10 mg L^{-1} carboxin 48 h after whole cell transformation of* U. trichophora *RK089 with pNEBUC (incubated at 30 °C) C: plasmid map for vector pUTr01. Potef: otef promoter; gfp: green fluorescent protein gene; Tnos: nos-terminator; ori ColE1: origin of replication in E. coli; ampR: ampicillin resistance cassette; ip^R: carboxin resistant ip-locus of U. trichophora D: fluorescence microscopic image of* U. trichophora *RK089 cells expressing pUTr01 after 24 h of cultivation in MTM medium.*

These first tests of molecular tools and methods resulted in cbx, hyg, nat, and phl as applicable antibiotics for selection after transformation at concentrations of 10 mg L^{-1}, 300 mg L^{-1}, 100 mg L^{-1}, and 100 mg L^{-1}, respectively. Additionally, two different transformation protocols, via protoplasts and via whole cells, and a protocol for plasmid rescue could be established.
The tested plasmids pNEBUC, pNEBUN and pNEBUP are self-replicating plasmids. For industrial application, however, a plasmid that integrates into the genome is favorable, since no addition of antibiotics into the medium is needed for plasmid maintenance. The plasmid pSMUT randomly integrates into the genome. With site-specific integration, screening efforts can be reduced, since unspecific integration likely results in random disruption of unknown genes, or site-specific variation of the expression level. In *U. maydis*, the plasmid pUMa43 otef gfp-nos cbx confers resistance to carboxin by site-specific integration into the ip^S-locus [247]. The transformation of this plasmid into *U. trichophora* RK089 resulted in positive clones containing the plasmid. However, this construct was not integrated site-specifically.
Since the integration method relies on homologous recombination, it is likely that the 88 % DNA sequence homology between *U. maydis* (sequence donor) and *U. trichophora* (sequence acceptor) is too low to ensure site-specific integration. Thus, the ip^R-locus from *U. maydis* on the plasmid pUMa43 Otef-gfp-nos cbx was exchanged with the ip^R-locus from *U. trichophora* RK089 giving plasmid pUTr01 (Figure 19C). Site-specificity was confirmed by Southern Blot and PCR. Since this vector harbors a Gfp-gene under control of the otef promoter, which is known to promote medium overexpression in *U. maydis* [159, 161], we tested the resulting colonies for fluorescence in microplates and with fluorescence microscopy. All colonies showed strong fluorescence (Figure 19D), while the reference strain without plasmid did not, confirming the activity of the Potef promoter. Thus, the function of all relevant elements of pUTr01 was confirmed, and this plasmid enables overexpression of target genes through site-specific genomic integration in *U. trichophora*.

2.4.4.2. Overexpression of malate dehydrogenase, pyruvate carboxylase and malic acid transporter genes in *U. trichophora* TZ1

With the established tools and methods, optimization of malic acid production by overexpression of expected bottleneck genes in *U. trichophora* TZ1 became possible. As targets we chose all putative enzymes in the reductive tricarboxylic acid cycle (rTCA) leading from pyruvate to malic acid. Thus, we compared [254] the sequences of pyruvate carboxylase UMAG_01054 (Pyc), the two malate dehydrogenases UMAG_11161 (Mdh1) and UMAG_00403 (Mdh2), and the two enzymes related to malic acid transport proteins UMAG_05271 (Ssu1) and UMAG_05764 (Ssu2) from *U. maydis* (MUmDB) against the recently published genome of *U. trichophora* [241]. The search on protein level yielded one hit for Pyc (97 % homology), Mdh1 (97 % homology), Mdh2 (94 % homology), Ssu1 (89 % homology), and Ssu2 (68 % homology). For the malate dehydrogenases N-terminal targeting sequences were analyzed using a combination of Signal-3L and TargetP 1.1 [248]. The resulting localization for Mdh1 (UT11161) was the cytosol and for Mdh2 (UT00403) the mitochondrion. The N-terminal mitochondrial targeting sequence for *mdh2* was either removed or retained during the cloning process, resulting in the protein versions Mdh2 (m) and Mdh2 (c), which are likely targeted to the mitochondria (m) and the cytosol (c), respectively. All genes were cloned under control of the otef promoter into vector pUTr01, by replacing the Gfp-gene. The resulting constructs were transformed into *U. trichophora* TZ1.

A first screening in MTM containing 200 g L^{-1} glycerol in 24-deep well plates revealed a broad variety among resulting mutants after 384 h of cultivation concerning growth, malic acid production and glycerol uptake (data not shown). From this screening the two best transformants for each gene were chosen for more detailed shake flask investigation (Table 7). Genomic integration of the constructs was verified by PCR using the primer pair fwd-ampII/rev-ampII.

Table 7: Production values for U. trichophora *TZ1 overexpressing* mdh1, mdh2, pyc, ssu1, ssu2. *Two individual transformants were tested for each strain, ±-values indicate deviation from the mean of biological replicates of each transformant (n=3).*

Strain	final OD_{600}	mal. titer [g L^{-1}]	mal. prod. rate [g L^{-1} h^{-1}]	gly. uptake rate [g L^{-1} h^{-1}]	yield [g_{mal} g_{gly}^{-1}]
U. trichophora TZ1	53 ± 2	86 ± 7	0.22 ± 0.02	0.52 ± 0.01	0.43 ± 0.04
U. trichophora TZ1 pUTr01-Mdh1 (1)	39 ± 2	119 ± 9	0.31 ± 0.02	0.46 ± 0.02	0.67 ± 0.03
U. trichophora TZ1 pUTr01-Mdh1 (2)	39 ± 1	114 ± 5	0.27 ± 0.01	0.48 ± 0.00	0.56 ± 0.02
U. trichophora TZ1 pUTr01-Mdh2 (m) (1)	38 ± 2	103 ± 9	0.27 ± 0.02	0.51 ± 0.01	0.53 ± 0.05
U. trichophora TZ1 pUTr01-Mdh2 (m) (2)	38 ± 4	109 ± 3	0.28 ± 0.01	0.46 ± 0.02	0.61 ± 0.01
U. trichophora TZ1 pUTr01-Mdh2 (c) (1)	35 ± 2	42 ± 10	0.10 ± 0.03	0.38 ± 0.03	0.29 ± 0.05
U. trichophora TZ1 pUTr01-Mdh2 (c) (2)	31 ± 4	56 ± 1	0.15 ± 0.00	0.28 ± 0.00	0.51 ± 0.01
U. trichophora TZ1 pUTr01-Pyc (1)	36 ± 0	88 ± 3	0.23 ± 0.01	0.35 ± 0.02	0.66 ± 0.03
U. trichophora TZ1 pUTr01-Pyc (2)	31 ± 2	29 ± 5	0.07 ± 0.01	0.26 ± 0.03	0.30 ± 0.07
U. trichophora TZ1 pUTr01-Ssu1 (1)	34 ± 2	96 ± 9	0.25 ± 0.02	0.50 ± 0.05	0.51 ± 0.01
U. trichophora TZ1 pUTr01-Ssu1 (2)	40 ± 4	112 ± 20	0.29 ± 0.05	0.50 ± 0.01	0.58 ± 0.10
U. trichophora TZ1 pUTr01-Ssu2 (1)	32 ± 2	111 ± 3	0.29 ± 0.01	0.50 ± 0.01	0.59 ± 0.01
U. trichophora TZ1 pUTr01-Ssu2 (2)	32 ± 3	112 ± 2	0.29 ± 0.00	0.48 ± 0.02	0.61 ± 0.03

Both strains overexpressing pyruvate carboxylase and both strains overexpressing malate dehydrogenase *mdh2* (c) showed lower or similar malic acid production compared to the reference strain (Table 7). This hints at naturally strong activity of pyruvate carboxylase in *U. trichophora* TZ1 and a bottleneck in another step of the production pathway. Further, the fact that malic acid production did not improve upon overexpression of the malate dehydrogenase without N-terminal targeting sequence likely indicates that this shorter version of the gene either lacks activity or is no longer localized in the correct compartment. In general, microbial malic acid production is possible via four different pathways, which have been described previously for *S. cerevisiae* (Figure 20) [115].

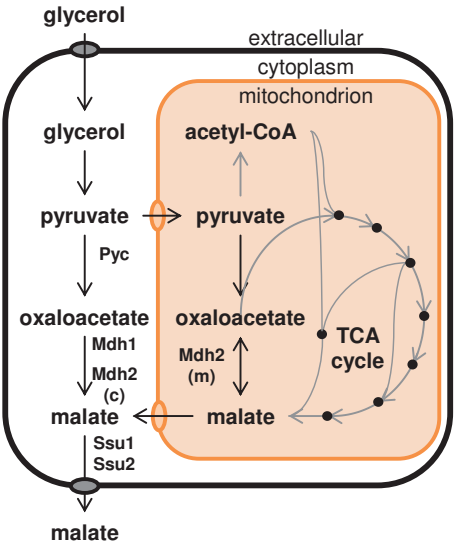

Figure 20: Possible microbial malic acid production pathways. Overexpression targets are indicated by gene names.

These pathways do not only differ in the enzymes involved, but also in their compartmentation. However, the cytosolic rTCA-cycle comprising the reaction of pyruvate to malic acid via oxaloacetate with the help of pyruvate carboxylase and malate dehydrogenase has been reported to be the predominant pathway for extracellular malic acid accumulation in many different organisms [54, 115, 117, 120-122], likely also being predominant in *U. trichophora*. Yet, this does not exclude the possibility of an activity of the mitochondrial alleles of malate dehydrogenase, especially, since not only overexpression of the gene encoding the cytoplasmic isoenzyme Mdh1 but also of the gene encoding the mitochondrial one, Mdh2 (m), resulted in increased malic acid formation in *U. trichophora* TZ1 (Table 7, Figure 21). Additionally the obtained yields are quite low compared to the theoretical yield possible with exclusive activity of rTCA (1 mole malate per mole glycerol), which further contributes to the assumption of activity in other pathways. The theoretical yields for the other pathways are lower with 0.5 mol_{mal} mol_{gly}^{-1} for the TCA cycle and cyclic glyoxylate route and 1.3 mole malate per 2 mole glycerol for the non-cyclic glyoxylate route.
Besides overexpression of the genes encoding malate dehydrogenases Mdh1 and Mdh2 (m), overexpression of the genes encoding the two transporters Ssu1 and Ssu2 resulted in increased malic acid production (Figure 21, Table 7).
For mutants overexpressing the genes encoding the putative malic acid transport proteins Ssu1 and Ssu2, malic acid titer and production rate were increased by up to 30 % and 38 % compared to the control strain *U. trichophora* TZ1, respectively. Mutants overexpressing malate dehydrogenases *mdh1* and *mdh2* (m) reached an up to 38 % and 27 % increased malic acid titer and rate, respectively. It has to be noted, that in general reached titers are low compared to published values with *U. trichophora* TZ1 cultivated under comparable conditions [195]. This phenomenon can be explained by differences in sample handling, resulting from the high amount of insoluble

compounds ($CaCO_3$, Ca-malate). However, the values within one experimental setup are comparable. Additionally to increased malic acid titer and production rate, optical density in all mutants was lower, than for the reference strain, resulting in drastically improved specific malic acid production ($g_{mal}\ OD_{600}^{-1}$). The reached yields were improved by 16-54 %, due to a simultaneously reduced glycerol uptake rate for nearly all mutants, reaching a maximal overall yield of 0.67 $g_{mal}\ g_{gly}^{-1}$ for one mutant overexpressing *mdh*1. In previous investigations with *U. trichophora* TZ1, we already observed, that even though product titer and production rate of the non-mutant strain could compete with values for highly engineered strains, the yield was low, leaving space for improvements by metabolic engineering [195, 222]. Clearly, the conversion of oxaloacetate to malate by malate dehydrogenase and the export of malic acid seem to be rate limiting steps. This has already been observed in *S. cerevisiae* and *A. oryzae*. Single overexpression of both, malate dehydrogenase and malate permease, resulted in a nearly 3-fold increased malic acid production in *S. cerevisiae* [115] and for *A. oryzae* overexpression of a C4-dicarboxylic acid transporter resulted in a 2-fold increase [117].

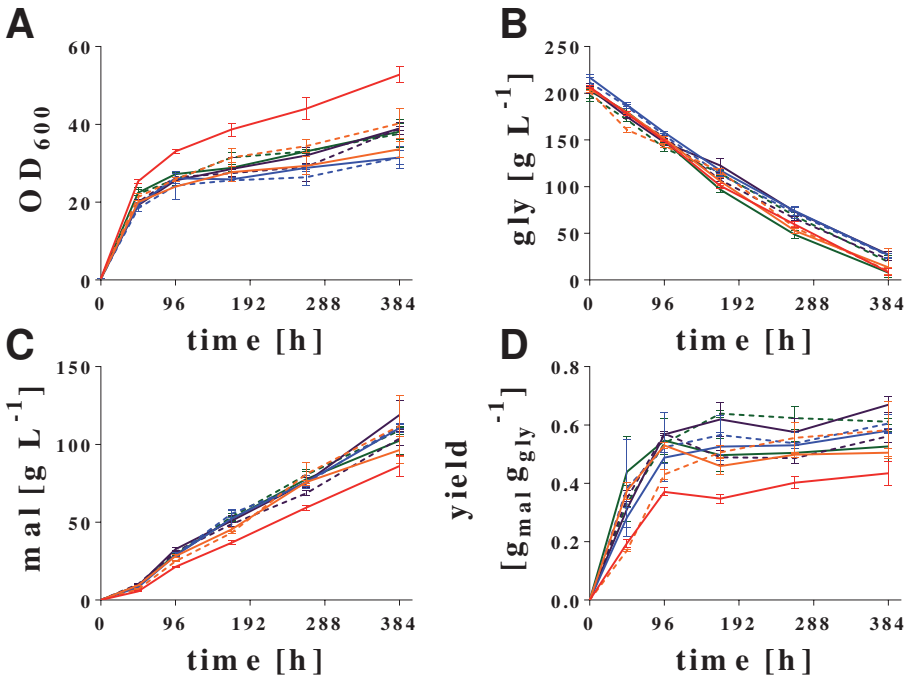

Figure 21: Shake flask cultivation of U. trichophora *TZ1 mutants in MTM with 200 g L^{-1} glycerol. Cultures contained 100 g L^{-1} $CaCO_3$. A: OD_{600}, B: glycerol concentration, C: malic acid concentration, and D: yield over time for* U. trichophora *TZ1 (red) and mutants overexpressing ssu1 (orange), ssu2 (blue), mdh1 (purple), mdh2 (m) (green). For each gene two individual transformants (dashed, solid) were investigated. Error bars indicate deviation from the mean of biological replicates of each transformant (n=3).*

To overcome known issues in shake flasks, such as insufficient mixing, oxygen supply and substrate depletion [195, 222], and to test whether the observed improvements would hold up under industrially more relevant conditions, mutants *U. trichophora* TZ1 overexpressing *ssu2* and *mdh2* (m) were cultivated in bioreactors containing doubled MTM with 200 g L^{-1} initial glycerol and 6.4 g L^{-1} NH_4Cl (Figure 22).

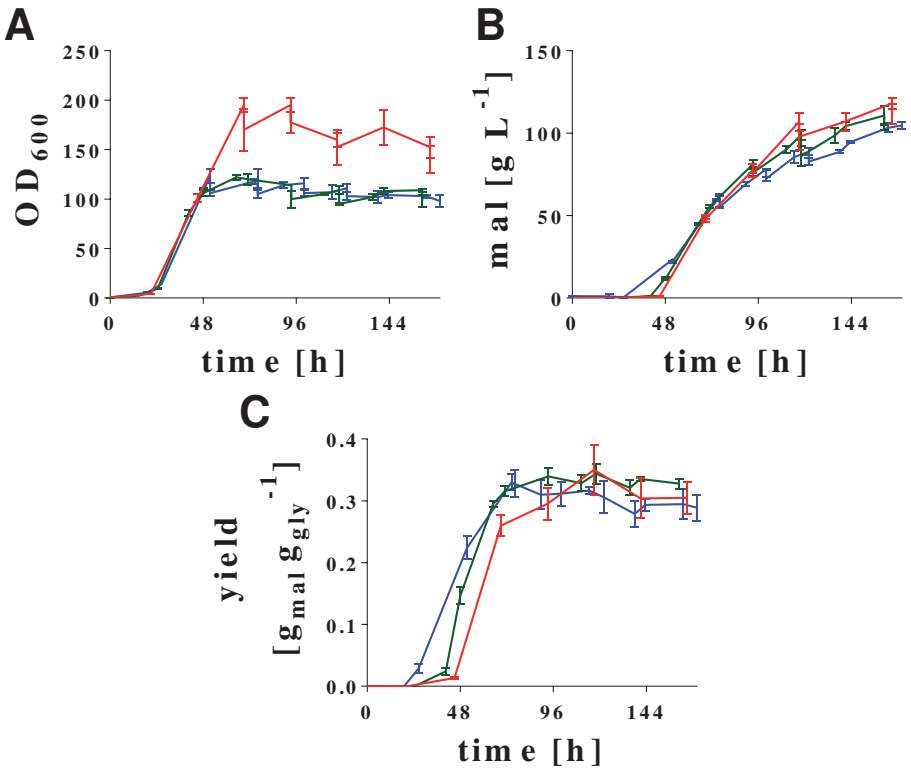

Figure 22: Fermentation of U. trichophora *TZ1 mutants in doubled MTM. A: OD_{600}, B: malic acid concentration, and C: yield for* U. trichophora *TZ1 (red)* [222], U. trichophora *TZ1 overexpressing* ssu2 *(blue), and* mdh2 *(m) (green) in controlled batch fermentations in MTM containing 200 g L^{-1} initial glycerol, 6.4 g L^{-1} NH_4Cl and doubled concentration of all other medium components at 30 °C with DO kept at 80 % and pH 6.5 kept constant at 6.5 by automatic NaOH addition. Error bars indicate deviation from the mean (n=2).*

Just as in shake flasks, optical density for mutant strains was drastically lower than for the reference strain (Figure 22A). The malic acid production rate however was not higher, but slightly lower (*mdh2* (m): 0.69 ± 0.03 g L^{-1} h^{-1}; *ssu2*: 0.63 ± 0.02 g L^{-1} h^{-1}) compared to the reference strain *U. trichophora* TZ1 (0.72 ± 0.02 g L^{-1} h^{-1}) (Figure 22B). However, this combination resulted in a 1.4-fold increased specific production rate (g_{mal} OD_{600}^{-1} h^{-1}). Strikingly, a higher product yield could

only be observed until approximately 72 h of cultivation, possibly resulting from an earlier onset of malic acid production (Figure 22C). The overall product yield for the mutant strains, however, was comparable to the one in the reference strain (Figure 22B), even though in shake flask cultivations it was drastically increased (Figure 21D).

This observation might be explained by the higher biomass formation, due to an elevated nitrogen concentration compared to shake flasks. Already in previous studies with *U. trichophora* TZ1 in bioreactors, 6.4 g L^{-1} NH_4Cl had a negative impact on the malic acid yield and only a slightly positive impact on the production rate compared to cultivations containing 3.2 g L^{-1} NH_4Cl [222]. The high concentration of all medium components, combined with the high biomass formation resulting from 6.4 g L^{-1} NH_4Cl, might trigger stress responses in the cells. This stress could result in lowered malic acid production, even though the specific production rate is still increased. To test this hypothesis, the bioreactor cultivation with *U. trichophora* TZ1 overexpressing *ssu2* was repeated with MTM containing 3.2 g L^{-1} NH_4Cl and the normal concentration for all other components (Figure 23).

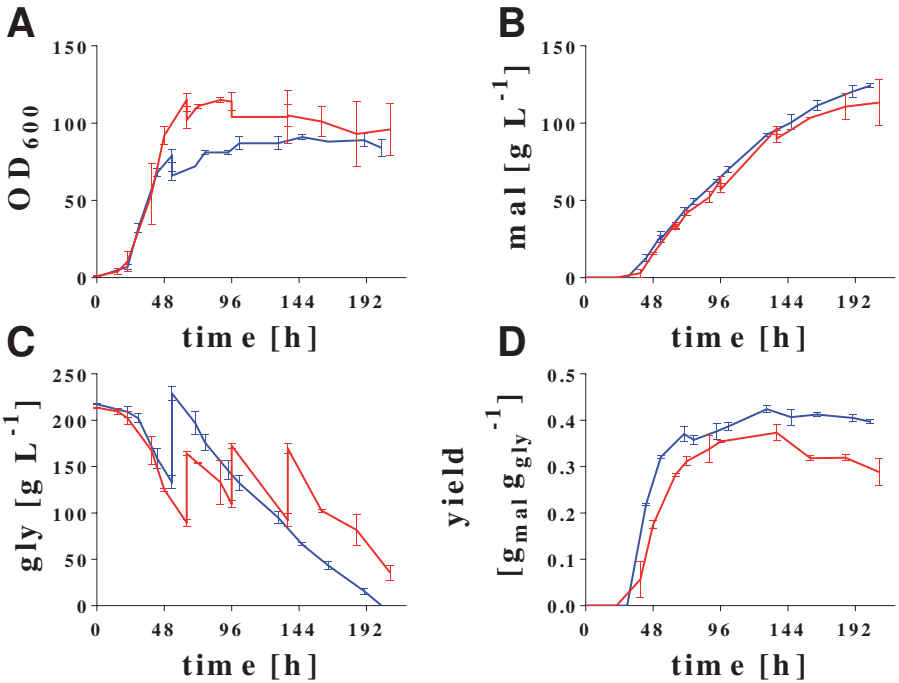

Figure 23: Fermentation of U. trichophora *TZ1 in single concentrated MTM. A: OD_{600}, B: malic acid concentration, C: glycerol concentration, and D: yield for* U. trichophora *TZ1 (red)* [222]*, and* U. trichophora *TZ1 overexpressing* ssu2 *(blue) in controlled batch fermentations in MTM containing 200 g L^{-1} initial glycerol, 3.2 g L^{-1} NH_4Cl at 30 °C with DO kept at 80 % and pH 6.5 kept constant at 6.5 by automatic NaOH addition. Error bars indicate deviation from the mean (n=2).*

Just as expected, with less NH_4Cl, the glycerol uptake in the mutant strain was lower than in the reference strain (Figure 23C), correlating with our observations in shake flask cultivations. Also in this cultivation, optical density was drastically lowered for the mutant strain (Figure 23A) resulting in a 29 % increased specific malic acid production rate. Yet, as previously discussed [119, 170, 195], a lower optical density for Ustilaginaceae does not necessarily imply a lower concentration of active biomass. However, the simultaneously reduced glycerol uptake strengthens the possibility of actually lowered active biomass, as already observed in shake flasks (compare Figure 21). Combined with a slightly increased malic acid titer (Figure 23B), the overall yield was improved by 1.4-fold to 0.40 $g_{mal}\ g_{gly}^{-1}$ (Figure 23B). Even though the yield could be improved, the value of 0.40 $g_{mal}\ g_{gly}^{-1}$ is just 27 % of the theoretical yield (1.46 $g_{mal}\ g_{gly}^{-1}$). Compared to the yield achieved with *A. oryzae* (0.69 % of the theoretical) [117], this value still indicates a huge loss of glycerol for formation of unwanted by-products. Since no significant amounts of other organic acids were found in HPLC analysis, the main by-products should be considered biomass, lipids and CO_2 formation. The strong intracellular and extracellular lipid formation for Ustilaginaceae has been reported previously [119, 141, 203, 204]. Since the lowered values for optical density in the mutant strains indicate a decrease in intracellular lipid formation, extracellular lipids should be considered the more interesting by-products to be investigated for an improved malic acid production with *U. trichophora* TZ1. The possibility of a lowered formation of the lipids ustilagic acid and mannosylerythritol has already been shown in *U. maydis*, by knockout of the responsive gene clusters [123, 141, 204]. Another possibility to further improve malic acid production with *U. trichophora* TZ1 would be, to take the process to bioreactors with $CaCO_3$ as buffering agent. We reported previously on the improvement of malic acid production with *U. trichophora* TZ1 by using $CaCO_3$ as buffering agent in bioreactors, which increased the product yield by 1.5-fold [222]. Possibly this improvement results from an additional CO_2 supply for pyruvate carboxylase and from a reduced stress level for the cells, due to a lower malic acid concentration in the medium. This lower malic acid concentration results from the reaction of produced malic acid with $CaCO_3$ forming insoluble Ca-malate [109, 117, 222]. Cultivation of the strain *U. trichophora* TZ1 overexpressing *ssu2* in bioreactors with $CaCO_3$ would likely result in a further improved malic acid yield, due to lower by-product formation and decreased stress levels for the cells. Further, the production rate could possibly be improved, due to the additionally supplied CO_2 for pyruvate carboxylase.

2.4.5. Conclusion

The potential of overexpressing rTCA in parts or in total for improved malic acid production has been shown consistently over the last years in different organisms. Also in *U. trichophora* TZ1 the single overexpression of genes from this pathway resulted in increased malic acid production, with simultaneously reduced glycerol uptake for nearly all mutants, thus improving the reached yield by more than 1.5-fold in shake flasks and 1.4-fold in bioreactors. Interestingly, overexpression of malic acid transporters and of malate dehydrogenases results in drastically lowered biomass formation, resulting in an improvement of the specific production rate of up to 1.4-fold. Further improvements can be expected by combinatorial overexpression of all three genes to further increase malic acid production with *U. trichophora* TZ1 from glycerol. With the tools established in this work, the possibility of multiple overexpressions in one strain is achievable. This would further contribute to establish an industrially feasible malic acid production process from glycerol with *U. trichophora* TZ1. With such a process, the efficiency of bio-diesel refineries could be further improved, thus contributing to the overall concept of a sustainable and innovative bioeconomy.

Efficient itaconic acid production from glycerol with *Ustilago vetiveriae* TZ1

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Contributions:

Thiemo Zambanini, Hamed Hosseinpour Tehrani, Elena Geiser, Dorothee Merker, Sarah Schleese and Judith Alferink performed the experiments. Thiemo Zambanini, Nick Wierckx, Joerg M. Buescher and Lars M. Blank analyzed results. Thiemo Zambanini and Nick Wierckx wrote the manuscript with the help of Lars M. Blank. Lars M. Blank, Nick Wierckx and Guido Meurer conceived and designed the project. We grategully acknowledge BioEton for providing the glycerol.

2.5. Efficient itaconic acid production from glycerol with *Ustilago vetiveriae* TZ1

2.5.1. Summary

The family of Ustilaginaceae is known for their capability to naturally produce industrially valuable chemicals from different carbon sources. Recently several Ustilaginaceae were reported to produce organic acids from glycerol, which is the main waste stream in biodiesel production.

In this study, we present *Ustilago vetiveriae* TZ1 as new production strain for itaconate from glycerol. In a screening of 126 Ustilaginaceae, this organism reached one of the highest titers for itaconate combined with a high glycerol uptake rate. By adaptive laboratory evolution, the production characteristics of this strain could be improved. Further medium optimization in 24-deep well plates resulted in a maximal itaconate titer of 34.7 ± 2.5 g L^{-1} produced at a rate of 0.09 ± 0.01 g L^{-1} h^{-1} from 196 g L^{-1} glycerol. Simultaneously, this strain produced 46.2 ± 1.4 g L^{-1} malate at a rate of 0.12 ± 0.00 g L^{-1} h^{-1}. Notably, an acidic pH-value of 5.5 resulted in decreased itaconate production, however, completely abolishing malate production. Strain optimization by overexpressing the regulator encoding gene *ria1* or the mitochondrial itaconate transporter encoding gene *mtt1* from the itaconate cluster of *U. maydis* resulted in higher itaconate production. Specifically, the production of itaconate in comparison to the wild type strain could be increased by up to 2.0-fold and 1.5-fold, respectively, simultaneously reducing malate production by 96 % and 61 %, respectively.

The observed production properties of *U. vetiveriae* TZ1 already make this strain a promising candidate for microbial itaconate production. The outcome of the overexpression experiments, which resulted in reduced malate production in favor of an increased itaconate titer, clearly strengthens its potential for industrial itaconate production from a major side stream of biodiesel production.

2.5.2. Introduction

The switch from a mainly petroleum based, to a bio-based, ecologically viable and responsible industry has become omnipresent over the last years. Consequently, research is focusing on the development of biotechnological production processes, resulting in bio-chemicals, able to compensate for petrochemicals. One group of these chemicals is organic acids, such as itaconate, malate or succinate.

Especially the C5-dicarboxylic acid, itaconate, has gained great interest, due to many different possible applications in carbon fiber technology, rubber industry, anti-scaling polymers in water treatment, cement additives, surface active agents, plastics, and dye intermediates [34, 77, 78]. Additionally, it can be converted into different profitable molecules, due to its multiple functional groups [34] or be used for self-polymerization to poly-itaconate, which has the potential to replace a broad range of different polymers [76, 92, 255]. In 2004, itaconate was announced one of the top twelve building block chemicals, to be produced from renewable biomass, by the U.S. Department of Energy [34]. Even though, the contemporary market for itaconate is rather small with about 41 kt a^{-1} in 2013, corresponding to a market value of approximately US$ 74.5 million, it is predicted to reach US$ 570 million by 2020 [79]. This huge increase in the expected market volume is based on the possibility to substitute existing chemicals, if an improved production process can be developed that would lower the price for itaconate.

Chemical synthesis of itaconate was first reported in 1836 [80-82] and in 1931, *Aspergillus itaconicus* was the first organism to be found to produce itaconate [84]. In contrast to many other chemicals, contemporary itaconate production is completely achieved by biotechnological processes [77]. These processes rely on improved *A. terreus* strains. The first *A. terreus* strain producing itaconate was discovered in 1939 [85] and since then, the use of this organism has been investigated and improved intensively for the production of itaconic acid [100, 256, 257]. Over the years, many different organisms have been found to produce itaconate, including several species of *Pseudozyma* [101, 258], *Ustilago* [102, 104, 145], *Candida* [105], and *Rhodotorula* [106].

Many of these production strains are a member of the family of Ustilaginaceae, which is a promising fungal family for biotechnological applications [142, 144, 197]. Recently, the itaconate production pathway in *U. maydis* has been clarified, allowing for targeted metabolic engineering of itaconate production in this host [232].

The family of Ustilaginaceae is generally known for combining natural production of many different industrially relevant products, such as organic acids, polyols, and lipids from a broad range of different substrates, with favorable characteristics for biotechnological processes, such as a yeast-like morphology, insensitivity towards medium impurities and tolerance to high product titers [43, 76, 104, 144, 145, 170, 173, 197-199, 259]. Especially the broad substrate range attracted interest in this group of organisms. As plant pathogens, Ustilaginaceae are able to degrade a broad range of polymers from biomass, such as cellulose, hemicellulose or xylan [171, 172, 260]. Recently, malate production from bio-diesel derived glycerol has been demonstrated with *U. trichophora* TZ1 [195, 222].

The use of glycerol as substrate for microbial conversion has been discussed frequently over the last years. In a follow-up study of the landmark 2004 DoE report [34] glycerol is still considered one of the 10 most promising building blocks to be produced [52]. However, increasing biodiesel production has resulted in a huge waste-stream of (crude) glycerol. During the production process of biodiesel, 10 % (w/v) of glycerol are produced. With 123 million tons of biodiesel per year predicted

for 2016 [177], 19 million tons of crude glycerol will flood the market, further lowering the price, while simultaneously decreasing the profit margin for the biodiesel production process itself. Consequently, valorization of this huge waste-stream has been discussed intensively, resulting in several microbial production processes for different products starting from glycerol [36, 37].
Here we present *U. vetiveriae* TZ1 as promising production organism for organic acids from glycerol, reaching high total acid titers with itaconate and malate as main products. Further, we demonstrate that by single overexpression of two different genes, the acid production profile can be drastically influenced in favor of itaconate.

2.5.3. Material and Methods

2.5.3.1. Strains and culture conditions

The 76 strains belonging to the family Ustilaginaceae screened by Zambanini *et al.* [195] plus 50 additional strains were screened in this study (see Table 8, supplement).
As standard medium, MTM was used according to Zambanini *et al.* [195].
Adaptive laboratory evolution was performed for 62 days as described previously [195].
Medium optimization was performed in 24 deep well plates (System Duetz®) with 1.5 mL MTM containing 200 g L^{-1} glycerol, $CaCO_3$ and differing concentrations of $FeSO_4$, $MgSO_4$ and KH_2PO_4 incubated at 30 °C (relative air humidity = 80 %) shaking at 300 rpm (shaking diameter = 50 mm). As nitrogen source different concentrations of NH_4Cl, yeast extract or peptone were used.
Shake flask experiments were conducted as described previously [195].
All batch cultivations were performed in New Brunswick BioFlo® 110 bioreactors (Eppendorf, Germany) with a total filling volume of 2.5 L and 1.25 L working volume. As medium MTM containing different amounts of glycerol and NH_4Cl or yeast extract was used. The temperature was maintained at 30 °C. The pH was controlled automatically with 10 M NaOH at different values. To prevent foam formation, antifoam 204 (Sigma Life Science, USA) was added automatically using level sensor control. The aeration rate was set to 1.25 L min^{-1} (1vvm) and the dissolved oxygen tension (DOT) was kept at 80 % saturation by automatically adjusting the stirring rate. Pre-cultures were prepared as described previously [222].

2.5.3.2. Analytical methods

All experiments were performed in duplicates. Shown is the arithmetic mean of the duplicates. Error bars and ± values indicate deviation from the mean.
When using $CaCO_3$ as buffer, 1 mL of culture broth was taken for OD_{600} determination and HPLC-analysis. The $CaCO_3$ was dissolved with HCl prior to further measurements. OD_{600} was determined in an Ultrospec 10 cell density meter (Amersham Biosciences, UK). Samples were diluted to an OD_{600} between 0.1 and 0.8.
From fermenters 5 mL of culture broth was taken for OD_{600} and cell dry weight determination and HPLC-analysis. For cell dry weight determination, 3 mL of the sample were filtered through pre-dried and -weighed Whatman paper, which was subsequently incubated at 60 °C for 72 h prior to weighing.
For HPLC analysis centrifuged samples (13,000 g, 5 min) were filtered through cellulose acetate filters (diameter 0.2 µm, VWR, Germany) and subsequently diluted 1:10 with distilled water.

Glycerol and organic acids were analyzed on a Dionex Ultimate 3000 HPLC (Dionex, USA) with an Organic Acid Resin column (CS-Chromatographie, Germany) kept at 75 °C, with a constant flow rate of 0.8 mL min^{-1} of 5 mM sulfuric acid as eluent. For detection, a Shodex RI 101 detector at 35 °C and a variable wavelength UV detector (Dionex, USA) at 210 nm were used.
Ammonium concentration was determined by a colorimetric assay according to Willis [182].

2.5.3.3. Cloning procedures

For overexpression of *ria1* and *mtt1*, the overexpression constructs generated by Geiser *et al*. were used [232].
All cloning procedures with *U. vetiveriae* were performed as described previously for *U. trichophora* (chapter 2.4.3.3).

2.5.4. Results and Discussion

2.5.4.1. Liquid screening reveals *Ustilago vetiveriae* as promising itaconate producer

Recently, we reported on an *U. trichophora* strain, which was found in a broad screening of Ustilaginaceae, to naturally produce malate from glycerol [195]. The primary screening in this study was performed on agar plates with a pH-indicator, only resulting in a qualitative indication of growth and semi-quantitative indication concerning total acid production. Only the best strains in terms of growth and acid production were selected for a second screening in liquid medium. However, due to the generally high malate production of many Ustilaginaceae [145], this method is less suited for finding producers of other organic acids, such as itaconate. Consequently, we performed a complete screening of 126 Ustilaginaceae cultivated in 24-deep well plate liquid cultures [261] in MTM containing 50 g L^{-1} glycerol, 0.8 g L^{-1} NH_4Cl and 100 g L^{-1} $CaCO_3$. After 355 h, the culture supernatants were initially evaluated for glycerol uptake (Figure 24A) and strains with the highest glycerol uptake rate were selected for further analysis.
As reported previously for Ustilaginaceae [145], a broad phenotypic distribution was observed. Some strains consumed all carbon while others did not grow at all on glycerol (Figure 24A). Although organic acid production was observed with a broad diversity, titers were generally low due to the low concentration of glycerol used. Hence, we chose the 24 strains with fastest glycerol uptake (indicated by the red box in Figure 24A), to investigate in a second 24-deep well plate screening with a higher initial glycerol concentration of 100 g L^{-1} (Figure 24B). From this screening the six strains with best itaconate and malate production properties and highest glycerol uptake (indicated by arrows in Figure 24B) were chosen and cultivated in shake flasks containing 200 g L^{-1} glycerol. Only three of these strains, 2220, 2221, and 2383 were able to produce itaconate, reaching a titer of 17.5 ± 1.9 g L^{-1}, 20.1 ± 4.6 g L^{-1}, and 8.5 ± 1.8 g L^{-1}, respectively (Figure 24C), while all strains produced malate with titers between 10.5 ± 0.7 g L^{-1} and 63.1 ± 0.3 g L^{-1} (Figure 24D). Even though these malate titers are rather high for wildtype strains, they stayed below the titer reached with the previously published *U. trichophora* TZ1 [195, 222] or *A. oryzae* [117, 194]. Consequently we focused on the strains producing itaconic acid, since these titers are relatively high, given the applied conditions, even though reached production rates are lower than for *A. terreus* [262] and *P. antarctica* [101] on glucose.

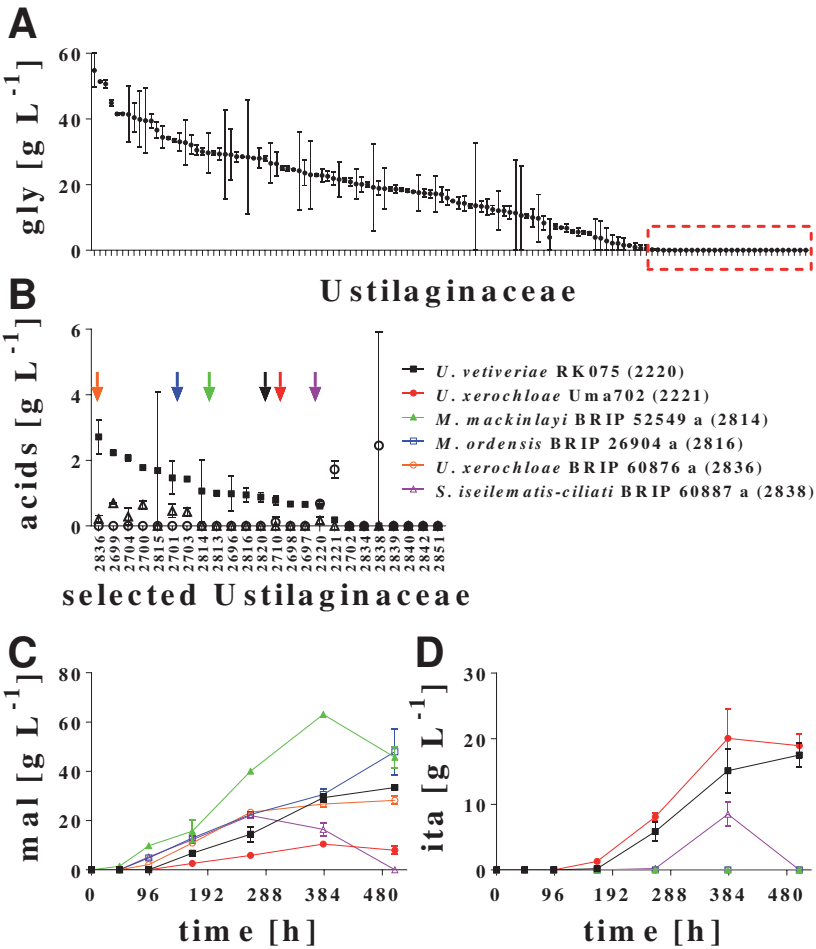

Figure 24: Screening for the production of organic acids from glycerol. A: Rank-ordered glycerol concentration after 355 h of cultivation in 24-deep well plates containing MTM with 50 g L^{-1} glycerol, 0.8 g L^{-1} NH_4Cl and 100 g L^{-1} $CaCO_3$ for 126 different Ustilaginaceae. Strains with high glycerol consumption (indicated by the red box) were re-screened for acid production. B: Rank-ordered malate concentration (closed squares), itaconate concentration (open circles) and succinate concentration (open triangles) after 383 h of cultivation in 24-deep well plates containing MTM with 100 g L^{-1} glycerol, 0.8 g L^{-1} NH_4Cl and 100 g L^{-1} $CaCO_3$ for 24 selected Ustilaginaceae. Strains with good production (indicated by arrows) were evaluated in detail. C: Malate concentration and D: itaconate concentration for different Ustilaginaceae cultivated in shake flasks containing MTM with 200 g L^{-1} glycerol, 0.8 g L^{-1} NH_4Cl and 100 g L^{-1} $CaCO_3$. The relation of glycerol concentration and strain numbers for a) is given in Table 8 (supplement). Error bars indicate deviation from the mean (n=2). Adapted from [263].

2.5.4.2. Adaptive laboratory evolution elevates growth and acid production on glycerol

The concept of adaptive laboratory evolution (ALE) is known to be suitable for improvement of specific characteristics by adaptation to environmental factors [183, 184]. Recently, we were able to drastically improve organic acid production, growth rate, and glycerol uptake in *U. trichophora* by applying this concept with growth rate as driving force [195]. Hence we used the same re-inoculation scheme in shake flasks for all six strains from the last screening round in duplicates (Figure 25A).

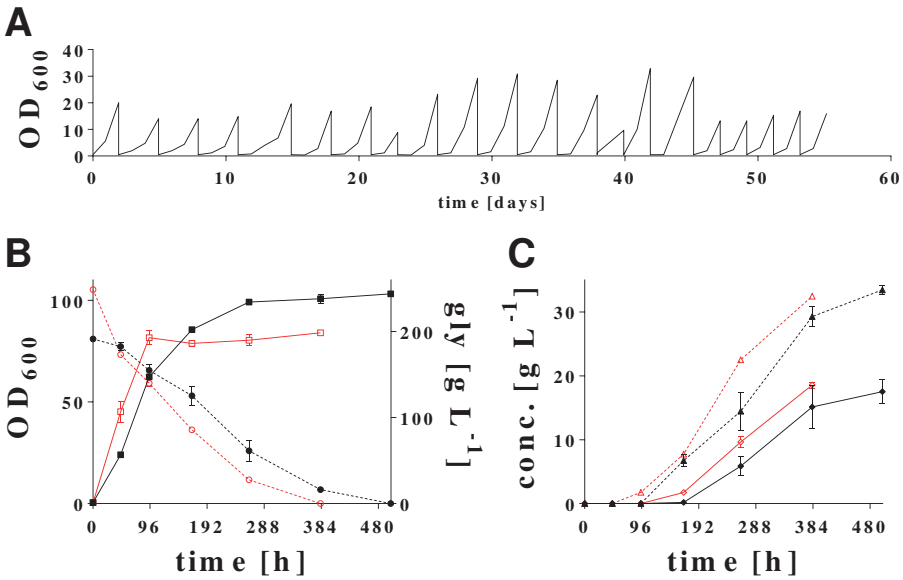

Figure 25: Adaptive laboratory evolution for U. vetiveriae. *A: ALE on glycerol as carbon source. A single representative culture is shown. B: Comparison of growth (squares, solid lines) and glycerol consumption (circles, dashed lines). C: Comparison of itaconate (diamonds, solid lines) and malate (triangles, dashed lines) production for* U. vetiveriae *RK075 (black, closed symbols) and* U. vetiveriae *TZ1 (red, open symbols). Error bars indicate deviation from the mean (n=2). Adapted from* [263, 264].

In all strains glycerol uptake and growth rate increased over time. However, all itaconate producers eventually stopped production of itaconate after different rounds of evolution. This fact might be explained by the strong generation of NADH by both, growth and itaconate production, which needs to be re-oxidized. Since the applied factor during ALE was growth, itaconate might be detrimental and as a trade-off, faster growth results in lowered or completely abolished itaconate production. This is also strengthened by the fact that malate production is improved in all cultures, a production pathway which re-oxidizes one NADH to NAD^+. Even though *U. vetiveriae* stopped itaconate production after 25 re-inoculations during ALE, it was still able to produce considerable amounts of itaconate after 21 re-inoculations. The best single colony (*U. vetiveriae* TZ1) isolated

from the 21st re-inoculation, which corresponds to about 105 generations, produced 18.9 ± 0.6 g L^{-1} itaconate within 383 h at a production rate of 0.05 ± 0.00 g L^{-1} h^{-1} (Figure 25C). In comparison, the reference strain (before ALE) produced 17.5 ± 1.9 g L^{-1} at a production rate of 0.03 ± 0.00 g L^{-1} h^{-1} (Figure 25C). Additionally, the malate production in this strain was improved reaching 32.5 ± 0.33 g L^{-1} product at a rate of 0.09 ± 0.00 g L^{-1} h^{-1}, whereas the reference produced 33.5 ± 0.6 g L^{-1} at a rate of 0.06 ± 0.00 g L^{-1} h^{-1}. Even though the acid titer could not be increased, the glycerol uptake rate reached 0.65 g L^{-1} after ALE compared to 0.38 g L^{-1} in the wildtype strain (Figure 25B). This hints at a limitation in the itaconic acid production pathway rather than the glycerol uptake.

2.5.4.3. Medium optimization enhances itaconate production with *U. vetiveriae* TZ1

The influence of differing concentrations of medium components on microbial production processes, for organic acids, biomass and proteins, has been shown consistently in literature for different organisms, such as *Aspergilli* [87, 257, 265, 266], *Corynebacterium glutamicum* [188], and Ustilaginaceae [145]. Additionally, the used concentration of nitrogen and the used nitrogen source itself (ammonium chloride, yeast extract, peptone) drastically changed acid production in different organisms [101, 119, 170, 195]. Consequently, we tested changing concentrations of ammonium chloride (0.8, 1.6, 3.2 g L^{-1}), $FeSO_4$ (3, 13, 53, 103 mg L^{-1}), KH_2PO_4 (0.125, 0.25, 0.5, 1 g L^{-1}), and $MgSO_4$ (0.1, 0.2, 0.5 g L^{-1}), while keeping the concentration of all other components in the MTM unaltered. Additionally, we used peptone (2 g L^{-1}) or yeast extract (2.4, 4.8 g L^{-1}) instead of ammonium chloride. The nitrogen (N) content of the different nitrogen sources corresponds to 0.26 g_N (0.8 g L^{-1} NH_4Cl), 0.52 g_N (1.6 g L^{-1} NH_4Cl), 1.05 g_N (3.2 g L^{-1} NH_4Cl), 0.26 g_N (2.0 g L^{-1} peptone), 0.26 g_N (2.4 g L^{-1} yeast extract), and 0.52 g_N (4.8 g L^{-1} yeast extract) [267].

Altered concentrations of $FeSO_4$, KH_2PO_4, and $MgSO_4$ did not change organic acid production with *U. vetiveriae* TZ1. Changing the nitrogen concentration or source itself, however, drastically improved growth, glycerol uptake, and organic acid production (Figure 26).

As expected, a higher ammonium chloride concentration increased final OD_{600} and volumetric glycerol uptake rates. However, growth of *U. vetiveriae* was delayed, possibly due to higher stress levels resulting from high ammonium concentrations. This effect was previously observed using *U. trichophora* [222]. As expected, total acid production rates improved with increasing NH_4Cl concentrations. However, with 3.2 g L^{-1} NH_4Cl, the final titer was reduced compared to 1.6 g L^{-1}. Even though the estimated amount of supplied nitrogen was the same for 0.8 g L^{-1} NH_4Cl, 2 g L^{-1} peptone, and 2.4 g L^{-1} YE, itaconate production was greatly improved with the latter two N-sources. In contrast to NH_4Cl, the use of these complex N-sources resulted in an earlier onset of growth, consequently also resulting in an earlier production. Likely, YE and peptone are less toxic to the cells in the initial growth stage, and their uptake and incorporation into biomass is energetically better for *U. vetiveriae*. While ammonium assimilation is dependent on NADH, transaminase activity is not. Additionally, the uptake of di-, tri-, and possibly even oligopeptides is more efficient, since energy is spend for the uptake of one molecule, however, several ammonium molecules can be generated. The resulting surplus of energy leads to an overall improved biomass yield. The highest itaconate titer of 34.7 ± 2.5 g L^{-1} was reached with 4.8 g L^{-1} yeast extract produced at a rate of 0.09 ± 0.01 g L^{-1} h^{-1}. Simultaneously 46.2 ± 1.4 g L^{-1} malate was produced.

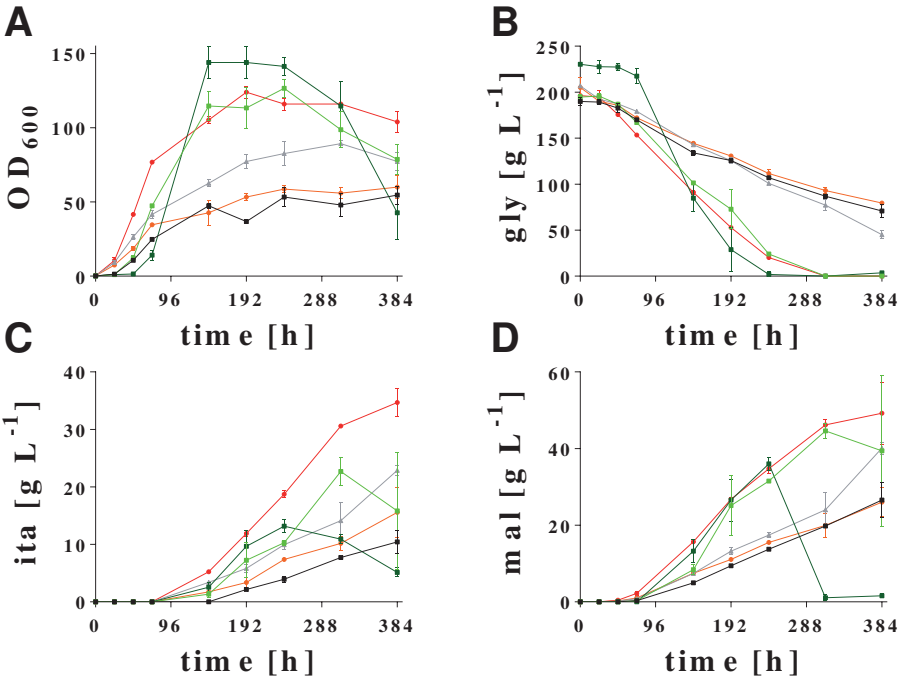

Figure 26: Medium optimization for U. vetiveriae *TZ1. A: OD_{600}, B: glycerol concentration, and C: itaconate concentration, and D: malate concentration for* U. vetiveriae *TZ1 cultivated in 24-deep well plates containing MTM with 200 g L^{-1} glycerol, 100 g L^{-1} $CaCO_3$ and 0.8 g L^{-1} (black), 1.6 g L^{-1} (light green), 3.2 g L^{-1} NH_4Cl (dark green), 2.4 g L^{-1} (orange), 4.8 g L^{-1} yeast extract (red) or 2.0 g L^{-1} peptone (grey). Error bars indicate deviation from the mean (n=3). Adapted from [264].*

2.5.4.4. Product inhibition by itaconic acid is stronger than product inhibition by malic acid

For malate production with *U. trichophora* TZ1, a drastic increase in production rate could be achieved in controlled bioreactors [222]. Hence, we also investigated itaconate production with *U. vetiveriae* TZ1 in fed-batch cultivations with 200 g L^{-1} initial glycerol. Using 3.2 g L^{-1} NH_4Cl or 5 g L^{-1} yeast extract resulted in a production rate of 0.06 ± 0.0 g L^{-1} h^{-1}, which is similar to the values observed in 24-deep well plates (Figure 27B). Surprisingly, the titer (about 24 g L^{-1}), was not increased for both cultures, even though additional glycerol was fed throughout the fermentation. The experiments were repeated and parameters were changed but in all bioreactor cultivations a titer of about 24 g L^{-1} itaconate could not be exceeded. In $CaCO_3$-buffered shake flasks, in contrast, higher concentrations were reached. This hints at product inhibition by itaconic acid concentrations above 24 g L^{-1}.

We described this effect previously in the context of malic acid formation with *U. trichophora* TZ1, where in $CaCO_3$-buffered shake flasks and bioreactors, a concentration of about 200 g L^{-1} was

reached, while in NaOH-buffered bioreactors a concentration of about 140 g L^{-1} was limiting [222]. The concentration of dissolved malic acid in the culture broth of $CaCO_3$-buffered cultures was determined to be about 15 g L^{-1} throughout the cultivation, consequently lowering the osmotic stress for the cells [195]. For itaconic acid, the concentration of dissolved itaconic acid in $CaCO_3$-buffered water was determined to be about 11 g L^{-1} (Tim Massmann, personal communication) with precipitation of the residual itaconate as calcium-itaconate, strengthening the hypothesis of product inhibition of higher dissolved itaconic acid concentrations in NaOH-titrated bioreactors. Since also for *U. vetiveriae* TZ1 malic acid concentrations of up to 60.0 ± 10.4 g L^{-1} were reached in bioreactors, product inhibition by itaconic acid seems to be stronger than by malic acid. Consequently, for a feasible production process, *in-situ* product removal would be needed. The possibility of continuous itaconic acid production with Ustilaginaceae using integrated cell retention and simultaneous product recovery in a membrane bioreactor has already been shown [259]. Another possibility would be further ALE on higher itaconic acid concentrations in the medium, to obtain a strain, which is less sensitive towards this product.

Apart from the observed product inhibition, the nitrogen source has a strong influence on fermentation performance. As expected, a lowered NH_4Cl concentration (1.6 g L^{-1} NH_4Cl) resulted in a lowered itaconate and malate production rate (Figure 27B), due to lower biomass formation (Figure 27A). In contrast to results observed with *U. trichophora* TZ1 for malate production [222], 6.4 g L^{-1} NH_4Cl combined with a doubled concentration for all other medium components resulted in neither rate nor titer improvement, but prolonged the growth phase, however reaching lower OD_{600}-values when compared to 3.2 g L^{-1} NH_4Cl (Figure 27A). In all, it seems that *U. vetiveriae* TZ1 is less tolerant to higher concentrations of ammonium or other medium salts than *U. trichophora* TZ1. In order to achieve higher cell-densities and thus production rates, strains with improved tolerance towards higher salt concentrations can likely be isolated by additional ALE selection under ammonium stress, or an ammonium-fed process could be investigated. The adaptation of *U. vetiveriae* TZ1 towards a higher NH_4Cl tolerance would greatly contribute to an improved production process, since the use of other nitrogen sources, such as yeast extract, which at the moment clearly improve the production, could be avoided.

The use of 5 g L^{-1} yeast extract resulted in the same production values as the use of 3.2 g L^{-1} NH_4Cl, even though only about half the nitrogen is supplied (0.55 g_N vs. 1.05 g_N). However, the use of yeast extract in the production of bulk fermentation products is often a cost-prohibitive factor. Likely, even with a higher concentration of NH_4Cl or another ammonium salt, such as $(NH_4)_2SO_4$, the overall process would be more cost-efficient. Additionally, cultures grown in bioreactors with yeast extract showed an earlier onset of the growth and production phase, just as in shake flasks. Besides yeast extract, other nitrogen sources, such as nitrate or urea, could be tested. Cultivations with *U. maydis* using these nitrogen sources resulted in high acid titers compared to acidic nitrogen sources, such as $NH_4H_2PO_4$ or NH_4Cl, even though the main effect was argued to result from higher final pH-values in barely buffered shake flask cultivations [103]. Yet, these observations would also correspond to first results with *U. vetiveriae* TZ1 cultivated in bioreactors at pH 4.5 and 5.5 (data not shown). At pH 4.5, no itaconate and malate production could be observed, while at pH 5.5 itaconate was still produced at a low titer of 8.0 ± 0.8 g L^{-1}. Notably, in this cultivation, no malate was produced, suggesting a strategy for single product formation.

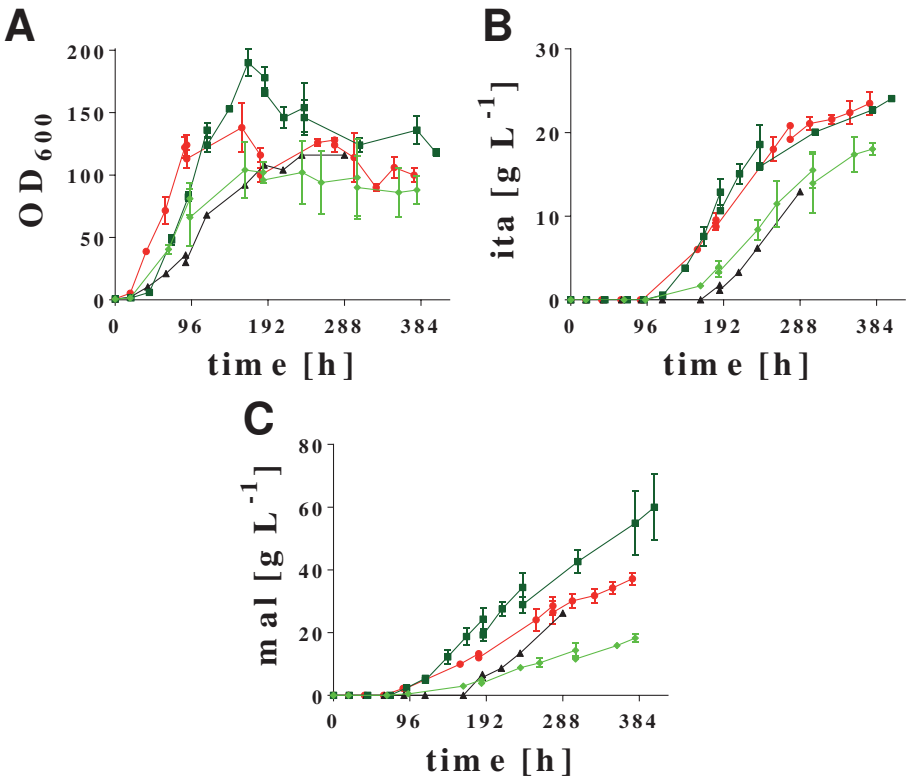

Figure 27: Controlled batch cultivations of U. vetiveriae *TZ1 with different nitrogen concentrations. A: OD_{600}, B: itaconate concentration, and C: malate concentration, for cultures in MTM containing 200 g L^{-1} initial glycerol at 30 °C and pH 6.5 with DO kept at 80 %. Colors indicate different initial nitrogen concentrations: 5 g L^{-1} yeast extract (circles, red), 1.6 g L^{-1} NH_4Cl (diamonds, light green), 3.2 g L^{-1} NH_4Cl (squares, dark green), and 6.4 g L^{-1} NH_4Cl with doubled concentration of all medium components except glycerol (triangles, black). Error bars indicate deviation from the mean (n=2). Adapted from [268].*

Even though itaconate production could not be improved in bioreactors, malate production was elevated. With 3.2 g L^{-1} NH_4Cl the malate titer increased to 60.0 ± 10.4 g L^{-1} produced within 403 h (Figure 27C). Just as for itaconate, malate production was reduced both with higher and lower NH_4Cl concentration. With yeast extract malic acid production was improved compared to production with 1.6 g L^{-1} NH_4Cl, which corresponds to about the same amount of nitrogen. These high values for malic acid underline the higher tolerance of Ustilaginaceae against malic acid compared to itaconic acid, even though, a specific production process for itaconic acid without by-product formation would be preferred.

2.5.4.5. Metabolic engineering shifts organic acid production towards itaconate

Product specificity and hence product yield on substrate is an important factor in microbial production processes. The simultaneous production of several organic acids in one strain results in a lowered titer for the desired product. Additionally, product recovery is more complex with similar compounds in the medium [60]. Thus a strain producing one organic acid with high specificity is desirable. As already mentioned the drastically increased glycerol uptake for *U. vetiveriae* TZ1 after ALE combined with only slightly improved acid production suggests a bottleneck in the specific itaconate production pathway, rather than glycerol uptake. Metabolic engineering of the itaconic acid production pathway could help to overcome this limitation. In a previous study on itaconate production from glucose with *U. maydis* MB215, overproduction of the mitochondrial transporter (Mtt1) and the regulator of the itaconate gene cluster (Ria1) led to improved itaconate production [123]. Consequently, we created mutants of *U. vetiveriae* RK075 overexpressing either *mtt1* or *ria1* from *U. maydis* MB215. For overexpression we used a plasmid for *U. maydis*, which confers resistance to carboxin by site-specific integration into the *ip*^{R}-locus. Previously we showed that this plasmid can also confer resistance to carboxin in other Ustilaginaceae, such as *U. trichophora*, even though site-specificity is not given. Additionally, all contained elements, such as promoter and terminator, were functional (compare chapter 2.4).

Cultivation of the *U. vetiveriae* overexpression mutants in 24-deep well plates containing MTM with 0.8 g L^{-1} NH_4Cl, 200 g L^{-1} glycerol and 100 g L^{-1} $CaCO_3$ resulted in a 1.5-fold and 2.0-fold increased itaconate production after 384 h for *U. vetiveriae* pETEF-UmagMtt1-CBX#2 and *U. vetiveriae* pETEF-UmagRia1-CBX#2, respectively (Figure 28C). Malate production was reduced to 4 % for *mtt1* and 39 % for *ria1* (Figure 28C). It has to be noted that the organic acid titers for both, *U. vetiveriae* RK075 and *U. vetiveriae* TZ1 under the same cultivation conditions in 24-deep well plates and shake flasks differ from previous experiments. This phenomenon may be explained by differences in sample handling, resulting from the high amount of insoluble compounds ($CaCO_3$, Ca-itaconate, Ca-malate) or handling of the precultures. In most experiments 24-deep well plates with many different strains were used, making the inoculation process laborious. Consequently, it might slightly differ. However, the values within one experimental setup are comparable but require validation in further experiments.

This shift of organic acid production in favor of itaconate upon overexpression of either *ria1* or *mtt1* is comparable to the one in *U. maydis* MB215 both on glucose [123] and glycerol (data not shown). From these results it was assumed, that the mitochondrial transporter Mtt1 is the bottleneck of itaconate production in *U. maydis*, which can be overcome directly by overexpression of *mtt1*, or indirectly by overexpression of *ria1* [123]. This bottleneck seems also to be present in *U. vetiveriae*. Interestingly, the regulator of the itaconate cluster from *U. maydis* is functional in *U. vetiveriae* and even other *Ustilago* strains, such as *U. xerochloae* (data not shown), even though the similarity of Ria1 from *U. maydis* on protein level is only 57% for *U. vetiveriae* and 44% for *U. xerochloae*. For the mitochondrial transporter, in contrast, a similarity of 87% (*U. vetiveriae*) and 71% (*U. xerochloae*) was found. A close genetic relation between different *Ustilago* strains and the functionality of genes from one strain in another strain has been shown for *U. trichophora* and *U. maydis* with the plasmid pUMa43, as already mentioned. Additionally, a genome comparison between *U. trichophora* and *U. maydis* revealed around 90% homology on DNA level for all genes involved in malate production from glycerol [241] (compare chapter 2.4). A comparison of the whole

itaconic acid cluster from *U. maydis* to the genome of *U. vetiveriae* revealed a homology of about 70 to 90% on protein level for all genes in the itaconic acid cluster. This strengthens the hypothesis of itaconic acid production in *U. vetiveriae* via the same pathway as in *U. maydis* [232].

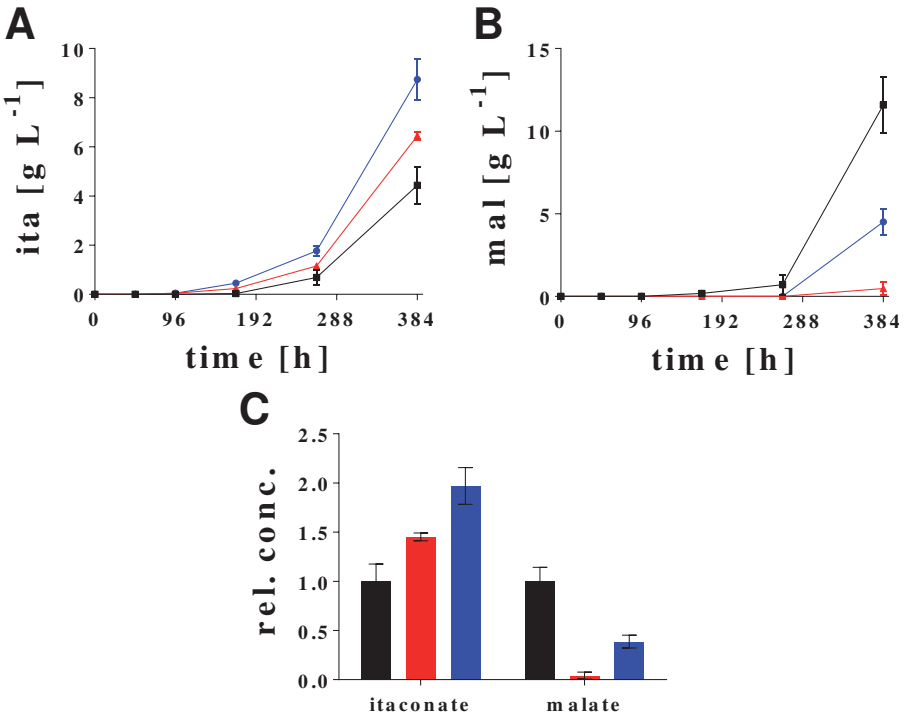

Figure 28: Overexpression of ria1 *and* mtt1 *in* U. vetiveriae *RK075. A: itaconate concentration B: malate concentration, and C: relative itaconate and malate concentration after 384 h for* U. vetiveriae *RK075 (black, squares),* U. vetiveriae *Petef-UmagMtt1-CBX#2 (red, triangles), and* U. vetiveriae *Petef-UmagRia1-CBX#2 (blue, circles) cultivated in 24-deep well plates containing MTM with 200 g L^{-1} glycerol, 100 g L^{-1} $CaCO_3$, and 0.8 g L^{-1} NH_4Cl. The concentration of* U. vetiveriae *RK075 was set to 1. Error bars indicate deviation from the mean (n=3).*

In all, we were able to present *U. vetiveriae* TZ1 as promising production organism for itaconic acid from glycerol. First metabolic engineering attempts revealed the possibility to improve the product specificity by down-regulation of by-products in favor of itaconate. With the genomic sequence and tools for genome editing in hand further metabolic engineering strategies will allow optimization of itaconate production from glycerol.

2.5.5. Conclusion

Valorization of glycerol from bio-diesel production has been a research focus for many years. The identified and improved *U. vetiveriae* strain TZ1 contributes a novel strategy, since it is able to produce high titers of organic acids from glycerol. Concentrations above 25 g L^{-1} itaconate seem to be inhibiting, consequently lowering the reached titers in NaOH titrated bioreactors compared to $CaCO_3$-buffered shake flasks, in which the product precipitates as calcium salt. The use of $CaCO_3$ as buffering agent in bioreactors might help to overcome this limitation. Additionally, single-gene metabolic engineering allowed a reduction in the main by-product malate, drastically increasing the itaconate titer. Multi gene target metabolic engineering harbors huge potential to improve strain performance of *U. vetiveriae*. Finally, *U. vetiveriae* could be transformed into a chassis for the production of different organic acids by deletion of the itaconate production cluster and subsequent channeling of the flux towards the desired organic acid.

Chapter 3

General discussion and outlook

3. General discussion and outlook

3.1. Conclusion

In this thesis, the potential of Ustilaginaceae as industrially applicable producers for malic and itaconic acid from biodiesel derived crude glycerol has been demonstrated. By an iterative approach, starting with a screening of the biodiversity within the fungal family of Ustilaginaceae, followed by strain improvement via adaptive laboratory evolution, medium optimization, and process investigation, malic acid production with an *Ustilago trichophora* strain and itaconic acid production with an *U. vetiveriae* strain could be drastically enhanced. Undergoing a next round of improvement, the production values of the resulting strains *U. trichophora* TZ1 and *U. vetiveriae* TZ1 were further optimized by application of newly developed molecular tools for the overexpression of different genes. In this, a special focus laid on the optimization of the product yield by decreasing the by-product formation and simultaneously increasing the flux towards the desired product. This goal was achieved for the malic acid producer by single overexpression of the underlying pathway genes comprising pyruvate carboxylase, malate dehydrogenase, and malic acid transport proteins. In the itaconic acid producer an enhanced product yield resulted from the single overexpression of the mitochondrial transporter *mtt1* and the regulator of the itaconic acid gene cluster *ria1* from *U. maydis*. The fact, that overexpression of single genes already results in favored production values, demonstrates the great potential for further research on these strains. Especially the malic acid titer and production rate for *U. trichophora* TZ1, reaching values of 200 g L^{-1} and 2 g L^{-1} h^{-1}, were already after the first round of optimization among the highest reported for microbial production processes. Additionally, these values were not only reached from pharma-grade glycerol but actually from biodiesel-derived crude glycerol without prior purification further extending the potential for industrially feasible production processes. In order to develop the full potential of the production organisms, a further optimization in terms of titer, rate, and yield can be envisaged. With superior valorization of glycerol the overall biodiesel bio-refinery will increase commercial benefit. Thus the here presented results contribute to the development of a sustainable, ecologically friendly bio-economy.

3.2. Optimization by metabolic engineering

In order to further improve the values for malic acid production with *U. trichophora* TZ1 and itaconic acid production with *U. vetiveriae* TZ1, different approaches are possible. One approach is the optimization via metabolic engineering. As already shown in many studies, the product titer, production rate, and product to substrate yield can be increased by upregulation of the corresponding metabolic production and substrate uptake pathways themselves and downregulation or complete deletion of by-product formation.

Potential targets for a reduced by-product formation by deletion of the corresponding pathways are the glycolipids ustilagic acid and mannosylerythritol lipids, since they are storage compounds produced generally at high concentrations [141, 204]. A deletion would shift the carbon usage towards product formation (malic acid, itaconic acid) and simultaneously also improve the sampling process, as the presence of glycolipids results in an increased viscosity of the culture broth and clogging of filters. In *U. maydis* it was already shown that the single knock-out of *cyp1*, coding for a P_{450} monooxygenase in ustilagic acid biosynthesis, or *emt1*, which codes for a glycosyltransferase

in the mannosylerythritol lipid biosynthesis, results in the abolishment of production of the respective glycolipid [269]. A double deletion mutant lacking both genes produced no extracellular lipids, however, organic acid production was not improved [123]. Yet, the sample handling was facilitated, due to the reduced glycolipid content in the culture broth. Especially for diafiltration, a significant decrease in filter clogging was the result [123]. These observations already indicate the potential of targeting glycolipid production as unwanted by-product stream, even if in these first experiments just on the basis of sample handling.
Besides the extracellular lipids, intracellular lipid formation is known for Ustilaginaceae. These lipids are mainly produced under nitrogen limitation and thus under organic acid producing conditions [76]. Their formation results in enormous cell swelling after nutrient dependent limitation of growth, thus wasting carbon source. However, for these lipids, the responsible genes are not yet known, making a knock-out more difficult. Metabolic engineering of *U. trichophora* TZ1 by overexpression of malate dehydrogenases and malic acid transport proteins (chapter 2.4), however, resulted in drastically reduced optical densities, besides an improved malic acid yield. The lowered optical densities can likely be explained by a reduced formation of intracellular lipids. Knock-out of *cyp1* and *emt1* in these overexpression mutants, expected to result in a combined deletion/downregulation of intra- and extracellular lipid formation, might further enhance malic acid production. To estimate the actual potential of deletion/downregulation of lipid formation, a closed carbon balance, for which the biomass composition is determined, would help. However, the changing biomass composition during growth phase and production phase has been reported previously [76, 170], possibly making an exact determination difficult.
Another suitable approach to reduce by-product formation is the knock-out or downregulation of other organic acids, such as malic acid secretion in the itaconate producing *U. vetiveriae* TZ1 or succinate production in both strains. However, it has to be taken into consideration that some organic acids are also required as intermediates of the TCA-cycle, making a knock-out detrimental. Consequently, fine-tuned down-regulation might be an option. Especially for malic acid a high interdepence with itaconic acid production as important intermediate is expected, which will be discussed in the following paragraphs. Besides, malic acid is not only produced by *U. trichophora* TZ1 but also *U. vetiveriae* TZ1 in significant amounts, especially after adaptive laboratory evolution. Consequently, metabolic engineering for increased production of these two products will be analyzed in two steps starting with the production of malic acid (chapter 3.2.1). Based on these discussions, a second paragraph will elaborate on metabolic engineering of itaconic acid production (chapter 3.2.2) while a sufficient precursor (malic acid) supply has to be guaranteed. This can be achieved by the same means as will be discussed in the first paragraph for malic acid production, apart from improved export out of the cell. However, for initial improvements of itaconic acid production, this supply is already considered sufficient in *U. vetiveriae* TZ1, since 2- to 3-fold more malic acid than itaconic acid is produced and a secretion of malic acid under itaconic acid producing conditions clearly demonstrates a surplus of malic acid.

3.2.1. Elevating malic acid production

By single overexpression of two different genes encoding malate dehydrogenases and two different genes related to malic acid transporters in *U. trichophora* TZ1, the malic acid production could be drastically improved in this study. Especially the yield was increased by overexpression of these

genes. This hints at a bottleneck in the corresponding conversion steps. The same observations were made for *Aspergillus oryzae* and *Saccharomyces cerevisiae*, with elevated malic acid production upon overexpression of these genes [115, 117]. For both organisms the best production values were achieved, by combined overexpression of the whole metabolic pathway (pyruvate carboxylase, malate dehydrogenase, malic acid transporter). Simultaneous overexpression of these three genes resulted in a >10-fold and 2.6-fold increased titer for *S. cerevisiae* and *A. oryzae*, respectively, while single overexpression had a smaller effect [115, 117]. Thus, the combined overexpression of the whole pathway seems to be a promising approach also for *U. trichophora* TZ1.

Interestingly, first investigations in microtiter plates within this study with the wildtype *U. trichophora* RK089 resulted in a positive effect upon overexpression of pyruvate carboxylase, while overexpression of malate dehydrogenase and malic acid transporter had a minor effect. Even though these data have to be further investigated and confirmed by shake flask and bioreactor cultivations, first hints are given on the possible effect of the adaptive laboratory evolution. While conversion of pyruvate to oxaloacetate seems to be rate limiting in the wildtype strain *U. trichophora* RK089 before ALE, this step is enhanced during the evolutionary rounds resulting in a new bottleneck in one of the consecutive steps for *U. trichophora* TZ1. This hypothesis is further strengthened by the fact that also for *U. vetiveriae* TZ1 malic acid production is drastically enhanced compared to the not evolved wildtype strain *U. vetiveriae* RK075. One general effect of ALE while screening for higher growth rates on glycerol seems to be improved malic acid production. This increase likely reflects the $NADH/NAD^+$-ratio, which is high in fast growing organisms. Malic acid production via the cytosolic rTCA-cycle in contrast is an NADH-dependent pathway, due to the activity of malate dehydrogenase. Consequently, elevated malic acid production might counteract the increased NADH-generation resulting from enhanced growth as additional NADH-sink, allowing for higher growth rates. Additionally, malic acid production as NADH-sink might have a positive effect on itaconic acid production, since this microbial production process results in a high $NADH/NAD^+$-ratio, just as growth. However, this will be discussed in the following chapter.

For optimization of malic acid production, a clarification of the underlying production pathway would be of great help. The different possible production pathways for malic acid in *S. cerevisiae* have been discussed by Zelle *et al.* [115]. Depending on the production route, the reached yields can differ drastically from 1 to 2 mol mol^{-1} on glucose, which corresponds to 0.5 to 1 mol mol^{-1} on glycerol. Additionally, the compartimentation would be of great interest, since the rTCA-cycle is known to be present as cytosolic and mitochondrial version with differing isoenzymes catalyzing the reactions and involvement of an additional mitochondrial malic acid transport protein for the mitochondrial pathway. Depending on the exact underlying production pathway and the localization, different overexpression strategies involving different enzymes would be suitable elevating flux through the (r)TCA-cycle, or glyoxylate shunt.

3.2.2. Elevating itaconic acid production

As mentioned earlier, itaconic acid production is closely related to malic acid production with several interferences between these two metabolites. In order to simplify the connection, itaconic acid production will be regarded as several consecutive steps following malic acid production, with malic acid as intermediate. The production pathway for itaconic acid has been investigated

intensively in *A. terreus*. It starts from two molecules of pyruvate, which are converted via oxaloacetate and acetyl-CoA to citrate in the TCA-cycle. The citrate is metabolized to *cis*-aconitate, which is converted to itaconic acid [84, 88-90, 262]. In comparison to *A. terreus*, itaconic acid production in *U. maydis* comprises one additional step, the conversion of *cis*-aconitate to *trans*-aconitate, which is then converted to itaconic acid [232].

Since for itaconic acid a flux through glycolysis (from glucose) or parts of glycolysis (from glycerol) and through the TCA-cycle is needed, the production results in a high $NADH/NAD^+$-ratio. This NADH-surplus can be coped with by different possibilities, such as the alternative electron transport chain or the simultaneous activity of an NADH-depending pathway. As already discussed for elevated malic acid production to oxidize NADH resulting from faster growth (see chapter 3.2.1), an increased activity of the cytosolic malate dehydrogenase likely helps to reduce the cellular NADH-level, independent from its origin (faster growth, increased itaconic acid production). As a consequence and in analogy to the previous argumentation, the increased activity of a pathway resulting in high NADH-levels (itaconic acid production), becomes possible upon high activity of malate dehydrogenase, which contributes to close the redox balance and regenerate NADH. Yet, the production of malic acid in order to close the redox balance results in a loss of carbon. Consequently, overexpression of the natural NAD^+ regeneration mechanism, namely the (alternative) electron transport chain, might trigger the same effect as malic acid production, without squandering carbon source. While the mitochondrial electron transport chain, active under growth conditions, creates a proton gradient during the oxidation of NADH to NAD^+ and is consequently used for the generation of energy, the alternative electron transport chain is active under production conditions after the growth phase and able to regenerate NAD^+ without the translocation of protons. Thus, this pathway can be used for the sole purpose of closing the redox balance and regenerating NAD^+, with heat as a by-product [270].

For *Ustilago*, the alternative electron transport chain consists of an external NADH dehydrogenase (Ndh-2) and an alternative oxidase (Aox), which in combination transfer the electrons from cytosolic NADH to oxygen as final electron acceptor [271, 272]. This translocation to oxygen explains the high oxygen demand of *Ustilago* during the growth and acid production phase and the observation, that even brief phases of oxygen limitation in the culture broth result in an impaired redox balance with irreversibly lowered acid production [103]. This is also known for other organisms, such as *Aspergilli* [95, 271]. Overexpression of both, the mitochondrial and the alternative electron transport chain might have a beneficial effect on itaconic acid production. While overexpression of Ndh-2 and Aox would on the one hand clearly help to regenerate NAD^+ and close the redox balance during the itaconic acid production phase, overexpression of the mitochondrial electron transport chain, on the other hand, would reduce the NADH-level during the growth phase. Both systems would therefore diminish the need for malic acid production and increase the available amount of carbon for itaconic acid production, assuming that sufficient oxygen is supplied. A similar effect might be achieved by addition of protonophoric uncouplers, such as 2,4-dinitrophenol (DNP). By addition of DNP, the proton gradient across the microbial and mitochondrial membrane is partially dissipated, consequently increasing the flux through the electron transport chain. This method, however, would not be feasible in an industrial production process, since addition of DNP would not only make the production process more expensive, but also hampers it. However, first experiments performed within this study already indicate the possibility of improved production characteristics by addition of DNP.

Instead of engineering the homologous regeneration system, an alternative approach would be the physiological engineering of the redox cofactor balance by heterologous expression of an NADH oxidase (Nox). The water-forming NADH oxidase from *Lactococcus lactis* has been found to be effective in generating higher NADH/NAD^+ conversion rates improving the metabolic rate [273, 274]. Additionally, this enzyme has already been employed in the reduction of overflow metabolism in *S. cerevisiae*, improved conversion of glycerol to dihydroxyacetone in redox-engineered *Escherichia coli*, increased acetaldehyde production in *L. lactis* and decreased lactate production resulting in simultaneously increased pyruvate content in *L. lactis*. [118, 275-277]. Since this Nox is a potent tool interfering with the cellular redox balance and thus with the whole metabolism of the organism, it is essential to strictly control the expression in terms of induction time and strength. A constitutive expression would likely result in reduced or no growth, since NADH needed to generate energy would be wasted. Consequently, expression under control of an inducible promoter conferring activity upon acid production would be appreciated. Preferably this promoter should not need to be activated by manual addition of an inducer but be auto-induced coupled to acid production.

Within this context, first investigations concerning the establishment of suitable promoters have been conducted. Four promoters from the recently discovered itaconic acid cluster [232], were cloned in front of a Gfp-gene and the activity was analyzed. As comparison, the well-known otef promoter was used [159]. The promoter of the mitochondrial *trans*-aconitate transporter (*mtt1*) was found to be highly active with a low basal expression. In contrast, the promoter of the *trans*-aconitate decarboxylase (*tad1*) was found to be tightly regulated with a mediocre expression level. For both promoters, a clearly increased expression level upon nitrogen limitation could be shown. In first expression experiments with the Nox in *U. maydis* under control of these promoters, the production of itaconic acid (*tad1*) and malic acid (*mtt1*) could be enhanced, strengthening the possibility of improved acid production by redox perturbation. Additionally, these experiments clearly showed the different effect of higher and lower NADH-levels within the cell, resulting from stronger (*mtt1*) and weaker (*tad1*) expression of the Nox, on malic acid and itaconic acid production further supporting the hypothesis of malic acid production as mean to close the redox balance.

Besides lowering malic acid production and thereby increasing the available amount of carbon source for itaconic acid production, an increased regeneration of NAD^+ by overexpression of the homologous electron sinks, addition of protonophoric uncouplers and the expression of a heterologous NADH-oxidase can help to directly drive the itaconic acid production. This driven-by-demand concept would contribute to an increased flux through NADH-generating pathways, in this case itaconic acid production, to cope with the lowered NADH-level, likely resulting in higher itaconic acid production levels [278].

3.3. Generation of a genome scale metabolic model

Even though, malic acid production with *U. trichophora* TZ1 and itaconic acid production with *U. vetiveriae* TZ1 has already been improved not only by ALE, medium optimization and process optimization, but also by metabolic engineering, knowledge on underlying pathways and reactions is scarce. This knowledge would greatly contribute to further metabolic engineering approaches, as already discussed in the previous chapters. For malic acid production in *U. trichophora* it is not yet clarified, whether cytosolic or mitochondrial production is predominant or a mixture of different

production pathways is responsible for the accumulation of high malic acid titers. For *S. cerevisiae* malic acid is produced via the cytosolic rTCA-cycle [115]. Also for other organisms, such as *Rhizopus oryzae* and different *Aspergillus* species, this pathway has been shown to be predominant [54, 117, 120-122]. For *U. trichophora*, however, this pathway does not seem to be the exclusive one in malic acid accumulation, especially since the reached yield is far from the theoretical one of 1 mole malate per mole glycerol and the overexpression of the mitochondrial allele encoding malate dehydrogenase yielded higher malic acid production (see chapter 2.4).

Also for itaconic acid production, the underlying pathway and the corresponding clustered genes have just recently been identified in *U. maydis* [232]. It was shown that itaconic acid is produced via the unusual intermediate *trans*-aconitate [232]. This clearly indicates a lack of knowledge concerning the molecular basis of the exact synthesis pathways in *Ustilago*. To overcome such knowledge gaps and predict biological capabilities, a genome-scale network reconstruction can be applied [279]. Different examples in literature have shown that the generation of a genome-scale metabolic model can help to improve production properties of microorganisms by the possibility to simulate fluxes. Further it can help to understand the whole metabolism. A genome-scale model for *E. coli* K12, for instance, enabled the design of lactic acid production in-silico [280], an increased malonyl CoA biosynthesis [281] and an improved L-threonine production by a systems metabolic engineering approach [282]. For *S. cerevisiae*, the sensitivity towards different nitrogen sources has been investigated on a global transcriptional and physiological level [283]. Furthermore, the role of transcriptional regulation in central carbon metabolism fluxes [284] and a regulatory-metabolic network as novel regulatory mechanism have been identified [285].

First attempts on the construction of a genome-scale stoichiometric model for Ustilaginaceae have already been undertaken in the course of this thesis based on the annotated genome sequence of *U. maydis* 521. With this first functional genome-scale stoichiometric model for *U. maydis* the demonstration and prediction of fluxes was possible. Yet, the predicted values for the growth rate on glucose (0.397 h^{-1}) and glycerol (0.227 h^{-1}) still differed when compared to measured values from experimental results with 0.138 h^{-1} and 0.126 h^{-1} for glucose and glycerol, respectively. However, this is not striking, since the model is just a preliminary draft version and curation is ongoing. A curated version of the model might become a powerful tool for the in-silico analysis of Ustilaginaceae allowing the determination of flux distributions to locate possible targets for further improvement.

Since the broad biodiversity within the family of Ustilaginaceae is known [145], a focus has to be laid on the implementation of different species, for correct prediction. This first genome-scale metabolic model reconstruction was based on the genome sequence of *U. maydis* 521, since it is fully annotated. In order to adapt the model to *U. trichophora* TZ1 or *U. vetiveriae* TZ1, it should be based on the corresponding genome sequences. For *U. trichophora* TZ1, the genome sequence is already available (see chapter 2.3). However, the genome, transcriptome, and proteome are not yet fully annotated. For *U. vetiveriae* TZ1, a first draft of the genome is available as well, however without any annotation so far.

Another possibility would be the generation of a pan-genome model, since the general biochemistry of the different Ustilaginaceae is likely similar. However, differences in specific production and degradation pathways have to be taken into account depending on the application of the model. For instance, not all Ustilaginaceae are able to produce itaconic acid (chapter 2.3). Consequently, to model the flux in *U. trichophora*, itaconic acid production pathways would need to be excluded.

With a fully functional metabolic model, the iterative process of rational whole-cell biocatalyst and bioprocess design could be further followed (Figure 8). By this, the strains and processes, which to a certain extend resulted from random optimization and screening, can be further improved on a fully rational level, with strongly interacting engineering and analysis, until a feasible bioprocess for industrial implementation is generated [139].

3.4. Optimization by process development

Besides optimizing the processes by engineering the production organisms themselves, the investigation of the production process in terms of medium composition, production parameters (pH, temperature, aeration), the setup of the process (batch, fed-batch, continuous, cascade), scale-up to an industrial level and the downstream process is an important factor for microbial production processes, which has to be taken into account.

3.4.1. Medium composition

The influence of the medium composition on microbial production processes, especially organic acid production, has been extensively investigated for different organisms. In *A. terreus* it is known, that the concentration of different metal ions, such as copper, iron, manganese, magnesium, zinc and calcium, drastically influences itaconic acid production as co-factors for enzymes [87, 96, 188, 286]. The influence of these medium components on malic acid production with *U. trichophora* TZ1 and itaconic acid production with *U. vetiveriae* TZ1 has already been investigated to some extend (see chapters 2.1 and 2.5). In these investigations, however, differing concentrations of magnesium and iron did not influence the production values. Yet, the influence might become apparent in a scaled-up production process or in combination with differing concentrations of other nutrients. For instance, an increase of NH_4Cl from 0.8 to 1.6 and 3.2 g L^{-1} resulted in higher biomass formation and consequently higher production rates. A further increase to 6.4 g L^{-1} NH_4Cl, in contrast, resulted in reduced biomass formation and production values in comparison to 3.2 g L^{-1} NH_4Cl. This negative effect was overcome by a simultaneous increase of all other medium components, while a simultaneous doubling of single compounds (KH_2PO_4, $FeSO_4$), did not suffice. This clearly shows the impact of the specific combination of medium components. A reduced usage of single compounds due to an exactly determined medium composition would not only result in an improved production but also in a cheaper and environmentally friendlier production process.

The concentration of nitrogen and the nitrogen source itself, definitely also have to be taken into focus in further optimization of the production processes. High nitrogen content results in higher biomass formation consequently increasing the production rate. The trade-off, however, is a lower yield. Yeast extract can help to supply different precursors and additional carbon source, which might beneficially influence the production, by a shorter growth phase. Yet, it is an expensive substrate, thus possibly lowering the profit margin depending on the improved values.

Apart from metal ions and nitrogen, the buffer system is a key feature of every medium in microbial production processes. While in many studies, just as in the beginning of this study, MES-buffer as soluble medium component is advantageous for screening due to an easier handling and higher reproducibility, the low buffer capacity makes high product concentrations impossible [145]. The enormous effect of $CaCO_3$ as buffering agent on both, malic acid and itaconic acid production has been shown in chapters 2.1, 2.2, and 2.5. This effect does not solely result from the higher buffer

capacity, but also from the reaction of calcium with the formed acid. The precipitation of the insoluble calcium-salts lowers the product concentration in the culture broth to around 13 g L^{-1} for both itaconic acid and malic acid, thus lowering the stress level for the cells. However, as insoluble medium component, $CaCO_3$ hampers the production process. Soluble $CaCl_2$ might be an alternative also able to form precipitating organic acid salts. An additional positive effect of the precipitation of the product as calcium-salt without other solid medium components would be an easier product recovery. Besides, not only the effect of buffer capacity, but also the pH-value itself is important for microbial organic acid production. Since, however, in industrial-scale bioreactors the pH is normally controlled via titration this factor will be discussed within the context of process parameters in chapter 3.4.2.

The reported production processes for malic acid and itaconic acid start from glycerol as carbon source. For *U. trichophora* TZ1 it could be shown, that the production values are stable not only with pharma-grade glycerol but also crude glycerol without prior purification. However, it has to be taken into account that the composition of bio-diesel derived crude glycerol drastically differs depending on the origin [36, 37, 220]. This differing composition can have a negative effect on the organisms, as shown for *Yarrowia lipolytica*, for instance [46]. Yet, Ustilaginaceae are generally known to be robust production organisms able to cope with high medium impurities. Consequently, they might not be influenced by the differing composition of the used crude glycerol. This, however, has to be further evaluated by the investigation of different crude glycerol batches. A production process with an organism tolerant to differing crude glycerol compositions, however, might be directly implemented into a biodiesel bio-refinery, in order to use the resulting 10 % (w/v) crude glycerol waste stream independent from the composition on-site without the need for prior transport. By this not only the production costs for this process, but for the biodiesel production process as a whole can be lowered, thus contributing to an improved bio-economy.

Besides further investigation of production from biodiesel derived crude glycerol, alternative substrates, such as lignocellulosic hydrolysates, should also be considered as feedstock. The use of inedible plant residues, which result from agricultural processes as waste stream, has attracted more and more attention in recent years. *Ustilago* is known to grow on many substrates, such as glucose, (crude) glycerol, saccharose, arabinose, xylose, xylan, CM-cellulose, poly-galacturonic acid and as plant pathogen, possesses a versatile set of lignocellulose-degrading enzymes [119, 144, 170-173, 260, 287]. Such production processes would clearly contribute to make *Ustilago* an industrial workhorse, with the possibility to be applicable not only for one substrate but many different ones. In close relation to this aspect, not only the used carbon source itself but also the initial amount is of importance for the production process, as already shown for both, *U. trichophora* TZ1 and *U. vetiveriae* TZ1 (see chapter 2.1, 2.2, and 2.5). Since this, however, is also closely linked to the setup of the bioreactor (batch, fed-batch, continuous, cascade) it will be discussed within this context in the following paragraph.

3.4.2. Production parameters

As already shown for itaconic acid production with *U. vetiveriae* TZ1 and malic acid production with *U. trichophora* TZ1, production parameters, such as the pH-value, temperature, and the CO_2- and O_2-supply are critical factors for improved production processes. Consequently, a further investigation of these harbors great potential.

For organic acid production with filamentous fungi, such as *Aspergilli*, acidic pH-values are generally preferred. For *A. terreus* a pH range between 2.0 [208, 209] and 5.9 [96] was investigated with differing results. Thus, in 2014 Hevekerl *et al*. investigated the influence of pH on itaconic acid production with *A. terreus* concluding, that a pH of 3 is best for itaconic acid production [100]. Also in other fungi, such as *Saccharomyces* and *Yarrowia*, low pH-values improved organic acid production [210, 211]. For Ustilaginaceae, however, mainly near neutral pH-values are best for organic acid production [170], even though some exceptions have been reported [145]. Also for malic acid production with *U. trichophora* TZ1 and itaconic acid production with *U. vetiveriae* TZ1 near neutral pH-values resulted in the best titer, rate and yield. Acidic pH-values, in contrast, had a negative effect on both production processes, with lowered production at a pH of 5.5 for malic and itaconic acid and nearly (malic acid) and completely (itaconic acid) abolished production at a pH of 4.5. Even though quite often production at low pH is considered the preferred condition for microbial production processes, both, high and near neutral pH-values have their inherent advantages and disadvantages. On the one hand, a near neutral pH-value lowers the stress level for the microbes resulting from undissociated acids and a higher H^+-concentration, consequently contributing to an elevated production. Additionally, a facilitated export of produced acids is the result and strategies, such as simultaneous saccharification and fermentation (SSF) become possible [16, 57, 119]. On the other hand, especially in industrial scale bioreactors, a low pH-value can lower the risk of contamination and enable different downstream methods, such as evaporation-crystallization, salting or cooling [197, 213].

Another factor, that greatly influences production processes, is the temperature. Both, for Ustilaginaceae and for *Aspergilli*, it was shown, that growth and organic acid production drastically differ depending on the applied temperature [103, 207]. Generally, growth was improved by elevated temperatures up to about 37 °C, with a drastic reduction exceeding 40 °C. Itaconic acid production in contrast, was only elevated at higher temperature using *Aspergillus* [207]. With *U. cynodontis*, the lowest tested temperature (25 °C) resulted in the highest acid titer [103]. Especially in advanced production processes, a different temperature for the growth and production phase can help to improve the production process. This was also shown in malic acid production from crude glycerol with *U. trichophora* TZ1, with a temperature of 28 °C for the growth phase and an elevated temperature of 37 °C during the production phase, clearly elevating the production (Figure 18). Besides these differences in reached production values, the process costs are directly linked to the process temperature. While already in lab-scale bioreactors, the heat produced from the process itself is enough to require a cooling system, in industrial-scale processes a high-end cooling system is inevitable. Depending on the process, the costs for cooling can make up the major part of the overall costs. Consequently, a production operable at higher temperatures would make the overall production process more feasible.

The supply of CO_2 and O_2 is known to be an important factor in microbial organic acid production, depending on the produced acid. Especially for itaconic acid production, a sufficient supply of oxygen as terminal electron acceptor is essential, to oxidize the large NADH surplus (compare chapter 3.2.2). For malic acid production, in contrast, a higher concentration of CO_2, as co-substrate for pyruvate carboxylase, seems to be beneficial. An increased CO_2-supply from $CaCO_3$ as buffering agent resulted in a clearly increased yield (chapter 2.2) and also for *S. cerevisiae*, CO_2 enrichment of the sparging gas up to 15 % improved malic acid production [189]. However, concentrations exceeding 15 % for CO_2 enrichment lowered malic acid production [189].

Consequently, a fine tuning of supplied oxygen and CO_2 is needed. Within this thesis, no additional CO_2-supply was tested, besides the use of $CaCO_3$. Aeration with different mixtures of oxygen and CO_2 would shed light on the needed amounts. Especially in industrial-scale processes, an efficient supply of oxygen throughout the culture broth is a challenging topic, closely related to the setup of the process.
In general, the scale-up of a process to an industrial level is a critical issue in biotechnology. As already shown in the scale-up from 24-deep well plates with 1.5 mL to shake flasks with 50 mL and bioreactors with 1 L working volume, huge differences can result from this change. Important topics in scale-up among others are the supply of nutrients and oxygen, the mixing of the culture broth and the temperature. Consequently, the most promising setup at lab-scale might be eliminated at industrial-scale, requiring a different setup of the process or different process parameters.
Besides the just discussed single process parameters, the general process setup is one of the most important questions in biotechnological production processes. Batch, fed-batch, continuous or cascade as just some examples. All these setups harbor their own inherent opportunities, depending on a variety of variables, such as organism, substrate, product and production values. Generally, the use of continuous processes with feed during cultivation is a clear advantage, as also shown for Ustilaginaceae in this study (compare chapter 2.2). The additional supply of substrate allows higher product titers and the longer operation time with shorter downtime helps to reduce non-profit times and thus increases the profit margin. Additionally, in continuous processes, the production can be kept at the maximum rate, without reaching the maximum titer. This can help to lower product toxicity and improve the space-time yield [76]. Another advantage of continuous processes is the recycling of nutrient-rich waste streams, and active biomass. The possibility of continuous itaconic acid production with Ustilaginaceae using integrated cell retention and simultaneous product recovery in a membrane bioreactor, for instance, has already been shown reaching a production rate of 0.8 g L^{-1} h^{-1} [259]. Compared to continuous processes using *A. terreus* without cell retention, which only reached values below 0.5 g L^{-1} h^{-1}, this production rate seems promising [288, 289]. However, in order to be able to set up a continuous process, the integration of the downstream process is inevitable. In general, the process setup is closely linked to the downstream process, since different techniques are suitable for different process types and scales.

3.4.3. Downstream process

The investigation and optimization of the downstream process and its adapation to the upstream process or the product itself are imparative for microbial chemical production, since depending on the process the recovery from the culture broth can make up the major part of the costs. After fermentation, the culture broth comprises, besides the desired product, cells, residual medium compounds, such as salts or unused substrate, and by-products, such as organic acids, proteins, and lipids. Generally downstream process techniques can be divided into two major groups. In the first group of methods, the upstream and the downstream process are seperated. In the second group, the downstream technology is implemented into the fermentation. The degree of implementation, however, can differ.
Generally in the first group, the purification starts with the separation of the cells and other solid compounds from the culture broth. In this step, washing of the solids might be important, since the cells can retain large amounts of the product. For citric acid, for instance, this fraction can make up

15 % of the product [60]. In the following steps, the product is separated from by-products and is further purified. For this, several methods are possible, such as precipitation, solvent extraction or anion-exchange absorption as classical methods [60]. The standard method, which is best known for citric acid production, precipitation, relies on the generation of calcium salts, which precipitate from the culture broth after reaction of dissolved citric acid with $CaCO_3$. By several successive crystallization steps, a high purity can be obtained.

Within this work, it was shown, that also for malic acid (compare chapters 2.1 and 2.2) and itaconic acid (compare chapter 2.5) production with *Ustilago*, precipitation as calcium salt is a suitable method for downstream processing. When using $CaCO_3$ as buffering agent, the formed malic acid reacts with the $CaCO_3$ to form insoluble calcium-malate. Isolation of all solid components from the culture broth after autoclavation without any purification resulted in 90 % pure calcium malate. Since the demonstrated downstream process relies on the precipitation as calcium-salt during fermentation, it belongs to the second group of downstream processing techniques, with an implemented method. However, it would also be possible to treat the culture broth from an NaOH titrated bioreactor with $CaCO_3$ after fermentation as a seperated downstream process. Independent from the integration or separated application of precipitation the use of calcium for purification of organic acids has its drawbacks. The main disadvantage is the generation of a large stream of gypsum as by-product. Even though it can be used for the generation of plasterboard, the major part has to be disposed as environment unfriendly pollutant. This drawback is circumvented by methods, such as solvent extraction. For this method, the organic acid is extracted from the culture broth with a solvent, which is (nearly) insoluble in the medium [290-292]. It is important to choose a solvent, which extracts a maximum of the desired product and a minimum of impurities. The desired product can afterwards be extracted from the product-enriched solvent by distillation [60]. However, this method also relies on the use of environmentally unfriendly solvents.

In general, the second group of methods, implemented downstream processes, has the advantage of resulting in continuous processes with advanced in-situ product recovery (ISPP) techniques, such as electrodialysis, ultra- and nanofiltration, application of liquid membrans or simulated moving bed (SMB) [60, 212, 293]. These techniques do not only purify the product from the culture broth, but tend to improve the overall production process by increased operation times of the bioreactors, shorter down-times and reduced toxicity to the organisms, due to a permanently lowered product concentration. By this, they can positively influence the production process itself, as already discussed in context of calcium-salt formation.

The removal of toxic compounds from the culture broth during the production process is generally known as possibility to increase microbial production lowering the metabolic burden. Especially for aromatic compounds, the application of two-phase cultivations with a second, liquid, insoluble organic phase in which the product has a higher solubility can help to lower toxicity. However, several drawbacks, such as issues with emulsification, increased hazard and phase toxicity [293, 294], are known. Besides, this method is not suitable for removal of organic acids, since their solubility in aqueous medium is far higher than in organic solvents. Yet, an implemented downstream process, resulting in a lower concentration would greatly improve the production, since for itaconic acid concentrations exceeding 25 g L^{-1} were found to be inhibiting (chapter 2.5) [119] and malic acid becomes inhibiting at concentrations exceeding 140 g L^{-1} (chapter 2.2). Consequently, besides all investigation and improvement of the organism and the production process, an implemented downstream process, which goes hand in hand with the upstream process, is essential.

Appendix

Table 8: 126 Ustilaginaceae screened for organic acids with final glycerol concentration.

no.	name	origin	glycerol [g L^{-1}]
1949	*Ustilago maydis* DSM 3121	DSM 3121	36,611
1950	*Ustilago maydis* DSM 4500	DSM4500	11,369
1951	*Ustilago maydis* DSM 14603	DSM 14063	39,497
2133	*Ustilago maydis* Nr. 195	Prof. M. Bölker, Philipps University Marburg, Germany	20,850
2134	*Ustilago maydis* Nr. 196	Prof. M. Bölker, Philipps University Marburg, Germany	17,408
2135	*Ustilago maydis* Nr. 197	Prof. M. Bölker, Philipps University Marburg, Germany	32,072
2136	*Ustilago maydis* Nr. 198	Prof. M. Bölker, Philipps University Marburg, Germany	24,576
2137	*Ustilago maydis* Nr. 199	Prof. M. Bölker, Philipps University Marburg, Germany	17,260
2138	*Ustilago maydis* Nr. 200	Prof. M. Bölker, Philipps University Marburg, Germany	21,885
2139	*Ustilago maydis* Nr. 201	Prof. M. Bölker, Philipps University Marburg, Germany	17,031
2140	*Ustilago maydis* Nr. 202	Prof. M. Bölker, Philipps University Marburg, Germany	8,303
2141	*Ustilago maydis* Nr. 203	Prof. M. Bölker, Philipps University Marburg, Germany	3,960
2142	*Ustilago maydis* Nr. 204	Prof. M. Bölker, Philipps University Marburg, Germany	29,073
2143	*Ustilago maydis* Nr. 205	Prof. M. Bölker, Philipps University Marburg, Germany	18,126
2144	*Ustilago maydis* Nr. 206	Prof. M. Bölker, Philipps University Marburg, Germany	29,696
2145	*Ustilago maydis* Nr. 207	Prof. M. Bölker, Philipps University Marburg, Germany	24,202
2146	*Ustilago maydis* Nr. 208	Prof. M. Bölker, Philipps University Marburg, Germany	21,592
2147	*Ustilago maydis* Nr. 209	Prof. M. Bölker, Philipps University Marburg, Germany	20,089
2148	*Ustilago maydis* Nr. 211	Prof. M. Bölker, Philipps University Marburg, Germany	13,586
2149	*Ustilago maydis* Nr. 212	Prof. M. Bölker, Philipps University Marburg, Germany	18,649
2150	*Ustilago maydis* Nr. 213	Prof. M. Bölker, Philipps University Marburg, Germany	39,392
2151	*Ustilago maydis* Nr. 214	Prof. M. Bölker, Philipps University Marburg, Germany	18,425
2152	*Ustilago maydis* Nr. 215	Prof. M. Bölker, Philipps University Marburg, Germany	18,605
2153	*Ustilago maydis* Nr. 462 a1bA	Prof. M. Bölker, Philipps University Marburg, Germany	28,521
2154	*Ustilago maydis* Nr. 463 a1bB	ATCC 22882	17,817
2155	*Ustilago maydis* Nr. 465 a1bD	ATCC 22884	30,038
2156	*Ustilago maydis* Nr. 466 a1bE	ATCC 22885	33,030
2157	*Ustilago maydis* Nr. 467 a1bF	ATCC 22886	29,631
2158	*Ustilago maydis* Nr. 469 a1bH	ATCC 18604	14,384
2159	*Ustilago maydis* Nr. 470 a1bI	Prof. M. Bölker, Philipps University Marburg, Germany	25,011
2160	*Ustilago maydis* Nr. 471 a1bJ	ATCC 22889	5,443
2161	*Ustilago maydis* Nr. 473 a1bL	ATCC 22891	0,022
2162	*Ustilago maydis* Nr. 474 a1bM	ATCC 22892	1,462
2163	*Ustilago maydis* Nr. 476 a1bO	ATCC 22894	10,518
2164	*Ustilago maydis* Nr. 477 a1bP	ATCC 22895	34,436
2165	*Ustilago maydis* Nr. 478 a1bQ	ATCC 22896	18,886
2166	*Ustilago maydis* Nr. 479 a1bR	ATCC 22897	14,310
2167	*Ustilago maydis* Nr. 480	ATCC 22899	11,999
2168	*Ustilago maydis* Nr. 481	ATCC 22900	17,558
2169	*Ustilago maydis* Nr. 482	ATCC 22901	29,357
2170	*Ustilago maydis* Nr. 483	ATCC 22902	32,788
2171	*Ustilago maydis* Nr. 484	ATCC 22903	40,438
2172	*Ustilago maydis* Nr. 485	ATCC 22904	28,038
2173	*Ustilago maydis* Nr. 487	ATCC 22906	21,402

no.	name	origin	glycerol [g L^{-1}]
2174	*Ustilago maydis* Nr. 488	ATCC 22907	34,179
2175	*Ustilago maydis* Nr. 489	ATCC 22908	26,360
2176	*Ustilago maydis* Nr. 490	ATCC 22909	22,790
2177	*Ustilago maydis* Nr. 491	ATCC 22910	13,179
2178	*Ustilago maydis* Nr. 492	ATCC 22911	17,197
2179	*Ustilago maydis* Nr. 495	ATCC 221914	11,563
2196	*Ustilago maydis* FB1 a1b1	Banuett & Herskowitz, 1989, Minnesota USA	28,372
2197	*Ustilago maydis* FB2 a2b2	Banuett & Herskowitz, 1989, Minnesota USA	2,123
2198	*Ustilago maydis* RK 122	[242]	22,963
2199	*Ustilago maydis* RK 123	[242]	24,866
2203	*Ustilago maydis* RK 134	[242]	28,064
2204	*Ustilago maydis* RK 139	[242]	15,940
2205	*Ustilago maydis* RK 212	[242]	41,546
2206	*Ustilago maydis* RK 213	[242]	20,004
2207	*Ustilago maydis* RK 214	[242]	18,753
2208	*Ustilago maydis* RK 215	[242]	26,500
2209	*Macalpinomyces eriachnes*	CBS 131454	0,000
2210	*Sporisorium consanguineum*	CBS 131456	19,178
2211	*Sporisorium cruentum* UMa920 MAT1	CBS 133249	23,552
2212	*Sporisorium exsertum*	CBS 131457	13,586
2213	*Sporisorium scitamineum* UMa698, Sscl4, JS109, MAT1	CBS 131462	6,893
2214	*Sporisorium walkeri*	CBS 131464	3,684
2215	*Ustanciosporium gigantosporum* UMa706	CBS 131478	12,425
2216	*Ustilago avenae*	CBS 131466	41,405
2217	*Ustilago cynodontis* UMa709	CBS 131467	0,611
2218	*Ustilago filiformis* UMa701	CBS 131469	28,518
2219	*Ustilago trichophora* RK089	CBS 131473	0,000
2220	*Ustilago vetiveriae*	CBS 131474	0,000
2221	*Ustilago xerochloae* UMa702	CBS 131476	0,000
2229	*Ustilago maydis* MB215	DSM 17144	20,218
2269	*Ustilago hordei* Uh4875-4 Mat1	[181]	22,557
2672	*Ustilago maydis* HB1990	Biotechnology And Information Research Network AG, Zwingenberg, Germany	10,606
2696	*Pseudozyma hubeiensis* NBRC 105053	NBRC 105053	0,000
2697	*Pseudozyma hubeiensis* NBRC 105054	NBRC 105054	0,000
2698	*Pseudozyma hubeiensis* NBRC 105055	NBRC 105055	0,000
2699	*Ustilago trichophora* NBRC 100155	NBRC 100155	0,000
2700	*Ustilago trichophora* NBRC 100156	NBRC 100156	0,000
2701	*Ustilago trichophora* NBRC 100157	NBRC 100157	0,000
2702	*Ustilago trichophora* NBRC 100158	NBRC 100158	0,000
2703	*Ustilago trichophora* NBRC 100159	NBRC 100159	0,000
2704	*Ustilago trichophora* NBRC 100160	NBRC 100160	0,000
2705	*Ustilago cynodontis* NBRC 7530	NBRC 7530	33,481
2706	*Ustilago cynodontis* NBRC 9727	NBRC 9727	29,236
2707	*Ustilago cynodontis* NBRC 9758	NBRC 9758	23,000

no.	name	origin	glycerol [g L^{-1}]
2708	*Ustilago rabenhorstiana* NBRC 8995	NBRC 8995	30,500
2709	*Pseudozyma antarctica* NBRC 10260	NBRC 10260	0,953
2710	*Pseudozyma tsukubaensis* NBRC 1940	NBRC 1940	0,000
2813	*Sporisorium lanigeri* BRIP 27609a	Queensland Plant Pathology Herbarium, Australia	0,027
2814	*Macalpinomyces mackinlayi* BRIP 52549a	Queensland Plant Pathology Herbarium, Australia	0,000
2815	*Sporisorium cenchri-elymoidis* BRIP 26491a	Queensland Plant Pathology Herbarium, Australia	0,000
2816	*Macalpinomyces ordensis* BRIP 26904a	Queensland Plant Pathology Herbarium, Australia	0,108
2817	*Ustilago schmidtiae* BRIP 26906a	Queensland Plant Pathology Herbarium, Australia	13,391
2818	*Sporisorium bothriochloae* BRIP 26908a	Queensland Plant Pathology Herbarium, Australia	0,000
2819	*Sporisorium themedae* BRIP 26917a	Queensland Plant Pathology Herbarium, Australia	5,646
2820	*Sporisorium tumiforme* BRIP 26919a	Queensland Plant Pathology Herbarium, Australia	27,890
2821	*Ustilago curta* BRIP 26929a	Queensland Plant Pathology Herbarium, Australia	0,037
2822	*Ustilago triodiae* BRIP 26907a	Queensland Plant Pathology Herbarium, Australia	0,000
2823	*Sporisorium setariae* BRIP 26910a	Queensland Plant Pathology Herbarium, Australia	9,980
2824	*Ustilago cynodontis* BRIP 28040a	Queensland Plant Pathology Herbarium, Australia	5,213
2825	*Sporisorium caledonicum* BRIP 28043a	Queensland Plant Pathology Herbarium, Australia	39,849
2826	*Ustilago lituana* BRIP 46795a	Queensland Plant Pathology Herbarium, Australia	41,544
2827	*Sporisorium ovarium* BRIP 26909a	Queensland Plant Pathology Herbarium, Australia	6,751
2828	*Ustilago porosa* BRIP 26920a	Queensland Plant Pathology Herbarium, Australia	44,882
2831	*Sporisorium modestum* BRIP 26928a	Queensland Plant Pathology Herbarium, Australia	51,392
2832	*Sporisorium iseilematis-ciliati* BRIP 60429a	Queensland Plant Pathology Herbarium, Australia	0,186
2833	*Macalpinomyces spermophorus* BRIP 60430a	Queensland Plant Pathology Herbarium, Australia	7,301
2834	*Macalpinomyces tubiformis* BRIP 60434a	Queensland Plant Pathology Herbarium, Australia	0,000
2835	*Macalpinomyces spermophorus* BRIP 60448a	Queensland Plant Pathology Herbarium, Australia	3,928
2836	*Ustilago xerochloae* BRIP 60876a	Queensland Plant Pathology Herbarium, Australia	0,000
2838	*Sporisorium iseilematis-ciliati* BRIP 60887a	Queensland Plant Pathology Herbarium, Australia	0,000
2839	*Anthracocystis sehimatis* BRIP 60890a	Queensland Plant Pathology Herbarium, Australia	1,556
2840	*Anthracocystis caledonica* BRIP 60892a	Queensland Plant Pathology Herbarium, Australia	0,000
2841	*Anthracocystis heteropogonicola* BRIP 60896a	Queensland Plant Pathology Herbarium, Australia	2,824
2842	*Anthracocystis bothriochloae* BRIP 60901a	Queensland Plant Pathology Herbarium, Australia	0,631
2844	*Cintractia sp*. BRIP 60413a	Queensland Plant Pathology Herbarium, Australia	12,069
2845	*Cintractia sp*. BRIP 60422a	Queensland Plant Pathology Herbarium, Australia	9,725
2846	*Cintractia axicola* BRIP 26922a	Queensland Plant Pathology Herbarium, Australia	54,790
2847	*Cintractia mitchellii* BRIP 26923a	Queensland Plant Pathology Herbarium, Australia	2,167
2848	*Cintractia lipocarphae* BRIP 26925a	Queensland Plant Pathology Herbarium, Australia	50,676
2849	*Cintractia lipocarphae* BRIP 26927a	Queensland Plant Pathology Herbarium, Australia	15,029
2850	*Sporisorium aristidicola* BRIP 26930a	Queensland Plant Pathology Herbarium, Australia	5,563
2851	*Ustilago egenula* BRIP 60884 a	Queensland Plant Pathology Herbarium, Australia	0,000

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Publications

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Poster presentations

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