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Malic acid production by *Aspergillus oryzae*

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Cover: Picture of *A. oryzae* conidia taken with an environmental scanning electron microscope.

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Abstract

Malic acid is a C_4 dicarboxylic acid which is used as an acidulant in food and beverages. It is also considered as a bio-building block to replace petrochemically derived compounds in the post oil era. This organic acid can be biotechnologically derived from fermentation using renewable feedstocks as carbon source. Aspergilli are among the best producers of organic acid and *A. flavus/oryzae* is the best natural producer of malic acid.

The mechanism of malic acid production in *A. oryzae* was first assessed by transcriptome analysis. A nitrogen starvation response, probably regulated by a transcription factor related to the *S. cerevisiae* Msn2/4 transcriptional activator of stress related genes, was found to result in high malic acid production. Furthermore the pyruvate carboxylase reaction was identified as a metabolic engineering target. This gene, together with the malate dehydrogenase and a malic acid exporter was overexpressed in the strain 2103a-68, which was characterized in a second project. The overexpression led to an 80% increase in yield during the starvation phase (1.49 mol (mol gluc)$^{-1}$) and a triplication of the specific production rate. The increase in citric acid production in the engineered strain and its evaluation through model simulations led to the curation of the *A. oryzae* GEM. The existing model was curated with special emphasis on the mitochondrial transport reactions and let to a more defined network around the production of organic acids. Furthermore, the performance of the strain 2103a-68 on xylose as carbon source was evaluated as well and the good results led to the final project of manipulating the carbon source utilization by deleting the carbon catabolite repressor CreA.

This work contributed to the understanding of the regulation of malic acid production. This knowledge was used for the development of *A. oryzae* as an organic acid producer through metabolic engineering. Furthermore, the evaluation of xylose as an alternative carbon source paved the way towards the use of lignucellulosic feedstocks and showed the suitability of *A. oryzae* for the biorefinery of the future.

Keywords: systems biology, *Aspergillus oryzae*, malic acid, metabolic engineering, fermentation, C_4 dicarboxylic acids

List of publications

This thesis is based on the following publications:

I. **Knuf C**, Nookaew I, Brown SH, McCulloch M, Berry A, Nielsen J. 2013 Investigation of Malic Acid Production in Aspergillus oryzae under Nitrogen Starvation Conditions. Appl Environ Microbiol **79**(19):6050-6058 doi:10.1128/aem.01445-13 (published)

II. **Knuf C**, Nookaew I, Remmers I, Khoomrung S, Brown S, Berry A, Nielsen J. (2014) Physiological characterization of the high malic acid-producing Aspergillus oryzae strain 2103a-68. Appl. Microbiol. Biotechnol.:1-11 (Epub ahead of print, doi: 10.1007/s00253-013-5465-x)

III. **Knuf C**, Nookaew I, Nielsen J. (2014) The effect of *creA* deletion on the metabolism of a malic acid producing *Aspergillus oryzae* strain (manuscript)

IV. **Knuf C**, Nielsen J. 2012. Aspergilli: Systems biology and industrial applications. Biotechnology Journal **7:**1147-1155. (Review, published)

Additional publications and patent applications not included in this thesis:

V. Scalcinati G, **Knuf C**, Schalk M, Daviet L, Siewers V, Nielsen J. Modified microorganisms and use thereof for terpene production. United States patent application filed on June 27, 2011 and *PCT Patent Application EP11171612.2* filed June 28, 2011

VI. Scalcinati G*, **Knuf C***, Partow S, Chen Y, Maury J, Schalk M, Daviet L, Nielsen J, Siewers V. 2012. Dynamic control of gene expression in *Saccharomyces cerevisiae* engineered for the production of plant sesquitepene α-santalene in a fed-batch mode. Metab. Eng. **14:**91-103.

(*equal contribution)

Contribution summary

I. Designed the study, conducted the experiments, analyzed the data and wrote the manuscript

II. Designed the study, conducted the experiments, analyzed the data and wrote the manuscript

III. Designed the study, conducted the experiments, analyzed the data and wrote the manuscript

IV. Reviewed the current literature and wrote the manuscript

Preface

This thesis is submitted for the partial fulfillment of the degree doctor of philosophy. It is based on work carried out between 2009 and 2013 at the Department of Chemical and Biological Engineering, Chalmers University of Technology, under the supervision of Professor Jens Nielsen. The research was funded by Novozymes Inc., Davis, California, and the European Research Council.

Christoph Knuf

February 2014

bbl	barrel
BLAST	basic local alignment search tool
bp	base pairs
DW	biomass dry-weight
GEM	genome scale metabolic model
GO	gene ontology
GOI	gene of interest
h^{-1}	per hour
PCR	polymerase chain reaction
TCA	tricarboxylic acid cycle
WTI	West Texas Intermediate
3HP	3 hydroxypropionic acid

Contents

1 Introduction

This thesis is combining the findings and methods that emerged and are still under development in the fields of metabolic engineering and systems biology. The term metabolic engineering was shaped in the visionary papers of Bailey [8] and Stephanopoulos and Valliano [140]. Bailey defined metabolic engineering as follows:

> "Metabolic engineering is the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology." [8]

This definition did not change much in the last two decades. Furthermore Bailey explained the usual procedure of metabolic engineering, called the metabolic engineering cycle. This cycle consists of "a genetic change, an analysis of the consequences and a design of a further change", as depicted in the center of Figure 1. The shown metabolic engineering cycle was extended by the action of systems biology. Systems biology describes the approach of integrating different omics data in order to describe or predict the behavior of a biological system. The function of systems biology in the metabolic engineering cycle is to allow a faster and broader analysis of the system under investigation and through integration of the obtained data predict the outcome of the genetic modification, thereby saving time and money in the wet lab.

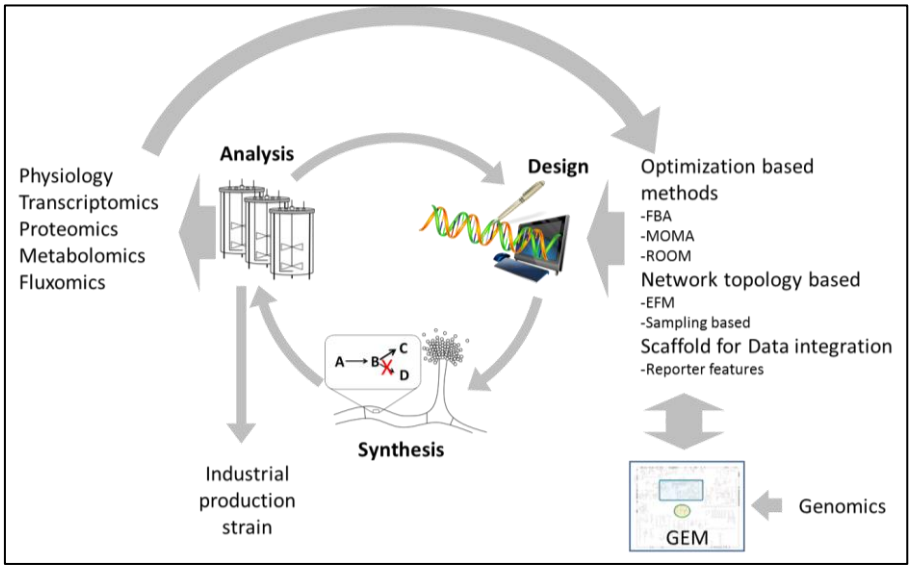


Figure 1: The metabolic engineering cycle (after Nielsen 2001 [96]), extended by the actions of systems biology, which integrates data from –omics studies and thereby facilitate the analysis and design part of the cycle. EFM, elementary flux mode; FBA, flux balance analysis; MOMA, minimization of metabolic adjustments; ROOM, regulatory on –off minimization

The aim of the cycle is an industrial production strain, which matches the industry's goals concerning yields, rates and titers. This strain can then be used in a biorefinery, which is in analogy to the petroleum refinery producing fuels, power and chemicals, just that the

biorefinery is not using crude oil as substrate, but fermentable sugars derived from biomass. As the metabolic engineering approach relies on the genetic modification of microorganisms, this field was initially applied using model organisms like *E. coli* and *S. cerevisiae* [97], which also made them the platform organisms for the first biotechnological production processes made possible through metabolic engineering. The same organisms were as well first used during the initial phase of systems biology, with the first genome scale metabolic models developed for these organisms in the early 2000s (*E. coli* [35]; *S. cerevisiae* [42]). With the availability of the genome sequences of several biotechnologically interesting Aspergilli (*A. nidulans* [43], *A. niger* [112], *A. oryzae* [83]), applying systems biology approaches in the field of filamentous fungi became possible. In case of *Aspergillus oryzae*, the first genome scale metabolic model was published in 2008 [153] and a microarray allowing for transcriptome analysis became available in the same year. Over the years, the molecular biology toolbox for Aspergilli was filled up with optimized transformation protocols and expression/deletion cassettes, so that rational metabolic engineering based on a detailed analysis through systems biology became possible.

1.1 Aspergilli: Industrial workhorses

Aspergilli have long been harnessed by mankind for human interests. *A. oryzae* for example, the subject of this study, has been used in the production of Japanese fermentation foods like Miso (soybean paste), Sake (rice wine) and Shoyu (soy sauce) for centuries. A patent on one of the first industrial enzymes produced by *Aspergilllus oryzae* was issued in 1895 to Jokichi Takamine who is known as the father of American biotechnology. This diastatic enzyme called Taka-Diastase (US Patent 525,823) was one of the first enzymes marketed in the United States of America. Since then the product range originating from Aspergilli largely expanded. There is the big field of organic acid production with the most dominant example of citric acid, which is naturally produced by *Aspergillus niger* and mainly used as an acidulant in food and beverages. Another major product area comprises of heterologous enzymes like e.g. lipases for the detergent industry produced by *A. niger* and *A. oryzae*. These two fields demand high volume production, especially citric acid with an estimated market capacity of about 1,600,000 t per year [132]. On the other hand Aspergilli have the ability to produce a wide variety of secondary metabolites, of which some are used in the pharmaceutical industry e.g. the cholesterol lowering polyketide lovastatin that was isolated from *A. terreus* [14], but others are extremely carcinogenic, like aflatoxin, which is produced by *A. flavus* [53]. Though there are some Aspergilli that can be potentially harmful for humans, the industrially relevant ones, *A. niger and A. oryzae*, are in general considered as safe, as both have a long history of safe use in the fermentation industry [9, 136]. In the case of *A. niger*, some strains have the potential to produce ochratoxin and therefore it is needed to check strains for their ochratoxin production potential before further developing new isolates to production strains [136].

Another advantage of Aspergilli is the fact that they are able to utilize a broad substrate range. In nature Aspergilli live a saprophytic livestyle, meaning that they are able to thrive on dead biomass. In order to have greatest flexibility Aspergilli are able to efficiently metabolize the monosaccharides abundant in this environment, like glucose, ribose, arabinose, xylose, rhamnose, mannose, galactose and fructose [36, 111]. This is already a big advantage over the model organisms *E. coli* and *S. cerevisiae*, which have to be engineered and evolved in the laboratory to be able to utilize all these sugars. These monosaccharides are not readily available in e.g. plant cell walls, but are incorporated in polymers like cellulose (glucose), starch (glucose) and hemicellulose (xylose, arabinose). For the hydrolysis of those polymers, the Aspergilli possess a big toolbox that enables them to find the right enzyme for breaking most bonds in the biomass. Among these enzymes are glucanases and xylanases, which are mainly responsible for the degradation of the most abundant polymers, cellulose and hemicellulose, repectively.

The availability of systems biology tools, the possibility of metabolic engineering, the current knowledge on large scale fermentation technology, the generally recognized as safe (GRAS)

status of many applications of Aspergilli, their ability to utilize and degrade a great variety of carbon sources and the natural ability of producing acids and enzymes makes them the perfect organism for the biorefinery of the future.

1.1.1 *A. niger* and citric acid production

Citric acid is a well-known organic acid that is extensively used in the food and beverage industry as it combines a pleasant taste with low toxicity and palatability. It serves several functions in the food formulation, like sterilization, flavor fixation and enhancement, bacterial stabilization, and standardization of acid levels. Furthermore it can be used as a chelating agent as it efficiently complexes metal ions. As the acid was initially extracted from Italian citrus fruits, the market became an Italian monopoly. At around 1880 the firm Charles Pfizer developed a chemical synthesis route. In the early 20^{th} century, surface culture methods using *Aspergillus niger* were implemented and in the middle of the century further developed towards a submerged process. The approximate yearly production in 2007 was 1.6 million tons. The majority of this is produced via fermentation using *A. niger* [60], but processes with *Yarrowia lypolytica* are reported as well [107].

The foundation for submerged cultures for citric acid production with *A. niger* were laid in 1948 [138], when the media composition promoting citrate accumulation was published. During the years the process was more and more defined and certain conditions for promoting citric acid accumulation were found. Firstly the carbon source should be provided in high initial concentration, as a direct correlation between the carbon source concentration and the specific production rate of citric acid was found [106]. Another important factor is the nitrogen source, which in industrial settings is usually provided through molasses that are used as carbon sources. For most studies in lab scale the medium is containing ammonium salts, like ammonium nitrate or ammonium sulfate [85]. In case of pH an optimum of around pH 2 or lower was found *in vivo* and *in silico* [6]. The aeration has an impact as well, as high aeration is lowering the CO_2 partial pressure in the medium. This leads to a reduction of available substrate for the carboxylation reaction of pyruvate to oxaloacetic acid, which is needed to replenish the carbon in the TCA cycle for high citric acid production. Though a certain amount of CO_2 is needed, sparging with CO_2 in the initial phase of citric acid fermentations has a negative influence on citric acid production [87]. Besides the hitherto discussed influences, trace-metals have to be present only in limiting conditions. Among those are manganese [64], zinc and iron [138]. Besides those external factors, it was reported that the cell morphology has an influence as well. Concerning the question whether pellet or filamentous growth promotes citric acid production the results are contradictory and probably strain specific. It was shown, that high concentrations of spores used for inoculation most likely yield in the dispersed morphology [105]. Common characteristics of the micro-morphology are short, swollen branches with swollen tips [104]. Through high shear forces the number of metabolically active tips can be increased, as

filaments that consist of old, vacuolated and metabolically inactive cells are more likely to break [106].

Though generations of researchers have investigated the interesting phenomenon of citric acid accumulation, Karaffa and co-authors came in 2003 to the conclusion that “only pieces of the puzzle are understood”[60]. The biochemical reactions that are leading towards citric acid accumulation were discovered in the 1950s. It was shown that the carbon is passing through the glycolysis, resulting in 2 moles pyruvate per mole glucose. One mole of pyruvate enters the mitochondrion and acetyl-CoA is formed in the pyruvate dehydrogenase reaction, releasing one CO_2. In order to reach a molar yield of citric acid per glucose of above 66%, this CO_2 has to be recovered. That reaction takes place in the cytosol, where one mole of pyruvate is carboxylated to oxaloacetic acid, which subsequently is reduced to malic acid. The cytosolic malic acid can then be exchanged for a mitochondrial citric acid molecule that is hereby transported across the mitochondrial membrane. Once the malate is in the mitochondrion it is used to fuel the TCA cycle. It is oxidized to oxaloacetate, which condensates with the acetyl CoA to form citrate. During growth part of the citrate has to be used to generate cytosolic acetyl-CoA for lipid biosynthesis, but during non-growth conditions the molar yield of citric acid on glucose can be one (Figure 2).

By using this route, which recycles the lost CO_2 from the pyruvate dehydrogenase reaction from the mitochondrion in the cytosol, the whole process seems more like a bioconversion of glucose to citrate with maximum yields of about 95%. The above described pathway shows the origin of pyruvate as a direct result of glycolysis. But it was shown that considerable amounts of citric acid must have been formed from previously accumulated glycerol and erythritol, which most likely were a result of carbon overflow during the initial phase of citric acid fermentation [111]. To obtain high yields of citrate on glucose there is a net production of NADH which can be oxidized in the respiratory system. However, this results in ATP production and alternative oxidation of NADH is therefore important for obtaining high citrate yields on glucose [7, 120].

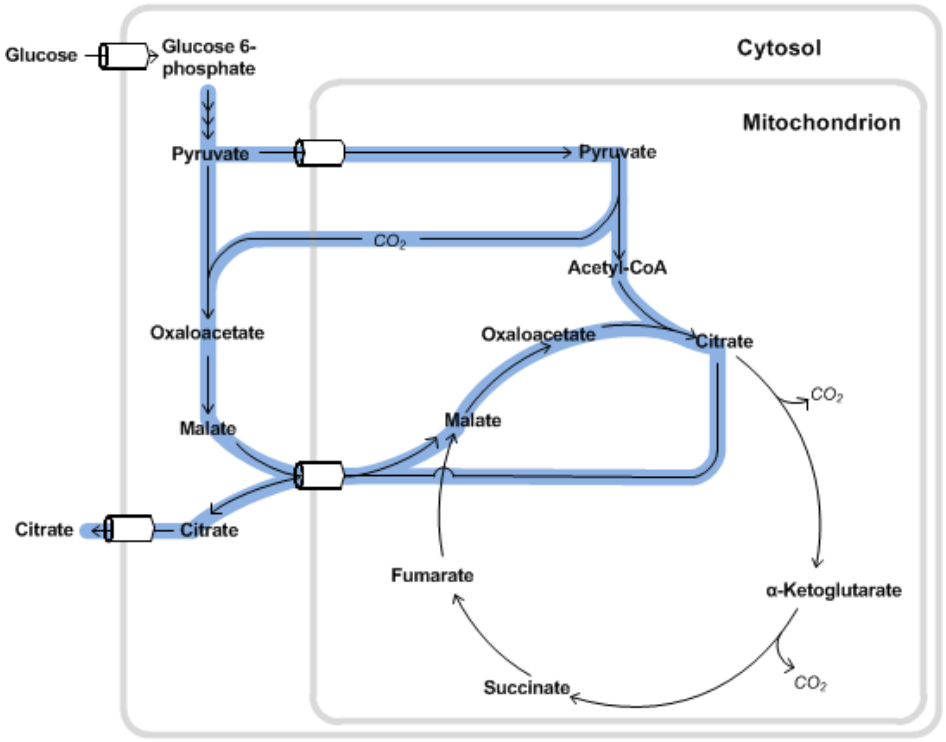


Figure 2: Central carbon metabolism and biochemical reactions leading to citric acid (after [60]).

A modelling approach identified the export of citrate from the mitochondrion as important factor for citric acid production in *A. niger* [44]. It is generally believed that this transport is based on the tricarboxylate transporter (TCT), which in yeast and mammalian cells exchanges cytosolic malate for mitochondrial citrate [38]. Therefore the malic acid accumulation that was measured prior to citrate accumulation in *A. niger* [129] might be a stimulus for the TCT to be more active. As the K_m for malic acid (K_m = 0.25mM) is 10 times higher than for internal citrate (K_m = 0.027 mM) [103], quite high cytosolic malate concentrations are needed for efficient export of citrate. Once this transporter is active, it competes favorably with aconitase whose K_m for citrate (3.2 mM) is significantly higher than the one of the TCT [48], thereby pulling the citrate out of the TCA cycle without any further constraints needed downstream in the TCA cycle to force citrate accumulation.

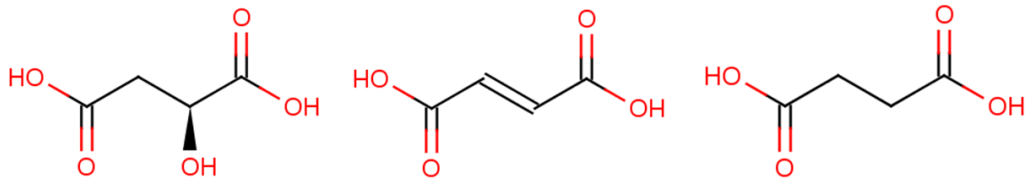
This phenomenon was observed for *S. cerevisiae*, where it was shown, that increased titers of dicarboxlyc acids in the cytosol positively influence the export of citrate from the mitochondrion [131]. Furthermore, the overexpression of malate dehydrogenase in *S. cerevisiae* increased not only the final malic acid titers significantly, but also increased the citrate titer by 33% [118]. 3D-structural predictions showed that there were similarities especially in the cytosol facing part between the *S. cerevisiae* and the *A. niger* putative TCT [34]. In order to utilize this mechanism for increased citrate production in *A. niger*, de Jongh et al. overexpressed heterologous genes of the reductive TCA branch in the cytosol in order to increase the cytosolic flux towards dicarboxylic acids. It was shown that the insertion of cytosolic malate dehydrogenase improved the production of citric acid and the glycolytic flux

[34]. Through the overexpression of Frds1 and fumRs, the yield could be improved to reach 0.9 g Citrate (g glucose)$^{-1}$ in the later stage of the cultivation and even the abundance of Mn^{2+} ions did not prevent the accumulation of citrate.

As the pH of the environment is an important factor for citric acid production, the influence of pH changes was investigated using model predictions and transcriptional analysis. The first studies on modelling the citrate production were conducted in the 1990s when Torres used a model of the carbohydrate metabolism of *A. niger* for the prediction of citric acid production [144, 145]. Over the years the modelling approach was optimized with the first genome scale metabolic model of *A. niger* as an important milestone [7]. Using the genome scale modelling approach and combining it with the proton production capability, the production of organic acids was investigated. It was successfully shown that the secreted acids at certain pH values were the most effective for *A. niger* [6]. Furthermore by using transcriptional analysis, it was shown that 109 genes were directly corresponding to pH and candidate orthologues of the Pal/PacC pH controlling pathway known from *A. nidulans* were identified. The findings led to the conclusion, that the aggressive acidification of the microenvironment in combination with the storage of gluconic acid was an evolved strategy among *Aspergilli* in order to outcompete rival microorganisms [6].

1.1.2 C_4 dicarboxylic acids

The group of C_4 dicarboxylic acids comprises of malate, fumarate and succinate (Figure 3). These acids, known as intermediates of the TCA cycle, are structurally very similar and can easily be interconverted chemically [151].



	L-Malic acid	Fumaric acid	Succinic acid
Molecular weight (g mol^{-1})	134.09	116.07	118.09
pK_A1	3.40	3.03	4.20
pK_A2	5.20	4.44	5.6

Figure 3: The group of C4 dicarboxylic acids, L-malic acid, Fumaric acid and Succinic acid, including their chemical properties.

The current way of producing C_4 dicarboxylic acids consists mainly of chemical conversion of petrochemically derived maleic acid or its acid anhydride, maleic anhydride. Succinic acid is derived by catalytic hydrogenation of maleic anhydride to succinic anhydride and

subsequent hydration to succinic acid [73]. In the case of fumaric acid, maleic acid is converted using catalytic isomerization. This fumaric acid can then be converted by hydration to malic acid. The same conversion method can be directly applied to maleic acid to derive malic acid. The drawback of the synthetic method towards malic acid is the production of a racemic mixture, which is not desired for food purposes and it causes problems in further polymerization processes. Therefore a biological production of L-malic acid is preferred. In order to achieve this a common method is the enzymatic hydration using Brevibacteria species which are able to convert petrochemically derived fumarate using the fumarase reaction to L-malate and secrete this back into the medium [47]. Another way of producing L-malic acid is fermentation of sugars, which can be used to produce bio succinic and fumaric acid as well and which will be discussed in the next section.

The C_4 dicarboxylic acids have broad application possibilities and overlapping application fields, as they are easily interconvertible. Succinic acid or derivatives thereof are used for example in dairy products and fermented beverages, but can also be applied as specialty chemicals in polymers, food, pharmaceuticals, and cosmetics. Through catalytic processes it is possible to transform succinic acid into 1,4 butanediol, tetrahydrofuran or γ-butyrolactone, which are nowadays mainly derived from fossil resources. Furthermore succinate can be esterified to dimethyl succinate, which is known as an environmentally friendly solvent. Fumaric acid can be used in chemical synthesis, for polyester and other synthetic polymers and resins, as well for production of biodegradable polymers. Specialized applications are in the treatment of the skin condition psoriasis [4] and in cattle feed, where the addition of fumaric acid has achieved a decrease of methane emissions of up to 70% [86]. Malic acid can be used in biodegradable polymers as well, but the biggest market is currently the food industry where it is used as an acidulant and flavor enhancer. Malic acid produced through chemical synthesis is a racemic mixture, but for the flavor enhancement as well as for the polymerization application it is important to have an isomeric homogeneous product, preferably the L- isomer as this is the natural occurring form which can be obtained through fermentation. In a review from 2008 [132], the annual production of C_4 dicarboxylic acids was estimated to range between 10 000 t in case of malic acid, 12 000 t for fumaric and 16 000 t for succinic acid, all produced from petrochemicals. In the same study the annual market volume, if low cost biobased production could be established, was projected to be higher than 200 000 t for fumaric and malic acid and more than 270 000 t in case of succinic acid.

The possibility of microbial production of organic acids, especially the C_4 dicarboxylic acids, has been proven. The general argument that oil is getting scarce and a future society will need alternatives for petrochemical based materials is a generally accepted prophecy and therefore a good reason for funding agencies to sponsor public research in this field. But if scientists want to see their processes emerging in an industrial setting, it has to become interesting for the biotech companies, which means making profit, in order to invest in a process. This profitable efficiency has to happen in the next 20 years, which is the run time

of a patent. Therefore the important point concerning large scale production of bulk chemicals from renewable resources is the short term economic feasibility. The fabrication of a product from renewable chemicals in which the production process even binds CO_2, which is generally demonized as the climate killer, is without a doubt desirable for our environment and climate and can be achieved through C_4 dicarboxylic acid production. Nevertheless the companies will only be able to sell hundreds of thousands of tons of a bio-based chemical if the price is comparable with the petrochemically derived product.

Many factors play a role in order to reach the bio-based/petrochemical prize equilibrium. On the one hand is the prize for crude-oil which has been rising over the last years. The bio-based equivalent of crude oil is glucose or other carbon sources, as it is usually used as the carbon source for a fermentative process. Therefore the prize of the carbon source is the first position to keep in mind for the cost calculation of a fermentative process. The refining of crude oil towards chemicals is a long optimized and established process and therefore the cost is more or less constant on that side. In case of the renewable building blocks this is the part the scientists have to optimize. There are numerous points to consider, some being the choice of organism, the mode of operation, the down-stream processing or the use of carbon sources.

In an attempt to calculate the feasibility of fumaric acid production, the authors of a study from 1990 came to the conclusion that the process will be viable in case the oil price reaches beyond 61 US$ bbl^{-1}. They assumed a productivity of 1.2 g L^{-1} h^{-1} and a yield of 0.74 g (g glucose)$^{-1}$). The 2012 average oil prices for WTI (~94.05$ bbl^{-1}) and Brent oil (111.67$ bbl^{-1}) (http://www.eia.gov/todayinenergy/detail.cfm?id=9530) were both far beyond that threshold. Furthermore, current technology led to better fermentation performance, which should give an even better advantage for the bio-based production.

A more recent attempt of a techno-economic analysis of white biotechnology products from 2008 by Hermann and Patel [54] came to the conclusion that succinic acid among other bulk chemicals like 1,3 propanediol, polytrimethylene therephtalate (PPT) and ethanol would be economically viable for a crude oil price of 25$ bbl^{-1}. They also calculated a production costs plus profits (PCPP) ratio of the biological process over the petrochemical process of about 100% at a glucose price of 135€ t^{-1} and a crude oil price of 25$ bbl^{-1}. That means at those oil and glucose prices the biological and petrochemical way are even at former technology stage. Unfortunately the glucose price elevated to a value in the range of 250.20 to 327.02 US$ t^{-1} (Last 52 weeks, from 15.01.2014 backwards, US sugar #11, http://www.investing.com/commodities/us-sugar-no11, 1$=0.736€), meaning that it doubled. But luckily for the white biotechnology, the current oil price is 4 times higher than the value that was considered in the study. Nevertheless, a thorough calculation taking current state of the art and raw material prices into account is needed for a proper analysis of the opportunities for white biotechnology products like the C_4 dicarboxylic acids.

1.1.2.1 Microbial C_4 dicarboxylic acid production

A wide range of microorganisms naturally produces the C4 dicarboxylic acids (Table 1) and the development of fermentative production processes dates back several decades. Initially researchers looked for natural producers and optimized the cultivation methods for these. During the development of metabolic engineering and molecular biology, the standard model organisms like *E. coli*, *C. glutamicum* and *S. cerevisiae* were targets of metabolic engineering. Heterologous gene expression and blocking of pathways by gene knock-out enabled those organisms to accumulate C_4 dicarboxylic acids. In parallel molecular biology tools were developed for the natural producers and the existing metabolic networks were optimized for organic acid production.

The best natural producers for **succinic acid**, [*Actinobacillus*] *succinogenes* [49], *Mannheimia succiniciproducens* [74] and *Basfia succiniciproducens* [70], were all isolated from bovine rumen, but the species affiliation is still questionable for [*Actinobacillus*] *succinogenes* and *Mannheimia succiniciproducens*. The gram negative, coccoidal, non-motile bacteria *B. succiniciproducens* was taxonomically classified as belonging to the family of *Pasteurellaceae* [70]. For *B. succiniciproducens*, a glycerol based fed-batch process has been developed which allows succinate production at a steady state rate of 0.094 g L^{-1} h^{-1} and a yield of 1.02g (g glycerol)$^{-1}$ for more than 80 days [135]. In wild-type *B. succiniciproducens* acetate and formate were identified as significant carbon sinks and therefore the pathway towards their formation was blocked by deleting the pyruvate formate lyase (*pflD*). This strategy worked out, but the flux was not completely directed towards succinate, but to a higher degree to lactate. This by-product was decreased by deleting lactate dehydrogenase as well. The double deletion strain showed a 45% increase in the molar yield of succinate on glucose (1.08 mol mol^{-1}) [12].

As mentioned above, not only natural producers were used for succinate production, but model organisms were extensively engineered in order to secrete organic acids as well. The hitherto most engineered organism for succinate production is a *C. glutamicum* strain that carries 4 deletions and 6 over-expressions. The deletions were aiming at diminishing the secretion of by-products like acetate (Δ*pqo*, pyruvate:menaquinone oxidoreductase; Δ*cat*, Acetyl-CoA:-CoA transferase; Δ*ackA*, acetate kinase) and lactate (Δ*ldhA*, L-lactate dehydrogenase). In order to enhance the production of succinic acid, the oxaloacetate pool (↑*pyc*, pyruvate carboxylase; ↑*ppc*, phosphoenolpyruvate carboxylase) was increased, the glyoxylate pathway reconstructed (↑*aceA*, isocitrate lyase (ICL); *aceB*, malate synthase (MS)), the initial reaction of the TCA cycle (↑*gltA*, citrate synthase) enhanced and the export of succinate from the cell (↑*sucE*, putative exporter) optimized [166]. All these modifications, together with a dual phase fermentation, characterized by an initial aerobic cultivation in shake-flasks and production in an anaerobic fed batch process, led to a final titer of 109 g L^{-1}, a volumetric production rate of 1.11 g L^{-1} h^{-1} and an average yield of 1.32 mol (mol glucose)$^{-1}$.

Table 1: Overview of natural C_4 dicarboxylic acid producers and their production performance regarding final yields, final titers, specific and volumetric production rates and the substrate used for the respective studies

Acid	Organism	Yield [g g^{-1}]	Titer [g L^{-1}]	r_p [g (g DW)$^{-1}$ h^{-1}]	r_p [g L^{-1} h^{-1}]	C-source	comments
Succinate	*Mannheimia succiniciproducens* LPK7	0.86	15.4	-	-	Glucose	Batch, MBEL55E delta: ldhA, pflB, and pta-ackA [98]
	Mannheimia succiniciproducens MBEL55E	0.69	-	-	3.9	Whey	Continuous culture[75]
	*Basfia siccinicipro*ducens DD1	1.02	5.21	0.375	0.094	glycerol	Continuous culture [135]
	Anaerobiospirillum succiniciproducens	0.99	32			glucose	Non-ruminal, CO_2 sparging [95]
	*Basfia siccinicipro*ducens DD1 engineered	0.708	-	1.027	-	glucose	Batch Δ*ldhA* Δ*pfl*D [12]
	E. coli SBS550MG	1.14	-	-	1.21	glucose/ fructose	Δ*ldhA,* Δ*adhE*, Δ*iclR,* Δ*ack-pta,* ↑PYC *L. lactis* [148]
	E. coli AFP111-*pyc*	1.1	99.2	-	1.3	glucose	Δ*ldhA,* Δ*pfl,* Δ*pts*G, [149]
	C. glutamicum	0.87	109	-	1.11	glucose	4 deletions, 6 over-expressions [166]
Fumarate	*Rhizopus oryzae*	0.78	~25	-	-	glucose	↑PEPC [164]
	S. cerevisiae FMME-001		3.18	-	-	glucose	↑PYC2, ↑RoMDH [154]
	E. coli CWF812	0.389	28.2	-	0.448	glucose	Fed batch, 8 deletions + ↑*ppc* [139]
	R. oryzae ATCC 20344,	0.85	92	-	4.25	glucose	Immobilized cells [21]
	Rhizopus arrhizus NRRL2582	0.65		-	-	glucose	20 L tank [125]
	Rhizopus arrhizus NRRL1526	0.8	97	-	-	glucose	[62]
Malate	*A. flavus* ATCC13697	0.938	113	-	0.59	glucose	[10]
	A. oryzae SaMF2103a-68	1.027	154	-	0.94	glucose	↑C4T318, ↑*pyc*, ↑*mdh* [17]
	Rhizopus arrhizius and *Paecilomyces varioti*	0.603	48	-	0.34	glucose	[142]
	Monascus araneosus	0.372	28	-	0.23	glucose	[81]
	Schizophyllum commune	0.357	18	-	0.16	glucose	[61]
	Zygosaccharomyces. rouxii	0.387	75	-	0.54	glucose	[141]
	Saccharomyces cerevisiae	0.313	59	-	0.19	glucose	↑*mae1*, ↑*pyc*, ↑*mdh3* [161]
	E. coli XZ658	1.057	34	-	0.47	glucose	11 deletions, Two stage fermentation [165]
	Toluopsis glabrata T.G-PMS	0.19	8.5		0.18	glucose	↑RoPYC, ↑*RoMDH,* ↑*SpMAE1* [26]

The first fermentation processes for the production of **fumaric acid** were established in the 1940 in the US, but they were soon after replaced by chemical synthesis [126]. But as the oil prizes increase significantly and rising environmental awareness called for sustainable production processes, the interest in fermentation processes for fumarate were revived [46]. *Rhizopus* species are known to be the best natural producers for fumaric acid, with *Rhizopus oryzae* being the best reported producer so far [21]. Metabolic engineering approaches helped to increase the production of fumaric acid in comparison to the wild-type strain, overexpression of *pepc* in *Rhizopus oryzae* improved the fumaric acid yield (0.76 g g-1 vs. 0.62 g g-1 WT), whereas *pyc* overexpression hampered cell growth and decreased fumarate yield. Instead the malate yield in the latter strain was increased 3 fold. And of course the model organisms *E. coli* and *S. cerevisiae* were metabolically engineered for fumarate production [139, 154].

1.1.2.2 *A. flavus / oryzae* and malic acid production

Malic acid was first isolated in 1785 by carl Wilhelm Scheele [88] from unripe apples, hence the name malic from the latin word for apple, *malum*. Since then it was found in many living cells, as it is an important intermediate in cellular metabolism and a constituent of the TCA cycle. Four major metabolic routes exist towards malic acid (Figure 4). The first one involves the carboxylation of pyruvate to oxaloacetate and subsequent reduction to malate, which leads to the highest theoretical yield of 2 moles per mole glucose. The second route involves the classic TCA cycle. As two carbon dioxide molecules are cleaved off the six-carbon backbone of citrate during its course through the oxidative TCA cycle, the theoretical yield drops to only 1 mole per mole glucose. The third possible pathway utilizes the reactions of the glyoxylate shunt. This can either be cyclic in case the oxaloacetate is replenished by malate and result in a yield of 1 mol mol^{-1} glucose or non-cyclic, which leads to a maximum yield of 1.33 mol mol^{-1}, through replenishing the oxaloacetate by pyruvate carboxylation.

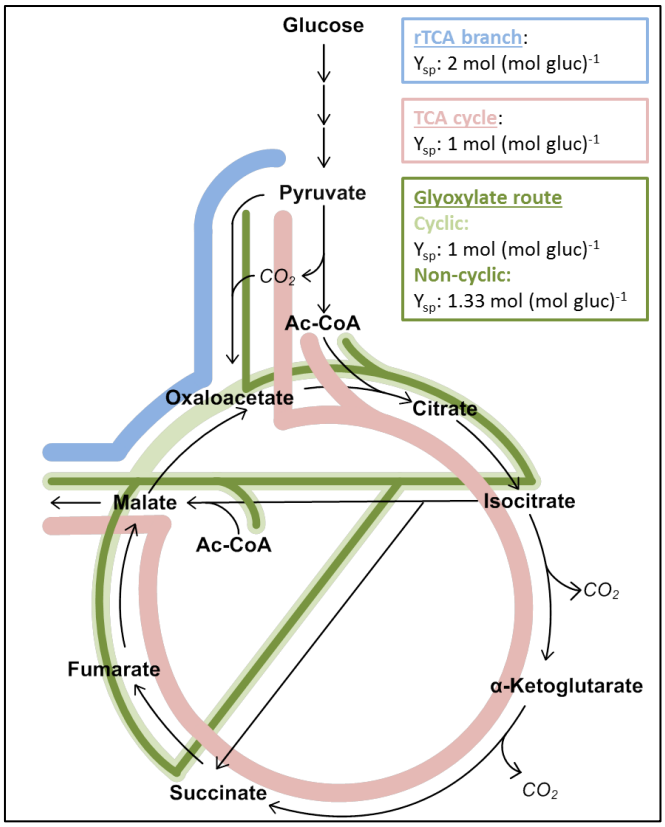


Figure 4: Possible pathways towards malic acid from glucose, the reductive branch of the TCA cycle, the oxidative TCA cycle and two versions of the glyoxylate cycle, cyclic and non-cyclic. Ac-CoA: Acetyl-CoA; Y_{SP}: maximal malic acid yield per substrate

Probably the first mentioning of malic acid as a product of microbial fermentation dates back to the year 1924 (Figure 5) when Dakin noticed malic acid as a by-product during ethanol fermentation [31] and also gave a hint towards the conditions for malic acid accumulation in microorganisms: “Some degree of nitrogen starvation seems to favor the production of malic acid.” In the following years malic acid was mainly mentioned in physiological studies of cell metabolism, which led to the conclusion that malic acid is an important constituent of the TCA cycle [67]. In the early 1960s industrial interest increased and a patent was filed on microbial malic acid production [1]. Several Aspergilli were screened and *Aspergillus flavus* was found to be the best producer in an initial screening. Furthermore, the limitation of nitrogen was applied as well, with $(NH_4)_2SO_4$ at a concentration of 0.2% as the best ammonium salt, yielding in malic acid concentrations of 30.4 g L^{-1} after 7 days and up to 38.2 g L^{-1} after 9 days of cultivation. The highest reported production rate was 3.28 mmol L^{-1} h^{-1}, which was achieved with *A. parasiticus* after media optimization and a subsequent feed of $CaCO_3$ [1]. A lot of research involving *A. flavus* was conducted in the late 1980s. This not only continued with further process optimization, but also elucidated the pathways leading to malic acid in *A. flavus* during intensive physiological studies. The first publication in the series from the Goldberg lab dealt with the biochemical aspects of acid biosynthesis.

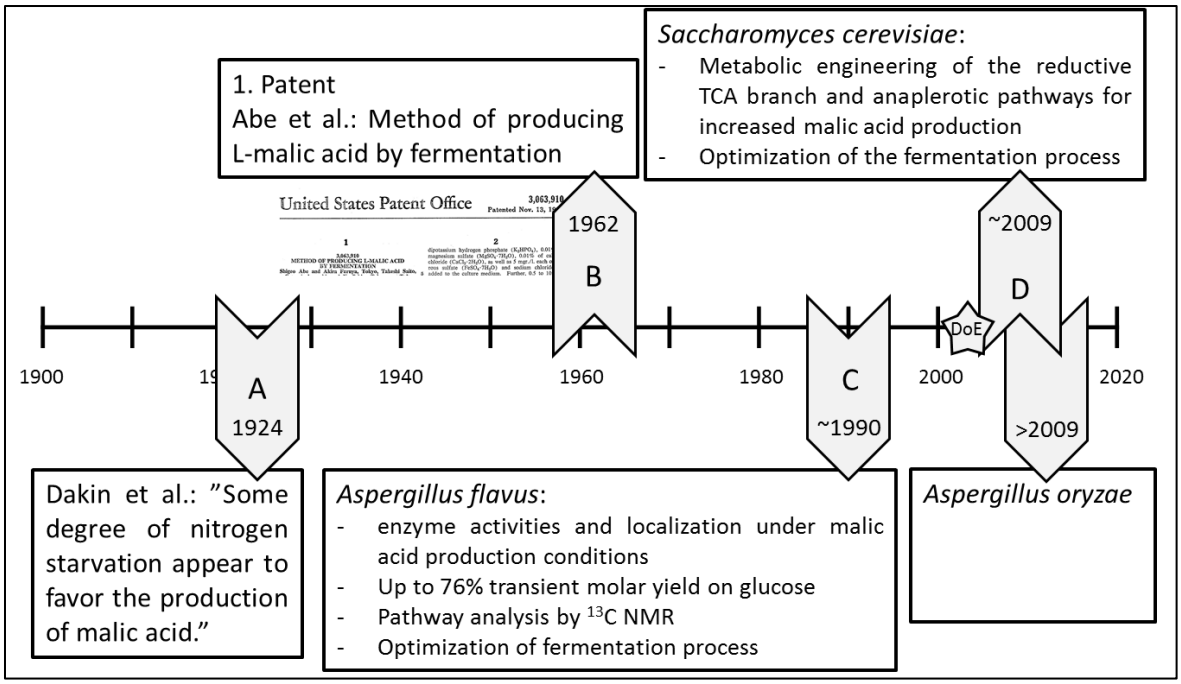


Figure 5: Historical timeline of research activities related to microbial malic acid production. A: [31]; B: [1]; C: [10, 13, 113, 114, 115, 117]; DoE: [151]; D: [160, 161, 162, 163]

In fermenters an overall molar yield of all C_4 dicarboxylic acids of 0.68 mol mol^{-1} glucose was obtained. In addition, it was shown that malate dehydrogenase (Mdh) activity increased up to 10 fold and that this increase is due to *de novo* protein synthesis [117]. This led to the conclusion that malic acid production in *A. flavus* is a result of significant changes in the metabolic network due to nitrogen starvation and that the reductive cytosolic branch of the TCA cycle is carrying the main flux towards malic acid. This result was further confirmed by ^{13}C NMR measurements, which confirmed that the labeling of [1-^{13}C] glucose was mainly incorporated in the third position of malate. This result proved, that malate originated directly from oxaloacetate and did not take the route through the TCA cycle [113]. Another indication for that was given after further process optimization, which resulted in overall C_4 dicarboxylic acid yields of 1.55 mol mol^{-1} glucose (malate yield of 1.28 mol mol^{-1} glucose), which is beyond the 1.33 mol mol^{-1} that could be achieved through the non-cyclic glyoxylate route. Along with the increased yield, the production rate was increased to reach 0.59 g L^{-1} h^{-1} (4.4 mmol L^{-1} h^{-1}). The main factors that increased the yield and titer of C_4 dicarboxylic acids were an increased agitation and Fe^{2+} ion concentration [10]. During fermentations yielding in high concentrations of C_4 dicarboxylic acids it was also observed that crystals were forming during the fermentation process, which consist of calcium salts of the secreted acids [114]. This fact shows the importance of the excess calcium carbonate. Through the formation of calcium acid salts, the product is constantly removed from the solution. This facilitates the transport of the acids out of the cells, as they do not have to pump the acid against a concentration gradient.

In order to better understand the metabolic network around the C_4 dicarboxylic acids the localization of enzymes was evaluated as well. In the case of *A. nidulans* pyruvate

carboxylase was found to be localized in the cytosol. Malate dehydrogenase and fumarase on the other hand showed unique isoenzymes in the cytosol and mitochondria. The presence of pyruvate carboxylase and malate dehydrogenase in the cytosol led to the postulation of the reductive cytosolic pathway to malic acid [101]. But the investigated *A. nidulans* strain did not produce malic acid under the given conditions. Later the presence of unique isoenzymes for malate dehydrogenase and fumarase were also confirmed in the malic acid producing *A. flavus* strain Kyowa A-114 (ATCC 13697) [113]. The presence of pyruvate carboxylase in *A. flavus* was investigated in a study with several Aspergilli. For the majority of the tested Aspergillus species, among them *A. flavus*, pyruvate carboxylase activity was only found in the cytosol fraction. For *A. oryzae* activity was detected in both, the cytosol and mitochondria [13]. The difference in localization of pyruvate carboxylase in *A. flavus* and *A. oryzae* is puzzling, as genome sequencing of both species indicate an extremely close relation between these two and it is even suggested, that the two are ecotypes of which one was selected by mankind (*A. oryzae*) to produce foodstuffs because of its inability to produce the carcinogen aflatoxin [109].

After metabolic engineering [8, 140] took off during the 1990s, pathways towards malic acid were first manipulated in model organisms like *S. erevisiae* and *E. coli*. The first modification in *S. cerevisiae* was the overexpression of the *FUM1* gene under the control of the strong inducible *GAL10* promoter [116], which enabled *S. cerevisiae* to efficiently convert fumarate into malate. In order to produce malate from glucose the cytosolic malate dehydrogenase (MDH2) was overexpressed [118]. The expression of MDH under the control of the GAL10 or the PGK promoter led to a 6 to 16 fold increase in cytosolic MDH activity and a 3.7 fold increase of malic acid accumulation in production medium. This relatively simple engineered strain was able to accumulate up to 11.8 g L^{-1}. A modern highly engineered *S. cerevisiae* strain overexpressing pyruvate carboxylase, malate dehydrogenase and a malate exporter in a pyruvate decarboxylase deletion background produced 59 g L^{-1} with a malate yield of 0.42 mol mol^{-1} glucose and a productivity of 0.19 g L^{-1} h^{-1} [161]. These values were obtained in $CaCO_3$ buffered shake-flasks. While scaling up the process, the culture pH and CO_2/O_2 concentrations were identified as key process parameters. An optimization of these parameters led to a 19% improvement of the malate yield on glucose [160]. In the case of *E. coli* metabolic engineering for malic acid production got a kick start by adding modifications on a strain that was already engineered for the production of succinic acid. The final malic acid production strain was able to accumulate malate to a final titer of 32 g L^{-1},in a two stage fermentation process, which is significantly lower than the titer obtained with *S. cerevisiae*, but the yield (1.42 mol mol^{-1}) was more than three times as high and the productivity (0.47 g L^{-1} h^{-1}) twice as high [165]. Another *E. coli* strain was engineered in a way as to generate ATP during the production process by overexpressing the *Mannheimia succiniciproducens* PEP carboxykinase [94], which enable this strain to produce malic acid to a final titer of 9.25 g L^{-1} after 12h of aerobic cultivation. Concerning the yield (0.75 mol mol^{-1}), this strain could not compare with the previously mentioned *E. coli* strain, but the increase in production rate (0.74 g L^{-1} h^{-1}) was a significant improvement.

1.1.2.3 Downstream processing

The downstream processing in which a biotechnological product is recovered from the fermentation broth is roughly estimated to account for 50% to 70% [11] of the total production cost and therefore has a high impact on the economic feasibility of the whole process. In order to obtain C_4 dicarboxylic acids in high purity from fermentation broth, three main steps are undertaken. In the first step the biomass is separated mainly by membrane filtration or centrifugation. The second step comprises the removal of impurities and primary separation. The last step finally purifies the acid by vacuum evaporation and crystallization. The main approaches for the separation of C_4 dicarboxylic acids are from the fermentation broth include direct crystallization, precipitation, membrane separation, solvent extraction, chromatography, and *in situ* separation [27], these are briefly discussed below using succinic acid as an example.

Direct crystallization aims at directly purifying the acid of choice from fermentation broths. When using different temperatures, 60°C for by-product removal through vacuum distillation and 4°C for the actual crystallization of succinic acid, a yield of 75% and a purity of 97% was obtained when using a simulated broth. Applying this method to real fermentation broths, the yield and purity dropped to 45% and 28%, respectively [78, 82]. Another approach used the different dissociation states of the organic acids in the fermentation broth and reached a yield of 70% and a purity of 90% [77]. This method is probably the oldest and a fairly efficient process, but the purity will have to be improved if the product is to be used for polymerization.

The **Precipitation** method uses different ions, mainly calcium, but also ammonium, in order to precipitate the acid from the fermentation broth. This method has been applied successfully in the purification of citric acid and lactic acid. During the process the acid is first precipitated by the addition of Ca(OH)2, CaO or ammonium and then the salt is separated by filtration. The filtrate reacts with sulfuric acid and the free organic acid is obtained. The drawback of using calcium ions is the production of gypsum in equal molar amounts to the organic acid [32]. An advantage is the already existing infrastructure and knowledge on this process. The use of ammonium has the advantage that it can be partly recovered after the process, though the recovery by pyrolysis uses a lot of energy. The recovery yield with ammonium is reported to be 93.3% [156].

Another approach is **membrane separation**, including membrane filtration, like microfiltration, ultrafiltration and nano-filtration, or electro-dialysis. Yao et al. were able to obtain a purity of >99.5% with a yield of 75% in a process that applied micro-centrifugation, ultrafiltration and active charcoal adsorption, followed by further purification and final crystallization [155]. Using two stages of electro-dialysis, Zeikus et al. achieved a yield of 60% [159]. Nevertheless, the drawback of this method is the cost of the device and a relatively low yield.

The development of a simple **solvent extraction** method turned out not to be satisfactory [63]. Nevertheless a reactive extraction method in which the product is first converted into a compound without carboxylic groups and then recovered was developed and the use of aliphatic amines yielded in 95% recovery for a simulated solution and 78%-85% using *E. coli* fermentation broth [72]. By using acidification and esterification, recovery yields of 95% were obtained for both model solutions and actual fermentation broth [100].

Ion exchange resin, alumina, silica, and zeolite molecular sieve adsorption are some of the methods used for **chromatography** and recovery yields of >95% were reported for e.g. the resin XUS 40285 and when applying a method involving alkaline-type anion exchange resins the yield was 99% [27]. The challenge in method development here lies in the evaluation of the ideal sorbent that shows a high capacity, a complete and stable regenerability and specificity for the desired acid.

A method that would be desirable to apply in case the product inhibits the growth of the production host is ***in situ*** **product recovery (ISPR)**. A successful implementation of this strategy, using the anion exchange resin NERCB 09, was reported for an *E. coli* process. The production process was extended from 48h (fed-batch process) to 126h. Though the yield and rate was slightly lower for the integrated fermentation system, 1.3 g L^{-1} h^{-1} and 0.52 g g^{-1} compared to 1.54 g L^{-1} h^{-1} and 0.57 g g^{-1} in the fed batch case, stable production could be achieved over a longer period, leading to the overall production of 145.2 g L^{-1} [76].

Though the above mentioned results all refer to succinic acid as the target product, the same methods can be applied to fumaric and malic acid as well. For the fermentation broth containing mainly malic acid obtained after cultivation of *A. oryzae* a procedure was patented that uses concentrating electro-dialysis in a first step and bipolar electro-dialysis in order to convert the acid salt into the free acid [57].

1.2 Aspergilli and molecular biology

In order to improve the production performance of a microbial host strain through metabolic engineering one needs molecular biology tools for directed modifications. Whereas the molecular biology studies and the resulting possibilities for the standard microbial hosts like *E. coli* and *S. cerevisiae* are numerous and easy to apply, the development of molecular biology tools for Aspergilli is lagging behind. Nevertheless, some strategies were inferred from other organisms and optimized for an *Aspergillus* host. Important aspects for efficient strain construction are the use of the transformation strategy, marker genes, plasmid availability and promoters and terminators. In order to introduce genetic material into the cell, different **transformation** strategies are available, among these are *Agrobacterium* mediated transformation [91], electroporation [25], biolistic methods [41] and probably mostly used protoplast mediated transformation [50].

In order to select for positive transformants **selection markers** are needed. Those markers can be divided into dominant and auxotrophic selection markers. Frequently used dominant markers are the prokaryotic markers conferring resistance against hygromycin B (*hph*, *E. coli*) [121] and phleomycin (*ble*, *Streptoalloteichus hindustanus*). Another dominant nutritional marker which can be used to select not only for the existance of a marker, but also for the frequency of integration is acetamidase (*amdS*, *A. nidulans*) [29]. Among the auxotrophic markers, orotidine-5'-phosphate decarboxylase (*pyrG*, *A. niger/oryzae*) [52, 84] and orotidine-phosphoribosyl transferase (*pyrE*, *A. niger*) [150] are well known and resemble the *URA3* auxotrophic marker from *S. cerevisiae* [15]. Both enzymes are part of the pyrimidine biosynthesis pathway and thereby involved in the *de novo* UMP biosynthesis and *de novo* pyrimidine base biosynthesis, which makes mutants auxotroph for uracil or uridine. Transformants can be selected on uracil-lacking minimal medium. Another advantage of using *pyrG* or *pyrE* markers is that they can be counter-selected for by 5-FOA (5-fluoroorotic acid). 5-FOA will be converted to the toxic compound fluorouracil through the pyrimidine pathway, thereby allowing only cells to survive which lack the *pyrG* or *pyrE* gene. The positive and negative selection makes it also possible to rescue the marker and thereby allows recycling of it. In order to do so, there are two strategies available. In the first case about 300 bp of direct repeat are flanking the marker gene. This sequence is a target for spontaneous recombination, which leaves only one copy of the flanking sequence in the genome [68]. In the second approach, the marker gene is flanked by loxP sites (34bp) [40], which recombine after recognition and action of the Cre recombinase. Other than the fairly easy approach of looping out the marker gene through direct repeats, the loxP/Cre system requires additional expression of the Cre recombinase. Though advanced transformation cassettes have been developed [93], it takes additional work and time until the next round of modification can take place. Other markers providing both positive and negative selection are *niaD* (selecting for growth without nitrate, negative selection using chlorate toxicity) [20] and *sC* (selecting for growth without sulfate, negative selection using selenite toxicity) [19].

In order to facilitate the cloning process, a range of **plasmids** was developed as well, which are mainly integrative plasmids, designed for integration into the genome. The drawback of Aspergilli concerning strain construction is their low frequency of homologous recombination. The random integrations of the DNA fragments into the genome make targeted gene deletion and specific integration of overexpression fragments into highly transcribed sequences troublesome. In some strains regularly used for strain construction components of the non-homologous end joining (NHEJ) process were deleted, which led to significantly increased homologous recombination in *Neurospora crassa* and was also adapted for *A. oryzae* [92]. Another obstacle for integration of DNA fragments is the fact that Aspergilli are multinucleate. Staining of conidia of the *A. oryzae/flavus* group showed that they are multinucleate [158]. The appearance of multinuclear cells and the high frequency of random integrations call for an additional confirmation of correct integration of the desired fragment into all nuclei by Southern blot analysis, even after confirmation of the integration of the deletion cassette at the desired locus by analytical PCR. An interesting and fast cloning method was developed and applied in *A. nidulans*. The method is based on USER cloning and allows for expression in a combined targeting-expression cassette. The vector set is designed for defined integration into the genome and expression of the GOI, either under the constitutive *gpdA* or the inducible *alcA* promoter [51].

For the overexpression of a gene or a whole pathway it would therefore be desirable to use episomal plasmids. Unfortunately episomal vectors do not naturally occur in Aspegilli, furthermore it was speculated that Aspergilli do not even possess the enzymatic machinery for handling small circular DNAs [3]. Nevertheless it was shown for *A. nidulans*, that genes could be expressed from episomal plasmids that contain an AMA1 sequence, which represents an inverted repeat of mobile Aspergillus transformation enhancers (MATEs) [2, 45]. Those plasmids are structurally stable and do not recombine with the chromosome. While comparing the transformation efficiencies of these plasmids with integrative plasmids, a 1000 times higher transformation efficiency was reported for the episomal plasmids (50.000 colonies (ng DNA)$^{-1}$ against 70 colonies (ng DNA)$^{-1}$). It was also shown, that there are about 10 copies of the plasmid per nucleus [3], nevertheless, long term stability could not be proven for these vectors, which makes them less useful for an industrial application in which the productivity is supposed to last for many generations [39].

In order to control the expression values, the choice of **promoter** is important as well. A great variety of promoters has been evaluated and used for protein expression in Aspergilli. On the one hand **inducible promoters** like P*glaA* and P*alcA* are used in case the expression of the desired protein has to happen at a certain stage of the cultivation. The expression of *glaA* is repressed on xylose, but highly induced when cultivated on maltose or starch. A CCAAT box has been identified which led to a gradual increase in transcriptional activity when multiple copies were added in the promoter region [79]. The *alcA* gene expression is

induced by various substrates like e. g. ethanol or threonine and strongly repressed by the presence of glucose. Expression of the *alcA* gene is highly depending on the activator AlcR [71]and a co-inducer. The performance of the *alcA* promoter for high level protein expression was enhanced by expression of AlcR under control of a constitutive promoter and multiple integrations of the *alcR* gene in the expression strain. Furthermore, the inhibition mediated through CreA was reduced through binding site modifications in the promoter [39]. Other inducible promoters are P*alcC* which controls the *A. nidulans* alcohol dehydrogenase gene, P*exlA* from *A. awamori* which is xylose inducible, *A. oryzae*'s thiamine dependent *thiaA* promoter [102] or the promoter controlling *A. niger*'s *sucA*, which is inulin or sucrose inducible. An interesting artificial promoter system is the Tet-On system which is based on the *E. coli* tetracycline resistance operon. It was evaluated in *A. nidulans* and has shown tight regulation, fast response within minutes after addition of the inducer and the ability of being fine-tuned depending on the inducer concentration [90]. On the other hand, **constitutive promoters** allow a stable expression of the gene of interest during the cultivation. Examples for constitutive promoters are the *A. nidulans* P*gpdA* (glyceraldehyde-3-phosphate), P*adhA* controlling expressing of aldehyde dehydrogenase and the mid-level expression promoter of the *tpiA* gene encoding triosephosphate isomerase. Furthermore *A. niger*'s *pkiA* (protein kinase A), the glutamate dehydrogenase A promoter (P*gdhA*) from *A. awamori* and the mitochondrial promoters of *oliC* (ATP synthase) from *A. nidulans* and *A. oryzae*'s P*tef1* (translation elongation factor Ia) are known to be constitutive promoters.

Promoters that have been successfully used in *A. oryzae* for high protein expression are P*amyA* (*taka*) [143], P*glaA* [146], P*sodM* [56] and P*tef1* [65]. A constitutive promoter used for metabolic engineering of *A. oryzae* is P*pgk* (phosphoglycerate kinase) [17, 130]. It showed stable and high expression during a 150 h cultivation for malic acid production in a wild type strain and was therefore used for the overexpression of the reductive TCA pathway towards malic acid [17].

2 Malic acid production

The first questions posed during my thesis work was **IF** *A. oryzae* is producing malic acid to same high amounts as *A. flavus* under nitrogen starvation and if it does **WHY** is it doing so. The project was therefore kicked off with a detailed study on the physiological response of *A. oryzae* under nitrogen starvation conditions, which were as well suggested for fumaric acid production using *Rhizopus nigricans* [127]. In order to study this, the wild-type strain NRRL3488 was cultivated in batch mode, supplied with a limited amount of either peptone or ammonium sulfate as nitrogen source and glucose in excess. The reason for choosing these two nitrogen sources was that peptone as a complex carbon source is closer to the natural environment of Aspergilli. They grow on decaying biomass, which supplies nitrogen in the form of polypeptides which have to be hydrolyzed by the cells in order to be usable for peptide synthesis. Peptone is available in varying qualities from different sources and is a relatively expensive nitrogen source, which is a disadvantage for the use in industrial large scale fermentations. Ammonium sulfate on the other hand is a defined and cheap nitrogen source.

2.1 Physiology

The use of nitrogen as the limiting substrate leads to a fermentation profile which in theory is divided into two stages, an initial stage of biomass formation, until the nitrogen source is depleted in the medium, and a second phase in which the existing biomass is converting the remaining glucose into malic acid and a small fraction into energy for maintenance of the cellular functions. With this setup, the overall malic acid yield is supposed to be increased, as carbon is not “wasted” for the synthesis of cell constituents and the associated increased energy demand. The expected fermentation profile can be seen in Figure 6. This figure also includes the concentration of ammonia measured over the time course of a representative cultivation to show the nitrogen limitation during the second phase (stationary/starvation phase) of the cultivation.

The question if *A. oryzae* is producing malic acid in the same conditions than shown before for *A. flavus* was investigated by cultivating two different wild-type strains in shake-flasks with peptone as nitrogen source and 50 g L^{-1} glucose. Both strains were accumulating malic acid in the fermentation broth, whereas NRRL3488 was secreting malic acid at a volumetric rate almost double of NRL3485 with values of 0.563 ± 0.020 g L^{-1} h^{-1} and 0.299 ± 0.011 g L^{-1} h^{-1}, respectively. The higher volumetric production rate was also reflected in the final titers of 38.86 ± 2.8 g L^{-1} for NRRL3488 and 23.12 ± 0 g L^{-1} for NRRL3485.

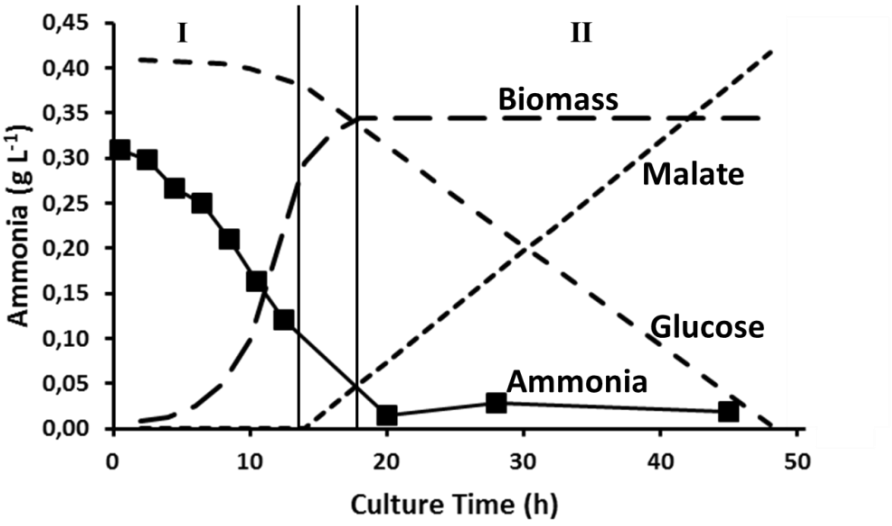


Figure 6: Theoretical fermentation profile for malic acid production and measured Ammonia concentrations of a representative fermentation. I: exponential phase; II: stationary phase

The better producing strain NRRL3488 was chosen for further investigation of the malic acid production mechanisms in *A. oryzae* and therefore cultivated in fermenters with either ammonium sulfate or peptone as nitrogen source and 50 g L^{-1} glucose as carbon source. The initial concentrations of the nitrogen source were adjusted in order to obtain a similar exponential growth phase, which led to initial concentrations of 6 g L^{-1} peptone and 1.4 g L^{-1} ammonium sulfate. The fermentation profiles of the quadruplicate cultivations of each condition can be seen in Figure 7. From the steeper slope of the glucose graph in the peptone fermentation one can see that the glucose uptake rate is significantly higher than in the ammonium sulfate condition (exact data can be seen in Table 2).

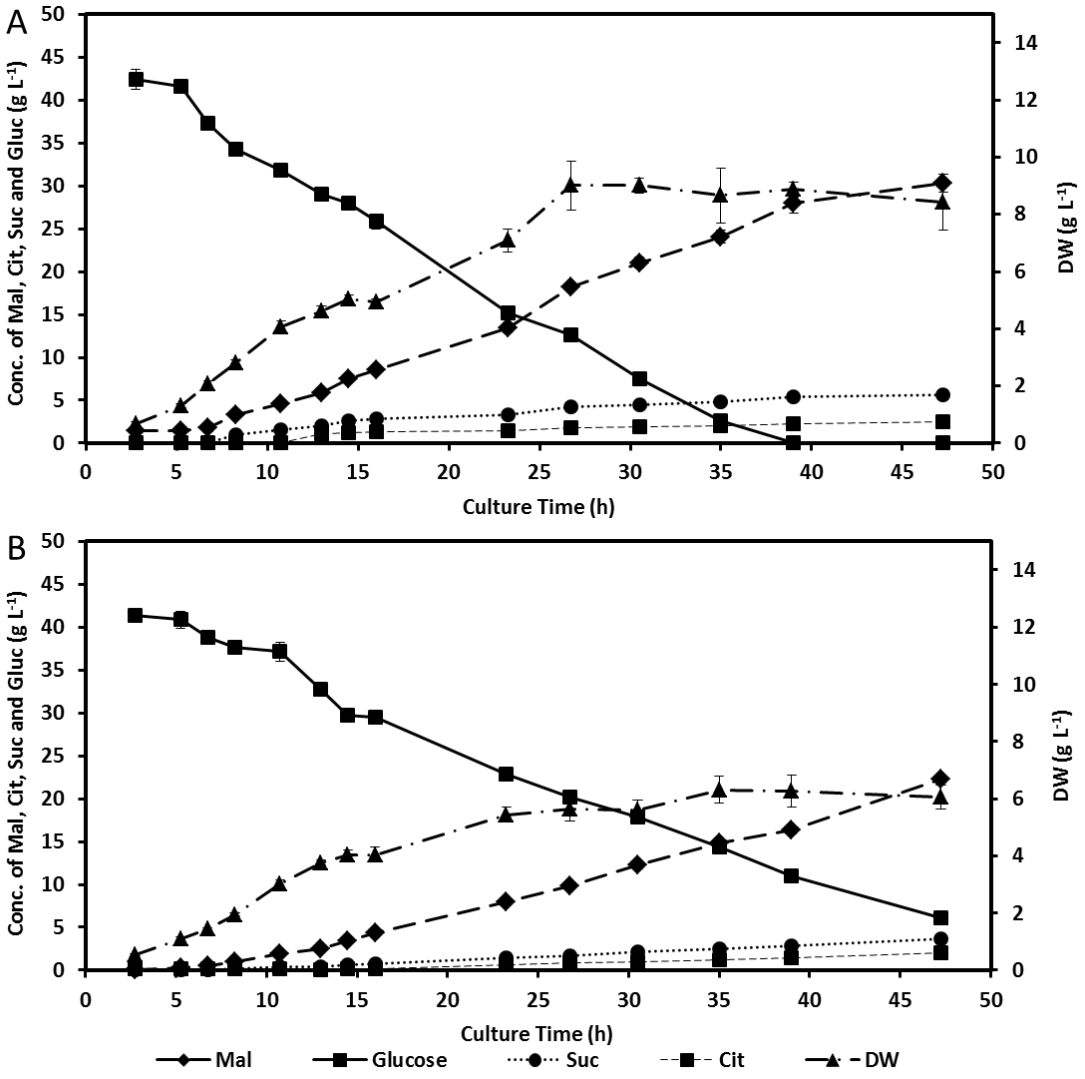


Figure 7: Profiles of malic acid production cultivations of NRRL3488 in MAF medium. The shown extracellular metabolite profiles are averages and standard deviations of 4 reactors each. The upper part shows the fermentation results on peptone (A) and ammonium sulfate (B). Mal, malate; Cit, citrate; Suc, succinate; Gluc, glucose and DW, dry weight.

Malate was detected in significant amounts in both fermentations. The volumetric production rate of malic acid was lower in the ammonium sulfate condition. But it showed the same trend in both fermentations, the volumetric malic acid production rate during the stationary phase is increased in comparison to the exponential phase. When it comes to the molar yields on a glucose basis, the values are almost the same for both nitrogen sources. In both conditions the yield increased from about 0.33 mol mol^{-1} to around 1 mol mol^{-1}. The second most abundant acid in the fermentation broth was succinic acid, which is following the same trend than malic acid, but in much lower concentrations. As a result of the increased volumetric glucose uptake and malic acid secretion rate, the final titers of malic acid in the fermentation broth were significantly higher for the peptone condition, 30.27±1.05 g L^{-1}, in comparison to the ammonium sulfate cultivations, 22.27±0.46 g L^{-1}.

Table 2: Physiological data for NRRL3488 grown on MAF medium supplemented with either peptone or ammonium sulfate as nitrogen source.

Nitrogen source	final titer of malate ($g\ L^{-1}$)	Phase[a]	μ_{max}[b] (h^{-1})	r_{malate}[c] ($mmol\ L^{-1}\ h^{-1}$)	r_s[d] ($mmol\ L^{-1}\ h^{-1}$)	Yields on glucose ($mol\ mol^{-1}$)			
						Citrate	Malate	Succinate	Pyruvate
peptone	30.27±1.05	exp.	0.23±0.01	4.22±0.25	8.10±0.81	n.d.[e]	0.33±0.05	n.d.	n.d.
		stat.	--	6.61±0.57	6.13±0.34	0.03±0.01	0.98±0.13	0.14±0.03	0.02±0.01
ammonium	22.27±0.46	exp.	0.21±0.05	1.59±0.15	3.71±0.44	0.01±0.01	0.34±0.06	0.07±0.02	n.d.
		stat.	--	4.36±0.14	3.92±0.10	0.07±0.01	1.09±0.05	0.20±0.01	0.03±0.00

The numbers stated are means of four individual bioreactors ± standard errors.
a) exp.: exponential growth phase; stat.: stationary phase
b) μ_{max}: maximum specific growth rate
c) r_{malate}: specific malate production rate
d r_s: substrate consumption rate
e) n.d.: not determined

In comparison to *A. flavus*, the results concerning the ammonium fermentation are comparable in case of the volumetric rates (0.59 g L^{-1} h^{-1}, *A. oryzae*; 0.58 g L^{-1} h^{-1}, *A. flavus*), whereas the yield is significantly lower in *A. oryzae* (1.09 mol mol^{-1}, *A. oryzae*; 1.26 mol mol^{-1}, *A. flavus*). Yield and titer are both higher than for an engineered *S. cerevisiae* strain [161] and concerning *E. coli* strains either higher in yield compared to WGS-10 [94] or higher in the production rate compared to XZ658 [165].

2.2 Transcriptome analysis

After verifying *A. oryzae*'s ability to secrete high amounts of malic acid into the fermentation medium, especially under nitrogen starvation conditions, the further underlying mechanisms were investigated that lead to the optimization of the metabolism towards malic acid production. Therefore the transcriptional state of the cells was analyzed using Aspergillus tri-species Affymetrix microarrays.

The first approach to assess the transcriptional state of the cell was the use of the reporter feature algorithm of the Biomet toolbox concerning reporter metabolites and biological process GO-term analysis Figure 8. The reporter metabolites analysis revealed 59 metabolites with significant transcriptional changes around them (distinct directional *P*-value <0.001). Among the up-regulated reporter metabolites, intra- and extracellular ammonia, end-products of purine metabolism, allantoate and urate, and three metabolites of the glutathione metabolism were found. The 51 metabolites connected to down-regulation contained for example metabolites from the amino acid synthesis, TCA cycle metabolites and energy or reduction equivalents.

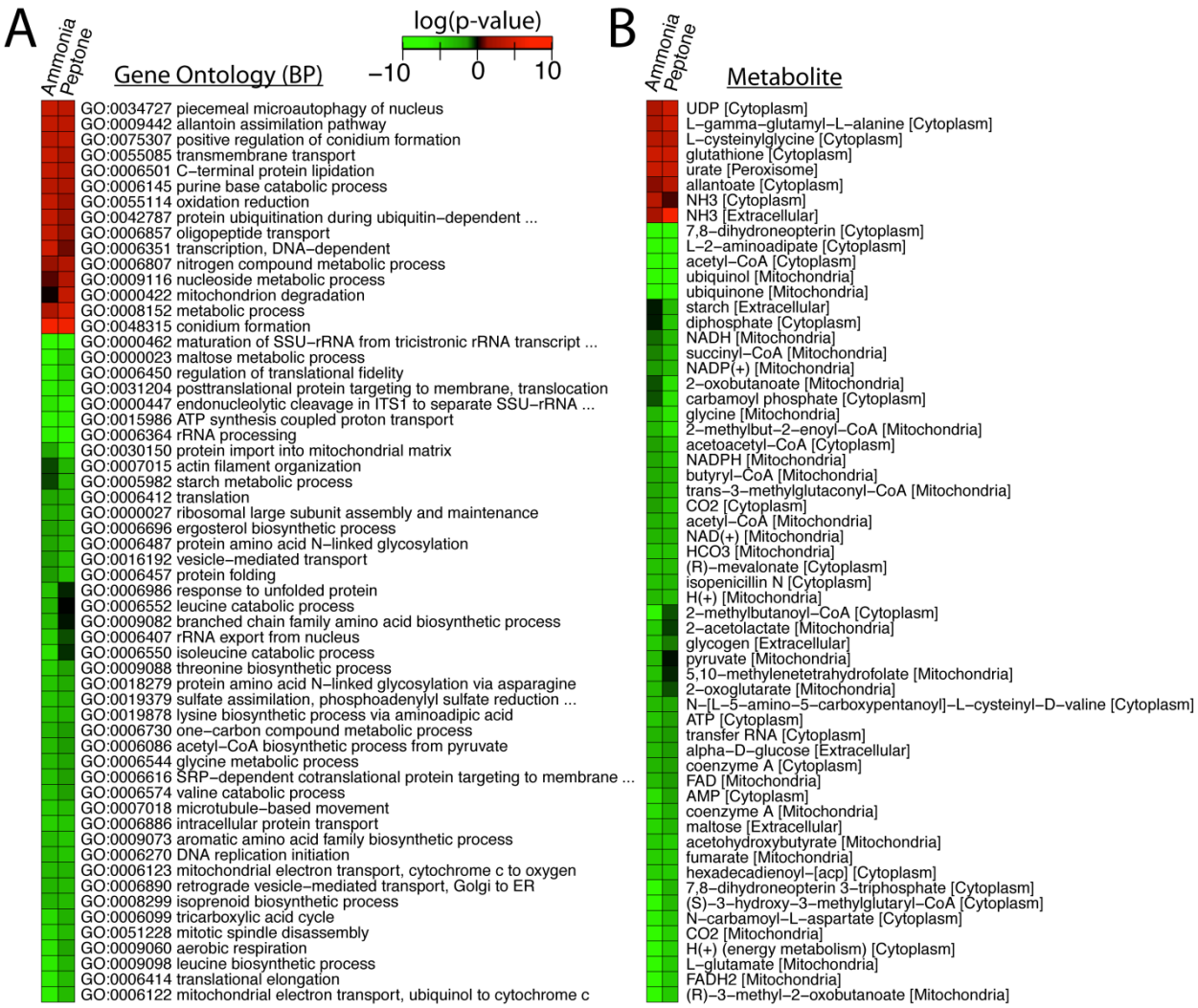


Figure 8: Heat maps of overrepresented GO-terms concerning biological processes (bp)(A) and reporter metabolites (B) depicting changes in transcription from the growth phase to starvation phase. The p-values of the shown GO-terms and reporter metabolites are smaller than 0.005 in either of the conditions (ammonium or peptone). SSU, small subunit; CoA, coenzyme A; SRP, signal recognition particle; Golgi, Golgi apparatus; ER, endoplasmic reticulum; acp, acyl carrier protein

The analysis concerning biological process GO terms revealed 15 GO terms that show a positive distinct directional p-value of less than 0.001 under at least one of the two conditions. Among the GO terms that are characterized by general up-regulation of the corresponding genes under nitrogen starvation are piecemeal microautophagy of the nucleus, purine base catabolic process, protein ubiquitination, or conidium formation. On the down-regulated side, amino acid synthesis related GO terms, as well as protein synthesis, translation/translational elongation, protein folding and intracellular protein transport, are found. As the largest sink of energy was removed through the stop of cellular growth, energy supplying processes like aerobic respiration, mitochondrial electron transport, or ATP synthesis coupled proton transport were correlated with transcriptional down-regulation.

Taken together, both, the reporter metabolite and GO term analysis, indicate that the cells are degrading cellular components and nitrogen containing compounds in order to recycle the nitrogen, furthermore the incorporation of nitrogen was reduced by general down-regulation of the protein production machinery. The up-regulation of the conidium formation and autophagy of the nucleus GO term lead to the assumption that the cells are in a severely stressed condition in which they struggle to survive. In this context it is even more intriguing that they continue taking up glucose from the medium and convert it to malic acid with a yield of about 1 mol mol^{-1}. Though the pathway to malic acid must be extremely active during nitrogen starvation conditions, no indication could be found using the initially discussed methods of transcriptome analysis.

Therefore a more targeted approach was chosen and the *P*-values and directions of transcriptional changes were plotted onto the central carbon metabolism network depicted in Figure 9. Following the color code (red & up-arrow, up regulated; green & down-arrow, down regulated), it becomes obvious, that on a transcriptional level the glycolysis is up-regulated. On the other hand, the TCA cycle seems to be down-regulated, which is in accordance with the results of the GO-term analysis. The connection of transcriptional regulation and malic acid production can be established by looking at the values for the pyruvate carboxylase and malate dehydrogenase reaction, which form the cytosolic reductive TCA branch from pyruvate via oxaloacetate to malic acid. The genes encoding for the reductive TCA branch are as well up-regulated, which forms a generally up-regulated direct connection between glucose and malic acid. In order to understand the regulation mechanism behind this, the promoter sequences of the up-regulated genes were searched for conserved sequences.

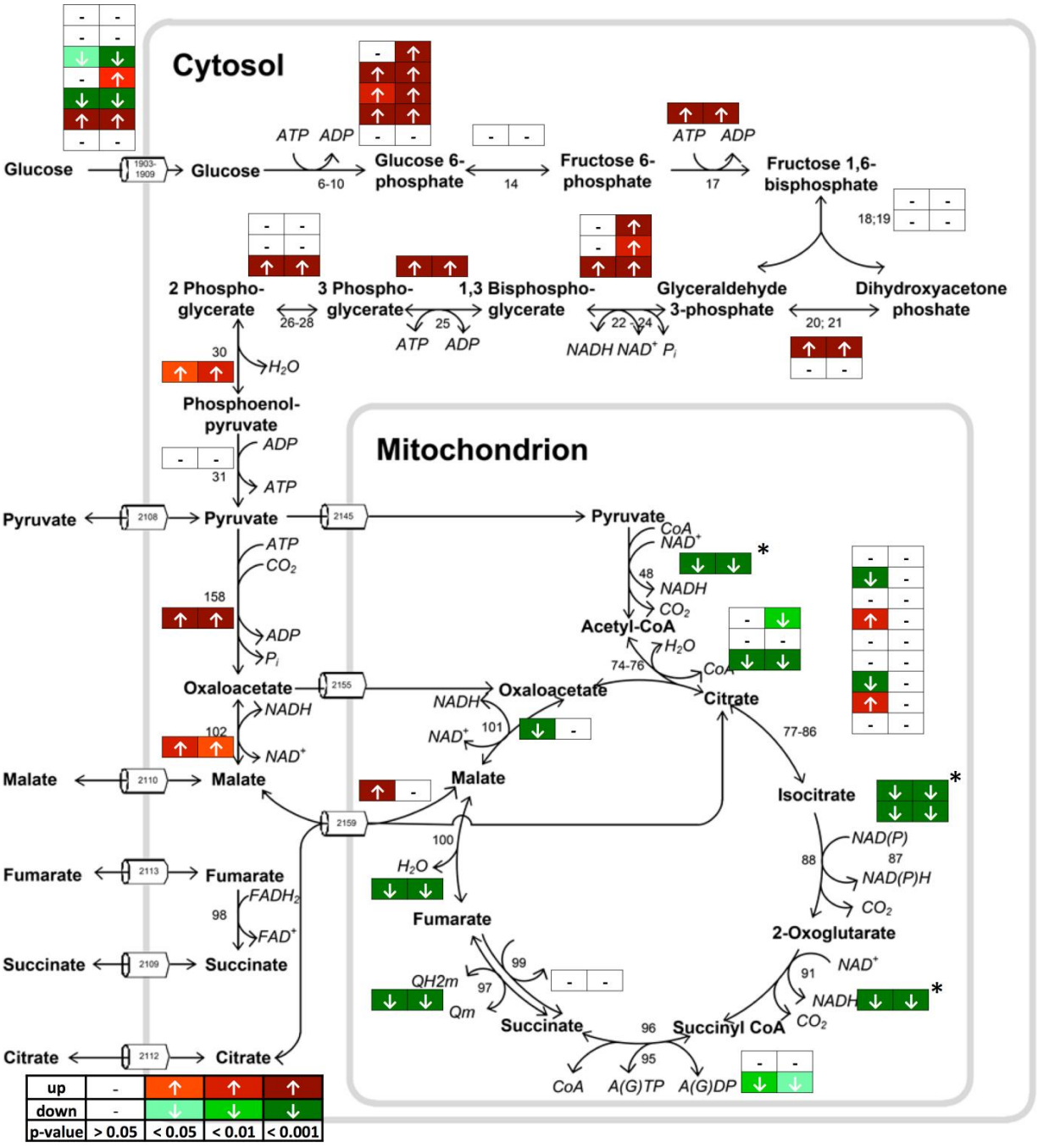


Figure 9: Schematic drawing of the central carbon metabolism of *A. oryzae* and reactions related to the production of malic acid. Transcriptional changes of the genes encoding for enzymes catalyzing the depicted reactions are shown in the boxes next to the reaction. The darker the color, the more significant is the transcriptional change between the stationary and the exponential phase of the cultivation. The direction of changes is indicated in the color and the direction of the arrow in the boxes. Red and up arrow: transcriptionally up-regulated in stationary phase; green and down arrow: transcriptionally down-regulated in the stationary phase. Asterisks, the shown data is taken from the most significantly changed genes of an enzyme complex.

One pattern that came out of the analysis was the motif CCCTC, which showed an occurrence *P*-value of 6.6E-06. This motif is the recognition site of the *S. cerevisiae* transcription factor Msn2/4. This transcription factor is known to be a transcriptional activator of the multi-stress response [134] and concluding from the results obtained here, is

likely to be responsible for the switch between ATP generation under unlimited growth and malic acid production under nitrogen starvation in *A. oryzae*. This direct conversion of glucose to malic acid makes sense from an ecological and evolutionary point of view, as (i) the glucose availability can be reduced, as glucose consumption via the NADH and ATP neutral reductive pathway can continue even without growing. (ii) *A. oryzae* shows optimal growth over a wide range of pH (pH 3-7) [23] and can therefore outgrow competing mircoorganisms, whose growth is oppressed by low pH. (iii) It has been shown by sequence analysis, that *A. oryzae* shows the largest extension for hydrolytic enzymes, working at low pH, in comparison to *A. nidulans* and *A. fumigatus* [83].

Having established an explanation of the regulatory mechanism leading to malic acid production above, the transcription data was also used for the identification of metabolic engineering targets to further enhance the production capacity. Therefore the transcriptional changes were linked to changes in reaction rates, which were calculated through a random sampling approach, using physiological data from the ammonium fermentation as constraints. One of the reactions identified was the carboxylation of pyruvate to oxaloacetate. In order to further check the feasibility of the overexpression of the pyc gene, the enzyme activities of pyruvate carboxylase and malate dehydrogenase (the two enzymes in the reductive TCA branch) were investigated in cells harvested from shake-flask cultivations during the exponential growth phase and the starvation phase. The activity of pyruvate carboxylase increased from 0.024 ± 0.004 units mg^{-1} total protein to 0.033 ± 0.007 units mg^{-1} total protein. Malate dehydrogenase activity decreased from 4.848 ± 0.828 units mg^{-1} total protein to 4.304 ± 0.358 units mg^{-1} total protein. As the increase of pyruvate carboxylase activity was expected to be higher, the possibility of increased protein degradation of pyruvate carboxylase was investigated. As seen in the GO term analysis, the protein ubiquitination was subject to transcriptional up-regulation, therefore the UbPred programme [122] was used to predict the ubiquitination sites of *pyc*, *mdh* and *mae3*. Two sites were predicted with high confidence for *pyc*, whereas none was predicted for *mdh* or *mae3*. As the enzyme activity of Pyc is significantly lower than for Mdh, this step is considered to be the flux controlling step of the reductive TCA branch. Furthermore, the maximum flux that can be achieved with the above mentioned enzyme activity during the stationary phase was calculated to be 1.01 mmol $(g\ DW)^{-1}\ h^{-1}$. This correlated well with the calculated malic acid production rate during that phase and supports the theory of Pyc being the flux controlling step in the pathway towards malic acid and therefore makes overexpression of *pyc* a promising target.

3 Engineering of the reductive TCA branch

After the natural ability to secrete high amounts of malic acid has been proven in the previous section, the engineered *A. oryzae* strain 2103a-68 was further investigated, which carries, among the overexpression of malate dehydrogenase and the malate exporter, the suggested strategy of overexpressing the cytosolic pyruvate carboxylase. The effect of overexpressing the reductive TCA branch and the malate exporter was investigated using glucose as carbon source. As a first step to using lignocellulosic material as carbon source for renewable chemical production, the engineered strain was then further analyzed on xylose containing medium and a mixture of glucose and xylose as carbon source.

The wild-type strain NRRL3488 and the engineered strain 2103a-68 were first compared concerning the expression of the additionally expressed genes and the enzyme activity of pyruvate carboxylase and malate dehydrogenase.

The strain 2103a-68 was created by transformation with DNA fragments containing the genes of interest under control of the phosphoglycerate kinase (*pgk*) promoter [130] and the *glaA* terminator. After transformants grew on selective plates, they were screened for the fastest acidification of the medium and 2103a-68 was the best performing transformant. The integration of all three fragments was confirmed by PCR, but the integration events were not quantified. Therefore a first approach to compare the engineered to the wild-type strain was the quantification of transcripts of each gene of interest and also the enzyme activity of pyruvate carboxylase and malate dehydrogenase.

As the focus is now on the production stage, the relative transcription of the GOIs was determined during the stationary phase (48h). Though all genes were under the control of the same promoter, the transcription level varied in relation to the wild-type strain. In case of *pyc*, the transcriptional level was 3.6 time higher in the engineered strain, *mdh* transcription was increased 9.6 times and the malate exporter was transcribed 7 times more frequent than in the wild-type. Though the transcription relies to a great extent on the location on the genome, one could speculate, that the *pyc* fragment was integrated once, the transporter fragment twice and the *mdh* fragment three times, as the relative transcription increases in increments of about 3.5.

In order to check if the increased transcription of the GOI translates into enzyme activity, pyruvate carboxylase and malate dehydrogenase activities were evaluated during exponential growth and starvation phase in both, the wild-type and engineered strain, from shake-flask cultures. Whereas the enzyme activities were not differing significantly during the exponential growth phase, the activity increased significantly in the starvation phase samples. The pyruvate carboxylase activity was twice as high in 2103a-68 compared to the wild-type and the malate dehydrogenase activity increased even four times, which is consistent with the trend of the relative transcription shown in Figure 10.

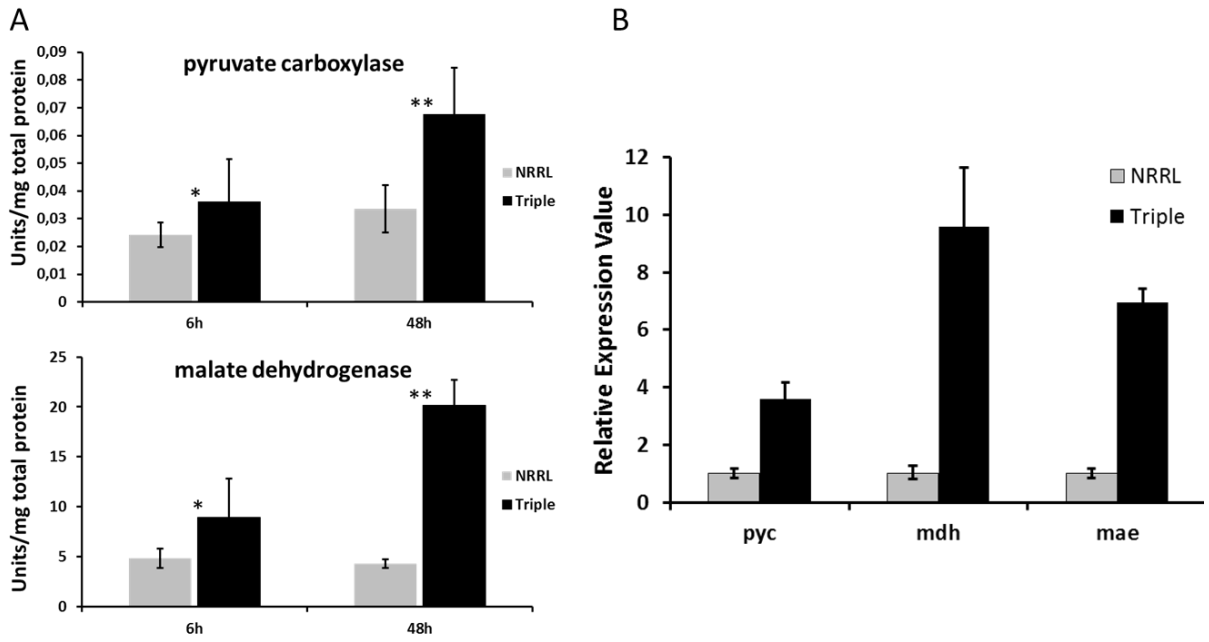


Figure 10: Enzyme activities (A) of pyruvate carboxylase (upper part) and malate dehydrogenase (lower part) and relative expression levels (B) of *pyc*, *mdh* and *mae* after 48h.

In the previous study it was already determined, that the enzyme activity of pyruvate carboxylase is two orders of magnitude lower than for malate dehydrogenase, this ratio is still unchanged and got even slightly worse. Therefore the pyruvate reaction is still considered to be the flux controlling step. And again, the overall specific production rate that was calculated using the enzyme activity (NRRL3488, 0.842 mmol (g DW)$^{-1}$ h^{-1}; 2103a-68, 1.709 mmol (g DW)$^{-1}$ h^{-1}) correlated well with the physiological data obtained during lab-scale cultivations Table 3. Though it was argued before, that the resulting variation of integration events and the subsequent selection of the best acidifying strain results in the optimal expression ratio of the GOIs [17], the pyruvate carboxylase step still seems to be the flux controlling step, and it would be worthwhile to consider additional integration of *pyc* expression fragments.

As the increased activity of the reductive TCA branch and over-expression of the malate transporter were proven by transcription analysis and enzyme assays, the final proof of the engineering strategy followed in lab-scale fermenters. The engineered strain and the wild-type were cultivated in MAF medium containing 100 g L^{-1} glucose as carbon source and ammonium sulfate as nitrogen source. As can be seen in Figure 11, the glucose uptake rate is increased in the engineered strain and the most significant difference is the steep slope of the malic acid graph. The wild-type and the engineered strain produced malate to final concentrations of 26.77 ± 0.197 g L^{-1} and 66.3 ± 2.36 g L^{-1}, respectively.

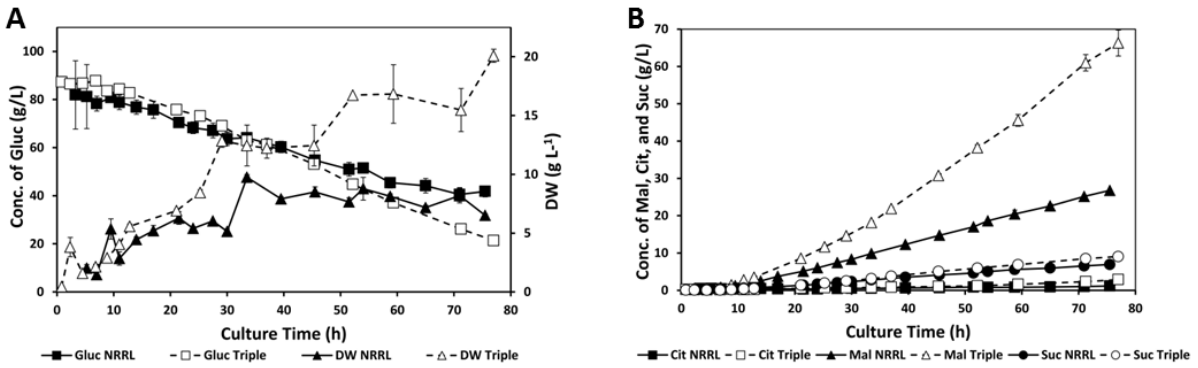


Figure 11: Fermentation profiles of NRRL3488 and 2103a-68 in MAF medium containing glucose from triplicate cultivations. Dry weight and carbon source concentration (A) and extracellular metabolite concentrations (B). Gluc, glucose; DW, dry weight; Cit, citrate; Mal, malate; Suc, succinate; NRRL, NRRL3488; Triple, 2103a-68.

The significantly higher final concentration in 2103a-68 was a result of a 70% increased glucose uptake rate and an 80 % increased malate yield of the engineered strain of 1.49 mol (mol glucose)$^{-1}$. These factors led to a malic acid production rate of 1.87 mmol (g DW)$^{-1}$ h^{-1} in the engineered strain, which is tripled compared to the parental strain. The malic acid production performance of the engineered strain exceeds all values obtained with other organisms and even beats most strains when comparing the 2103a-68 malic acid values with succinic acid production performance. The only strains performing better are the *Mannheimia* and *Basfia succiniciproducens* wild-type and engineered strains.

As the results obtained with glucose as carbon source were so promising, the performance of the engineered strain using xylose and a glucose/xylose mixture was investigated as well (Figure 12). In the xylose only case, the condition were exactly as mentioned above, just that 100 g L^{-1} xylose were used instead of glucose. The malic acid production rates in this setting cannot reach the high values obtained on glucose, but are still higher than for the wild-type strain cultivated on glucose. The cultivation on the glucose/xylose mixture was a first attempt to move towards a process for second generation biochemical production using lignocellulosic feedstocks, which contain glucose and xylose as main sugars. In the fermentation profile in Figure 12 it can be seen that glucose is the preferred carbon source, as it was first taken up and xylose consumption followed. The rates and yields were calculated for the two carbon sources separately. Though the values are not as divergent as shown before for the single carbon source cultivations, the values represent the same trend, the strain performs better on glucose. During the glucose phase (16-35h), the engineered strain showed lower values compared to the single carbon source, but still produces malic acid at a rate of 1.46 mmol (g DW)$^{-1}$ h^{-1}. The values for the xylose phase (43-66h) on the other hand are slightly increased compared to the xylose only cultivation and reached a malate production rate of 1.08 mmol (g DW)$^{-1}$ h^{-1}. The interesting part is the carbon uptake rate, which stayed almost constant for the glucose, xylose and glucose/xylose mixture cultivations.

Table 3: Physiological data for NRRL3488 and 2103a-68 (Triple) grown in malic acid fermentation medium with varying carbon sources during acid production phase.

Strain	Carbon-source	Specific rates [mmol (g DW)$^{-1}$ h^{-1}]				Volumetric rates [g L^{-1} h^{-1}]				Yields [mol mol^{-1} C-source]			Yields [mmol Cmol^{-1}]		
		r_{PMal} [a]	r_{PCit} [b]	r_{PSuc} [c]	r_S [d]	r_{PMal}	r_{PCit}	r_{PSuc}	r_S	Y_{SMal} [e]	Y_{SCit} [f]	Y_{SSuc} [g]	Y_{SMal}	Y_{SCit}	Y_{SSuc}
NRRL3488	Glucose	0.61±0.01	0.02±0	0.16±0.03	0.73±0.06	0.34±0.06	0.01±0	0.08±0.01	0.55±0.05	0.83±0.07	0.02±0	0.22±0.02	138.33±10.74	3.33±0.53	36.67±3.22
Triple	Glucose	1.87±0.23	0.06±0.01	0.28±0.04	1.26±0.11	1.05±0.13	0.05±0.01	0.14±0.02	0.95±0.08	1.49±0.05	0.05±0	0.22±0.01	247.67± 8.27	8±0.79	36.49±1.74
Triple	Xylose	0.89±0.14	0.05±0.01	0.16±0.02	1.27±0.09	0.5±0.08	0.04±0.01	0.08±0.01	0.8±0.06	0.7±0.06	0.04±0.01	0.12±0.01	140.83±12.54	7.57±1.53	24.75±2.04
Triple	Glucose phase (16-35h)	1.46±0.16	0.05±0.01	0.14±0.02	1.23±0.05	0.82±0.09	0.04±0.01	0.07±0.01	0.93±0.04	1.18±0.08	0.04±0	0.12±0.01	196.74±13.27	6.76±0.66	19.32±1.47
	Xylose phase (43-66h)	1.08±0.19	0.03±0.01	0.02±0.01	1.26±0.06	0.61±0.11	0.03±0.01	0.01±0.01	0.79±0.04	0.86±0.11	0.03±0	0.01±0.01	171.97± 4.59	5.25±0.19	2.9±0.41

The numbers stated are means of four individual bioreactors ± standard errors.

a) r_{PMal}: malic acid production rate

b) r_{PCit}: citric acid production rate

c) r_{PSuc}: succinic acid production rate

d) r_s: substrate consumption rate

e) Y_{SMal}: Yield of malate per substrate

f) Y_{SCit}: Yield of citrate per substrate

g) Y_{SSuc}: Yield of succinate per substrate

This is an important trait for the development of new strains for second generation chemical production, which is often limited by low carbon uptake rates. In this connection it is important that *A. oryzae* is not only able to metabolize xylose efficiently, but also to take it up from the medium with the same rate as glucose. Therefore *A. oryzae* does not need to be extensively engineered just to be able to utilize this pentose sugar, as the usual platform organisms like *E. coli*, *S. cerevisiae* and *C. glutamicum* needed to be [18, 80, 137].

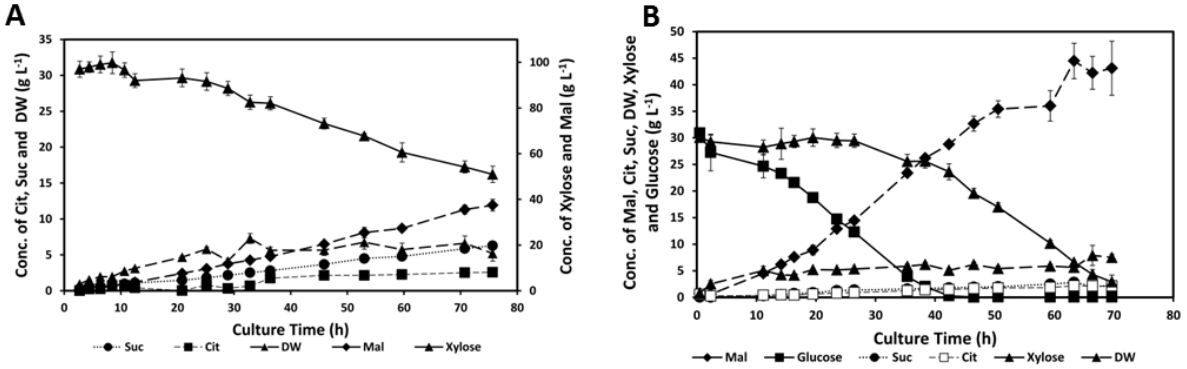


Figure 12: Fermentation profiles of 2103a-68 in MAF medium containing xylose (A) or a glucose/xylose mixture. DW, dry weight; Cit, citrate; Mal, malate; Suc, succinate.

In order to further characterize the engineered strain ^{13}C flux analysis was used, in which the intracellular fluxes are fitted towards the measured external metabolites and the labeling pattern of amino-acids, which was determined by GC-MS. As this method is limited to measuring *de-novo* synthesized amino-acids, intracellular fluxes could only be determined during the exponential growth phase. In order to obtain labeled samples from exponentially grown cells, shake-flask cultivations were performed and samples were taken after 7.5h of cultivation. In order to be able to fit the fluxes with special respect to organic acids, a compartmentalized flux model needed to be constructed. As the existing *A. oryzae* flux model [133] was not compartmentalized, an *A. niger* model [89] was extended by the reductive TCA branch in the cytosol. The so calculated fluxes show an increase of carbon flow through the rTCA branch in the cytosol and an increased flux of malate and oxaloacetate into the mitochondrion, in order to fuel the TCA cycle (Figure 13). These results show that the overexpression of the rTCA branch already has an impact on the malate production during the exponential growth phase and allows increased malate production compared to the wild-type during cellular growth. Though the efficiency of this strain is not optimal during the growth phase, the parallel growth and increased production allows for use of the engineered strain even in a continuous process. Thereby carbon containing waste streams could be used for the production of renewable chemicals.

Taken together, this strain allows for high level production of malic acid from both, glucose and xylose. Therefore it is very well suited for the biorefinery of the future. Though it already performs very well concerning malic acid production, it might still be optimized through

metabolic engineering. One example might be the engineering of the pyruvate carboxylase step, which still seems to be a flux controlling step.

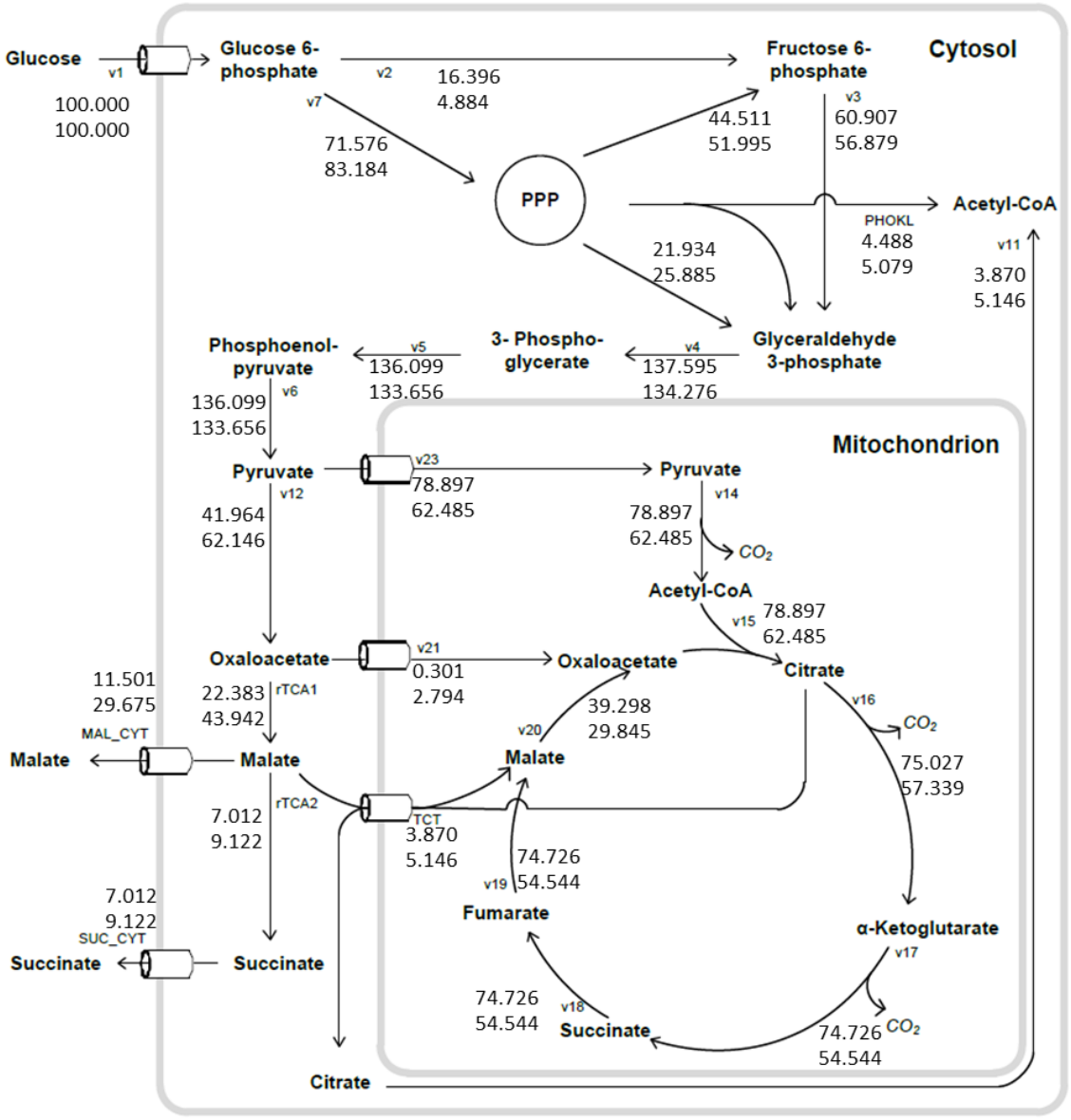


Figure 13: Central carbon metabolism of *A.oryzae* including the intracellular fluxes of NRRL3488 (upper values) and 2103a-68 (lower values). The samples were taken in mid exponential phase (7.5h) from shake-flask cultivations with MAF medium containing 25 g L^{-1} glucose (slightly modified after Knuf et al. [66]).

4 *A. oryzae* GEM update

Genome scale metabolic models (GEMs) are used for a great variety of systems biology applications. They can be used for data generation as well as interpretation. For transcription data for example, the metabolites that participate in certain reactions are linked through a GEM to the genes that encode for the reaction. Thereby the transcriptional changes around a certain metabolite can be determined and metabolic hot spots can be identified in the network [99, 108]. GEMs are available for the most important Aspergillli, *A. niger* [7], *A. nidulans* [33] and *A. oryzae* [153].

After the publication of the genome sequence of *A. oryzae* [83] in 2005 work on a GEM could start. As the annotation of only about 50% of the identified genes was too poor, this was improved by sequencing an expressed sequence tag (EST) library. After that it was possible to assemble a GEM. This first genome scale *A. oryzae* model *i*WV1314 was published by Vongsangnak et al. in 2008. The GEM was validated by comparing the model predictions for maximum specific growth rate (μmax; h^{-1}) in batch cultivations and biomass yield (Y_{SX}; g DW (mmol Substrate)$^{-1}$) during chemostat cultivations with experimental data. The model was able to accurately predict the growth rates for given carbon source uptake rates in batch cultivations using glucose, maltose, glycerol and xylose as carbon sources with an average accuracy of 98%. The biomass yields during chemostat cultivations could be calculated successfully as well. The model was used in a protein production project where it aided the investigation of amino acid requirements of an α-amylase overexpression strain [152]. As the requirements for tyrosine, aspartate cysteine and threonine significantly increased, the pathways leading to increased amounts of these amino acids were identified as possible targets for improving α-amylase production in an industrial setting. For this kind of simulation the model was well suited, as the reactions that are important for the amino acid and protein production are well annotated. On the other hand, information about transporters, especially the annotation of mitochondrial transport reactions is poor in almost any GEM. This is the same case in this model, as out of the 161 unique transport reactions only 53 are annotated, leaving the existence of 108 mitochondrial transport reactions open for speculation. Furthermore, most genes are annotated according to their degree of homology to *A. niger*, *A. fumigatus* or *S. cerevsiae* genes, which means, that the transport characteristics might vary as well.

In order to further elucidate the organic acid production potential and to find further engineering targets, an accurate model for this purpose is needed. The malic acid secretion is very much dependent on transport reactions, especially the exchange of metabolites between the mitochondrion and the cytosol. As mentioned above, one model that explains the high malic acid secretion obtained with *A. niger* is based on the assumption, that malic acid production in the cytosol is preceding citric acid production [69]. The malic acid titers in the cytosol act as a trigger for the tricarboxylate transporter [129].

During cultivations of the high malic acid producing strain 2103a-68 citric acid accumulation was detected along with an increased malic and succinic acid production. Assuming this mechanism to be active in *A. oryzae* as well, the knock-out of the TCT encoding gene in *A. oryzae* might diminish secretion of the by-product citric acid and thereby positively influence the yield of the desired C_4 dicarboxylic acids. The TCT reaction is one of the few mitochondrial membrane transport proteins that are annotated. According to the model the gene AO090020000012 encodes for the anti-port of malic acid and citric acid. In order to verify this strategy, knock out simulations were performed, in which the experimental external fluxes of malate, succinate and citrate were used as constraints. These calculations always resulted in a possible solution, indicating that the network around the organic acid transport between mitochondrion and cytosol offers too much flexibility. When looking at the mitochondrial membrane exchange possibilities in the model, it almost seemed as if there was no border for organic acids.

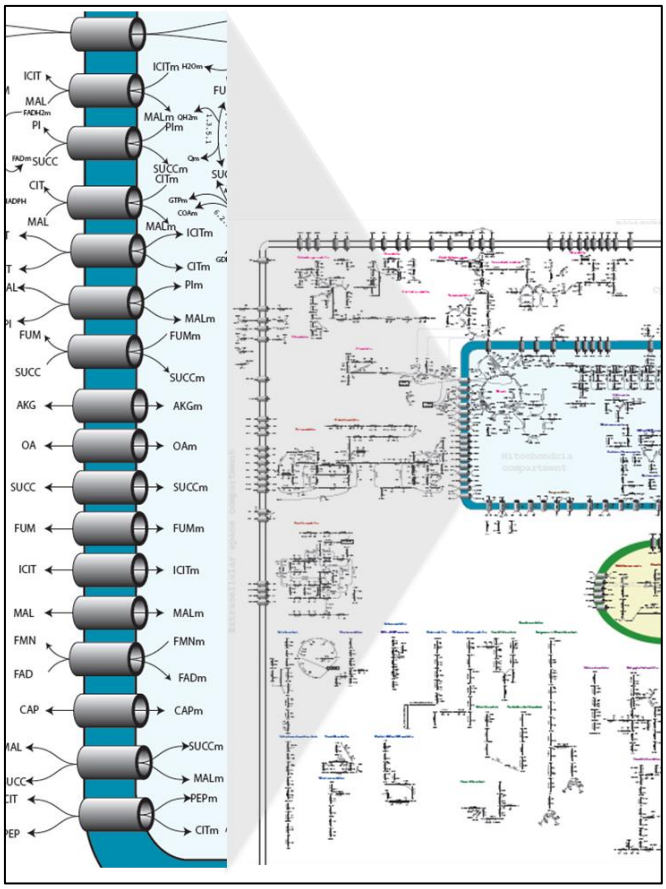


Figure 14: Detail of the graphical representation of the mitochondrial transport reactions of *i*WV1314.

As this would not be feasible in nature, the model was curated with special emphasis on the mitochondrial transporters. Through BLAST comparison of *A. oryzae* genes with other Aspergilli and *S. cerevisiae*, using KEGG [58], Uniprot [28], Cello [157] and NCBI BLAST [5], the latest annotations were taken into account for improved annotation of the *A. oryzae* model.

The resulting model *i*LC1407 contains net 18 additional reactions compared to the available model from the Sysbio Toolbox [30], which is an update in which some bugs of iWV1314 were fixed. Furthermore 38 gene annotations were added and 46 relocated. 28 of the transport reactions were removed, as no homolog genes were found in *A. oryzae* (Table 4).

Table 4: Model properties of iWV1314, the update available on Sysbio.se and the latest update iLC1407

Characteristics	***i*WV1314**	**Vongsangnak; Ågren Updates**	***i*LC1407**
Reactions	1846	2328	2346 (+18)
Metabolites	1073	1264	1265 (+1)
Genes	1314	1369	1407 (+38 46 relocated)
Transport reactions	281	457	429 (-28)
Compartments	4	4	4

Predictions of biomass yields on different carbon sources were made using *i*LC1407 and very good fits were obtained using pure and mixed carbon sources (Figure 15). Then the metabolic functions of *A. oryzae* and *A. niger* were analyzed in order to investigate the differences of both species that diverge the metabolism for the production of malic acid and citric acid under similar culture conditions. Through the analysis of gene orthologs, it has been observed that both organisms possess almost the same metabolic machinery including proton transport and electron transport chain. Hence the *in-silico* production of TCA intermediates of both GEMs showed no differences. These results suggest that regulatory functions or varying enzyme transport capabilities are related to the production of malic acid in *A. oryzae* and citric acid in *A. niger*.

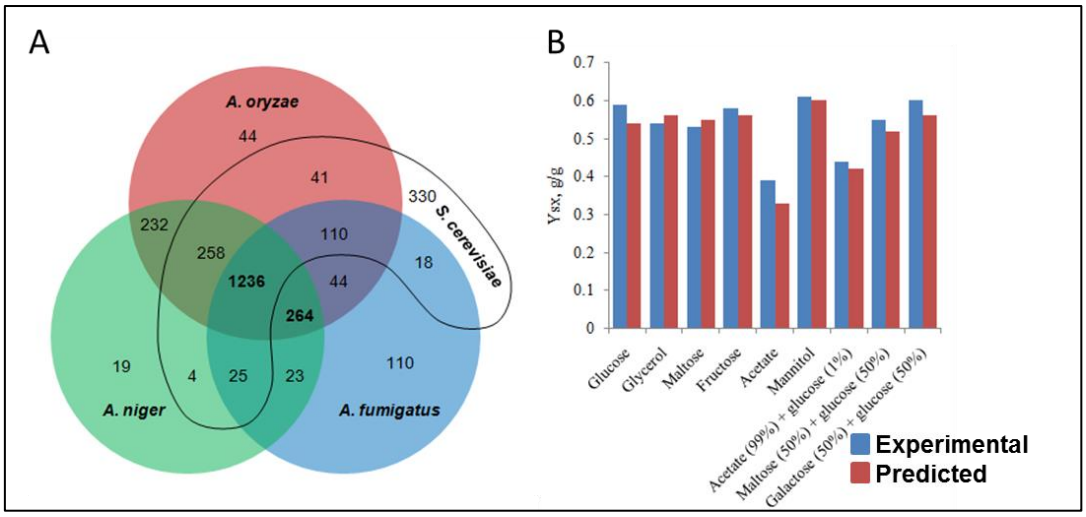


Figure 15: Venn diagram of homolog genes in *A. niger*, *A. oryzae*, *A. fumigatus* and *S. cerevisiae* (A) and comparison of predicted biomass yields (Y_{SX}) using the updated model *i*LC1407 and experimental data on different carbon sources [22, 110, 123]

Furthermore different knock out scenarios were calculated using the random sampling algorithm [16] and experimental data from malic acid fermentations that were used as external fluxes. *In silico* the TCT (citrate/malate antiporter), the aspartate/malate shuttle, the oxaloacetate/alpha keto glutarate transporter and combinations of these were knocked out. Other than expected, all *in silico* mutants were able to grow and fulfill the constraints. The growth ability should have been hampered significantly by the TCT deletion, as the cytosolic acetyl-CoA supply was supposed to be hampered. Cytosolic acetyl-CoA is needed for fatty acid and sterol synthesis and protein acetylation [55]. There are two main pathways considered for the supply of cytosolic acetyl-CoA. The first route is mainly used by e.g. *S. cerevisiae* or *Candida albicans*, which converts acetate that originates from pyruvate via acetyl-CoA synthetase to acetyl-CoA. The second route utilizes ATP-citrate lyase which converts cytosolic citrate to acetyl-CoA and oxaloacetic acid. As shown for *A. nidulans*, the deletion of the ATP-citrate-lyase, which converts citrate to acetyl-CoA, is greatly diminishing growth on carbon sources that do not result in cytosolic acetyl-CoA [55], indicating that the acetyl-CoA synthetase is not able to supply acetyl-CoA in e.g. glucose containing medium.

As the main transporter for citrate out of the mitochondrion into the cytosol was removed, there should be no possibility to generate cytosolic acetyl CoA in our calculations. But instead the model used the pyruvate decarboxylase route, as the enzymes needed for that route are present in *A. oryzae*. This result shows the limitations of FBA and stoichiometric models, they do not consider regulation, but give you the best solution possible with the set of reactions/genes the model/organism contains. On the one hand this could be assessed as a flaw, as it is not accurately reflecting the natural behavior. On the other hand it opens the possibility to speculate about evolutionary engineering a strain that is not able to generate acetyl-CoA as the citrate route is blocked to relief repression on the acetyl-CoA synthetase

route, thereby generating a strain that is not producing citrate, but increased malate and can still grow on glucose as sole carbon source.

The above mentioned tricarboxylate transporter is not described for Aspergilli, but the *S. cerevisiae* proteins Yhm2p and Ctp1p were shown to have citrate transport abilities [37]. Yhm2p is supposed to be a component of the citrate-oxoglutarate NADPH redox shuttle without malate transport capabilities and to play a role in replication and segregation of the mitochondrial genome [24]. For Ctp1p citrate and malate transport abilities have been proven, whereas the K_m is significantly higher than reported for mammalian systems [59]. A BLAST search of Cpt1p against the *A. oryzae* RIB40 genome returned several positive genes. Out of these, two genes, AO090020000012 and AO090102000454 showed a significant up-regulation comparing starvation against the growth phase (Data obtained in the first study, Table 5). As AO090020000012 was among the genes most probably encoding for a transporter with citrate-malate antiport function, this gene was deleted in the *A. oryzae* strain NRRL3488. But in initial shakeflask cultivations, the deletion strain did not show a significant difference in the profile of secreted organic acids.

Table 5: Comparison of transcriptional changes of *A. oryzae* genes, which show a certain degree of homology to the *S. cerevisiae* Ctp1p, a tricarboxylate transporter gene. Transcriptional comparison of expression levels between the starvation and the growth phase.

Gene	**Sequence identity to *S. cerevisiae* Ctp1p**	**adj,P,Val**	**logFC**
AO090020000012	49.8%	0.000317967	0.501386012
AO090023000454	49.0%	1.27854E-05	0.454117181
AO090005000048	39.1%	0.159778469	-0.061435612
AO090102000125	32.4%	0.436944134	-0.076882676

Another question that arose from the project on the engineered strain was the question of the origin of the detected succinate. 2103a-68 was not only producing increased levels of malate, but also increased amounts of succinate. This led to the speculation, that there is a direct continuation of the reductive TCA branch from malate via fumarate to succinate in the cytosol. This question was addressed by measuring the summed fractional labelling of the secreted malic acid and succinic acid by GC-MS. Furthermore the summed fractional labelling for cytosolic and mitochondrial derived malate and succinate were simulated (Figure 16).

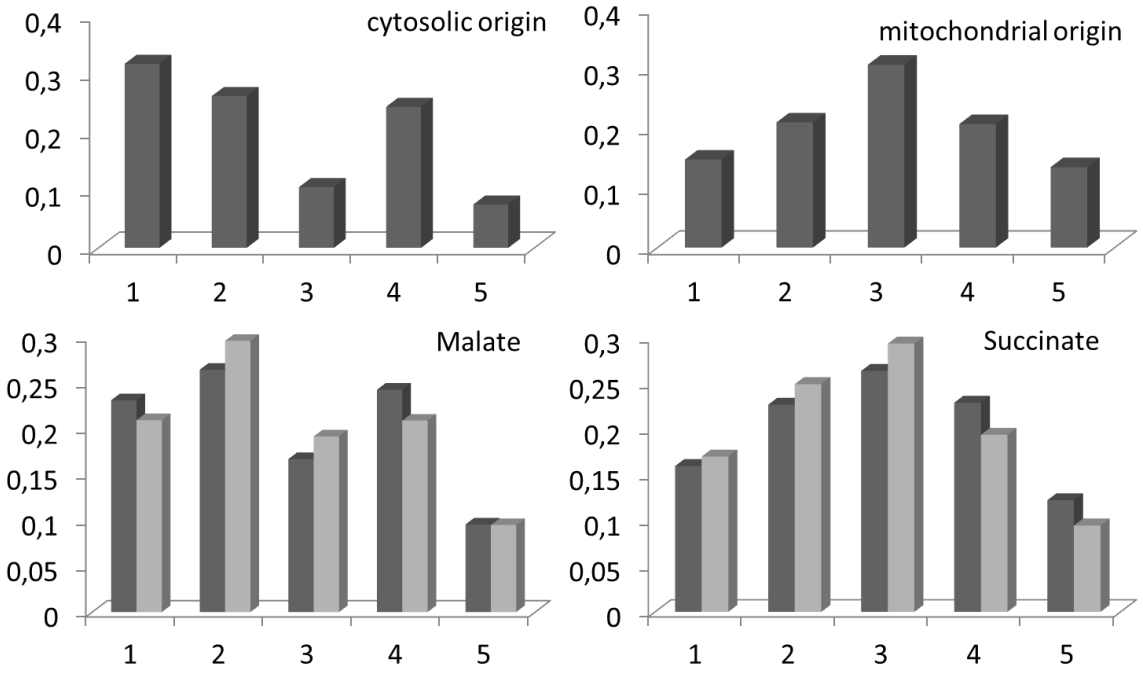


Figure 16: Pattern of the summed fractional labelling. Upper part, simulated pattern for cytosolic and mitochondrial derived malic acdi. Lower part, fitting of simulated and experimentally derived SFLs for malate and succinate. Dark bars, fitted SFLs; bright bars, measured SFLs.

The pattern of summed fractional labelling of malate resembled the one of the cytosolic route to a large extend, with minor contributions from the TCA cycle. The labelling pattern of the succinic acid mainly resembled the simulated pattern of the TCA cycle derived succinic acid. Therefore, the idea of a functional pathway from malic acid to succinic acid was dropped and the model structure concerning the reductive cytosolic TCA branch was kept as in iWV1314.

A simpler flux model with the summed fractional labelling of malate and succinate and the external fluxes for malate, succinate, citrate and glucose as input was used to calculate internal fluxes Figure 17. These indicate that 22% of the secreted malate originated from the mitochondrion.

In summary, the model curation led to a “cleaner” picture of the current knowledge on mitochondrial exchange reactions, it is important to stress the current knowledge part, as the knowledge about mitochondrial transport reactions in general and in *A. oryzae* in particular are not that well studied. Based on the current model, it was shown that metabolic possibilities of *A. oryzae* and *A. niger*, extrapolated from the data obtained from the corresponding GEMs, are not that different, which leads to the conclusion, that regulatory mechanisms or enzyme properties make the difference between malic acid and citric acid secretion.

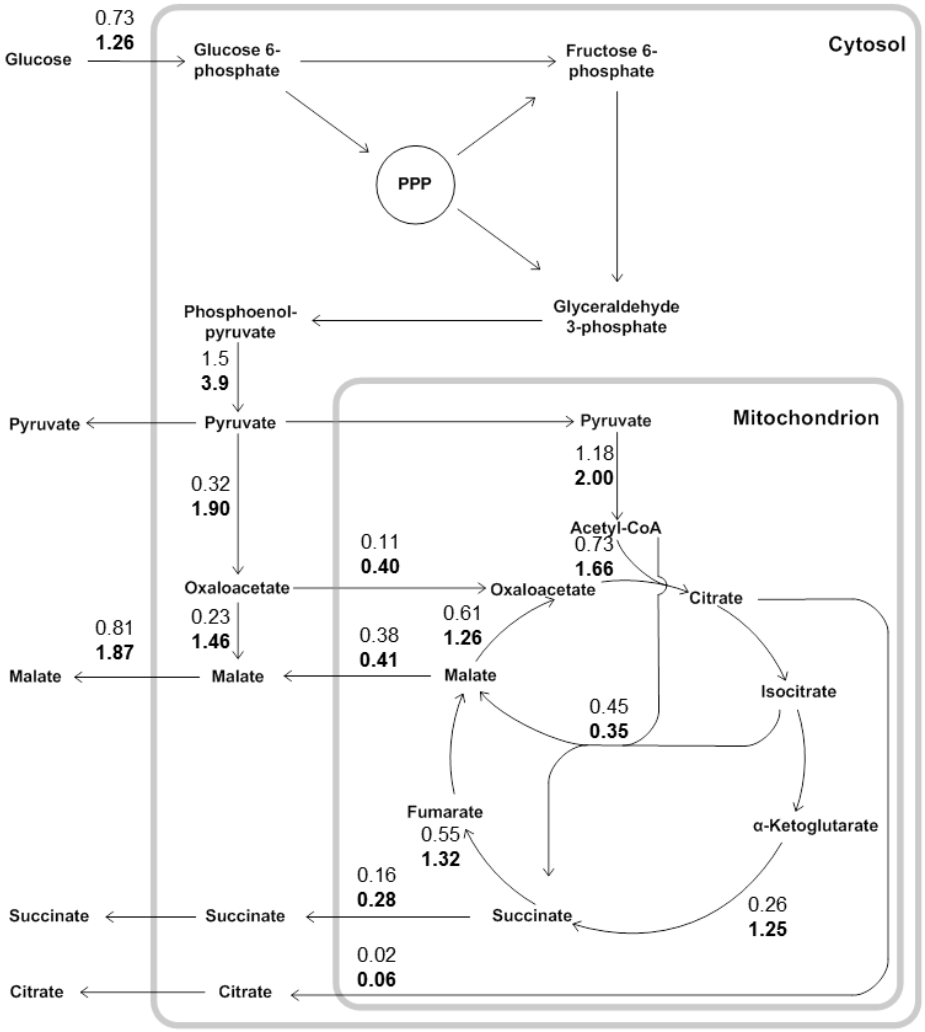


Figure 17: Central carbon metabolism of A. oryzae and internal fluxes calculated according to external fluxes determined for NRRL3488 and 2103a-68 during the stationary phase of a bioreactor cultivation and the summed fractional labelling of malate and succinate from the final sample of a shakeflask cultivation.

This allows for speculations about turning *A. niger*, which can grow at even lower pHs than *A. oryzae*, into an efficient malic acid producer. The deletion of the gene AO090020000012, which was identified to possibly encode for a TCT in *A. oryzae* , did not lead to significant changes in the organic acid production and will require future investigation. The additional deletion of AO090102000454, which might be another gene encoding for a TCT, could be an additional task for the future.

5 Manipulating the carbon source utilization

Most microorganisms prefer to metabolize glucose over any other carbon source [128] and so does *A. oryzae*, as shown in the section 3. Enzymes which are needed for the catabolism of less preferred carbon sources are usually transcriptionally inhibited. This inhibition also contains enzymes participating in the degradation of sugar polymers, like cellulose, hemicellulose and pectin. In Aspergilli, the transcriptional inhibitor CreA is known to be a major player in the complex regulation mechanisms [128]. CreA has been studied intensively in *A. nidulans*. Like *S. cerevisiae*'s Mig1, it contains zinc fingers of the Cys2His2 type, which probably bind to the 5'-SYGGRG-3' consensus sequence. When deleting CreA in *A. nidulans*, Prathumpai et al. [119] reported previously that the subsequently metabolized sugars glucose and xylose were consumed in parallel. Furthermore, culture supernatant of the mutant strain showed elevated xylanase activity, which would be beneficial for a simultaneous saccharification and fermentation process. This would also cut down costs, as the expenses for enzyme mixes for hydrolysis would be omitted.

As shown above, *A. oryzae* is able to consume xylose at high rates and efficiently converts this pentose to malate. Lignocellulosic feedstocks, which would be the preferred carbon source for a truly sustainable production of chemicals through a biorefinery, contain both glucose and xylose. In a batch-cultivation setup the subsequent utilization does not cause problems, but in a continuous cultivation setup, only the glucose fraction would be used for the conversion and the carbon from xylose will not be metabolized. This is of course a major drawback and a parallel utilization, as shown for the *A. niger* CreA mutant, would be desirable. Therefore the CreA gene was deleted in a uracil auxotrophic descendent of NRRL3488.

This pyrG deleted NRRL3488 mutant was transformed with the deletion cassette. This cassette contained the *A. niger* pyrG gene flanked by 1kb DNA fragments which are homologous to the upstream and downstream region of the creA gene. Prototrophic mutants were selected on minimal medium and colonies were subsequently purified on minimal medium. DNA from purified colonies was isolated and PCR was performed using the DNA as template. Primers were constructed to bind upstream and downstream of the integration cassette (Figure 18) and inside the pyrG sequence, running towards the ends. The two primer pairs for upstream and downstream verification amplified a 1.5 kb and 2 kb fragment, respectively. All but one of the in Figure 18 displayed mutants showed the expected bands. As Aspergilli are known to integrate DNA fragments in an ectopic manner, the correct single integration of the deletion fragment was furthermore verified by Southern blotting. As only mutant AOMCK01.09 showed a clear single band, this transformant was used for further analysis of the effect of the *creA* deletion in *A. oryzae*.

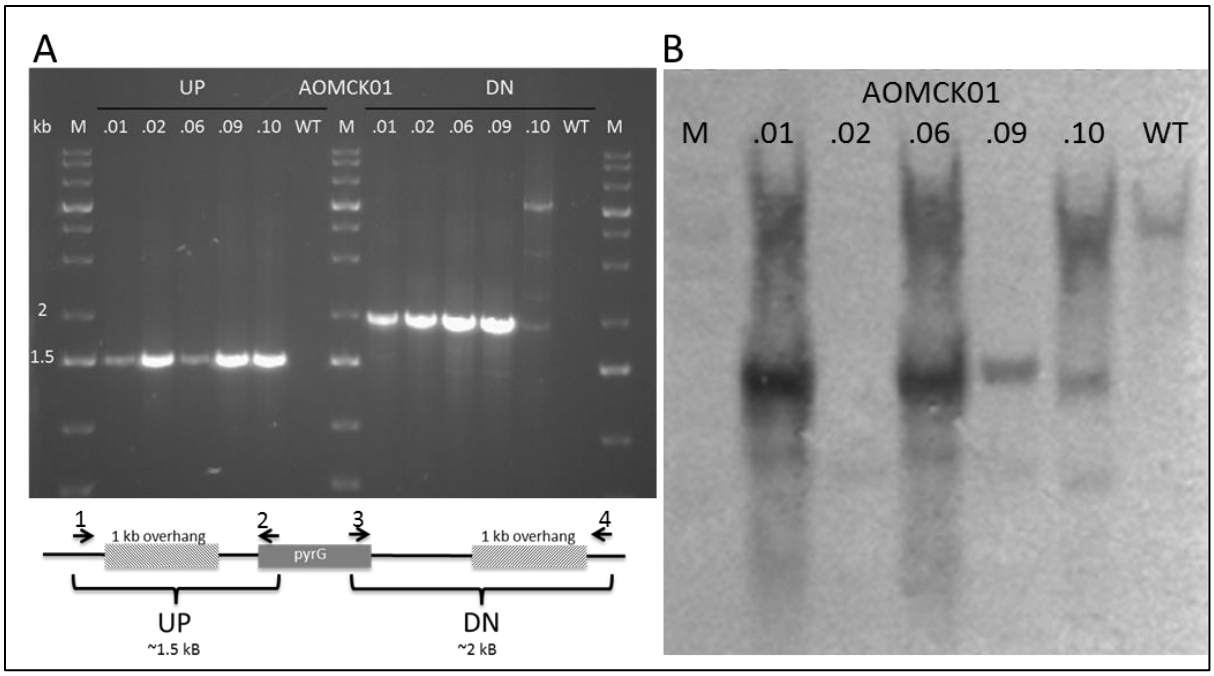


Figure 18: PCR (A) and southern blotting (B) confirmation of the integration of the deletion fragment. M: GeneRuler^{TM} 1 kb Plus Ladder; WT: NRRL3488

The resulting *creA* deletion strain showed reduced mycelial growth compared to the wild-type. The mycelium is very dense in the center of the colony and an uncoordinated network of hyphae is growing outwards, whereas the wild-type spreads evenly in straight radial hyphae Figure 19. Furthermore the mycelium is growing upwards as well in case of NRRL3488, whereas the mutant hyphae are flat on top of the agar. Concerning sporulation, AOMCK01.9 takes much longer time to sporulate and the spores are concentrated more towards the center, whereas in the case of the wild-type, sporulation occurs more evenly after around 4 days.

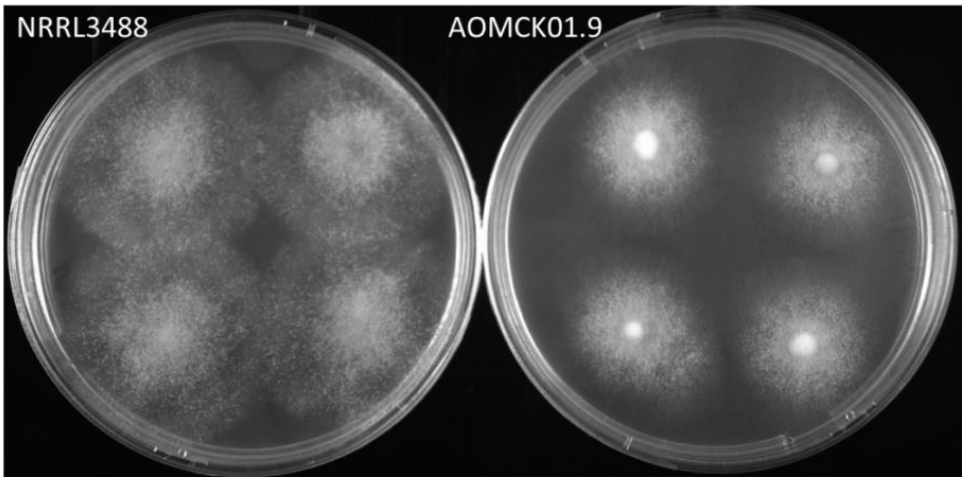


Figure 19: Growth comparison of NRRL3488 and AOMCK01.9 on spore propagation plates.

The first thing to check on the mutant strain was the effect of the *creA* deletion on the carbon source uptake. Therefore the wild-type and mutant strain were cultivated in MAF

medium containing increased nitrogen source, in order to exclude the effect of nitrogen starvation. Both strains consumed the glucose first (Figure 20), until a glucose concentration of about 2 g L^{-1} was reached. This happened at about 10h of cultivation, thereafter glucose and xylose were consumed in parallel and after 13h, when the glucose was completely exhausted, xylose consumption went on until about 25h of cultivation. This result is in disagreement with the results obtained in *A. nidulans*.

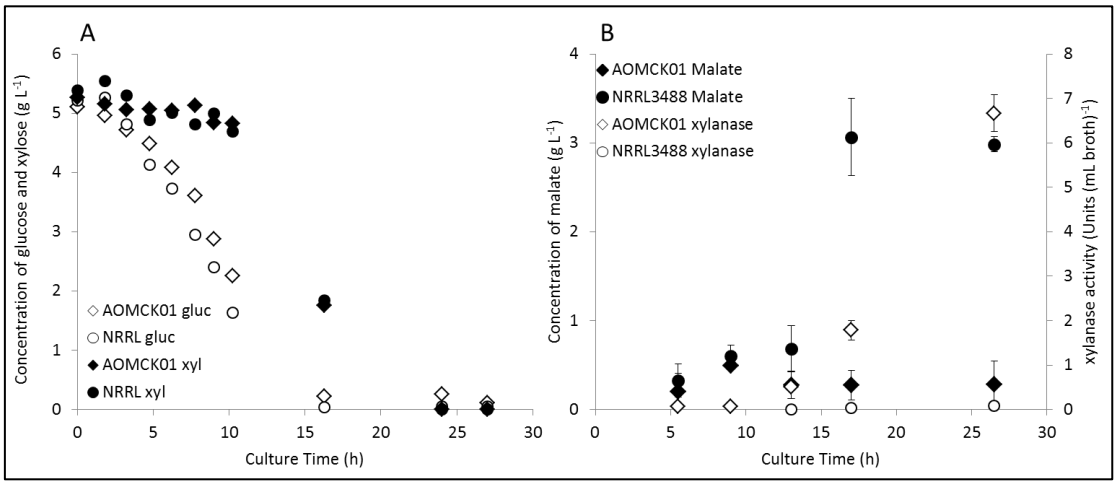


Figure 20: Fermentation profiles of NRRL3488 and AOMCK01.09 during batch cultivations in MAF medium containing 6.4 g L^{-1} ammonium sulfate and 5g of each carbon source. Carbon source concentration (A), malate concentration and xylanase activity (B).

Another trait previously found for an *A. nidulans creA* deletion mutant was the positive influence on the secretion of xylanases. In order to check for the same effect, the same fermentation conditions as above were applied and the xylanase activity of the supernatant was measured. For NRRL3488, no activity could be detected in the first samples, in the sample at 17h, the activity reached 0.045 Units mL^{-1}. In the following samples the activity kept rising to finally reach 0.090 Units mL^{-1}. For the mutant strain on the other hand, xylanase activity could already be detected after 13 h. From there on the activity increased constantly from 0.510 Units mL^{-1} to finally reach 6.668 Units mL^{-1}. This final activity is 74 times higher than measured for the wild-type cultivations. Though the deletion obviously affected the secretion of xylanases positively, the effect of glucose repression either on the xylanase secretion or on the xylose metabolism could not be confirmed.

The initial aim was to construct a strain that could be used in a continuous consolidated bioprocess. As the strain NRRL3488 was previously shown to be able to produce malic acid, the *creA* deleted strain was supposed to be used in a process to produce this C_4 dicarboxylic acid. Therefore the ability of AOMCK01.9 to produce malic acid was evaluated as well. As can be seen in Figure 20, there was hardly any malate detected in the supernatant of the mutant cultivation, whereas about 3 g L-1 were accumulated in the NRRL3488 cultivation. The initial characterization was performed using high amounts of nitrogen in the fermentation broth. In order to simulate the production medium and investigate the malic acid production potential further, the wild-type and the mutant strain were cultivated in MAF medium

containing the usual 1.4 g L^{-1} of ammonium sulfate. The carbon source composition was also varied. In the first case a glucose/xylose mixture, in the second pure glucose and in the third case pure xylose were used at initial concentrations of 30 g L^{-1}, each. The final concentrations of malate are shown in Figure 21. Though the final concentration of malate increased significantly and also the ratio towards the wild-type is not as disproportionate as in the initial comparison, the final malic acid concentration in the mutant broth is less than half of the wild-type.

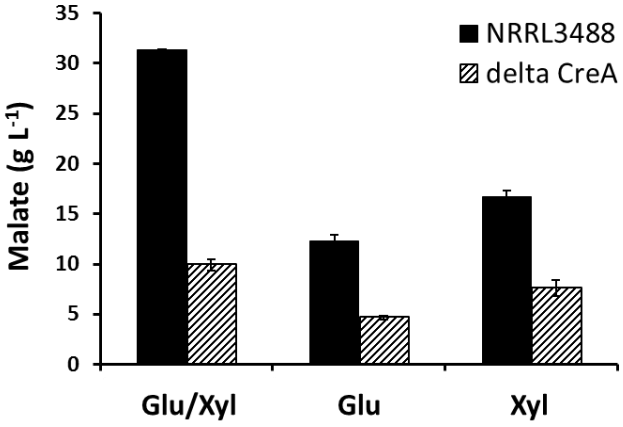


Figure 21: Final malate concentrations of NRRL3488 and AOMCK01.9 after cultivation in three different carbon source compositions. Glu/Xyl, glucose and xylose mixture; Glu, glucose only; Xyl, xylose only; intial concentration of wach carbon source was 30 g L^{-1}.

Taken together, the deletion of *creA* did not lead to the expected effect of creating a strain that can produce malic acid from parallel metabolized glucose and xylose. The strain AOMCK01.9 was producing less than half of the final titer of malate than the wild-type NRRL3488 and consumed glucose before xylose. But the strain showed significantly elevated xylanase activity in the fermentation broth. As the reduced malate production indicates an important role of the deleted gene AO090026000464 in the regulation of the central carbon metabolism, further investigation through for example transcriptome analysis of this strain, would be an interesting follow up project.

6 Conclusion

The objective of the work was to investigate the malic acid production potential of the filamentous fungus *A. oryzae*. Therefore the ability of the wild-type strain NRRL3488 concerning malic acid production was evaluated on different nitrogen sources and the transcriptional changes between the exponential growth phase and the malic acid production phase, which is characterized by nitrogen starvation, was evaluated (**Paper I**). Subsequently an engineered *A. oryzae* strain (2103a-68) was characterized in lab scale fermenters (**Paper II**). This strain also produced significant amounts of citrate as a by-product. In order to perform *in-silico* knock out evaluations the existing model needed to be updated and was refined concerning reactions connected to organic acid production. During the characterization of 2103a-68, the performance of this strain was not only evaluated on the commonly used carbon source glucose, but also on xylose. As the positive results for both carbon sources paved the way towards the use of *A. oryzae* as a platform organism for the production of renewable chemicals in the biorefinery of the future, the carbon source utilization was sought to be de-regulated by the deletion of AO090026000464, which is supposed to encode for the carbon repressor *creA* (**Paper III**).

From the first study we learned that *A. oryzae* wild-type strains have a natural ability to produce significant amounts of malic acid and that they convert glucose to malic acid with half of the maximum theoretical yield, when nitrogen becomes limiting. This is an important trait in order to reach high yields, as the carbon is not incorporated into biomass, but into the product, in this case malic acid. The regulation of this mechanism was investigated through transcriptome analysis, which revealed a general up-regulation of genes involved in the glycolysis, a synchronistic down-regulation of TCA genes and an up-regulation of the rTCA genes in the starvation phase compared to the growth phase. Binding sites for the *S. cerevisiae* transcriptional activator Msn2/4 were found when analyzing the up-stream sequences of the up-regulated genes of the glycolysis and rTCA. This suggests that *A. oryzae* uses malic acid production as a stress response towards nitrogen starvation. The metabolic changes evoked by the nitrogen starvation stress, leading to continued conversion of glucose to malate also opens possibilities to utilize this mechanism to produce other products. One could for example speculate about developing metabolic engineering strategies that divert the flux away from malic acid at the various branch-points on the way towards malic acid. The most important hub is the cytosolic pyruvate pool. A product easily derived from pyruvate with only one enzymatic reaction is lactate, for which the flux would just have to be diverted through the lactate dehydrogenase reaction. Another product with increased interest is 3 hydroxypropionic acid. There are several theoretical pathways from pyruvate to 3HP [147], for most of them the enzymes that would be needed were not reported to occur in nature, yet. One of the working pathways has been reconstructed in *E. coli* and goes in three steps from Pyruvate via Acetyl-CoA and Malonyl-CoA to 3HP [124]. A very interesting but so far only theoretical pathway employs a hitherto unknown malate decarboxylase. Once such an enzyme is found or engineered, it would allow production of 3HP in a single

step from malic acid and in this case the *A. oryzae* NRRL3488 strain would be the perfect production platform.

An even better strain to build on would be the engineered *A. oryzae* strain 2103a-68, which was further characterized in paper 2 and shown to be an even better malic acid producer than the wild-type. The overexpression of the cytosolic TCA branch and the malic acid transporter let to production rates, yields and final titers that are among the highest ever reported for a microbial system. Nevertheless, the pyruvate carboxylase reaction might still be the controlling step in this pathway. In order to overcome this problem, inserting more copies of the *pyc* expression cassette and resulting higher expression, or enzyme engineering might be helpful in this respect. Another observation was the increased citric acid production, which might be a result of the increased activity of the tri-carboxylate transporter, which is supposed to be triggered by high cytosolic malic acid concentrations. A deletion of the corresponding gene in *A. oryzae* could lead to diminishing the citric acid production and to higher yields of malate. During this second project, the performance of the engineered strain was furthermore evaluated on xylose and glucose/xylose mixtures and the positive results confirmed the suitability of this strain to be used in a biorefinery, using e.g. lingo-cellulosic biomass, which mainly consists of those two sugars, as carbon source. Pretreated renewable feedstocks usually contain additional inhibitors and Aspergilli are generally known to be more resistant than other organisms, nevertheless follow-up studies would have to confirm the positive results concerning malic acid production on pretreated biomass.

The issue of alternative carbon source utilization was also addressed in this thesis. As seen from the cultivations with the glucose/xylose mixture, the engineered strain prefers to consume glucose over xylose. For *A. niger* it was shown that the deletion of *creA*, a carbon catabolite repressor, let to simultaneous consumption of both sugars and increased secretion of hydrolytic enzymes. This effect could not be fully confirmed for the deletion of the gene AO090026000464, which is supposed to encode for CreA. The knock-out led to increased xylanase activity, but the carbon sources were still consumed subsequently and the malic acid production was also negatively affected. These results indicate that the regulation of the central carbon metabolism is affected in a way, as well as the carbon repression of secretion of hydrolytic enzymes. In order to further investigate the role of AO090026000464, transcription and metabolite analysis would be interesting.

The initial *A. oryzae* model was constructed with respect to protein production. In order to be able to confidently predict the metabolism of organic acid production, the transport reactions between the mitochondrion and the cytosol were of special interest. As these and other reactions connected to organic acid production were poorly annotated, the annotation and localization of reactions in the *A. oryzae* GEM were revised. This curation led to the deletion of 28 transport reactions for which no annotation could be found, annotation of 38 reactions and re-localization of 46 reactions. The resulting model was able to accurately

predict previously reported results and could be used as a scaffold for further development of metabolic engineering targets for not only protein production, but also organic acid production.

Nevertheless, the GEMs of the Aspergilli and the one of *A. oryzae* in particular are not as concise as the one of the model organisms like *S. cerevisiae* or *E. coli*. This is mainly due to bad annotation or annotation entirely based on homology to other organisms. Bad gene ontology definitions are a result of the incomplete or inaccurate gene annotation and this hampers high throughput analysis of transcription data. In order to bring systems biology in Aspergillli to a level seen in e.g. *S. cerevisiae*, the community has to make an effort in order to reach more reliable gene annotation and localization in the future.

Taken together, the presented work shows the great opportunities that *A. oryzae* offers for biotechnological applications. The work on malic acid production in this organism extended the possible use not only for the production of enzymes, but also bulk chemicals like organic acids. *A. oryzae* combines several advantages. It has been used safely for several centuries in the food industry. Furthermore large scale production processes have been established for the production of enzymes and the experience can be applied for the production of organic acids as well. In addition it is able to secrete large amounts of hydrolytic enzymes, which can help making the carbon from renewable feedstock accessible for conversion to the desired product. All these advantages make *A. oryzae* the organism of choice for the future biorefinery, which will aid in making the world a more sustainable place. People might think that *A. oryzae* is not as “sexy” as *E. coli* or *S. cerevisiae* and projects might take longer time, but *A. oryzae* has a great potential for the sustainable production of chemicals. After the era of “proof of principle” metabolic engineering in model organisms I hope that *A. oryzae* will be attended to as production host in order to achieve the required yields, titers and rates needed for the economic feasibility of industrial production processes.

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