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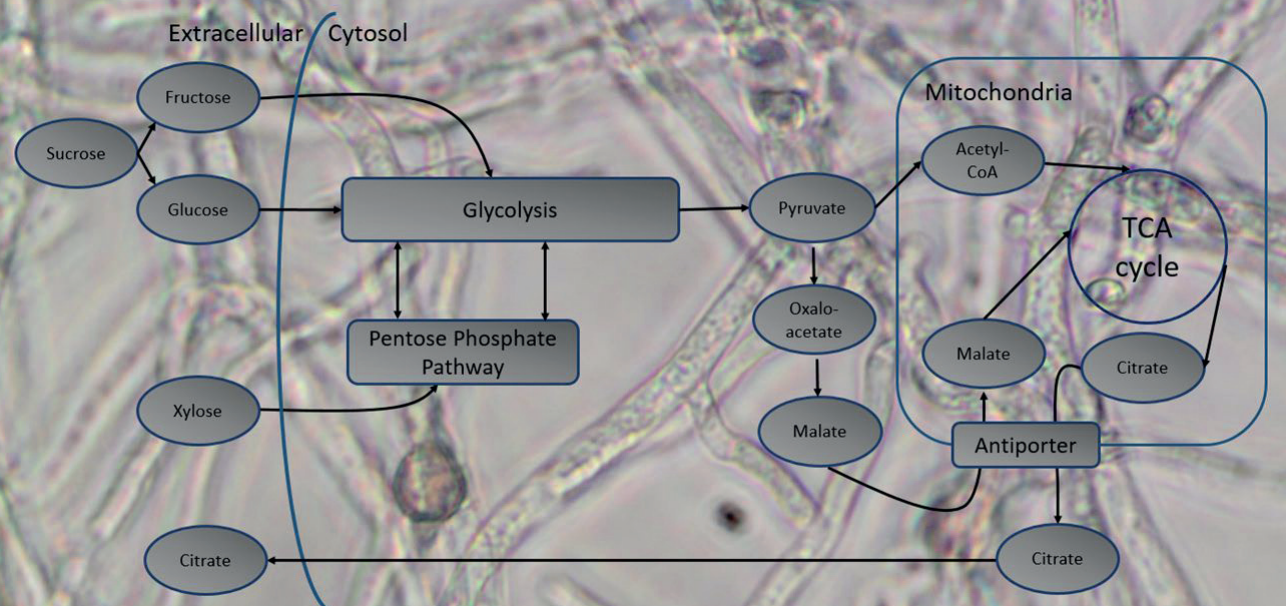

ENHANCEMENT OF ORGANIC ACID PRODUCTION FROM ASPERGILLUS CARBONARIUS USING A METABOLIC ENGINEERING APPROACH

BY
TORE LINDE

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AALBORG UNIVERSITY
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ENGLISH SUMMARY

Impact on the environment and on human health from the use of fossil fuels and fossil fuel dependency are some of the major problems of the 21st century. Biorefineries as a concept can be seen as one part of a solution as it gives the possibility of replacing oil refinery products with sustainable biomass derived substitutes. There are many types of biorefinery systems; the most described type is probably the enzymatic and microbial conversion of lignocellulose to different products, e.g. bioethanol. However, for this type of biorefinery to be economical competitive on the open market, emphasis on high value products like certain chemicals is essential. One way of producing high value products is to use specialized microbial cell factories for the conversion of substrate to products. There are many possible microbial organism that are good candidates as specialized microbial cell factories, *Escherichia coli*, *Saccharomyces cerevisiae, Aspergillus niger* just to mention a few.

The overall objective of the present PhD. study was to investigate and engineer microbial cell factory strains in *Aspergillus carbonarius,* a close relative to *A. niger*, with the aim of enhancing the organic acid production of the strain. The primary acids pursued were the high value malic and fumaric acid, however, citric acid also played an important role due to the natural citric acid producing nature of *A. carbonarius*. The PhD. thesis comprise four manuscripts, the first three describes metabolic engineering approaches altering the genome of *A. carbonarius* the fourth manuscript describes an optimization of a fermentation method.

Five genes were inserted in *A. carbonarius* with the aim of enhancing the cytosolic reductive TCA branch and thereby increasing the malic and fumaric acid production. The genes were the native pyruvate carboxylase gene (*pyc2*), a truncated version of the malate dehydrogenase from *Saccharomyces cerevisiae* (*mdh3*), a truncated version of the fumarase gene from *S. cerevisiae* (*fum1*), a truncated version of the fumarase gene from *Rhizopus oryzae* (*fumR*), and a malate transporter gene from *Schizosaccharomyces pombe* (*mae1*). Three of the enzymes had a significant positive effect on the malic acid production. Pyc2 alone increased the malic acid production with 28%, Pyc2 and Mdh3 increased malic acid with 32%, Pyc2, Mdh3 and Fum1 increased the malic acid with 57%. The results indicate that pyruvate carboxylase and malate dehydrogenase builds up the cytosolic pathway from pyruvate through oxaloacetate to malate and furthermore that fumarase converts cytosolic fumarate to malate, which further increases the accumulation of malate.

The role of the global regulator LaeA on citric acid and lignocellulose degrading enzymes was investigated in *A. carbonarius*. When the gene *laeA* encoding for LaeA was disrupted, the following fermentation experiments showed that citric acid production was decreased with 74-96% compared to the wild type, and

endoglucanase production was decreased with 51-78% compared to wild type. There were no changes in beta-glucosidase or xylanase production. Overexpression of *laeA* gave an increase of citric acid production of 113% compared to wild type, however no increase in endoglucanase production or other lignocellulose degrading enzymes was observed.

Citrate-malate mitochondrial antiporters are believed to control the flux of malate and citrate through the mitochondrial membrane; however, none of these has been identified in filamentous fungi. Seven putative antiporters were identified in the *A. carbonarius* genome and knocked out one by one to investigate their role on citric and malic acid accumulation. The hypothesis was that disruption of a true citrate-malate antiporter would result in a shift from citric to malic acid production. One of the seven putative antiporters showed to have an effect on citric and malic acid production, however, not statistically significant. In the antiporter knockout transformant, the average citric acid production was reduced with 30%, and the malic acid was increased (not quantifiable with percentage). Further investigation will have to be conducted to reach a conclusion as to whether the antiporter is in fact a mitochondrial citrate-malate antiporter.

Batch fermentation experiments are widely used in screening of mutant and transformant strains for phenotypic differences; however, problems with variations between seemingly identical samples are profound. 24-well culture plates were used in an attempt to optimize the fermentation method used in the thesis, with the aim of reducing unwanted variation. Wild type *A. carbonarius* was used as test organism in citric acid fermentation in Erlenmeyer flasks and 24-well plates and the relative standard deviation (RSD) was used as comparison factor. The average RSD from the Erlenmeyer experiment was 29% whereas the average RSD from the comparable 24-well experiment was 12%, which is a 2.4 fold reduction in variance.

The PhD. project successfully altered *A. carbonarius* in three different directions creating phenotypes with multiple altered organic acid production profiles. Especially was the production of malic and citric acid significantly enhanced. The knowledge assimilated from the work can potentially be used to support new projects in *A. carbonarius* or in *A. niger* aiming for organic acid production.

DANSK RESUME

Indvirkninger på miljøet og på menneskers sundhed, som følge af brugen af fossile brændstoffer, og afhængigheden af fossile brændstoffer, er nogle af de store problemer i det 21. århundrede. Bioraffinaderier som koncept, kan ses som en del af en løsning, da de giver mulighed for at erstatte olieraffinaderiprodukter med produkter fremstillet bæredygtigt fra biomasse. Der findes mange typer af bioraffinaderier, den mest beskrevne type er nok den enzymatiske og mikrobielle omdannelse af lignocellulose til forskellige produkter, f.eks. bioethanol. For at denne type bioraffinaderi kan være økonomisk konkurrencedygtig på det åbne marked, kræver det fokus på højværdiprodukter som f.eks. visse kemikalier. En måde at producere højværdiprodukter på, er at bruge specialiserede mikrobielle cellefabrikker til omdannelse af substrat til produkter. Der er mange mulige mikrobielle organismer der er gode kandidater som cellefabrikker, *Escherichia coli*, *Saccharomyces cerevisiae*, *Aspergillus niger* blot for at nævne et par stykker.

Det overordnede formål med dette Ph.d. projekt var at undersøge og fremstille mikrobielle cellefabrikker i den filamentiøse svamp *Aspergillus carbonarius*, en nær slægtning til *A. niger*. Formålet var at øge den organiske syreproduktion. De syrer som primært var eftersøgt var malat og fumarsyre, men citronsyre spillede også en vigtig rolle på grund af *A. carbonarius'* naturlige citronsyre-producerende karakter. Ph.d. afhandlingen består af fire manuskripter, de første tre beskriver forskellige forsøg med genetisk manipulation af *A. carbonarius'* genom, det fjerde manuskript beskriver en optimering af en fermenterings metode.

Fem gener blev indsat i *A. carbonarius* med det formål at øge den cytosoliske reduktive TCA gren og dermed øge malat- og fumarat-syreproduktion. Generne var *A. carbonarius'* eget pyruvatcarboxylase gen (*pyc2*), en trunkeret version af malat-dehydrogenase genet fra *Saccharomyces cerevisiae* (*mdh3*), en trunkeret version af fumarase genet fra *S. cerevisiae* (*fum1*), en trunkeret version af fumarase genet fra *Rhizopus oryzae* (*fumR*), og et malat transporter gen fra *Schizosaccharomyces pombe* (*mae1*). Tre af enzymerne havde en signifikant positiv effekt på malatsyreproduktion. Pyc2 alene øgede malatsyreproduktion med 28%, Pyc2 og Mdh3 øgede malatsyre-produktionen med 32%, Pyc2, Mdh3 og Fum1 øgede malatsyreproduktionen med 57%. Resultaterne indikerer, at pyruvatcarboxylase og malat-dehydrogenase opbygger en cytosolisk stofskiftevej fra pyruvat gennem oxaloacetat til malat og endvidere at fumarase konverterer cytosolisk fumarat til malat, hvilket yderligere øger akkumuleringen af malat.

Den globale regulator LaeA's betydning for citronsyre og lignocellulose-nedbrydende enzymer blev undersøgt i *A. carbonarius* ved hjælp af knockoutforsøg. Fermenteringsforsøg med stammer uden *laeA* genet viste, at citronsyreproduktion

blev reduceret med 74-96% i forhold til vildtypen, og endoglucanaseproduktion blev reduceret med 51-78% i forhold til vildtypen. Der var ingen ændringer i beta-glucosidase eller xylanaseproduktion. Overekspression af *laeA* gav en stigning af citronsyreproduktion på 113% sammenlignet med vildtypen, men der blev ikke observeret stigning i endoglucanaseproduktion eller andre lignocellulose-nedbrydende enzymer.

Mitokondrielle citrat-malat antiportere menes at styre strømmen af malat og citrat gennem mitokondriemembranen, men ingen af disse er blevet identificeret i filamentøse svampe. Syv formodede antiportere blev identificeret i *A. carbonarius'* genom og slået ud én efter én for at undersøge deres betydning for citronsyre og malatsyreproduktion. Hypotesen var, at slukker man for en ægte citrat-malat antiporter, ville det resultere i et produktionsskift fra citronsyre til malatsyre. En af de syv formodede antiportere havde en indvirkning på produktionen af citronsyre og malatsyre, dog var effekten ikke så stor at den var statistisk signifikant. Den gennemsnitslige citronsyreproduktion i denne type transformant var 30% mindre end vildtypen, og malatsyreproduktionen blev øget (ikke procentvis kvantificerbar). Yderligere undersøgelser bør gennemføres for at nå frem til en konklusion om, hvorvidt denne antiporter i virkeligheden er en mitokondriel citrat-malat antiporter.

Batch-fermenteringseksperimenter er meget udbredt i screening af mutant- og transformant-stammer for fænotypiske forskelle, men ofte er der problemer med variationer i resultaterne mellem tilsyneladende identiske prøver. 24-brønds dyrkningsplader blev anvendt i et forsøg på at optimere fermenteringsmetoden som blev anvendt i Ph.d. projektet. Formålet var at reducere uønsket variation. *A. carbonarius* blev anvendt som testorganisme i citronsyrefermentering i Erlenmeyer-kolber og 24-brøndsplader, og den relative standardafvigelse (RSD) blev anvendt som sammenligningsfaktor. Den gennemsnitlige RSD fra Erlenmeyer forsøgene var 29%, mens den gennemsnitlige RSD fra det sammenlignelige 24-brøndsforsøg var 12%, hvilket er en 2,4-gange reduktion i varians.

Det lykkedes i Ph.d. projektet at ændre *A. carbonarius* i tre forskellige retninger, og derved skabe fænotyper med forskellige organiske syreproduktionsprofiler. Specielt var produktionen af malatsyre og citronsyre væsentligt forbedret. Den viden der er samlet fra arbejdet kan potentielt anvendes til at understøtte nye projekter i *A. carbonarius* eller *A. niger*, der sigter efter organisk syreproduktion.

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LIST OF ABBREVIATIONS

A. carbonarius	*Aspergillus carbonarius*
A. niger	*Aspergillus niger*
ANOVA	Analysis Of Variance
BIOREF	biorefinery concept for integrated production of biomedical, biochemical, feed and fuels from selected plant materials
BLAST	Basic Local Alignment Search Tool
CTP	Citrate Transport Protein
GRAS	Generally Recognized As Safe
HPLC	High-Performance Liquid Chromatography
JGI	Joint Genome Institute
LaeA	Loss of Aflatoxin-related gene Expression-A
MycoFuelChem	MYCO-fuels and MYCO-chemicals: Consolidated bioprocessing of biomasses into advanced fuels and high value compounds in fungal cell factories
PCR	Polymerase Chain Reaction
RSD	Relative Standard Deviation
S. cerevisiae	*Saccharomyces cerevisiae*

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CHAPTER 1. THESIS OUTLINE

The present Ph.D. thesis is composed of an Introduction, a collection of Manuscripts and a summarizing Discussion.

The overall objective of the present Ph.D. project was to genetically alter ***Aspergillus carbonarius*** to construct a **microbial cell factory** strain with enhanced organic acid production capabilities. The main goal was to make a strain for the use in **biorefineries** that could produce the **high value chemicals, malic and fumaric acid**. The primary method applied for the work was **metabolic pathway engineering** using a molecular laboratory-based approach. The transformed strains were investigated using a **fermentation screening** approach and the results were analyzed using **well-established statistical methods**.

In the introduction is given a brief description of the above-mentioned concepts (bold). This will hopefully give both background knowledge and a perspective to appreciate the four manuscripts in the next section. Manuscript I, II and III are a description of three different metabolic engineering approaches on how to enhance or alter the organic acid production from *A. carbonarius*. Manuscript IV is a description of an optimization of the fermentation method used in this thesis work, with the aim of reducing unwanted variation in the results. The laboratory work behind the four manuscripts were finished in a different chronology than the one depicted here; the work behind manuscript IV was finished first, followed by I, II and then III. However, much of the work was timewise intertwined.

MANUSCRIPT I

Engineering of the reductive TCA branch for enhanced carboxylic acid production in *Aspergillus carbonarius*

Tore Linde, Niels Bjørn Hansen, Mette Lübeck, Peter Stephensen Lübeck

Manuscript in preparation

The first attempt to genetically engineer *A. carbonarius* and to alter the organic acid production was carried out in the footstep of especially two other successful studies, one in *S. cerevisiae* by (Zelle et al. 2008) and one in *A. niger* by (de Jongh & Nielsen 2008). The laboratory work was carried out over a period of two years, and included the handiwork of three laboratory technicians and one PhD student beside the undersigned. The setup was to insert four genes that encode for the enzymes, which are responsible for the conversion pathway from pyruvate to malic and fumaric acid, and one gene encoding for a malic acid transporter. The work was initially set out with two separate aims; (1) to make a strain that produces malic acid, and (2) to make a strain that produces fumaric acid. However, these two directions required so much of the same work that it made more sense to join the two attempts into one. In the end, only the malic acid direction succeeded. The fumaric acid direction was halted due to firstly not having enough of the right transformants, and secondly due to problems with the cloning of a new fumarase gene.

MANUSCRIPT II

The global regulator LaeA controls production of Citric acid and Endoglucanases in *Aspergillus carbonarius*

Tore Linde, Marta Zoglowek, Mette Lübeck, Peter Stephensen Lübeck

Intended for submission to Journal of Industrial Microbiology and Biotechnology

In manuscript I, the approach that was used to alter the metabolic pathway was that of an intuitive engineering approach. This means that if we want to make malic acid, we insert enzymes we know are responsible for converting other metabolites into malic acid. In manuscript II, a different and more indirect approach was used. From the University's associates from Washington State University, the undersigned was made aware that the global regulator gene *laeA* might somehow have an effect on citric acid production in *A. niger*. However, it was not clear how this effect worked. This information was used to make the work behind manuscript II. Knockout and overexpression of *laeA* in *A. carbonarius* gave a decrease and an increase in citric acid production, respectively. As a spinoff, a co-worker tested the positive transformants for other effects and found out that also endoglucanase production was decreased in the knockout transformants. The laboratory work originally included two different *laeA's* that was identified in the *A. carbonarius* genome. However, one of the genes turned out to be either an artefact in the genome assembly or a partly and non-functional gene and was therefore excluded from the manuscript.

MANUSCRIPT III

Knockout of putative Citrate-Malate antiporters in *Aspergillus carbonarius* and the effect on di- and tri-carboxylic acid production

Tore Linde, Marta Zoglowek, Mette Lübeck, Peter Stephensen Lübeck

Intended for submission to Journal of Industrial Microbiology and Biotechnology

A number of lessons were learned during the laboratory work conducted by either the undersigned or co-workers during this PhD period. When we attempted to alter the metabolic pathways to make other organic acids than citric acid, the outcome was very often that we only saw changes in citric acid production, and no other organic acid enhanced. It was hypothesized that the reason for this was that of a malate-citrate mitochondrial antiporter as has been suggested by several authors (Röhr & Kubicek 1981; de Jongh & Nielsen 2008). In Manuscript III, it was attempted to identify this antiporter and knock it out to see if that possibly could change the organic acid production in *A. carbonarius*. This was performed with some success. However, because of lack of time in the end of the Ph.D. period, the experimental work had to be stopped before a completely and clear conclusion could be made. A part of the experimental work also included overexpression of two of seven putative antiporter candidates. Yet, no useful results were obtained from these experiments. Therefore, this part of the work was excluded from the manuscript. The laboratory work was conducted together with a Master student as a part of her Master's thesis and a post doc in training.

MANUSCRIPT IV

Fermentation in 24-well plates is an efficient screening platform for filamentous fungi

Tore Linde, Niels Bjørn Hansen, Mette Lübeck, Peter Stephensen Lübeck

Published in Letters in Applied Microbiology

Manuscript IV is different from the other manuscripts in the way that it is not based on molecular engineering of *A. carbonarius* but on optimization of a fermentation method. Early in the Ph.D. period, it became clear that the fermentation screening method that was used gave high variations in the results even between wild type samples that were supposed to be identical. This was problematic since the fermentation screening was meant to be the primary test method for transformant and mutant strain screening. Together with another PhD. student, I investigated a method with fermentation screening in 24-well plates that turned out to have a significantly lower variation compared to the original Erlenmeyer flask setup. This 24-well screening method was afterwards used for fermentation screening in the following laboratory work leading to manuscript I, II and III.

CHAPTER 2. INTRODUCTION

One of the major global challenges of the 21st century is a growing energy demand, which is fueled by a fast growing population in the world and high rising economies especially in developing countries. This has a big impact on the global fossil fuel demand; the combined oil, coal, and gas usage for energy was 209.4 mboe/d (million barrels oil equivalent/day) in 2010 and is predicted to rise to a staggering 321.7mboe/d in 2040. Of this amount, the global oil usage for energy was 81.8 mboe/d in 2010 and is predicted to rise to 99.6mboe/d in 2040. If the usage of oil for other applications than fuel and energy is added to this prediction, the global oil demand will reach 111mboe/d in 2040 (Abdul-Hamid et al. 2014). A consequence of an increasing usage of fossil fuels is a growing impact on the environment. One of the main concerns is the global warming that comes from an increase in greenhouse gasses where the use of fossil fuels is a major contributor by emitting CO_2 into the atmosphere. The effects of global warming is predicted to be many folded, e.g. change the weather to be more extreme, increase the sea level due to melting of glaciers and permafrost, change the life of numerous plants and wildlife species due to changing of climate zones, and increase the epidemiology of infectious diseases. These are just a few of many hundreds predicted effects from global warming (VijayaVenkataRaman et al. 2012). The use of fossil fuels also have an effect on many other environmental factors, air pollution with carbon monoxide, nitrogen oxides, sulfur oxides and particles, water and land pollution from oil spills and mining activities, just to mention a few (Vallero 2014; Ivshina et al. 2015). Further, air pollution alone is believed to be the single greatest reason for premature death in the world; and the World Health Organization (WHO) estimated that 7 million people died as a result of air pollution in 2012 (World Health Organization 2014).

There are many solutions on how to deal with the environmental effects from the use of fossil fuels or how to avoid them, and many are indeed needed. In this thesis, the biorefinery concept is looked upon as a solution to lower the oil demand by substituting oil-derived products with biomass-derived equivalents. A great deal of global oil use is for energy and fuel, approximately 84-92 % (Abdul-Hamid et al. 2014; IEA 2014). However, today on the global market, there are more than 2500 different oil-based products. Almost everything we use in our life is to a lesser or greater degree dependent on organic carbon that being shelter, medications, tools, clothes, etc. (Hongbin & Wang 2013). The majority of these products can be substituted by biomass-based equivalents produced with building-block chemicals from biorefineries, and fuel and energy can be substituted with biogas, bioethanol and biodiesel (Bozell & Petersen 2010; Sauer et al. 2008). However, biorefineries are in many areas still in its developing stage and there are many challenges that have to be solved before global commercialization can be completed. Some of the major challenges are: Increasing feedstock yields, improving enzymes, optimizing

microbial cell factories, and integrating the products in to existing value chains (King et al. 2010). The aim of the research conducted in this thesis has been to optimize **microbial cell factories** for the use in **biorefineries**.

2.1. BIOREFINERY

The biorefinery as a concept, is regarded as a facility that converts biomass in to fuel, power, and chemicals by using processes and equipment (Demirbas 2009; Sauer et al. 2008). One of the main ideas behind the biorefinery is to be able to replace oil-derived products from the petrol-chemistry industry. Like with petroleum, biomass has a complex composition that – with appropriate separation and subsequent processing – leads to a whole palette of products, comparable to what is seen from a petroleum-refinery. However, it is believed that for a biorefinery to be economically feasible, the portion of high value chemicals relative to fuel and energy has to be higher than that of a petroleum-refinery (Figure 2.1) (Kamm & Kamm 2006).

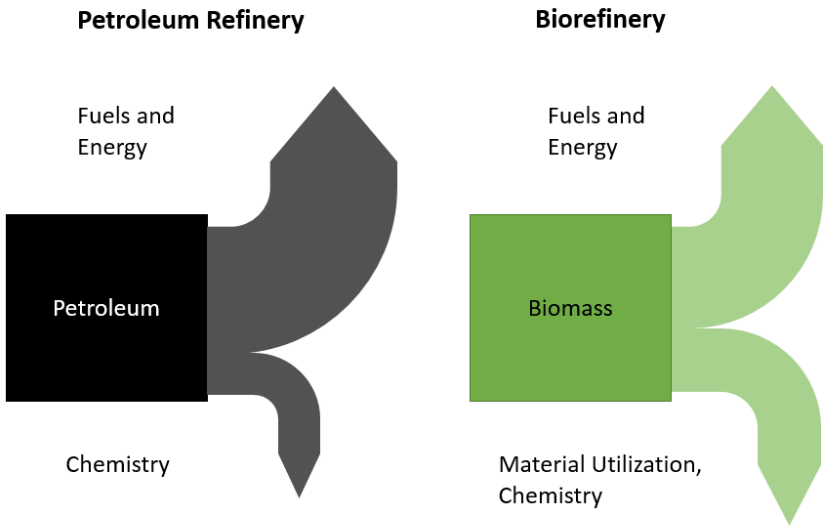

Figure 2.1 Comparison of the basic principles of biorefinery and petroleum-refinery (Kamm & Kamm 2006).

Descriptions of processes within biorefinery are very diverse. Examples are bio-oil production by pyrolysis, Fischer–Tropsch liquids from biomass, supercritical liquefaction, char production by pyrolysis, gaseous fuels from biomass, hydrothermal liquefaction of biomass, and biochemical processes of biomass (Demirbas 2009). Furthermore, Demirbas (2009) suggests that there are seven different types of

biorefineries, 1) fast pyrolysis-based biorefineries; 2) gasification based biorefineries; 3) sugar based biorefineries; 4) green biorefinery; 5) energy crops biorefinery; 6) oilseed biorefinery; and 7) forest based and lignocellulosic biorefinery. The type of biorefinery, which the research in this thesis is aimed for, is the lignocellulosic biorefinery (7) that uses biochemical processes for conversion of biomass. The concept is here briefly explained (Figure 2.2).

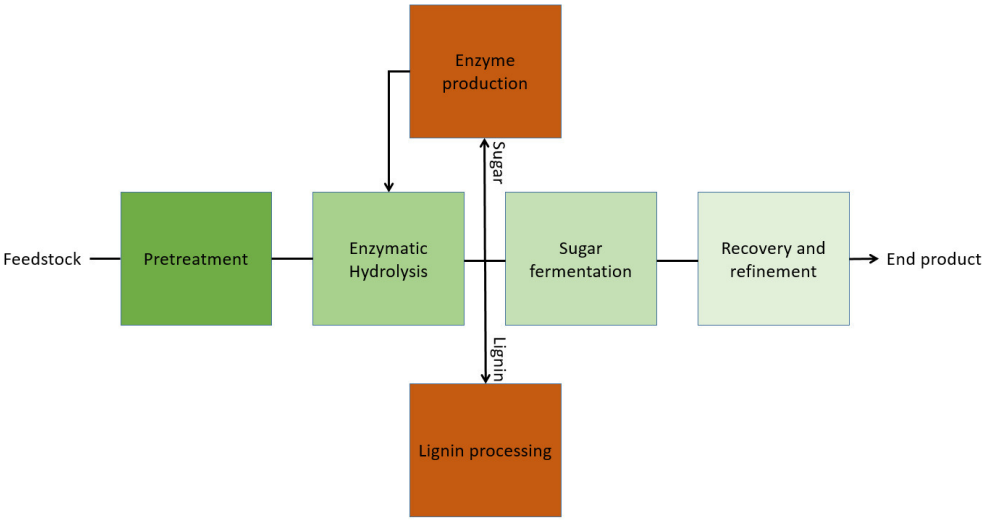

Figure 2.2 Simplified representation of the lignocellulosic biorefinery that uses biochemical processes for conversion of biomass (Tolan 2002).

A lignocellulosic feedstock uses e.g. wood or straw, and the first step is **pretreatment** of the feedstock in order to open up and increase the surface area of the material by destroying the fibrous structure. The pretreatment is often done mechanically, one example is milling followed by e.g. steam explosion with addition of acids. The pretreatment makes the cellulose, hemicellulose, and lignin accessible for the next step, the **hydrolysis**. Hydrolysis of cellulose and hemicellulose converts the long polysaccharides in to short C6 and C5 sugars respectively. Most often enzymatic hydrolysis with cellobiohydrolase, endoglucanase, and beta-glucosidase is used. The sugar fraction is separated from the lignin fraction and the sugar fraction is converted in to the primary product by **sugar fermentation** with microorganisms. Examples of primary product formation could be citric acid production by *Aspergillus niger,* ethanol production by *Saccharomyces cerevisiae* or cellulase production by *Trichoderma reesei*. Finally, the product is **recovered and refined** to the end-product (Tolan 2002; Goldberg et al. 2006; Jourdier et al. 2013). It is worth mentioning that the lignin fraction also serves as a feedstock for other types of production platforms. However, these technologies are still in their infant stages (Ragauskas et al. 2006).

Numerous variations exist within lignocellulosic biorefineries. Three examples of lignocellulosic biorefinery concepts that have been developed in collaboration with co-workers from Section of Sustainable Biotechnology, Aalborg University, are briefly mentioned here. 1) The patented MaxiFuel concept is a combined biological production of ethanol, methane and hydrogen from lignocellulosic materials. The key component of the concept is to minimize the waste-stream from the production by integrating the conversion of three products thereby maximizing the net energy production and the economic feasibility of a biorefinery (Ahring & Westermann 2007). 2) The BIOREF project is a biorefinery concept for integrated production of biomedical, biochemical, feed and fuels from selected plant materials. The production is focused on various high value products and the main development focus is on fungal enzyme production for enzymatic hydrolysis as well as genetically transformed fungal strains for organic acid production (Ahring et al. 2012). The BIOREF project was a precursor for 3) the MycoFuelChem project that also includes this present PhD. project. The MycoFuelChem biorefinery concept is based on consolidated bioprocessing of biomass into advanced fuels and high value compounds in fungal cell factories. The research focus was on developing transgenic fungal strains for three purposes. - Production of enzymes for enzymatic degradation of residual plant material, - production of biofuels, - and production of high value organic acids (Ahring et al. 2015).

In these examples of biorefineries, microorganisms carry out the sugar fermentation. These microorganisms are referred to as 'microbial cell factories' or just '**cell factories**'.

2.2. CELL FACTORIES

The name 'cell factory' can be traced back to at least 1981 where Günter J. Hammerling wrote about 'cell factories of the future' (Hammerling 1981). However, there is no clear definition in the literature of what a 'cell factory' is despite the fact that the concept is heavily used. There is consensus in the literature that a cell factory is a living organism that makes products, which the human society utilizes (Danchin 2004; Kavščček et al. 2015; Meyer et al. 2011). Further, there is a tendency towards an understanding that for a cell factory to exist it cannot be too big, thus production of compounds from plants or other higher eukaryotes are not often described as cell factories. However, occasionally terms like 'plant cell factories' are used (Verpoorte et al. 2000; Danchin 2004). In the other end of the size-scale, an organism does not have to be single-celled to receive the nametag 'cell factory', e.g. multicellular filamentous fungi are often referred to as cell factories (Meyer et al. 2011). To summarize, the cell factory concept encompasses 'living microbial organisms designed by human or nature able to produce compounds that are used by the human society'. Three different types of microbial cell factories can be deducted from literature. **The first type** is also the oldest one and refers to a naturally occurring microbial prokaryotic or eukaryotic cell with its genetic code intact. These types of

cell factories have been used for thousands of years to help the production of e.g. bread, beer, cheese, and wine, and are still used massively today (Kavšček et al. 2015; Danchin 2004). Today, these natural types of cell factories are used for hundreds of different production applications, e.g. production of citric acid from *A. niger*, production of ethanol from *S. cerevisiae*, production of penicillin from *Penicillium chrysogenum* and production of cellulases from *T. reesei* (Tolan 2002; Goldberg et al. 2006; Jourdier et al. 2013; Hoff et al. 2008). **The second type** of cell factory has only been around for a couple of decades and comprises all the microbial eukaryotic and prokaryotic cell factories that have been deliberately genetically altered or altered in the cellular mechanisms by other means. The first genetically altered strains date back to 1972 where the use of restriction enzymes and ligases made it possible to insert foreign DNA into living cells (Jackson et al. 1972; Mertz & Davis 1972). Today, these types of cell factories are used for numerous different applications, including the applications that years back were carried out by 'naturally occurring cell factories'. The products are often heterologous produced, and the microorganisms are very often optimized beyond what can be seen naturally occurring in our surroundings (Kavšček et al. 2015; Danchin 2004). Examples of heterologous protein expression are human interleukin 6 from *A. niger* and human serum albumin from *Pichia pastoris* (Nevalainen et al. 2005). **The third type** of microbial cell factories are the youngest, and the very first step was taken in 1989 where the first non-natural proteins were constructed by inserting non-natural amino acids in to polypeptides (Noren et al. 1989; Bain et al. 1989). These cell factories are the prokaryotic and eukaryotic microbial cells where the genetic code itself has been reprogrammed to look completely different from what we have seen before. This allows variations of the building blocks of macromolecules to go beyond what is naturally feasible, leading to fascinating new aspects of the cell factory concept (Hirao et al. 2002; Hohsaka & Sisido 2002). Arguably, a **fourth type** of microbial cell factory could be included into the list, protein assembly in vitro. Yet, this type of cell factory lacks an actual cell. For many decades it has been possible to amplify DNA, to synthesize RNA, and generate cDNA in vitro but the last step to a fully functional protein has not been possible outside of a cell. However, during the last two decades the methods for protein assembly in vitro has developed immensely and today it is possible to produce pure protein in a cell-free translation system that uses all the necessary translation factors in vitro (Swartz et al. 2004; Shimizu et al. 2001). In this thesis, the research has been aimed at constructing cell factories of 'type two' in *A. carbonarius* to enhance production of **high value chemicals.**

2.3. HIGH VALUE CHEMICALS FOR BIOREFINERY PRODUCTION

One of the issues of biorefineries is that there are so many different chemicals that potentially can be produced biologically that research aimed to develop the necessary biotechnology tools face the risk of being very widespread (Bozell & Petersen 2010). Hence, to narrow down the research and development field, effort has been put into

identifying the most economically and environmentally promising candidate chemicals. In 2004, the United States Department of Energy published a large study aimed to find building block chemicals that can be produced from sugars via biological or chemical conversion. The study aimed to identify top candidates and found among 12 other chemicals that the 1,4-dicarboxylic acids, malic, fumaric, and succinic acids were candidates to be high value chemicals (named 'Top value added chemicals') (Werpy et al. 2004). In 2006, this study was followed up by a report from European Commission's GROWTH Program that described bulk chemical candidates derived from biomass feedstocks produced by fermentation or enzymatic conversion. Again, the 1,4-dicarboxylic acids, malic, fumaric, and succinic acids were on the list, which among others also included citric acid (Patel et al. 2006). In 2010 and 2012 the 15 'top value added chemicals' were evaluated and adjustments were made, including removal of malic and fumaric acid from the list. However, the removal was primarily based on the fact that biological malic and fumaric acid production had received limited research activity and not because the market or the potential of malic and fumaric acid had changed (Bozell & Petersen 2010; de Jong et al. 2012). When looking at the global market demand of the 1,4-dicarboxylic acids, it is obvious to see that malic and fumaric acid is in high demand and that the market potential is equal to that of succinic acid. The global malic acid demand in 2012 was 63.7k tonnes and predicted to grow above 80k tonnes in 2020 (Grand View Research 2015b). In 2012, the global fumaric acid demand was 225.2k tonnes and predicted to grow above 330k tonnes in 2020 (Grand View Research 2015a). And for comparison in 2014, the global succinic acid demand was 47.5k tonnes and predicted to double in 2022 (Grand View Research 2015c).

The scope of the thesis was to develop a fungal strain with enhanced production capabilities of organic acid as part of a biorefinery program 'MycoFuelChem' lead by the Section of Sustainable Biotechnology, Aalborg University. The model organism for the PhD project was *A. carbonarius*, a natural efficient producer of **citric acid**. The organic acids that the project aimed for were **malic acid** and **fumaric acid**.

2.4. MALIC, FUMARIC AND CITRIC ACID

Malic, fumaric and citric acid are all intermediates in the TCA cycle and therefore ubiquitous throughout all aerobic organisms. Furthermore, these three acids are all used as pure chemicals by the industry for different purposes.

Malic acid is a 1,4-dicarboxylic acid. It is primarily used as an acidifier and a preservative in food where it has a big market share (Grand View Research 2015b). However, there are other possible applications for malic acid, e.g. it is possible to substitute malic acid for maleic anhydride, which is used to produce styrene for plastics, acrylic acids for detergents and polyester and alkyd resins that are used to produce fiberglass reinforced plastics (Lohbeck et al. 2012). If malic acid could be

produced at a price that are economically competitive with that of maleic anhydride, the potential market for malic acid could be 20 fold increased (Sauer et al. 2008). Originally malic acid was extracted from apple juice, but the concentration of malic acid in the apple juice was low, which made the production capacity small and advances in chemistry made the petrochemical method far more favorable (Buck & Mottern 1947). Malic acid is produced in the petrochemical method from a double hydration of maleic anhydride that are produced from oxidation of benzene or butane (Miltenberger 2012; Lohbeck et al. 2012). There are other ways to produce malic acid, one method is enzymatic conversion from fumaric acid (Rossi & Clementi 1985; Kajiwara & Maeda 1986). Another method is direct conversion from carbon sources using microorganism (Battat et al. 1991; Taing & Taing 2007), the latter being the one that is pursued in this thesis. Yet, the petrochemical method of producing malic acid and maleic anhydride is currently by far the most used method (Sauer et al. 2008).

Fumaric acid is like malic acid a 1,4-dicarboxylic acids. It is the isomer of maleic acid (not to be mistaken for malic acid). Fumaric acid can be used for many of the same applications as malic acid including substitution of maleic anhydride, which gives the possibility of expanding the market for the chemical tremendously (Sauer et al. 2008). However, the market for fumaric acid is already bigger than that for malic acid due to many different applications of the chemical (Grand View Research 2015a). Examples are paper resins, alkyd resins, polyester resins, plasticizers, and food and beverage additives (Roa Engel et al. 2008; Lohbeck et al. 2012). Originally, fumaric acid was produced from succinic acid involving an oxidation of furfural (Volhard 1892). Yet today, the majority of fumaric acid is produced by a petrochemical method where the first maleic acid is obtained by hydration of maleic anhydride followed by conversion of the maleic acid by thermal or catalytic isomerization to fumaric acid (Lohbeck et al. 2012). As with malic acid, fumaric acid can also be produced by direct conversion of carbon sources using microorganisms (Roa Engel et al. 2008), which is also what is pursued in this thesis.

Citric acid is a tricarboxylic acid that exist in great amounts in citrus fruits, thus the name of the acid. Citric acid is a bulk chemical produced and consumed throughout the world. Primarily, it is used in the food and beverage industry as an acidifier and preservative (Berovic & Legisa 2007). Furthermore, it can be used for cleaning where its chelating ability assists in removal of scale; as a detergent builder in liquid formulations; and as plasticizers for food-grade plastic containers. Originally, citric acid was produced from unripe citrus fruits by precipitation followed by crystallization. Starting from 1919, the production changed to be conversion of sugars by the filamentous fungi *A. niger* in submerged or surface fermentation (Verhoff 2005; Max et al. 2010). Globally, citric acid is produced in much higher amounts than malic or fumaric acid. In 2007, the global citric acid production was 1.6 million tonnes and rising, which is approximately 6 times more than the current global malic

and fumaric acid production combined (Berovic & Legisa 2007; Grand View Research 2015b; Grand View Research 2015a).

On the global market, the price for citric acid was approximately 600-850$/ton in 2015 (Made-in-China.com 2015). Malic, fumaric, maleic acid, and maleic anhydride are all chemically produced and very closely linked. A single chemical reaction can change one compound to the next one (Lohbeck et al. 2012; Miltenberger 2012). Because of this, the prices on the global market are in the same range, approximately 1000-1500$/ton (Made-in-China.com 2015). This is approximately double the price for citric acid. The maximum theoretical yield of citric, malic, and fumaric acid from 100 g of glucose through microbial fermentation is 107 g citric acid, 149 g malic acid, and 129 g fumaric acid, respectively. The main reason for the higher theoretical yield in malic and fumaric acid conversion being utilization of a different metabolic pathway and fixation of CO_2 (Roa Engel et al. 2008; Zelle et al. 2008; Goldberg et al. 2006). It is obvious to see that if only the substrate is taken in to consideration it would be cheaper to produce malic or fumaric acid than citric acid if the production could be made sufficiently efficient. If it were possible to use the highly efficient citric acid producing machinery of the microbial cell factory of *A. niger* or an equally efficient similar strain to produce malic or fumaric acid, it would be possible to compete and maybe even outmatch the petro chemically produced maleic anhydride, and malic, fumaric and maleic acid. This was what the thesis was aimed for, to make a malic or fumaric acid producing strain of ***Aspergillus carbonarius*** a very close relative to the highly efficient citric acid producer *A. niger* (Nielsen et al. 2009).

2.5. *ASPERGILLUS CARBONARIUS*

Aspergillus carbonarius is a filamentous fungus in the phylum of Ascomycota within the *Aspergillus* section *nigri*. It has white hyphae and black or dark conidia (Crespo-Sempere et al. 2013). The conidia are very large (7-9 µm), which is the largest within the section *nigri* and they have echinulate ornamentations. Some isolates of *A. carbonarius* are able to produce sclerotia in a pink to yellow color. The fungus can grow in the temperature range of 9-36 °C (Samson et al. 2004; Samson et al. 2007). The very large conidia are heterokaryons and contains 2-12 nuclei (Abarca et al. 2004; Kevei et al. 1996; Nødvig et al. 2015).

A. carbonarius is closely related to *A. niger* and it is like *A. niger* also a very good producer of citric acid and it is very tolerant to acid environments (Linde et al. 2014; Yang et al. 2015; Weyda et al. 2014). Investigations made at the Section for Sustainable Biotechnology at Aalborg University, Copenhagen, showed that *A. carbonarius* was among the very best citric acid producers among many *A. niger* relatives, and it was furthermore very efficient at producing different lignocellulosic degrading enzymes (Kolasa 2013; and unpublished results). Since 2009, the genome sequence of *A. carbonarius* ITEM 5010 has been made publicly available from JGI at http://genome.jgi.doe.gov/Aspca3/Aspca3.home.html, which greatly simplifies

genetically alterations of the strain. Further, it has been documented that it is possible to make protoplasts from *A. carbonarius* spores, which is a requirement for the protoplast transformation method that were used in this Ph.D. project (Yang et al. 2014). It should be noted that because conidia from *A. carbonarius* are heterokaryons in transformation of *A. carbonarius*, it is necessary to use several subsequent isolation steps to obtain pure homokaryotic mutant strains, in order to prevent a fallback to the mother-strain at a later stage (Nødvig et al. 2015). However, where *A. niger* is well known for its efficiency as a cell factory, *A. carbonarius* is known for its production of ochratoxin A, a mycotoxin that is considered to be nephrotoxic, immunosuppressive, teratogenic, and carcinogenic (Cabañes et al. 2002; Nielsen et al. 2009). *A. carbonarius* is mainly known to grow on grapes and vine-fruits but it has also been isolated from e.g. coffee, cocoa, and cereals (Crespo-Sempere et al. 2013; Bayman & Baker 2006). Between 25 and 100% of the *A. carbonarius* isolates that have been investigated, are producing ochratoxin A. Yet , *A. niger* is also known to produce ochratoxin A, and between 0.6 and 50 % of the investigated *A. niger* isolates, produces ochratoxin A (Abarca et al. 2004). Even though *A. niger* is known to produce ochratoxin A, it has been granted GRAS status by the US food and drug administration, as long as the used isolate is not producing ochratoxin A, or in amounts that are below the threshold set by authorities (Schuster et al. 2002). *A. carbonarius* does not have GRAS status and will most likely never receive one due to the high potential of ochratoxin A production of the strain. However, the GRAS status is only necessary for food additives, thus non-food chemicals produced from *A. carbonarius* are still viable for the market. Furthermore, it is possible to eliminate the production of ochratoxin A completely from *A. carbonarius* ITEM 5010 by inactivating a specific gene encoding for a non-ribosomal peptide synthase (Gallo et al. 2012).

In this thesis, **metabolic pathway engineering** was used to alter *A. carbonarius* with the aim of constructing a cell factory strain with enhanced organic acid production capabilities.

2.6. METABOLIC PATHWAY ENGINEERING IN THE *ASPERGILLUS* SECTION *NIGRI*

The focus of the thesis has been to alter the metabolic pathways of *A. carbonarius* with the aim of enhancing the organic acid production of the strain, especially malic and fumaric acid, and to some extent citric acid. To do this, an understanding of the metabolic pathways leading to organic acid production and information about what other studies have experienced in connection with these pathways are necessary. However, the number of studies made about metabolic engineering in *A. carbonarius* are very limited, which is why it was necessary to broaden the view to *A. carbonarius*' closest neighbors, *Aspergillus* section *nigri.*

The *Aspergillus* section *nigri* group, also called the black aspergilli contains 23 species, six of these are common: *A. niger, A. tubingensis, A. brasiliensis, A. acidus, A. carbonarius*, and *A. ibericus* (Nielsen et al. 2009; Varga et al. 2011). It should be noted though that new species are identified and added to the section with regular intervals. The most studied organism in *Aspergillus* section *nigri* is *A. niger* and thus most of the available information is from this organism. The primary organic acid product that studies within the *Aspergillus* section *nigri* group are focused on is citric acid due to the capabilities of *A. niger*. Still many other organic acids are produced by *Aspergillus* section *nigri* that are of importance for the industry: gluconic acid, oxalic acid, lactic acid, itaconic acid, malic acid, succinic acid, and fumaric acid. In the following section, an overview of the metabolic pathway from carbon source to organic acid in *Aspergillus* section *nigri* is given. Furthermore, a description of what metabolic engineering progresses research groups have made in their aim to enhance organic acid production from *Aspergillus* section *nigri* is described.

The pathway for citric acid production starts with the uptake of extracellular sugars. Then through glycolysis, where interconnections to the pentose phosphate pathway plays a role. The next step is the formation of acetyl-CoA, oxaloacetate, and malate, which in turn is transported into the mitochondria where the TCA cycle commence. Citrate is formed exported out of the mitochondria via antiporters to the cytosol and further out to the extracellular space (Figure 2.3) (Aanen et al. 2011; Alvarez-Vasquez et al. 2000).

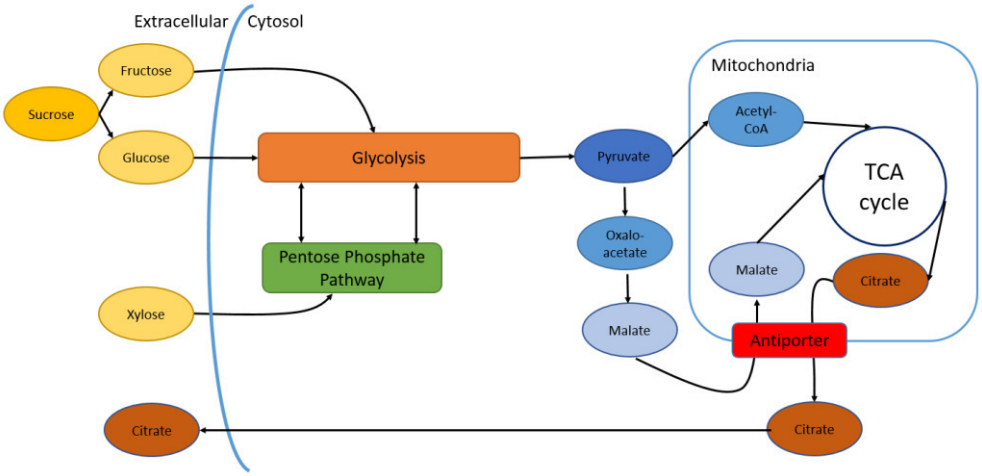

Figure 2.3 Simplified representation of the suggested metabolic pathway from carbon source to citric acid in *Aspergillus* section *nigri* organisms (Aanen et al. 2011; Alvarez-Vasquez et al. 2000).

2.6.1. SUGAR UPTAKE AND GLYCOLYSIS (EMBDEN MEYERHOF PARNAS PATHWAY)

Before glycolysis can commence, sugar molecules must be imported from the extracellular space. The uptake of sucrose molecules are done partly by the sucrose-proton symporters in *S. cerevisiae* (Santos et al. 1982). Yet, so far, no similar study has been done in filamentous fungi. Glucose and fructose are transported across the cell membrane, but a specific fructose transporter has not yet been identified in *A. niger*. Two glucose transporters are present in *A. niger*, one is constitutively expressed with high affinity and one is induced at high glucose concentrations with low affinity for glucose. Glucose molecules are used directly in the glycolysis whereas sucrose molecules firstly have to be hydrolyzed by invertase to glucose and fructose. In the first step of glycolysis, fructose is phosphorylated by hexokinase and glucose is phosphorylated by hexokinase or glucokinase (Magnuson & Lasure 2004; Ruijter et al. 1998). The name glycolysis literally means the lysis of glucose and is the metabolic pathway that converts glucose and fructose into pyruvate. As illustrated in figure 2.4, this conversion is done via 10 subsequent enzymatic steps, releasing free energy in the form of ATP and NADH. The total net production from one molecule of glucose is two molecules of pyruvate, two molecules of ATP, two molecules of NADH and two molecules of water. Glycolysis can run with or without oxygen and is the first part of cellular respiration (Aanen et al. 2011). Three of the ten enzymatic steps are irreversible: step one performed by glucokinase/hexokinase, step three performed by phosphofructokinase, and step ten performed by pyruvate kinase (Mlakar & Legisa 2006; Aanen et al. 2011). Researchers trying to alter the metabolic pathway in glycolysis have mainly focused on these three of the ten enzymatic steps, due to their irreversibility.

Trehalose-6-phosphate (T6P) is a sugar-signaling molecule of incipient significance. It works as a regulator of sugar utilization and starch metabolism in plants and interacts with other signaling pathways (Paul 2007). In *S. cerevisiae* and other eukaryotes, it has been shown that T6P works as an inhibitor of hexokinase, the first step of glycolysis. Hexokinase from *A. niger* showed to be weakly inhibited by T6P in vitro. In vivo, overexpression and disruption of the *ggsA* gene coding for trehalose-6-phosphate synthase A showed an effect on the citric acid production during high sucrose fermentation. The strain with the disrupted *ggsA* gene initiated citric acid accumulation earlier, whereas the strain overexpressing *ggsA* showed the opposite (Figure 2.4) (Arisan-Atac et al. 1996).

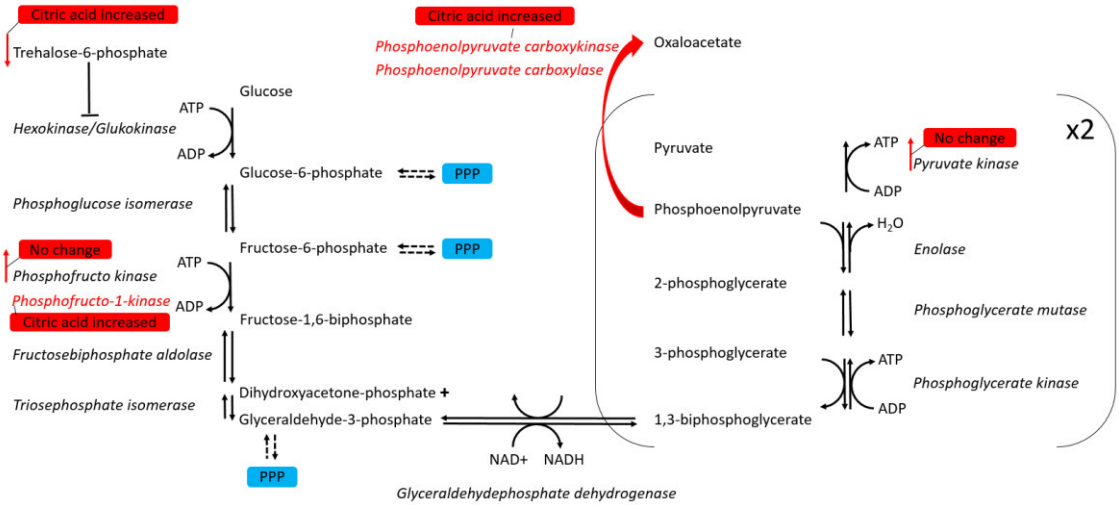

Figure 2.4 Glycolysis in *Aspergillus* section *nigri*. Enzymes are shown in *Italic*. PPP=Pentose phosphate pathway. Dotted arrows represent interconnections (Alvarez-Vasquez et al. 2000; Aanen et al. 2011). Genetically engineered alterations are shown in red, red boxes explains the outcome of the genetic alterations. Red arrows pointing up represent overexpression of an enzyme, red arrows pointing down represent downregulation of disruption of an enzyme (Arisan-Atac et al. 1996; Ruijter et al. 1997; Capuder et al. 2009; Yang et al. 2015).

As described above, the 3rd and the 10th step of glycolysis are catalyzed by phosphofructokinase and pyruvate kinase. Phosphofructokinase converts fructose-6-phosphate to fructose-1,6-biphosphate using an ATP molecule. Pyruvate kinase converts phosphoenolpyruvate to pyruvate and ATP. In a study by (Ruijter et al. 1997) phosphofructokinase and pyruvate kinase were moderately (3-5 fold) overexpressed in *A. niger*. The resulting strains both single and double overexpressing mutants did not increase citric acid production compared to wildtype. Furthermore, overexpression of these two enzymes did not change the activities of other enzymes in the glycolytic pathway nor did it change intermediary metabolite levels. However, in the strain overexpressing phosphofructokinase, a positive allosteric effector of phosphofructokinase, fructose-2,6-biphosphate, was reduced almost 2-fold. This decreases the specific activity of phosphofructokinase significantly and thereby the cell adapted to the overexpression (Ruijter et al. 1997). In a later study, the same phosphofructokinase was again targeted for molecular engineering. This time a mutated and truncated *pfkA* gene encoding for a modified 6-phosphofructo-1-kinase (PFK1) that was resistant to citrate inhibition and activated to a higher degree by fructose-2,6-biphosphate was inserted into the genome of *A. niger*. The result was an increased production of citric acid of approximately 1.75-fold due to a higher flux through the glycolysis (Capuder et al. 2009). Studies in mutagenized *A. niger* strains with elevated citric acid production shows that the uptake of sucrose was increased and the activity of hexokinase and

phosphofructokinase were increased, indicating the importance of these three steps (Figure 2.4) (Rørdam et al. 2010).

In a very recent study made in our laboratory at the Section for Sustainable Biotechnology at Aalborg University, Copenhagen, an attempt was made to circumvent the last step in the glycolysis in order of rerouting the carbon flux to the cytosolic reductive TCA branch instead of the mitochondrial TCA cycle. Phosphoenolpyruvate carboxykinase (*pepck*) from *Actinobacillus succinogenes* and phosphoenolpyruvate carboxylase (*ppc*) from *Escherichia coli* were inserted individually and in combination in the *Aspergillus carbonarius* genome. This was done to make an alternative cytosolic conversion pathway of phosphoenolpyruvate (PEP) to oxaloacetate and thereby enhance the carbon flux through the reductive TCA branch (rTCA) effectively bypassing pyruvate. The consequence was an elevated production of citric acid, both in glucose and xylose-based media at pH higher than three (Figure 2.4) (Yang et al. 2015).

2.6.2. PENTOSE CATABOLIC PATHWAY AND PENTOSE PHOSPHATE PATHWAY

In the *Aspergillus* section *nigri*, the conversion of xylose and arabinose are carried out via the pentose catabolic pathway. Xylose is taken up by the cell and converted in one enzymatic step into xylitol. Arabinose is taken up by the cell and converted in three enzymatic steps into xylitol. Xylitol is converted to xylulose, which in turn is converted by the only irreversible enzymatic step of the pentose catabolic pathway to xylulose-5-phosphate. This then enters the pentose phosphate pathway (Figure 2.5) (De Groot et al. 2007; Weyda et al. 2014).

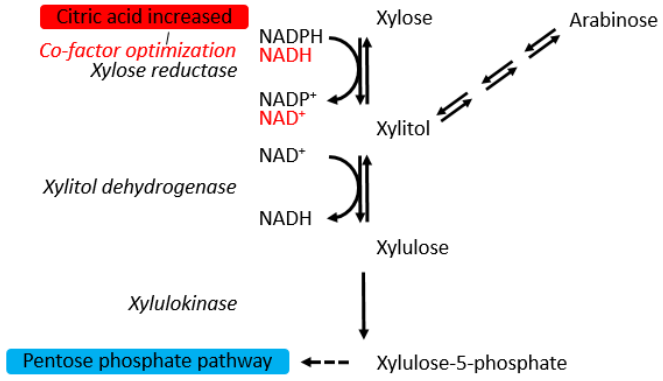

Figure 2.5 Pentose catabolic pathway. Enzymes are shown in *Italic* (De Groot et al. 2007; Weyda et al. 2014). Genetic alterations are shown in red (Weyda et al. 2014). The three double arrows represent the enzymatic steps that carry out the conversion from Arabinose to xylitol.

The pentose phosphate pathway has two separate phases. The first phase is the oxidative phase where the net outcome is generation of NADPH. Glucose-6-phosphate is in three enzymatic steps converted to ribulose-5-phosphate and two NADPH are generated. The second phase is the non-oxidative phase where pentoses are generated. Ribulose-5-phosphate can in five enzymatic steps be converted to glyceraldehyde-3-phosphate and fructose-6-phosphate. However, the intermediates of the non-oxidative phase are also of importance, with ribose-5-phosphate as the most important one, due to the use in synthesis of nucleotides and nucleic acids. The pentose phosphate pathway is interconnected with the glycolysis by glucose-6-phosphate, glyceraldehyde-3-phosphate, and fructose-6-phosphate (Figure 2.6) (R. Poulsen et al. 2005; Aanen et al. 2011).

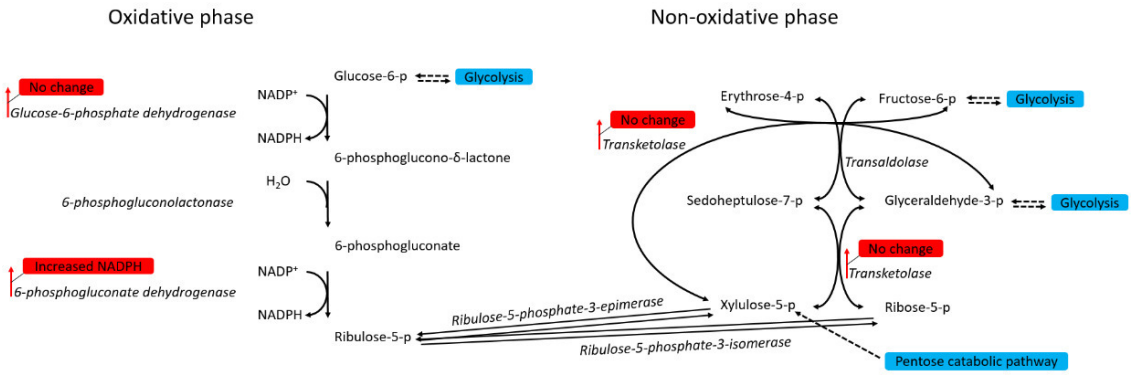

Figure 2.6 Pentose phosphate pathway. Enzymes are shown in *Italic* (R. Poulsen et al. 2005; Aanen et al. 2011). Genetic alterations are shown in red (R. Poulsen et al. 2005). p = phosphate.

When *Aspergillus carbonarius* is grown on xylose under oxygen-limiting conditions, xylitol is accumulated. A point mutation of a lysine residue to an arginine in the conserved Lys-Ser motif of the xylose reductase (XR) changed the XR from NADPH-dependent to NADH-dependent. This co-factor optimization reduced the xylitol accumulation 2.8 fold and increased the citric acid production 4.5-fold (Figure 2.5) (Weyda et al. 2014). In *A. niger* the genes encoding glucose 6-phosphate dehydrogenase (*gsdA*), 6-phosphogluconate dehydrogenase (*gndA*) and transketolase (*tktA*) were cloned and overexpressed in separate strains. This was done to increase the availability of intracellular NADPH and to increase the flux through the pentose phosphate pathway. Intracellular NADPH concentrations was increased two- to nine-fold as a result of a 13-fold overproduction of 6-phosphogluconate dehydrogenase. However, the other two enzymes did not change the intracellular concentration of NADPH. Concentrations of intermediary metabolites and polyols were changed in the overexpression transformants, but the study did not specify further (Figure 2.6) (R. Poulsen et al. 2005).

2.6.3. REDUCTIVE CYTOSOLIC TRICARBOXYLIC ACID (TCA) CYCLE BRANCH

The reductive TCA cycle – or the reverse TCA cycle – is as the name says the reverse of the normal oxidative TCA cycle. Where the normal TCA cycle uses complex carbon compounds primarily to make energy, the reverse cycle uses energy, CO_2 and H_2O to make carbon compounds. However, while the TCA cycle is situated in the mitochondria, the reductive cytosolic TCA cycle branch is situated in the cytosol. The reductive cytosolic TCA cycle branch is described as the metabolic pathway from pyruvate through oxaloacetate to malate and fumarate, and in some succinate overproducers (like *Actinobacillus succinogenes* and *Anaerobiospirillum succiniciproducens*) this pathway continues to succinate though this is not the case in *Aspergillus* section *nigri* (Figure 2.7) (Cheng et al. 2013; de Jongh & Nielsen 2008; Aanen et al. 2011; Susan Meijer et al. 2009).

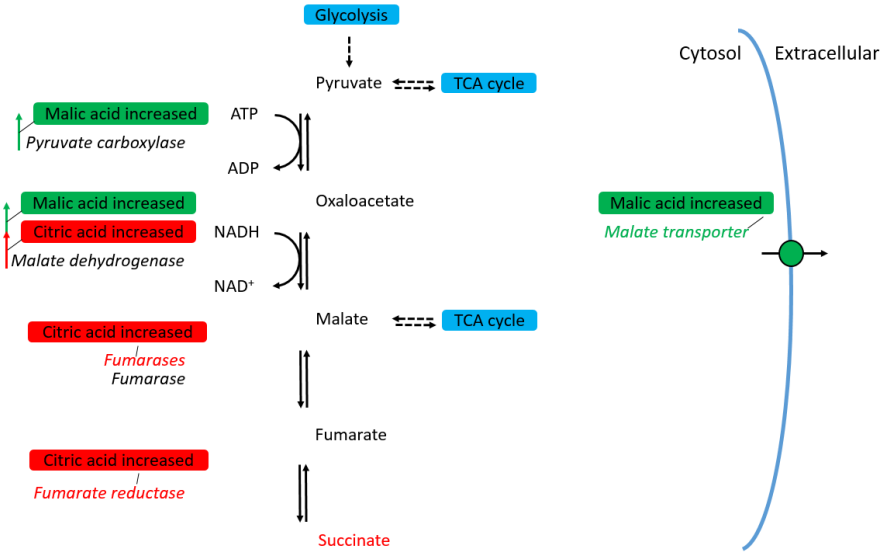

Figure 2.7 Reductive cytosolic TCA cycle branch. Enzymes are shown in *Italic*. Genetic alterations are shown in red (de Jongh & Nielsen 2008). Genetic alterations outside *Aspergillus* section *nigri* are shown in green (Zelle et al. 2008; Brown et al. 2013).

The reductive cytosolic TCA branch has received much attention from researchers trying to enhance organic acid production in *Aspergillus* section *nigri* and in other species. The following two examples in other species are outlined due to their connection to this PhD.

In *S. cerevisiae* Zelle et al. (2008) overexpressed the native cytosolic pyruvate carboxylase and introduced a cytosolic malate dehydrogenase together with a malate permease from *Schizosaccharomyces pombe*, and was able to increase malate production via the reductive cytosolic TCA branch (Zelle et al. 2008). The same setup was used in *Aspergillus oryzae*, a natural malic acid producer, where three native enzymes, a pyruvate carboxylase, a malate dehydrogenase and a C4-dicarboxylate transporter were targeted to the cytosol and overexpressed. This gave a 2.27-fold increase in malic acid production compared to the wild type (Brown et al. 2013). de Jongh and Nielsen (2008) made a study in *A. niger* where citrate production was enhanced through insertion of several genes in single or double insertions in the reductive TCA branch. The genes were malate dehydrogenase (*MDH2)*, fumarate reductase (*FrdsI)*, fumarase (*Fum1)* from *Saccharomyces cerevisiae*, and fumarase (*FumR)* from *Rhizopus oryzae*. Significantly higher citrate yields were seen when the two different cytosolic targeted fumarases, *fumI* and *fumR* were expressed compared to the wildtype and control strains. The highest increase in citric acid production of the single transformant strains was the overexpression of *frdsI*. Furthermore, this strain was enhanced with a double transformation with *fumR,* which increased the citric acid production even further. The insertion of either of the two fumarases *fumR* or *fum1* also resulted in an increased production of oxalate. Overexpression of the *MDH2* gene resulted in an increase of citric acid production; however, it was the smallest increase of the different types of transformants investigated. The increased production of citrate in the *MDH2* transformant were found especially in the initial citrate production rate compared to all other strains but had a slower overall citrate production rate (de Jongh & Nielsen 2008). (Röhr & Kubicek 1981) observed intracellular malate accumulation preceding the beginning of citrate production in *A. niger*. Therefore, they suggested that the accumulation of malate triggers citrate production through the malate-citrate antiporter. The study by de Jongh and Nielsen supports this theory but also suggests that other factors than cytosolic malate dehydrogenase plays a role in later fermentation (Figure 2.7) (de Jongh & Nielsen 2008).

2.6.4. TRICARBOXYLIC ACID (TCA) CYCLE

The tricarboxylic acid cycle or the citric acid cycle is a series of chemical reactions starting from the oxidation of acetyl-CoA. The primary function is the generation of energy in the form of GTP, but also NADH and certain precursors for amino acids are produced. The TCA cycle consist of nine chemical compounds and nine consecutive enzymatic reactions. As the name says, the citric acid cycle evolves around citrate, and is the source of the citric acid production in *Aspergillus* section *nigri*. Citrate is transported out of the mitochondria through an antiport mechanism where malate is transported in in exchange (Figure 2.8). Malate, fumarate, and succinate are – like citrate – intermediates in the TCA cycle and thus looked upon as a possible origin for malic, fumaric, and succinic acid production in many species (Ruijter et al. 2000; Kobayashi, Hattori, Hayashi, et al. 2014; Susan Meijer et al. 2009; Aanen et al. 2011). Very often, the pathway called the 'glyoxylate shunt' is described as a shortcut interconnected in the TCA cycle. However it should be noted that the TCA cycle enzymes are located in the mitochondria, whereas the enzymes active in the glyoxylate shunt are located in connection with the peroxisomal membrane and therefore not directly connected (Kunze et al. 2006).

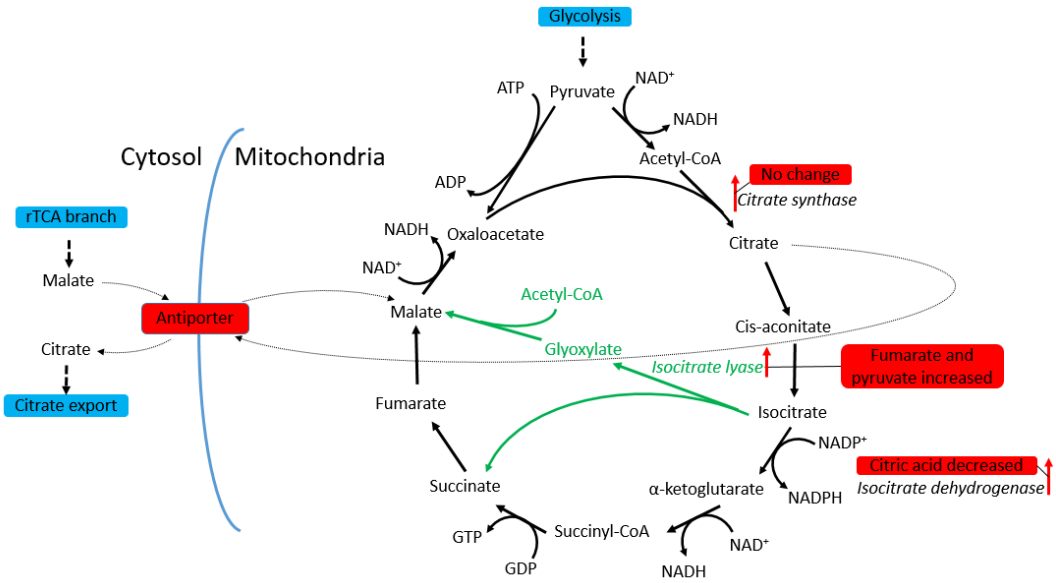

Figure 2.8 TCA cycle. Enzymes shown in *Italic*, only genetically altered enzymes are shown. Genetic alterations shown in red (Ruijter et al. 2000; Kobayashi, Hattori, Hayashi, et al. 2014; Susan Meijer et al. 2009). The glyoxylate shunt are shown in green and are not situated in the mitochondria (Kunze et al. 2006).

Enhancement of citric acid production through changes in the TCA cycle has not been very successful. An 11-fold increase of citrate synthase in *A. niger* did not change the production of citric acid under citric acid production conditions. The missing effect was explained by the fact that *A. niger* have a naturally large capacity for this enzymatic reaction (Ruijter et al. 2000). The enzyme isocitrate dehydrogenase (NADP+-ICDH) catalyzes oxidative decarboxylation of isocitric acid to form alfa-ketoglutaric acid with $NADP^+$ as a cofactor. In a *A. niger* strain the *icdA* gene was overexpressed and resulted in a strain where the amount of citric acid produced decreased by 19 %, and the amount of glucose consumed decreased by 11 % (Kobayashi, Hattori, Hayashi, et al. 2014). An attempt to improve the activity of the glyoxylate bypass pathway was done by overexpressing the isocitrate lyase gene (*icl*). The hypothesis was that the up-regulation of isocitrate lyase would increase the flux towards glyoxylate leading to increased formation of malate and succinate. However, the fermentation results showed an increased fumarate and pyruvate production instead of the hypothesized outcome. This outcome may be because the oxidative part of the TCA cycle is up-regulated instead of the glyoxylate bypass, and this creates a bottleneck in the TCA cycle, leading to fumarate accumulation (Figure 2.8) (S Meijer et al. 2009).

2.6.5. BYPRODUCT FORMATION

Byproduct formation during organic acid production can be many things. In this section, byproducts are understood as any product that are not the desired organic acid product from an industrial point of view. However, a byproduct in one production facility could be the main product in another facility; one example is the production of oxalic acid. From the microorganisms' point of view, these byproducts are obviously most often of importance and have different purposes, and therefore not considered byproducts. Genetic alterations to change byproduct formation can both have an effect on the specific byproduct and on other products due to changed carbon fluxes through desired pathways.

One of the problems for citric acid production in *A. niger* is the by-product formation of oxalic acid, gluconic acid, and polyols such as glycerol, which can cause problems with downstream processing. For that reason, studies have been made with elimination of oxaloacetate hydrolase and elimination of glucose oxidase (Rørdam et al. 2010). The oxalic acid production is due to the action of the oxaloacetate hydrolase encoded by the *oah* gene. The oxaloacetate hydrolase converts oxaloacetate to oxalate and acetate in the cytosol. In a glycoamylase-producing strain of *A. niger*, the *oah* gene was disrupted resulting in a strain that was unable to produce oxalic acid. The growth rate and citric acid production of the transformant and the wildtype were identical and the metabolic fluxes were almost identical. However, the glucoamylase yield was decreased by 50% in the transformed strain (Pedersen et al. 2000). Oxalic acid is not only a byproduct; it can also be used as a chelator, tanning agent, or detergent and are therefore produced industrially. A transformant of *A. niger* overexpressing the gene encoding for oxaloacetate hydrolase increased the production capacity of oxalic acid under citric acid fermentation conditions by approximately 1.85-fold (Kobayashi, Hattori, Honda, et al. 2014). In another study, conducted to increase oxalic acid production in *A. niger*, a knockout approach was done to identify oxalate overproducing phenotypes. The yield of oxalic acid was increased up to 158% compared to the wild type in the best performing strain. The corresponding transcription factor was named oxalic acid repression factor (OafA). The primary method for the mutant to perform better than the wild type was more efficient re-uptake of produced gluconic acid and therefore a higher flux through glycolysis (Figure 2.9) (Poulsen et al. 2012).

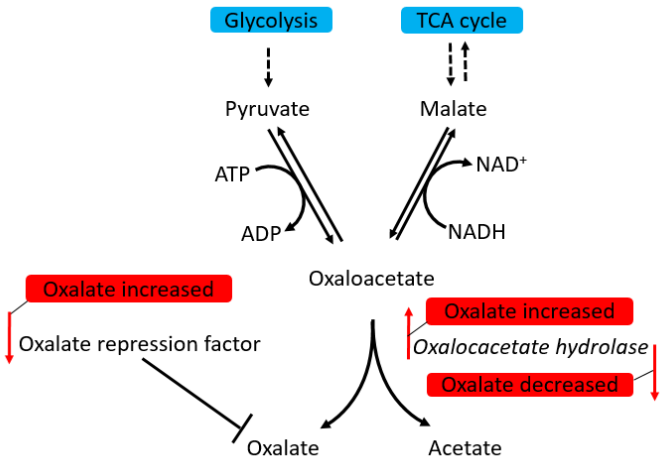

Figure 2.9 Oxalate production. Enzymes shown in *Italic,* only genetically altered enzymes are shown. Genetic alterations shown in red (Pedersen et al. 2000; Kobayashi, Hattori, Honda, et al. 2014; Poulsen et al. 2012).

A. niger is capable of converting glucose into gluconic acid outside the cell. This is done very fast because the glucose oxidase is localized in the extracellular matrix (Ruijter et al. 1998). In *A. carbonarius*, high amounts of gluconic acid is accumulated during citric acid fermentation when the pH is at 5.5 or above. The *gox* gene encoding for glucose oxidase was identified and disrupted in *A. carbonarius* with the aim of decreasing gluconic acid production and enhancing the flux towards other organic acids. The results showed that the gluconic acid accumulation was completely inhibited and instead an increased production of citric, oxalic, and malic acid was observed (Figure 2.10) (Yang et al. 2014).

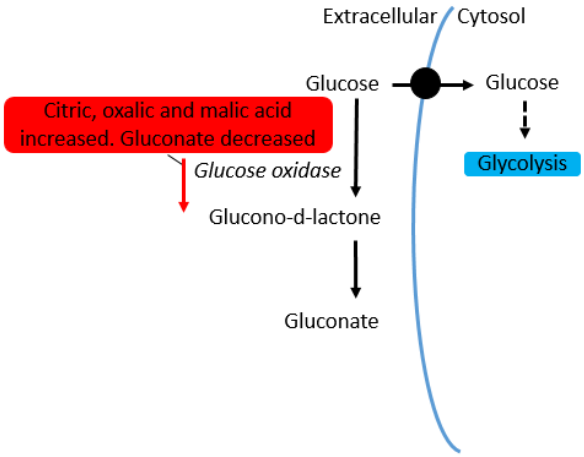

Figure 2.10 Gluconate production. Enzymes shown in *Italic,* only genetically altered enzymes are shown. Genetic alterations shown in red (Yang et al. 2014).

Elimination of glycerol accumulation as a by-product has been done in *S. cerevisiae*. Here the disruption of the isoenzymes glycerol-3-phosphate and dehydrogenase I and II successfully decreased the accumulation of glycerol (Nissen et al. 2000). This has not yet been attempted in *A. niger* but it has been suggested that this strategy would be promising for inhibiting the production of glycerol as a by-product in citric acid fermentation (Magnuson & Lasure 2004). A glycerol kinase deficient mutant was produced in *A. niger*, which was only able to grow weakly on glycerol. The strain was not tested in a citric acid production setup (Figure 2.11) (Witteveen et al. 1990).

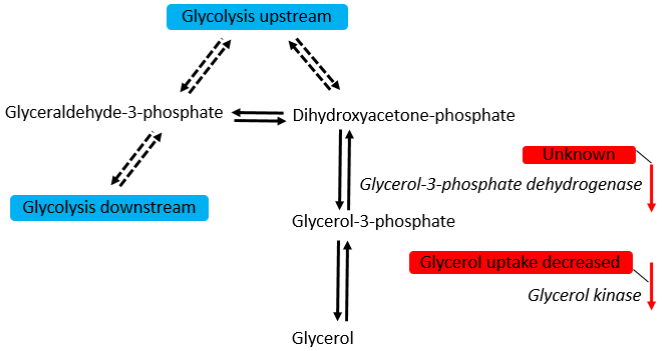

Figure 2.11 Glycerol production. Enzymes shown in *Italic,* only genetically altered enzymes are shown. Genetic alterations shown in red (Magnuson & Lasure 2004; Witteveen et al. 1990).

Another example of byproduct elimination was made by Meijer et al. (2009) in *A. niger*, where one of the pathways to the fatty acid biosynthesis was disrupted with the aim to increase the carbon flux towards succinic acid production. The enzyme 'cytosolic ATP: citrate lyase' catalyzes the conversion of citrate and coenzyme A to acetyl-CoA and oxaloacetate in the flux towards fatty acids. The gene coding for 'ATP: citrate lyase' was knocked out and it was found that the yield of succinic acid was increased three-fold and the total amount of organic acids produced was significantly increased. Two different fermentation conditions were used. Succinate was not produced when xylose was used as carbon source only when glucose was used. Fumarate production increased significantly in the mutant strain when xylose was used as carbon source, this was not observed when grown on glucose. It was unexpected that the production of fumarate and succinate were not correlated since they are very closely related in the metabolic pathway. This is an indication that *A. niger* uses two different pathways for succinic and fumarate production, respectively, and that the fumarate reductase most likely is either missing or not active. Succinate is most likely produced via the TCA cycle or the glyoxylate shunt, and fumarate is probably produced via the reductive TCA branch (Figure 2.12) (Susan Meijer et al. 2009; David et al. 2003).

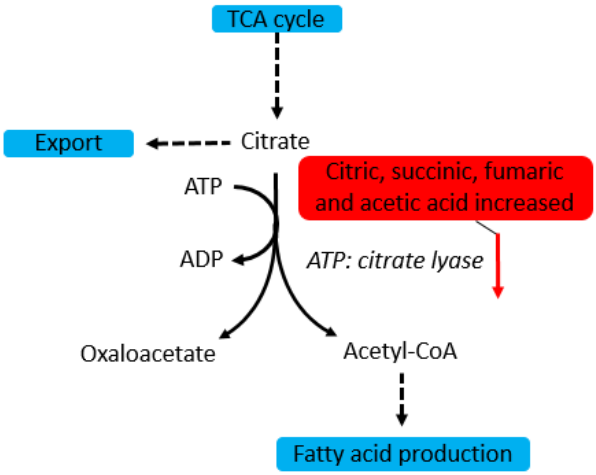

Figure 2.12 Fatty acid production. Enzymes shown in *Italic,* only genetically altered enzymes are shown. Genetic alterations shown in red (Susan Meijer et al. 2009; David et al. 2003).

2.6.6. HETEROLOGOUS PRODUCTION

There are examples of heterologous production of organic acids in *Aspergillus* section *nigri*, two of these are mentioned here. In *Rhizopus oryzae,* the NAD-dependent lactate dehydrogenase (LDH) is responsible for the conversion of pyruvate to lactate, and for the high amount of lactic acid produced from *R. oryzae*. The *ldhA* gene from *R. oryzae* that codes for LDH was successfully integrated into the *Aspergillus brasiliensis* genome producing a recombinant strain that are able to produce lactic acid in high amounts (titer 32.2g/L) (Liaud et al. 2015).

A. niger is not a natural producer of itaconic acid. However, heterologous expression of aconitase and *cis*-aconitate decarboxylase enables the conversion of citrate through cis-aconitate into itaconate and improves the production of itaconic acid in *A. niger*. These two enzymes can be targeted to the cytosol or the mitochondria. When targeted to the mitochondria the production of itaconic acid doubles compared to when they are expressed in the cytosol. When the two enzymes were present in both the mitochondria and the cytosol, the production of itaconic acid were even further increased. Aconitase is a component of the TCA cycle, and the enzyme is therefore already present in the mitochondria. Yet overexpression of only *cis*-aconitase decarboxylase does not produce itaconic acid in great amounts (Figure 2.13) (Blumhoff et al. 2013). The gene coding for *cis*-aconitate decarboxylase is in the natural itaconic acid producer *A. terreus* located in a gene cluster with two transporters. Overexpression of either of these two transporters in *A. niger* led to an increased itaconic acid production in *A. niger* (Figure 2.13) (Li et al. 2013).

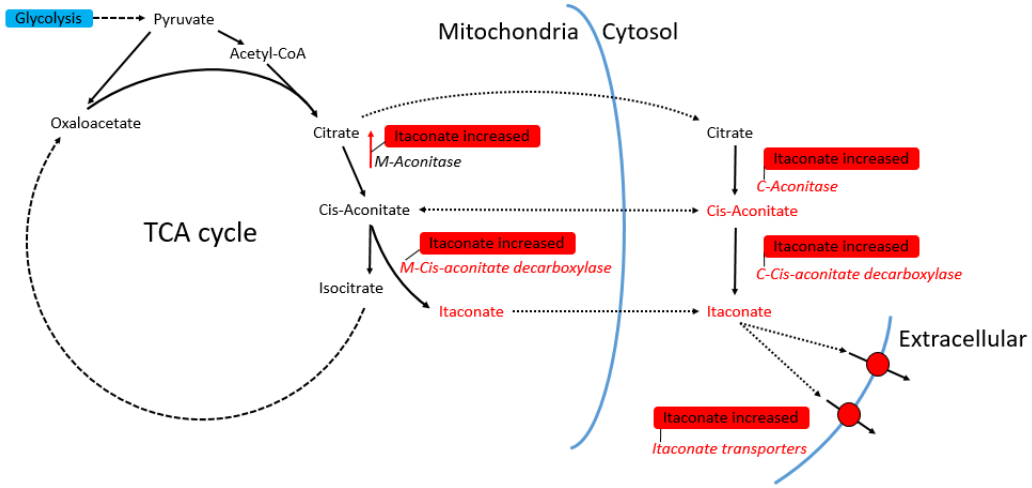

Figure 2.13 Heterologous production of itaconic acid. Enzymes shown in *Italic,* only genetically altered enzymes are shown. M=mitochondrial, C=cytosolic. Genetic alterations shown in red (Blumhoff et al. 2013; Li et al. 2013).

2.7. FERMENTATION, VARIATION AND STATISTICS

In this thesis, *A. carbonarius* was genetically altered to enhance its production of organic acids. One way to test for these phenotypic changes in mutant and transformant strains is by fermentation experiments where the altered strains production capabilities are compared with wild type. Fermentation screening experiments was the primary test method used in this thesis. Fermentation in classical terms refers to the anaerobic metabolic conversion of sugars to gases, alcohol or acids; one example is the conversion by *S. cerevisiae* of sugar to ethanol for the use in beer and wine. However, fermentation is also used as a broader term for microbial production of chemicals (Willey et al. 2014). In this thesis, fermentation is understood as a liquid submerged conversion of substrate in to products carried out by microorganisms in bioreactors. Laboratory bioreactors can be anywhere from 120 µl to 60 l, and possibly even smaller or bigger if it fits the process (Büchs 2001). The smallest bioreactors are microtiter plates and have the great advantage that numerous samples can be examined at the same time (Linde et al. 2014; Duetz & Witholt 2001). However, in the other end of the size-scale, the biggest lab-scale bioreactors have the advantage of being closer in size to pilot-plant and industrial bioreactors. Results from experiments carried out at this size are often more relevant from an industrial point of view (Liu & Hong 2001; Büchs 2001). There are many different types of bioreactors ranging from very simple ones, which are only a vessel able to contain liquid, to the very advanced bioreactors with stirring and control of numerous parameters. Usually the smallest ones are also the simplest, e.g. micro titer plates. In this thesis, fermentations were carried out in bioreactors of the simple kind on a small scale, and as shaking batch fermentations. Simple batch fermentations with shaking are in general used widely as screening and bioprocess developing tools for projects with filamentous fungi (Büchs 2001). Even though this type of shaking fermentation is described as simple, numerous parameters can be adjusted that potentially can have an impact on the result. A quick investigation of the methods used in five different research projects studying citric acid production from *A. niger* gave a list of 12 adjustable parameters: (1) flask type; (2) lid type; (3) media composition; (4) pH; (5) fermentation time; (6) relative head-space; (7) fungal mass or amount of spores in starting broth; (8) preparation of fungus before fermentation; (9) temperature; (10) evaporation during fermentation; (11) the shaking system's rpm; and (12) the shaking system's amplitude (Shu & Johnson 1948; Dhillon et al. 2011; Jaklitsch et al. 1991; de Jongh & Nielsen 2008; Sanchez-Marroquin et al. 1970).

With many adjustable parameters, or in some cases random alternating parameters, comes the risk of variation in the results. This is not something addressed by many scientists even though it appears to be a common problem both in research projects and in industrial production (Max et al. 2010; Büchs 2001; Linde et al. 2014). Some parameters have been deemed the most important factors to control, in order of avoiding variation in experiment results, among these are aeration and media composition. However, in some cases even seemingly insignificantly small

differences can lead to variation in results, e.g. shift in water source or small differences in lighting (Max et al. 2010; Büchs 2001; Linde et al. 2014; and unpublished results see appendix A). Optimization of a fermentation setup to decrease variation is a very important factor before screening multiple strains for phenotypic differences. Yet, eliminating variation completely is not possible. Therefore, it is important to use well-established statistical methods to be able to analyze results objectively.

In this thesis, the well-established student's t-test and ANOVA methods have been used for statistical analysis. ANOVA is a collection of statistical models that are used to analyze differences between group means. The student's t-test can be used to test if two sets of data are significantly different. The ANOVA methods can be used to test if more than two sets of data are significantly different. Both methods work by testing if a null-hypothesis can be rejected with a probability (p-value) less than the significance level. The null-hypothesis is set to be that all groups investigated are from one population (randomly picked). If the null-hypothesis is rejected it means that the groups investigated are not from the same population and different. Good science practice usually sets the significance level to be $p<0.05$. This means that 5% of a normal distributed sample from the same population are rejected and that 95% are accepted (Rice 2006; Gelman 2005; Rushing et al. 2013; Craparo 2007).

2.8. REFERENCES

Abarca, M.L. et al., 2004. Taxonomy and significance of black aspergilli. *Antonie van Leeuwenhoek*, 86, pp.33–49.

Abdul-Hamid, O.S. et al., 2014. *2014 World Oil Outlook*, Vienna, Austria.

Ahring, B.K., Lübeck, M. & Lübeck, P.S., 2012. Development of a biorefinery concept for integrated production of biomedical, biochemical, feed and fuels from selected plant materials - BIOREF. , p.1. Available at: http://vbn.aau.dk/da/projects/development-of-a-biorefinery-concept-for-integrated-production-of-biomedical-biochemical-feed-and-fuels-from-selected-plant-materials--bioref%28abd7a33c-2208-4c56-8973-24f1331852da%29.html [Accessed February 2, 2016].

Ahring, B.K., Lübeck, P.S. & Lübeck, M., 2015. MYCO-fuels and MYCO-chemicals: Consolidated bioprocessing of biomass into advanced fuels and high value compounds in fungal cell factories. , p.1. Available at: http://vbn.aau.dk/da/projects/mycofuels-and-mycochemicals-consolidated-bioprocessing-of-biomass-into-advanced-fuels-and-high-value-compounds-in-fungal-cell-factories%28b97b27d7-2005-4742-aee3-677999ca0743%29.html [Accessed February 2, 2016].

Ahring, B.K. & Westermann, P., 2007. Coproduction of bioethanol with other biofuels. In *Advances in Biochemical Engineering/Biotechnology*. pp. 289–302.

Alvarez-Vasquez, F., González-Alcón, C. & Torres, N. V, 2000. Metabolism of citric acid production by *Aspergillus niger*: model definition, steady-state analysis and constrained optimization of citric acid production rate. *Biotechnology and bioengineering*, 70(1), pp.82–108.

Arisan-Atac, I., Wolschek, M. & Kubicek, C., 1996. Trehalose-6-phosphate synthase A affects citrate accumulation by *Aspergillus niger* under conditions of high glycolytic flux. *FEMS Microbiology Letters*, 15(140), pp.77–83.

Bain, J.D. et al., 1989. Biosynthetic site-specific incorporation of a non-natural amino acid into a polypeptide. *Journal of the American Chemical Society*, 111, pp.8013–8014. Available at: http://pubs.acs.org/doi/abs/10.1021/ja00202a052.

Battat, E. et al., 1991. Optimization of L-malic acid production by *Aspergillus flavus* in a stirred fermentor. *Biotechnology and bioengineering*, 37(11), pp.1108–16. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18597343.

Bayman, P. & Baker, J.L., 2006. Ochratoxins: A global perspective. *Mycopathologia*, 162(3), pp.215–223.

Berovic, M. & Legisa, M., 2007. Citric acid production. *Biotechnology Annual Review*, 13, pp.303–343.

Blumhoff, M.L. et al., 2013. Targeting enzymes to the right compartment: metabolic engineering for itaconic acid production by *Aspergillus niger*. *Metabolic engineering*, 19, pp.26–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23727192 [Accessed October 23, 2014].

Bozell, J.J. & Petersen, G.R., 2010. Technology development for the production of biobased products from biorefinery carbohydrates—the US Department of Energy's "Top 10" revisited. *Green Chemistry*, 12(4), p.539.

Brown, S.H. et al., 2013. Metabolic engineering of *Aspergillus oryzae* NRRL 3488 for increased production of L-malic acid. *Applied microbiology and biotechnology*, 97(20), pp.8903–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23925533 [Accessed August 16, 2014].

Buck, R. & Mottern, H., 1947. L-Malic Acid as by-product in Apple Sirup Manufactured by Ion Exchange. *Industrial & Engineering Chemistry*, 39(9), pp.1087–1090.

Büchs, J., 2001. Introduction to advantages and problems of shaken cultures. *Biochemical Engineering Journal*, 7(2), pp.91–98.

Cabañes, F.J. et al., 2002. What is the source of ochratoxin A in wine? *International journal of food microbiology*, 79(3), pp.213–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12371656.

Capuder, M. et al., 2009. Highly active, citrate inhibition resistant form of *Aspergillus niger* 6-phosphofructo-1-kinase encoded by a modified *pfkA* gene. *Journal of biotechnology*, 144(1), pp.51–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19379783 [Accessed June 6, 2012].

Cheng, K.-K. et al., 2013. Improved succinate production by metabolic engineering. *BioMed research international*, 2013, p.538790. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3652112&tool=pmcentrez&rendertype=abstract.

Craparo, R.M., 2007. Significance level. In N. J. Salkind, ed. *Encyclopedia of Measurement and Statistics*. Thousand Oaks, CA: SAGE Publications, pp.

889–891.

Crespo-Sempere, A. et al., 2013. VeA and LaeA transcriptional factors regulate ochratoxin A biosynthesis in [I]Aspergillus carbonarius. *International journal of food microbiology*, 166(3), pp.479–86. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24041999 [Accessed October 20, 2014].

Danchin, A., 2004. The bag or the spindle: the cell factory at the time of systems' biology. *Microbial cell factories*, 3, p.13.

David, H., Åkesson, M. & Nielsen, J., 2003. Reconstruction of the central carbon metabolism of *Aspergillus niger*. *European Journal of Biochemistry*, 270(21), pp.4243–4253.

Demirbas, A., 2009. Biorefineries: Current activities and future developments. *Energy Conversion and Management*, 50(11), pp.2782–2801. Available at: http://dx.doi.org/10.1016/j.enconman.2009.06.035.

Dhillon, G.S. et al., 2011. Apple pomace ultrafiltration sludge – A novel substrate for fungal bioproduction of citric acid: Optimisation studies. *Food Chemistry*, 128(4), pp.864–871. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0308814611005085 [Accessed November 30, 2012].

Duetz, W.A. & Witholt, B., 2001. Effectiveness of orbital shaking for the aeration of suspended bacterial cultures in square-deepwell microtiter plates. *Biochemical engineering journal*, 7(2), pp.113–115. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11173298.

Gallo, A. et al., 2012. New insight into the ochratoxin a biosynthetic pathway through deletion of a nonribosomal peptide synthetase gene in *Aspergillus carbonarius*. *Applied and Environmental Microbiology*, 78(23), pp.8208–8218.

Gelman, A., 2005. Analysis of variance — why it is more important than ever. *The Annals of Statistics*, 33(1), pp.1–53. Available at: http://projecteuclid.org/Dienst/getRecord?id=euclid.aos/1112967698/.

Goldberg, I., Rokem, J.S. & Pines, O., 2006. Review Organic acids : old metabolites, new themes. *Journal of Chemical Technology and Biotechnology*, 1611(October 2005), pp.1601–1611.

Grand View Research, 2015a. *Fumaric Acid Market Analysis By Application (Food*

& Beverages, Rosin Paper Sizes, UPR, Alkyd Resins) And Segment Forecasts To 2020, San Francisco.

Grand View Research, 2015b. *Malic Acid Market Analysis By Application (Beverages, Confectionery & Food) And Segment Forecasts To 2020*, San Francisco.

Grand View Research, 2015c. *Succinic Acid Market Analysis By Application (1,4 BDO, Resins, Coatings, Dyes & Inks, Pharmaceuticals, Polyurethanes, Food, Plasticizers, Cosmetics, Solvents & Lubricants, De-icing Solutions) And Segment Forecasts to 2022*, San Francisco.

De Groot, M.J.L. et al., 2007. Regulation of pentose catabolic pathway genes of *aspergillus niger*. *Food Technology and Biotechnology*, 45(2), pp.134–138.

Hammerling, G.J., 1981. Hammerling, G. J. (1981) Cell Factories of the future. *Umschau in Wissenschaft und Technik*, 9, pp.259–263.

Hirao, I. et al., 2002. An unnatural base pair for incorporating amino acid analogs into proteins. *Nature biotechnology*, 20(2), pp.177–182.

Hoff, B., Pöggeler, S. & Kück, U., 2008. Eighty years after its discovery, Fleming's Penicillium strain discloses the secret of its sex. *Eukaryotic Cell*, 7(3), pp.465–470.

Hohsaka, T. & Sisido, M., 2002. Incorporation of non-natural amino acids into proteins. *Current Opinion in Chemical Biology*, 6(6), pp.809–815.

Hongbin, C. & Wang, L., 2013. Lignocelluloses feedstock biorefinery as petrorefinery substitutes. In *Biomass Now - Sustainable Growth and Use*. pp. 347–388.

IEA, 2014. *Key World Energy Statistics 2014*, Paris. Available at: http://www.iea.org/publications/freepublications/publication/KeyWorld2014.pdf.

Ivshina, I.B. et al., 2015. Oil spill problems and sustainable response strategies through new technologies. *Environmental science. Processes & impacts*, 17(7), pp.1201–19. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-84936937979&partnerID=tZOtx3y1.

Jackson, D. a, Symons, R.H. & Berg, P., 1972. Biochemical method for inserting new genetic information into DNA of Simian Virus 40: circular SV40 DNA

molecules containing lambda phage genes and the galactose operon of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America*, 69(10), pp.2904–2909.

Jaklitsch, W.M., Kubicek, C.P. & Scrutton, M.C., 1991. Intracellular location of enzymes involved in citrate production in *Aspergillus niger*. *Can. J. Microbiol.*, 37, pp.823–827.

de Jong, E. et al., 2012. *Bio-based Chemicals. Value Added Products from Biorefineries*,

de Jongh, W.A. & Nielsen, J., 2008. Enhanced citrate production through gene insertion in *Aspergillus niger*. *Metabolic engineering*, 10(2), pp.87–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18162426 [Accessed March 21, 2012].

Jourdier, E. et al., 2013. Cellulase activity mapping of *Trichoderma reesei* cultivated in sugar mixtures under fed-batch conditions. *Biotechnology for Biofuels*, 6(1), p.79. Available at: http://www.biotechnologyforbiofuels.com/content/6/1/79.

Kajiwara, S. & Maeda, H., 1986. Coimmobilization of malate dehydrogenase and formate dehydrogenase in polyethyleneglycol (# 4000) diacrylate gel by droplet gel-entrapping method. *Biotechnology and bioengineering*, 28(12), pp.1794–1800.

Kamm, B. & Kamm, M., 2006. Biorefineries - Multi product processes. *Advances in Biochemical Engineering/Biotechnology*, 105(January), pp.175–204. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17408084.

Kavšček, M. et al., 2015. Yeast as a cell factory: current state and perspectives. *Microbial Cell Factories*, 14(1), p.94. Available at: http://www.microbialcellfactories.com/content/14/1/94.

Kevei, F. et al., 1996. Molecular polymorphism and phenotypic variation in *Aspergillus carbonarius*. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, 70(1), pp.59–66. Available at: <Go to ISI>://A1996UZ29100007.

King, S.D. et al., 2010. *the Future of Industrial Biorefineries*, Geneva. Available at: www.weforum.org.

Kobayashi, K., Hattori, T., Hayashi, R., et al., 2014. Overexpression of the NADP + -specific isocitrate dehydrogenase gene (*icdA*) in citric acid-producing

Aspergillus niger WU-2223L. *Bioscience, Biotechnology, and Biochemistry*, 78(7), pp.1246–1253. Available at: http://www.tandfonline.com/doi/abs/10.1080/09168451.2014.918483.

Kobayashi, K., Hattori, T., Honda, Y., et al., 2014. Oxalic acid production by citric acid-producing *Aspergillus niger* overexpressing the oxaloacetate hydrolase gene oahA. *Journal of Industrial Microbiology & Biotechnology*, 41(5), pp.749–756. Available at: http://link.springer.com/10.1007/s10295-014-1419-2.

Kolasa, M., 2013. *Enzymes for efficient and cost-effective hydrolysis of pretreated biomass*. Aalborg University.

Kunze, M. et al., 2006. A central role for the peroxisomal membrane in glyoxylate cycle function. *Biochimica et Biophysica Acta - Molecular Cell Research*, 1763(12), pp.1441–1452.

Li, A. et al., 2013. Reduced by-product formation and modified oxygen availability improve itaconic acid production in *Aspergillus niger*. *Applied microbiology and biotechnology*, 97(9), pp.3901–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23397482 [Accessed October 22, 2014].

Liaud, N. et al., 2015. L-lactic acid production by *Aspergillus brasiliensis* overexpressing the heterologous *ldha* gene from *Rhizopus oryzae*. *Microbial Cell Factories*, 14(1), p.66. Available at: http://www.microbialcellfactories.com/content/14/1/66.

Linde, T. et al., 2014. Fermentation in 24-well plates is an efficient screening platform for filamentous fungi. *Letters in applied microbiology*, 59(2), pp.224–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24725208 [Accessed September 5, 2014].

Liu, C.M. & Hong, L.N., 2001. Development of a shaking bioreactor system for animal cell cultures. *Biochemical Engineering Journal*, 7(2), pp.121–125.

Lohbeck, K. et al., 2012. Maleic and Fumaric Acids. In *Ullmann's Encyclopedia of Industrial Chemistry*. Weinheim: Wiley-VCH Verlag GmbH & CO, pp. 145–155. Available at: http://onlinelibrary.wiley.com/doi/10.1002/14356007.a16_187.pub2/full.

Made-in-China.com, 2015. Made-in-China.com Product Directory. Available at: http://www.made-in-china.com/prod/catlist/ [Accessed December 10, 2015].

Magnuson, J.K. & Lasure, L.L., 2004. Organic Acid Production by Filamentous Fungi. In J. Lange & L. Lange, eds. *Advances in Fungal Biotechnology for Industry, and Medicine*. Kluwer Academic/Plenum Publishers, pp. 307–340.

Max, B. et al., 2010. Biotechnological production of citric acid. *Brazilian journal of microbiology*, 41(4), pp.862–75. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3769771&tool=pmcentrez&rendertype=abstract.

Meijer, S. et al., 2009. Gene deletion of cytosolic ATP: citrate lyase leads to altered organic acid production in *Aspergillus niger*. *Journal of Industrial Microbiology & Biotechnology*, 36(10), pp.1275–1280. Available at: http://link.springer.com/10.1007/s10295-009-0607-y.

Meijer, S. et al., 2009. Overexpression of isocitrate lyase - Glyoxylate bypass influence on metabolism in *Aspergillus niger*. *Metabolic engineering*, 11, pp.107–116. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19558969 [Accessed April 27, 2012].

Mertz, J.E. & Davis, R.W., 1972. Cleavage of DNA by R1 restriction endonuclease generates cohesive ends. *Proceedings of the National Academy of Sciences of the United States of America*, 69(11), pp.3370–3374.

Meyer, V., Wu, B. & Ram, A.F.J., 2011. *Aspergillus* as a multi-purpose cell factory: current status and perspectives. *Biotechnology Letters*, 33(3), pp.469–476. Available at: http://link.springer.com/10.1007/s10529-010-0473-8.

Miltenberger, K., 2012. Hydroxycarboxylic Acids, Aliphatic. In *Ullmann's Encyclopedia of industrial chemistry*. Weinheim: Wiley-VCH Verlag GmbH & CO, pp. 481–492.

Mlakar, T. & Legisa, M., 2006. citrate inhibition-resistant form of 6-phosphofructo-1-kinase from *Aspergillus niger*. *Applied and environmental microbiology*, 72(7), pp.4515–21. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1489355&tool=pmcentrez&rendertype=abstract [Accessed June 6, 2012].

Nevalainen, K.M.H., Te'o, V.S.J. & Bergquist, P.L., 2005. Heterologous protein expression in filamentous fungi. *Trends in Biotechnology*, 23(9), pp.468–474. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0167779905001551.

Nielsen, K.F. et al., 2009. Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. *Analytical and Bioanalytical Chemistry*, 395(5), pp.1225–1242.

Nissen, T.L. et al., 2000. Anaerobic and aerobic batch cultivations of *Saccharomyces cerevisiae* mutants impaired in glycerol synthesis. *Yeast*, 16(5), pp.463–474.

Noren, C. et al., 1989. A general method for site-specific incorporation of unnatural amino acids into proteins. *Science*, 244(4901), pp.182–188.

Nødvig, C.S. et al., 2015. A CRISPR-Cas9 System for Genetic Engineering of Filamentous Fungi. *PloS one*, 10(7), p.e0133085. Available at: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0133085.

Patel, M. et al., 2006. *Medium and Long-term Opportunities and Risks of the Biotechnological Production of Bulk Chemicals from Renewable Resources*, Utrecht. Available at: http://igitur-archive.library.uu.nl/chem/2007-0628-202305/UUindex.html.

Paul, M., 2007. Trehalose 6-phosphate. *Current Opinion in Plant Biology*, 10(3), pp.303–309. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1369526607000362.

Pedersen, H. et al., 2000. Construction and characterization of an oxalic acid nonproducing strain of *Aspergillus niger*. *Metabolic engineering*, 2(1), pp.34–41.

Poulsen, L. et al., 2012. Identification of a transcription factor controlling pH-dependent organic acid response in *Aspergillus niger*. *PloS one*, 7(12), p.e50596. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3520943&tool=pmcentrez&rendertype=abstract [Accessed September 5, 2014].

R. Poulsen, B. et al., 2005. Increased NADPH concentration obtained by metabolic engineering of the pentose phosphate pathway in *Aspergillus niger*. *FEBS Journal*, 272(6), pp.1313–1325. Available at: http://doi.wiley.com/10.1111/j.1742-4658.2005.04554.x.

Ragauskas, A.J. et al., 2006. The Path Forward for Biofuels and Biomaterials. *Science*, 311, pp.484–489.

Rice, J.A., 2006. *Mathematical Statistics and Data Analysis* 3rd ed., Duxbury Advanced.

Roa Engel, C.A. et al., 2008. Fumaric acid production by fermentation. *appl . microbiol. biotechnol*, 78, pp.379–389.

Rossi, J. & Clementi, F., 1985. L-Malic acid production by polyacrylamide gel entrapped *Pichia membranaefaciens*. *Biotechnology Letters*, 7(5), pp.329–334.

Ruijter, G.J.G. et al., 2000. Properties of *Aspergillus niger* citrate synthase and effects of *citA* overexpression on citric acid production. *FEMS Microbiology Letters*, 184, pp.35–40.

Ruijter, G.J.G., Panneman, H. & Visser, J., 1998. Metabolic Engineering of the Glycolytic Pathway in *Aspergillus niger*. *Food technol. biotechnol.*, 36(3), pp.185–188.

Ruijter, G.J.G., Panneman, H. & Visser, J., 1997. Overexpression of phosphofructokinase and pyruvate kinase in citric acid-producing *Aspergillus niger*. *Biochimica et Biophysica Acta - General Subjects*, 1334, pp.317–326.

Rushing, H., Karl, A. & Wisnowski, J., 2013. *Design and Analysis of Experiments by Douglas Montgomery: A Supplement for Using JMP(R)*, Cary, North Carolina.

Röhr, G. & Kubicek, C.P., 1981. Regulatory aspects of citric acid fermentation by *Aspergillus niger*. *Process Biochemistry*, 16(34).

Rørdam, M. et al., 2010. Metabolic Engineering of Filamentous Fungi 25.1. In *Developing Appropriate Hosts for Metabolic Engineering*. By Taylor & Francis Group, LCC, pp. 1–30.

Samson, R. a et al., 2007. Diagnostic tools to identify black aspergilli. *Studies in mycology*, 59, pp.129–45. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2275192&tool=pmcentrez&rendertype=abstract [Accessed March 26, 2012].

Samson, R. a. et al., 2004. New ochratoxin A or sclerotium producing species in *Aspergillus* section *Nigri*. *Studies in Mycology*, 50(1), pp.45–61.

Sanchez-Marroquin, A., Carreno, R. & Ledezma, M., 1970. Effect of trace elements on citric acid fermentation by *Aspergillus niger*. *Applied and environmental microbiology*, 20, pp.888–892.

Santos, E. et al., 1982. Uptake of sucrose by *Saccharomyces cerevisiae*. *Archives of biochemistry and biophysics*, 216(2), pp.652–660.

Sauer, M. et al., 2008. Microbial production of organic acids: expanding the markets. *Trends in biotechnology*, 26(2), pp.100–8. Available at:

http://www.ncbi.nlm.nih.gov/pubmed/18191255 [Accessed March 21, 2012].

Schuster, E. et al., 2002. On the safety of *Aspergillus niger* - a review. *Applied Microbiology and Biotechnology*, 59(4-5), pp.426–435. Available at: http://link.springer.com/10.1007/s00253-002-1032-6.

Shimizu, Y. et al., 2001. Cell-free translation reconstituted with purified components. *Nature biotechnology*, 19(8), pp.751–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11479568.

Shu, P. & Johnson, M.J., 1948. Production by Submerged Fermentation with *Aspergillus niger*. *Industrial & Engineering Chemistry*, 40(7), pp.1202–1205.

Swartz, J.R., Jewett, M.C. & Woodrow, K. a, 2004. Cell-free protein synthesis with prokaryotic combined transcription-translation. In *Methods in molecular biology (Clifton, N.J.)*. pp. 169–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15269424.

Taing, O. & Taing, K., 2007. Production of malic and succinic acids by sugar-tolerant yeast *Zygosaccharomyces rouxii*. *European food research and technology*, 224(3), pp.343–347.

Tolan, J.S., 2002. Iogen's process for producing ethanol from cellulosic biomass. *Clean technologies and environmental policy*, 3(February), pp.339–345.

Vallero, D., 2014. *Fundamentals of Air Pollution* 5th ed., Academic Press.

Varga, J. et al., 2011. New and revisited species in *Aspergillus* section *Nigri*. *Studies in Mycology*, 69, pp.1–17.

Verhoff, F.H., 2005. Citric Acid. In *Ullmann's Encyclopedia of Industrial Chemistry*. Weinheim: Wiley-VCH Verlag GmbH & CO, pp. 1–6.

Verpoorte, R., van der Heijden, R. & Memelink, J., 2000. Engineering the plant cell factory for secondary metabolite production. *Transgenic research*, 9(4-5), pp.323–343; discussion 321.

VijayaVenkataRaman, S., Iniyan, S. & Goic, R., 2012. A review of climate change, mitigation and adaptation. *Renewable and Sustainable Energy Reviews*, 16(1), pp.878–897. Available at: http://linkinghub.elsevier.com/retrieve/pii/S136403211100459X.

Volhard, J., 1892. Darstellung von Maleïnsäureanhydrid. *Justus Liebig's Annalen der Chemie*, 268, pp.255–256.

Werpy, T. et al., 2004. *Top Value Added Chemicals from Biomass Volume I — Results of Screening for Potential Candidates from Sugars and Synthesis Gas*, U.S. Department of Energy, Oak Ridge.

Weyda, I. et al., 2014. Point mutation of the xylose reductase (XR) gene reduces xylitol accumulation and increases citric acid production in *Aspergillus carbonarius*. *Journal of industrial microbiology & biotechnology*, 41(4), pp.733–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3953602&tool=pmcentrez&rendertype=abstract [Accessed October 27, 2014].

Willey, J.M., Sherwood, L.M. & Woolverton, C.J., 2014. *Prescott's Microbiology* 9th ed., McGraw-Hill Global Education Holdings, LLC.

Witteveen, C.F. et al., 1990. Characterization of a glycerol kinase mutant of *Aspergillus niger*. *Journal of general microbiology*, 136(1990), pp.1299–1305.

World Health Organization, 2014. Public health, environmental and social determinants of health (PHE). *Burden of disease from ambient and household air pollution*. Available at: http://www.who.int/phe/health_topics/outdoorair/databases/en/ [Accessed December 9, 2015].

Yang, L., Lübeck, M. & Lübeck, P.S., 2014. Deletion of glucose oxidase changes the pattern of organic acid production in *Aspergillus carbonarius*. *AMB Express*, 4(1), p.54. Available at: http://www.amb-express.com/content/4/1/54.

Yang, L., Lübeck, M. & Lübeck, P.S., 2015. Effects of heterologous expression of phosphoenolpyruvate carboxykinase and phosphoenolpyruvate carboxylase on organic acid production in *Aspergillus carbonarius*. *Journal of Industrial Microbiology & Biotechnology*. Available at: "http://dx.doi.org/10.1007/s10295-015-1688-4.

Zelle, R.M. et al., 2008. Malic acid production by *Saccharomyces cerevisiae*: engineering of pyruvate carboxylation, oxaloacetate reduction, and malate export. *Applied and environmental microbiology*, 74(9), pp.2766–77. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2394876&tool=pmcentrez&rendertype=abstract [Accessed March 6, 2012].

Aanen, D.K. et al., 2011. *Cellular and Molecular Biology of Filamentous Fungi* 1st ed. K. A. Borkovich & D. J. Ebbole, eds., Washington: American Society for Microbiology.

CHAPTER 3. MANUSCRIPT I – ENGINEERING OF THE REDUCTIVE TCA BRANCH FOR ENHANCED CARBOXYLIC ACID PRODUCTION IN *ASPERGILLUS CARBONARIUS*

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3.1. ABSTRACT

Citrate, fumarate and malate are naturally found as intermediates in the TCA cycle in all aerobic organism thus also in filamentous fungi, and they are all used as industrial chemicals. Citric acid is mainly produced as a bulk chemical biologically from the filamentous fungi *Aspergillus niger* whereas fumaric and malic acid are produced mainly by the petro chemistry industry. A promising way to produce malic and fumaric acid biologically is through the reductive cytosolic TCA-branch in fungi. The strategy is to build a reductive pathway in the cytosol by introducing genes that encodes enzymes targeted to this location in *Aspergillus carbonarius*, a close relative to *A. niger*. In this study the effect of co-transformation of five different genes were investigated. The genes were the native pyruvate carboxylase gene (*pyc2*), a truncated version of the malate dehydrogenase from *Saccharomyces cerevisiae* (*mdh3*), a truncated version of the fumarase gene from *S. cerevisiae* (*fum1*), a truncated version of the fumarase gene from *Rhizopus oryzae* (*fumR*), and a malate transporter gene from *Schizosaccharomyces pombe* (*mae1*). Eleven different genotypes were successfully constructed via co-transformation. The transformants were tested in fermentation experiments and the results showed that Pyc2 alone on average increased the malic acid production with 28%, Pyc2+MDH3 increased the malic acid production with 32%, and Pyc2+MDH3+Fum1 increased the malic acid production with 57%. There were no significant effect on citric acid production and fumaric acid was not detected in the experiments.

3.2. INTRODUCTION

The world that we live in today suffers from massive environmental problems due to the pressure we put on the earth because of the way that we spend the earth's natural resources. A great part of this arises because of our fossil fuel usage for numerous different products. Sustainable biotechnology has the tools to replace the majority of the fossil fuel-based products with biomass-based equivalents. The biorefinery concept can make a wide range of these products that could substitute petroleum-based products; however, the price of making these products is still not economically competitive with the petrochemical industry for the majority of products. One way of solving this issue is to develop the biological methods to produce high-value products and thereby increasing the profitability of the biorefinery concept (Hansen 2014). With the molecular engineering techniques that are available today, it is theoretically possible to design and construct microbial cell factories that are capable of producing specific natural products of industrial interest. Several natural products are proposed to substitute petro chemically made products thereby being of high economical value, among these are malic acid and fumaric acid (Werpy et al. 2004).

Malic acid is mainly used as an acidifier in food. Originally malic acid was produced by extraction from apple juice, however apple juice only contains between 4 to 7 g/l malic acid so the production capacity was small and advances in chemistry quickly made the petrochemical method far more favorable (Buck & Mottern 1947). In the petrochemical method, double hydration of maleic anhydride yields a racemic mixture that is separated by chiral resolution creating malic acid. The petrochemical method of producing malic acid is by far the most used method today. Other methods of producing malic acid involves enzymatic conversion from fumaric acid (Rossi & Clementi 1985; Kajiwara & Maeda 1986) or production directly from carbon sources (Battat et al. 1991; Taing & Taing 2007). Battat et al. (1991) reported the highest malic acid productivity ever from a wild type fungal strain. They produced 0.59 g/(l*h) malic acid during optimized submerged conditions with *Aspergillus flavus*, a natural producer of malic acid.

Introduction of genetic engineering has contributed to advances within the field of biological malic acid production. In a study made with *Saccharomyces cerevisiae* three genetic modifications were made: overexpression of the native pyruvate carboxylase encoded by *pyc2*; expression of *mdh3* that encoded a cytosol targeted malate dehydrogenase; and functional expression of the *Schizosaccharomyces pombe* malate transporter gene *mae1*. Single and double modifications increased the malate production compared to wild type but the highest increase in malate production was achieved with all three modifications. In this strain, a final production titer of 59 g/l was achieved with a malate yield of 0.42 mole/mole glucose. In this study it was argued that the high malic acid productivity was achieved due to an increase in the cytosolic flux from pyruvate to malic acid through oxaloacetate thus bypassing the TCA cycle and functionally introducing a reductive pathway in the cytosol (Zelle et

al. 2008). In a strain of *Aspergillus oryzae* modified with the same three genes, malic acid production rates reached 1.05 g/(l*h) and final titers reached 66.3 g/l after only 48 hours of fermentation (Knuf et al. 2014). These studies furthermore inspired a recent patent application in *A. oryzae* with a codon optimized malic acid transporter from *S. pombe* that showed a 40% increase in malic acid productivity (Mcfarland & Fischer 2013).

Fumaric acid is primarily used as an acidifier in food but it is also used as feedstock for the production of paper resins, unsaturated polyester resins, alkyd resins, plasticizers, and miscellaneous industrial usage. Fumaric acid is mainly produced by the petrochemical industry via a catalytic isomeration of maleic acid, a hydrolysis product of maleic anhydride, that are produced from benzene or butane products (Roa Engel et al. 2008). Fumaric acid is an intermediate in the TCA cycle and therefore ubiquitous throughout aerobic organisms, but only very few organisms excrete fumaric acid to its surroundings. One of the best natural producers of fumaric acid is the filamentous fungi *Rhizopus oryzae* (Goldberg et al. 2006). It is believed that the *fumR* gene encoding a fumarase in *R. oryzae* is at least partly responsible for the high production of fumaric acid by *R. oryzae* (Friedberg et al. 1995). In a study by de Jongh & Nielsen (2008), two truncated fumarase genes, *fum1* from *S. cerevisiae* and *fumR* from *R. oryzae,* were inserted in *A. niger* to enhance the production of citric acid. FumR from *R. oryzae* catalyzes the reaction of malate to fumarate, whereas Fum1 from *S. cerevisiae* catalyzes the opposite reaction from fumarate to malate. The altered strains were able to produce more citric acid than the control strain, but malic and fumaric acid accumulation was only observed intracellular in high amounts (de Jongh & Nielsen 2008).

A. niger is capable of producing citric acid in great amounts and it has furthermore shown the ability of producing malic acid in small amounts. The reductive pathway plays an important role during citric acid production. Formation of malate via the reductive pathway in the cytosol is believed to start and fuel the export of citric acid from the mitochondria to the cytosol via a citrate malate antiporter resulting in citric acid productivity higher than 2.00 g/(l*h) (Goldberg et al. 2006; Karaffa & Kubicek 2003). The malate present in the cytosol during citric acid production in *A. niger* could potentially give the opportunity to change *A. niger* into a malic acid producer or maybe even a fumaric acid producer by introducing the needed genetic elements thereby altering the reductive pathway in a desired way.

Aspergillus carbonarius is closely related to *A. niger* and the two fungi bear a strong resemblance (Samson et al. 2007). In the present study *A. carbonarius* was chosen as model organism for multiple reasons. In several studies it has been shown that *A. carbonarius* can produce citric acid in high amounts, it is very tolerant to acid environments and protoplasts can be made from its spores enabling the possibility of genetic manipulation (Linde et al. 2014; Weyda et al. 2014; Yang et al. 2015). Furthermore the *A. carbonarius* ITEM 5010 genome sequence has been made public

available through the Joint Genome Institute, which together with the vast information available about *A. niger* and the fact that the two species share great resemblance, simplifies genetic engineering of *A. carbonarius*.

In this study *A. carbonarius* was genetically altered with the main goal of making a strain capable of producing either malic or fumaric acid. Four genes encoding for enzymes targeted to the cytosol and one gene encoding a malate transporter were selected and inserted in *A. carbonarius* to introduce a full reductive pathway, including export out of the cell, from pyruvate to malate and fumarate thereby partly bypassing the TCA cycle (Figure 3.1). The five genes were 1) the native *pyc2* gene encoding a cytosolic pyruvate carboxylase, which catalyzes the carboxylation of pyruvate to oxaloacetate (Stucka et al. 1991). 2) a truncated version of the *mdh3* gene from *S. cerevisiae* encoding a malate dehydrogenase targeted to the cytosol, which catalyzes the reduction of oxaloacetate to malate (McAlister-Henn et al. 1995). 3) a truncated version of the *fum1* gene from *S. cerevisiae* encoding a cytosolic fumarase, which catalyzes the hydration of fumarate to malate (Pines et al. 1996). 4) a truncated version of the *fumR* gene from *R. oryzae* encoding a cytosolic fumarase, which catalyzes the dehydration of malate to fumarate (Friedberg et al. 1995). and 5) the *mae1* gene from *S. pombe* encoding a permease able to transport malate and other c_4 dicarboxylic acids like succinate and malonate (Grobler et al. 1995). Co-transformation was carried out with three different combinations of the five genes, 1) *pyc2+mdh3+mae1*, 2) *pyc2+mdh3+fum1*, 3) *pyc2+mdh3+fumR*. The hypothesis was that combination 1 would facilitate an increased flux from pyruvate via oxaloacetate to malate including active transport of malate out of the cell. Combination 2 would facilitate an increased flux from pyruvate via oxaloacetate to malate and furthermore convert fumarate in the cytosol in to malate increasing the amount of malate produced. Combination 3 would facilitate an increased flux from pyruvate via oxaloacetate to malate and one step further to fumarate. Single, double and triple transformation events with the five different genes created 11 different types of transformants with different parts of the reductive pathway inserted. The transformants were examined for genetic alterations by PCR and positive transformants were evaluated via citric, malic and fumaric acid production in fermentation experiments.

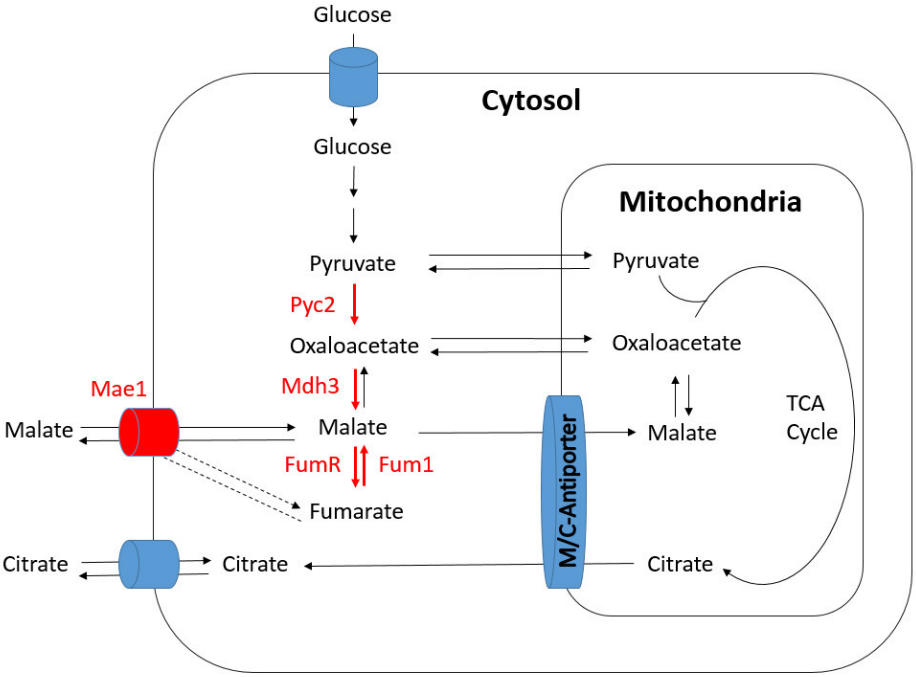

Figure 3.1 Simplified version of the reductive pathway and the TCA cycle in *A. carbonarius* with the genetic engineered elements shown in red. The double arrows from glucose to pyruvate represent the glycolysis.

3.3. MATERIALS AND METHODS

3.3.1. STRAINS AND GROWTH CONDITIONS

A. carbonarius ITEM 5010 originally isolated from grapes in Italy (www.ispa.cnr.it/collection) was kindly supplied from Prof. Jens Frisvad, Danish Technical University, Lyngby (Department of Systems Biology, Center for Microbial Biotechnology). *A. carbonarius* cultivation was done on potato dextrose agar (PDA; Scharlau Chemie S.A. Barcelona, Spain) at 30° C in darkness. *S. pombe* Eg545 was obtained from Professor Olaf Nielsen (Department of Biology, University of Copenhagen, Denmark) and cultivated on a medium consisting of yeast extract 5 g/l, adenine sulfate 0.1 g/l, uridine 0.1 g/l, L-histidine 0.1 g/l, L-leucine 0.2 g/l, H_2O 1 L, glucose 30 g/l, agar 20 g/l at 30°C. *R. oryzae* ATCC 20344 used in this study was cultured on YMP agar plates containing 3 g/L yeast extract, 3 g/L malt extract, 3 g/L peptone, 20 g/L glycerol and 20 g/L agar. *S. cerevisiae* DDSF 623 was from Danish Distillers, Aalborg, Denmark and cultivated on potato dextrose agar at 30°C in darkness. *Escherichia coli* strain DH5α was used as host for plasmid propagation. Spore suspension was stored as stock solution in 20% glycerol in -80°C

3.3.2. GENOMIC DNA EXTRACTION

Genomic DNA extraction was done with a CTAB method as described in chapter 4, manuscript II of this thesis, based on the method described by (Lee et al. 1988; Wu et al. 2001).

3.3.3. GENES

pyc2 encoding pyruvate carboxylase was amplified from genomic DNA from *A. carbonarius*. *mdh3* encoding a malate dehydrogenase was amplified from genomic DNA from *S. cerevisiae* and retargeted to the cytosol by deleting the C-terminal peroxisomal targeting sequence. The malate transporter encoded by the *mae1* gene was amplified from genomic DNA from *S. pombe*. *fum1* encoding a fumarase was amplified from genomic DNA from *S. cerevisiae* and truncated by deleting the initial 17 amino acids for retargeting to the cytosol. *fumR* encoding a second fumarase was amplified from genomic DNA from *R. oryzae* and truncated by deleting the initial 15 amino acids for retargeting to the cytosol.

3.3.4. PLASMIDS

In fungi, multiple genetic alterations can be introduced into the genome in a single transformation event. This is achieved with one selective plasmid carrying the selective marker and a desired gene. This plasmid is used in a co-transformation event with one or more co-plasmids only carrying elements needed for expression of a desired gene but no selective marker (Roberts et al. 1989; Wernars et al. 1987). In our study, co-transformation was used with one plasmid carrying hygromycin resistance as selective marker and *pyc2* together with two co-transformation plasmids. Three different combinations of genes were used in the co-transformation, 1) *pyc2*+*mdh3*+*mae1*, 2) *pyc2*+*mdh3*+*fum1*, 3) *pyc2*+*mdh3*+*fumR* (Table 3.1). The high copy number plasmid pJET2.1 (ThermoFisher Scientific) was used as backbone for all the plasmids used in this study. The selective plasmid with *pyc2* (pe1Pyc2) and the co-plasmid with *mdh3* (pCoMdh3) was constructed prior to this study by Hansen et al. (2014). pe1Pyc2 contains the following genetic elements: *RP27-hph-βT* for hygromycin resistance, origin of replication and ampicillin resistance for growth in *E.coli* and a *gpdA* promoter and a *trpC* terminator flanking the *pyc2* gene. pCoMDH3 contains the same elements except for the hygromycin resistance and that it contains *mdh3* instead of *pyc2*. pCoMae1, pCoFum1 and pCoFumR were constructed like pCoMdh3 as described by Hansen et al. (2014). Briefly, the genes were amplified from genomic DNA by PCR. Purified PCR products were inserted into the intermediate vector pSB30011 using Nicking cloning and transformed into chemically competent DH5α *E. coli* cells for further propagation using standard procedures (Sambrook & Russel 2001). Plasmid extraction was done using the GeneJET plasmid miniprep kit (ThermoFisher Scientific, Waltham, MA, USA)

according to the manufacturer's protocol. All plasmids were verified by Sanger sequencing using the sequencing service from StarSEQ (Mainz, Germany).

3.3.5. CO-TRANSFORMATION

The selection plasmid pe1Pyc2 was mixed with two co-plasmids in the mixing ratio 1:3:3 w/w to a final concentration of 1.5 µg DNA/µl and used in transformation of 100µl aliquots of protoplasts from *A. carbonarius* ITEM5010 as described by Yang et al. (2014). Transformants able to grow on hygromycin were isolated and transferred 3 times to new plates containing hygromycin in order to ensure that the transformants were stable. Spore-suspensions of each transformant in 20% glycerol were stored at -80°C for long-term storage. Table 3.1 shows the three different combinations of genes that *A. carbonarius* was transformed with including a fourth combination carried out in a previous study where the transformants were investigated in this present study.

Table 3.1 References to gene combinations used in this study

Gene combination	Transformation done by
pyc2+mdh3	(Hansen et al. 2014)
pyc2+mdh3+mae1	This study
pyc2+mdh3+fum1	This study
pyc2+mdh3+fumR	This study

3.3.6. IDENTIFICATION OF INSERTED DNA

Validation of inserted genes in the transformants were done using PCR with transformant genomic DNA as template. The PCR was made with a forward primer binding on the *gpdA* promoter and reverse primers binding at the end of one of the five target genes. This was done as to avoid seeing any background signals from native *A. carbonarius* genes since the *gpdA* promoter sequence does not exist in *A. carbonarius*. The primers used can be seen in table 3.2.

3.3.7. FERMENTATION SETUP AND CONDITIONS

Citric acid fermentation was done in 24-well plates and the setup were made following the principles of Linde et al. (2014). Citric acid production media from Shu & Johnson (1948) was used with no alterations (140 g/l sucrose, 2.5 g/l KH_2PO_4, 0.25 g/l $MgSO_4 \cdot 7H_2O$, 2.5 g/l NH_4NO_3, pH to 3.8, Cu^{2+} 0.06 mg/l, Zn^{2+} 0.25 mg/l, Fe^{2+} 1.3 mg/l). All transformant fermentation experiments were made in biological triplicates. Fresh spore-suspension was added to 10 ml fermentation media to a final concentration of $2*10^5$ spores/ml and mixed well. The fermentation media was distributed with 3 ml each in to three wells in the 24-well plate (UNIPLATE, 24 wells, 10 ml, Whatman). The plates were covered with silicone lids (BugStopper Venting Capmat, Whatman) and incubated for 5 days at 25°C and 420 rpm in a Glas-

Col model 099A DPMINC24 (Glas-Col, Terre Haute, IN). The incubator was kept at a constant high humidity to avoid excess evaporation by having open boxes with distilled H_2O mounted inside the incubator. Samples were taken at the end of the experiments, filtered through 0.45 µm HPLC-grade regenerated cellulose membrane filters and analyzed on High Performance Liquid Chromatography (HPLC Dionex Ultimate 3000-LC system Dionex Corporation, Sunnyvale, CA) with an Aminex HPX-87H column coupled to an RI-detector. The eluent was 4 mmol/l H_2SO_4 with a flow rate of 0.6 ml/min at 60°C. All chromatograms were integrated using the Chromeleon software (Dionex Corporation). Different from the rest of the experiments, the wild type acid profile (Figure 3.5) was made with six biological replicas and sampling was done every 24 hours for 12 days.

Malic and fumaric acid fermentation was done similar to the citric acid fermentation with the only exception that malic acid media was used instead of citric acid media. The malic acid media from (Peleg et al. 1988) was used with minor alterations (glucose 100 g/l, $(NH_4)_2SO_4$ 2 g/l, KH_2PO_4 0.15g/l, K_2HPO_4 0.15g/l, $MgSO_4 \cdot 7H_2O$ 0.1 g/l, $CaCl_2 \cdot 2H_2O$ 0.1 g/l, NaCl 0.005 g/l, $ZnSO_4$ 0.1 g/l, $FeSO_4 \cdot 7H_2O$ 0.005 g/l and $CaCO_3$ 30 g/l). The supernatant obtained from each malic acid fermentation was prepared as described by Goldberg et al. (1983). 1 ml samples were taken at day 5 and 50 µl 50% H_2SO_4 was added and the mixture was heated to 80°C and incubated for 15 minutes. The mixture was cooled to room temperature and the supernatant was filtered through 0.45 µM HPLC-grade regenerated cellulose membrane filters. 250 µl of the filtrate were analyzed for the content of glucose, fructose and citric acid by HPLC. 50 µl of the filtrate were analyzed for the content of malic acid using the Megazyme™ L-Malic Acid Kit (Megazyme, Wicklow, Ireland) according to manufacturer's instructions.

After the fermentation experiments, the biomass measurements was gathered, rinsed with plenty of water, dried at 105°C for 48h and weighed on a microscale.

3.3.8. DETERMINATION OF GENE EXPRESSION

Total RNA was extracted from 5-days old cultures of transformed and wild type *A. carbonarius* using the Total RNA Mini Kit (Cat # 031) according to manufacturer's protocol (A&A biotechnology). Following extraction and purification, DNA was removed from RNA samples using Fermentas' DNAse kit (#EN0521). cDNA was synthesized from DNase treated total RNA with reverse transcription (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems). Gene expression was tested by amplifying short fragments of the inserted genes from 10 times diluted cDNA with RUN polymerase (A&A biotechnology), RUN buffer (A&A biotechnology), 0.16 mM dNTP mix and 0.4 µM of each forward and reverse primers.

3.3.9. STATISTICAL ANALYSIS

Statistical comparison of transformant results against wild type samples were done by independent (unpaired) two-sample *t*-test with a significant level of $p<0.05$. Error bars on figures are standard deviation based on a sample or standard error of the mean.

Table 3.2 Primers used in this study

Name	No.	Sequence (5' → 3')	Annotation
Mae1 fw-U	1	AGAGCGAUCATGGGTGAACTCAAGGA	Amplifies *mae1*, contains uracil tail
Mae1 rv-U	2	TCTGCGAUTTAAACGCTTTCATGTTCA	Amplifies *mae1*, contains uracil tail
Fum1 fw-U	3	AGAGCGAUATGAACTCCTCGTTCAGAACTGAAAC	Amplifies *fum1*, contains uracil tail
Fum1 rv-U	4	TCTGCGAUTTATTTAGGACCTAGCATGTGTTCAGG	Amplifies *fum1*, contains uracil tail
FumR fw-U	5	AGAGCGAUATGAACAACTCTCCTCGTCTTTTCAG	Amplifies *fumR*, contains uracil tail
FumR rv-U	6	TCTGCGAUTTTAATCCTTGGCAGAGATCATATCTTC	Amplifies *fumR*, contains uracil tail
Contr fw	7	TCGTTGACCTAGCTGATTCTGG	Control of all inserts, binds on *gpdA*
Contr Pyc2 rv	8	CTAGGCCTTGGTGATCTTGC	Control of *pyc2* insert
Contr Mdh3 rv	9	TCAAGAGTCTAGGATGAAACTCT	Control of *mdh3* insert
Contr Mae1 rv	10	TTAAACGCTTTCATGTTCA	Control of *mae1* insert
Contr Fum1 rv	11	TTATTTAGGACCTAGCATGTGTTCAGG	Control of *fum1* insert
Contr FumR rv	12	TTTAATCCTTGGCAGAGATCATATCTTC	Control of *fumR* insert
Expr Pyc2 fw	13	AGGTTCGTCAGGTCAGTGTC	Verification of *pyc2* expression
Expr Pyc2 rv	14	GCCGAGATAACCATTTCCATCT	Verification of *pyc2* expression
Expr Mdh3 fw	15	AGATGAACGCCGGTATTGTC	Verification of *mdh3* expression
Expr Mdh3 rv	16	AGGTCAAGGTTCGTCACACC	Verification of *mdh3* expression
Expr Mae1 fw	17	TAATATTGCGCGTGGTGCTA	Verification of *mae1* expression
Expr Mae1 rv	18	GCTAACCATGGCGAGACAGT	Verification of *mae1* expression

3.4. RESULTS

3.4.1. VALIDATION OF TRANSFORMANTS AND SCREENING STRATEGY

Co-transformation successfully created 11 different genotypes and a total of 103 transformants that were verified as positive transformants by PCR (Table 3.3). Examples of positive transformants can be seen in figure 3.2, 3.3 and 3.4. *pyc2* was validated by a PCR fragment of 4.5kb. As it can be seen in figure 3.2, all the transformants and the wild type have a PCR fragment at approximately 3.5kb. This was identified through sequencing to be the native *pyc2* gene that was amplified even though the forward primer was not designed to bind on the genomic DNA. *mdh3* was validated by a PCR fragment of 1.9kb (Figure 3.3) and *mae1* was validated by a PCR fragment of 2.15KB (Figure 3.4). Examples of *fum1* (2.3kb) and *fumR* (2.9kb) are not shown.

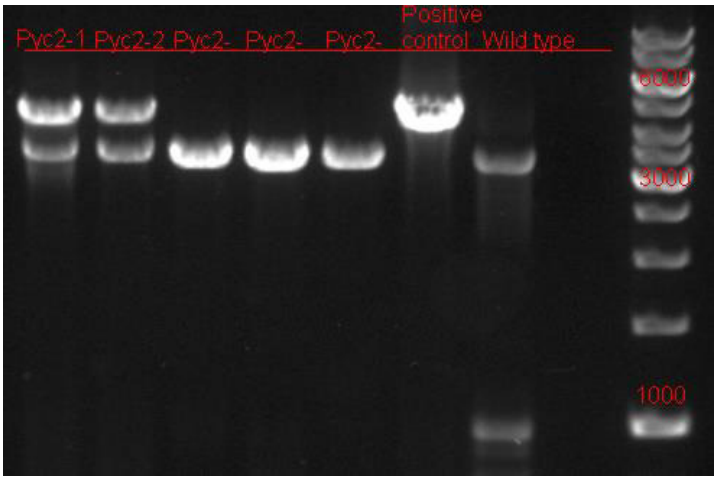

Figure 3.2 Example of identification of inserted DNA using gel-electrophoresis. Two transformants, Pyc2-1 and Pyc2-2 (lane 1 and 2) contained the inserted *pyc2*.

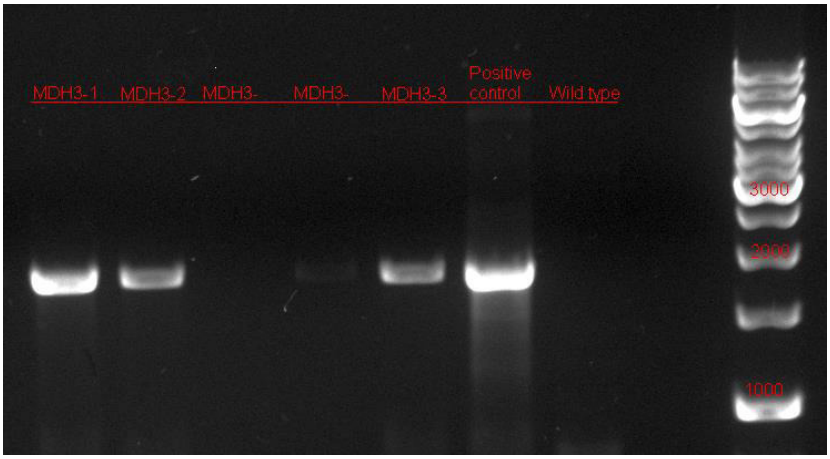

Figure 3.3 Example of identification of inserted DNA using gel-electrophoresis. Transformants Mdh3-1, Mdh3-2, Mdh3-3 (lane 1, 2 and 5) were chosen for further study.

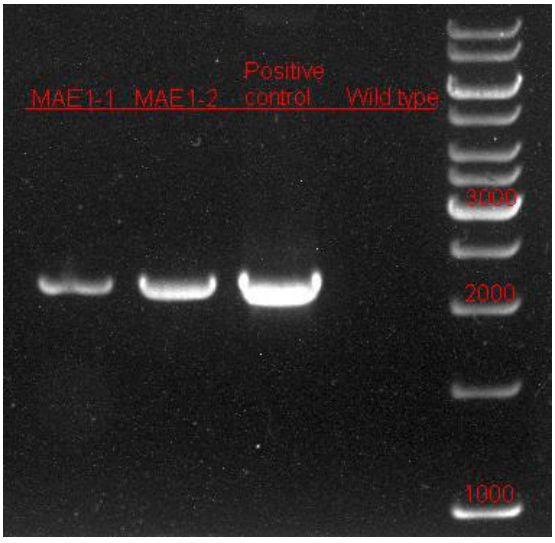

Figure 3.4 Example of identification of inserted DNA using gel-electrophoresis. Two transformants, Mae1-1 and Mae1-2 (lane 1 and 2) contained the inserted *mae1*.

RT-PCR was used to test eight of the transformants with three genes inserted to see if the gene expression was functional. All eight gave a positive signal with all three genes inserted verifying that all the tested genes were positively expressed (results shown in Hansen (2014)). The result was used as a random check of the whole sample size and the conclusion was that all transformants were functional. Fermentation experiments with these 11 different genotypes should provide amply information about how the genes influence the cytosolic reductive pathway and their effect on

citric, malic and fumaric acid production. Wild type *A. carbonarius* was used as control strain throughout the screening experiments. Before the transformants were tested, a time-course fermentation experiment was conducted with *A. carbonarius* to identify optimal sampling time and average citric and malic acid accumulation. The transformants were tested in citric and malic acid fermentation experiments at least one time and for many of the transformants two or three successive times. The malic acid fermentation media is very similar to the fumaric acid fermentation media used in fumaric acid production with *R. oryzae* with success (Ding et al. 2011; Fu et al. 2010). Fermentation with *R. oryzae* has been carried out in the malic acid media and fumaric acid was produced successfully (Results not shown). It was therefore assumed that the malic acid media was able to support fumaric acid production with the *A. carbonarius* transformants as well; however, no fumaric acid was detected in any of the experiments.

Table 3.3 Transformant types and number of positive transformants

Transformant type	Number of positive transformants
pyc2	30
pyc2+mdh3	19
pyc2+mae1	8
pyc2+fum1	3
pyc2+fumR	1
pyc2+mdh3+mae1	13
pyc2+mdh3+fum1	8
pyc2+mdh3+fumR	1
mdh3	12
mae1	6
mdh3+mae1	2
Total	103

3.4.2. TIME-COURSE FERMENTATION EXPERIMENT WITH *A. CARBONARIUS* WILDTYPE

A time course experiment was conducted with wild type *A. carbonarius* to produce knowledge about the development of the experimental parameters and products over time during one round of fermentation (Figure 3.5). The substrate sucrose was followed over time along with the primary products of relevance; citric and malic acid, whereas no fumaric acid was produced by the wild type *A. carbonarius* during these fermentation conditions. The main goal for the experiment was to select a time during the fermentation where the titers of the products were sufficient and

significant enough to use to compare results between transformants and wild type in later fermentation experiments. As it can be seen on figure 3.5, sucrose is consumed at an approximately constant rate during the full fermentation. Citric acid was produced at an approximately constant rate between 24 h and 168 h and to some extent the full fermentation period. Malic acid was produced at a constant rate between 24 h and 168 h, after 168 h the amount of malic acid in the media started to decline due to uptake from the fungus. Based on these results, with an included safety margin, an interval was chosen between 72 h to 144 h where it would be relevant to take samples and use as comparable results between transformants and wild type without missing information about production of metabolites and consumption of substrates.

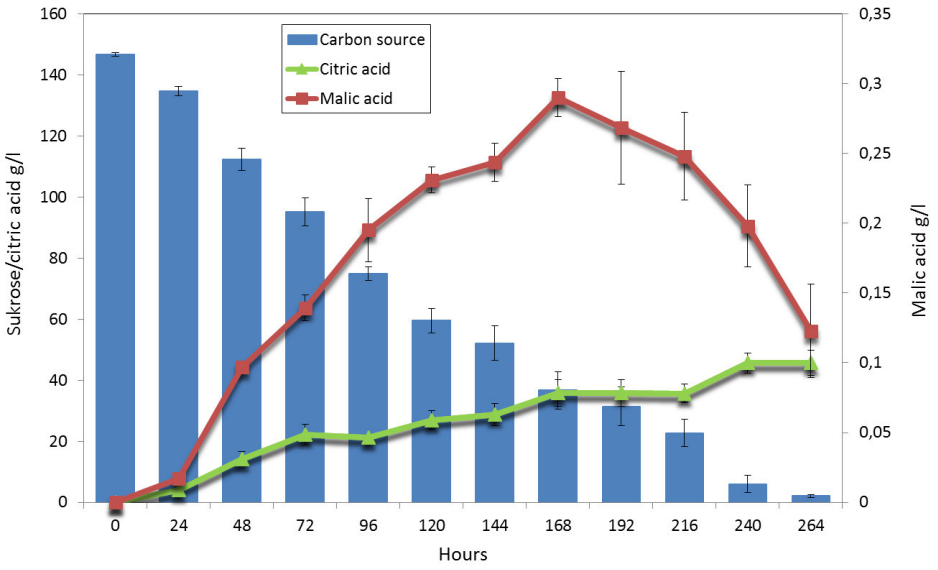

Figure 3.5 Time course experiment over 264 h. Remaining sucrose, accumulated citric acid and malic acid are shown. Error bars represent the sample standard deviation.

3.4.3. INVESTIGATION OF BIOMASS PRODUCTION

After finishing each fermentation experiment, the produced biomass was gathered, dried, and weighed. Figure 3.6 shows one representative fermentation experiment with selected transformants and the wild type *A. carbonarius* run for 5 days. The graphs show amount of sucrose consumed, amount of metabolites accumulated (citric acid, malic acid, glycerol and ethanol) and amount of biomass produced. In the experiment the sucrose consumption varied from 41 g/l to 56 g/l, the metabolite production varied from 15 g/l to 29 g/l and the biomass production varied from 9 g/l to 11 g/l. The significance of this result is that even though sucrose consumption and

especially metabolite production showed high variation in one experiment, the biomass production was close to similar among strains when looking at one experiment. This trait continued throughout the fermentation experiments showed in this chapter and throughout the experiments in the rest of the thesis unless otherwise specified. Thus, the biomass production has not been a parameter that received much focus during the experimental studies.

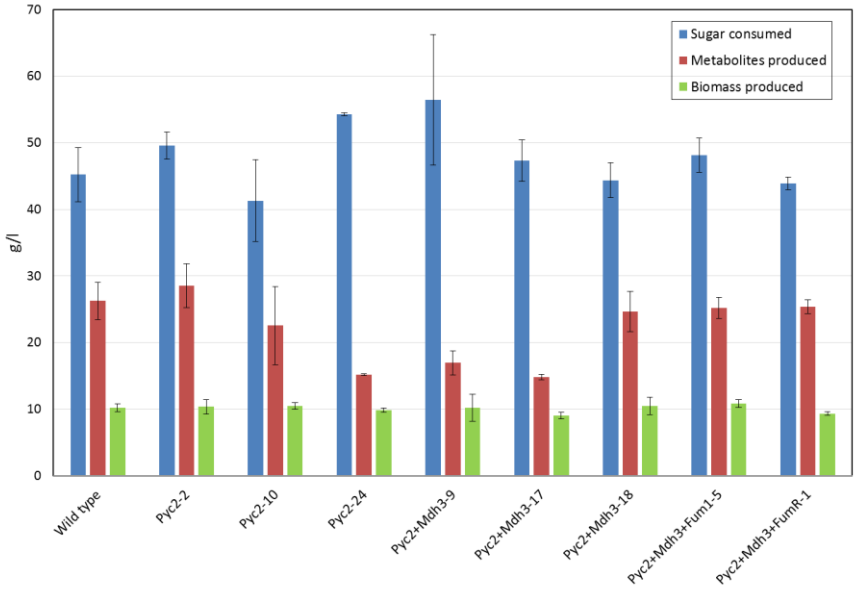

Figure 3.6 Sucrose consumption, metabolite (citric acid, malic acid, glycerol and ethanol) and biomass accumulation during 120 h citric acid fermentation. Error bars represent the sample standard deviation.

3.4.4. VARIATION BETWEEN FERMENTATION EXPERIMENTS

As described by Linde et al. (2014) (see chapter 6, manuscript IV) great variations were experienced during the fermentation experiments. However, the variation was most significant when comparing one experiment with the next one. Replicas of the same strain within the same experiment round did not show great variation. This led to the assumption that within one experiment the strains could be compared between each other and the wild type sample that was included in every experiment. To test this hypothesis, two successive rounds of fermentation with the exact same setup were conducted. The results from these experiments are shown in figure 3.7. Even though the general levels of glucose, fructose, glycerol and citric acid varied from the first replication to the second replication, the significant traits among these transformants can still be found. Pyc2-2 shows a greatly increased citric acid production in both experiments compared to wild type. Pyc2-10 is exactly similar to wild type in both experiments. Pyc2-18 has low glucose consumption and no citric

acid production in both experiments compared to wild type. Pyc2-24 has low glucose consumption, a very high fructose consumption, very high glycerol production and no citric acid production in both experiments compared to wild type. It should be noted that the variation from one experiment to the next one could be much higher than what is seen in these two examples. These results showed that even though there were great variations between experiments, the significant traits in transformants are still trustworthy as long as they are compared directly within one experiment with the wild type *A. carbonarius*.

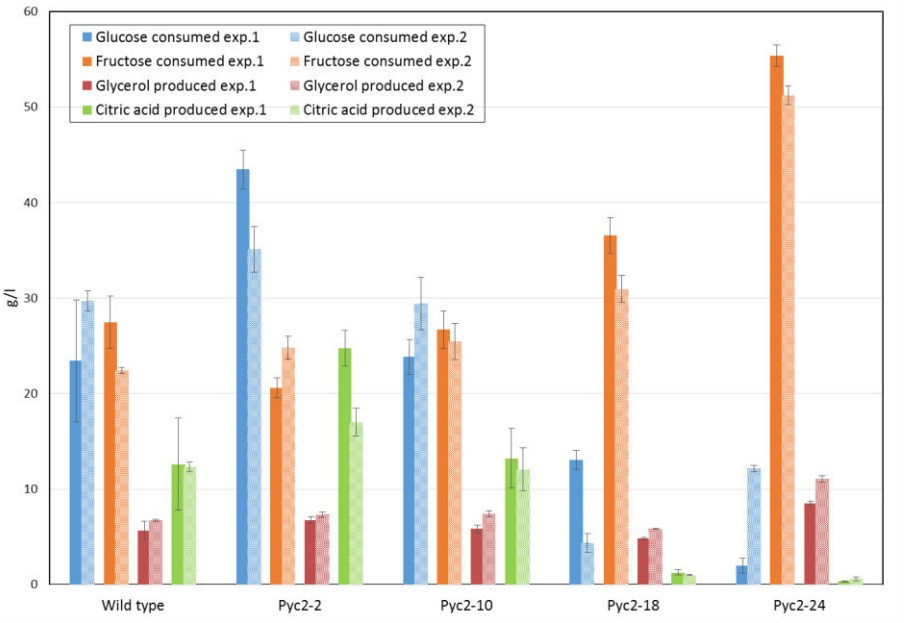

Figure 3.7 Glucose and fructose consumption, and glycerol and citric acid accumulation after 120 h of fermentation from two successive citric acid fermentation experiments. Results from experiment 1 are shown with solid bars; results from experiment 2 are shown with 50% pattern. Error bars represent the sample standard deviation.

3.4.5. VALIDATION OF SCREENING RESULTS

In this study 103 positive transformants were constructed. 78 of these transformants have been tested in citric acid fermentation and malic acid fermentation at least one time and most of them two times or more. Figure 3.8 and 3.9 show the results from these experiments. These results are the outcome of many separate experiments and as described before in this thesis, problems with great variations between experiments, make it impossible to compare the numbers directly. To be able to compare between experiments, the results for the citric and malic acid accumulation

have been calculated relative to the wild type sample that is included in each experiment. The wild type results are set to 100% and the transformant results are calculated relative to that. This representation of the results is valid in a study like the present one where it is differences from the wild type that are pursued, and not optimization of e.g. citric acid production to reach a specific value. The representation is furthermore validated by the description above (Figure 3.7), that transformants can be compared with each other and the wild type within one specific experiment.

3.4.6. CITRIC ACID FERMENTATION

The results shown in figure 3.8 are from one to three repeated experiments with biological triplicates in citric acid fermentation. The citric acid accumulation after 120 h varied from approximately 5 to 35 g/l throughout the experiments. Two different types of results were analyzed: 1) general trends in one type of transformants and 2) single outliers with great differences compared to wild type. General trends within a transformant type was investigated using t-test with a significant level of $p<0.05$ against wild type samples. None of the transformant types showed any significant differences from wild type. Average production was increased or decreased with less than 15% compared to the wild type. Outliers for citric acid production were defined as transformants that produced 30% or more citric acid than the wild type. There were 18 transformants matching these criteria. Out of these 18 transformants, six were tested in a second round of fermentation. The single highest producer of citric acid from the second round of fermentation, Pyc2-2, was selected for a third replication of the fermentation. In the third round of fermentation, Pyc2-2 consistently produced more citric acid compared to the wild type, on average 53% more. There are also outliers that produces significantly less citric acid than the wild type *A. carbonarius*. Four transformants, Pyc2-17, Pyc2-23, Pyc2+Mdh3-9 and Pyc2+Mdh3-17, showed significantly lower production than the wild type. All of the four transformants were tested in a second round of fermentation and they all consistently produced significantly less citric acid than the wild type.

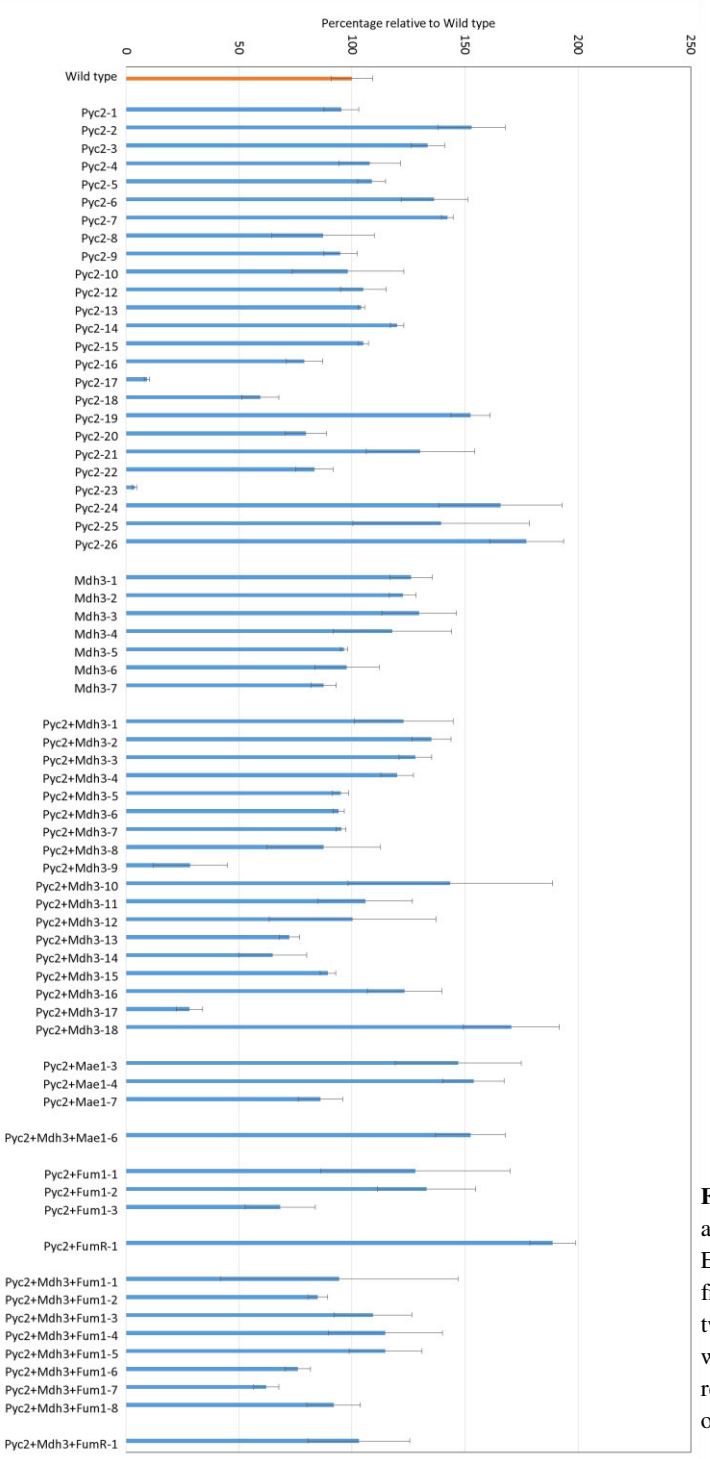

Figure 3.8 Citric acid accumulation relative to wild type. Each data point represent results from at least one and often from two or three repeated experiments with triplicate samples. Error bars represent standard deviation based on a sample.

3.4.7. MALIC ACID FERMENTATION

The results shown in figure 3.9 are from one to three repeated experiments with biological triplicates in malic acid fermentation. The malic acid accumulation after 120 h varies from approximately 100 to 800 mg/l throughout the experiments. As with the citric acid results, two different types of results were analyzed within the malic acid results, 1) general trends in one type of transformants and 2) single outliers with significant differences compared to wild type. The results from four types of transformants were verified by t-test with a significant level of $p<0.05$ to be significantly different from the wild type results, and the results from Pyc2+Mae1 and Pyc2+Mdh3+Mae1 were verified to be significantly lower than Pyc2 and Pyc2+Mdh3 respectively (Figure 3.10). Pyc2 produced on average 28 % more malic acid than the wild type. Pyc2+Mdh3 produced on average 32 % more than the wild type. Pyc3+Mdh3+Fum1 produced on average 57 % more than the wild type. Pyc2+Mae1 produced on average 30 % less than Pyc2. Pyc2+Mdh3+Mae1 produced on average 49 % less than Pyc2+Mdh3. Noteworthy, though not significant according to the statistical analysis, is it that all the transformant types with *mae1* inserted alone or together with other genes, produced on average 10 % less malic acid than the wild type. Also noteworthy is it that all the transformant types without *mae1* inserted produced on average 32 % more malic acid than the wild type. Outliers for malic acid production were defined as single transformants producing 50 % more malic acid than the wild type; this was found for 18 transformants. Out of these 18 transformants, four were tested in a second round of fermentation, Pyc2-23, Pyc2+Mdh3-9, Pyc2+Mdh3-17 and Pyc2+Mdh3+Fum1-5, and consistently showed an increased malic acid production compared to wild type. The best malic acid producer of the four, Pyc2-23, was tested in a third round of fermentation and consistently produced significantly more malic acid, on average 161 % more than the wild type.

It is noteworthy that of all the transformants tested, 41 % had both an increased production of citric acid and malic acid, 56 % had either an increased production of malic acid or citric acid and only 3% had a decreased production of both citric and malic acid. It is furthermore notable that the confirmed high citric acid producer Pyc2-2 with 53 % more citric acid produced compared to the wild type was the fourth poorest malic acid producer with 25 % less malic acid produced compared to wild type. Similarly, the confirmed highest malic acid producer Pyc2-23 with 161 % more malic acid produced compared to wild type was also the confirmed lowest citric acid producer with 96% less citric acid produced compared to wild type.

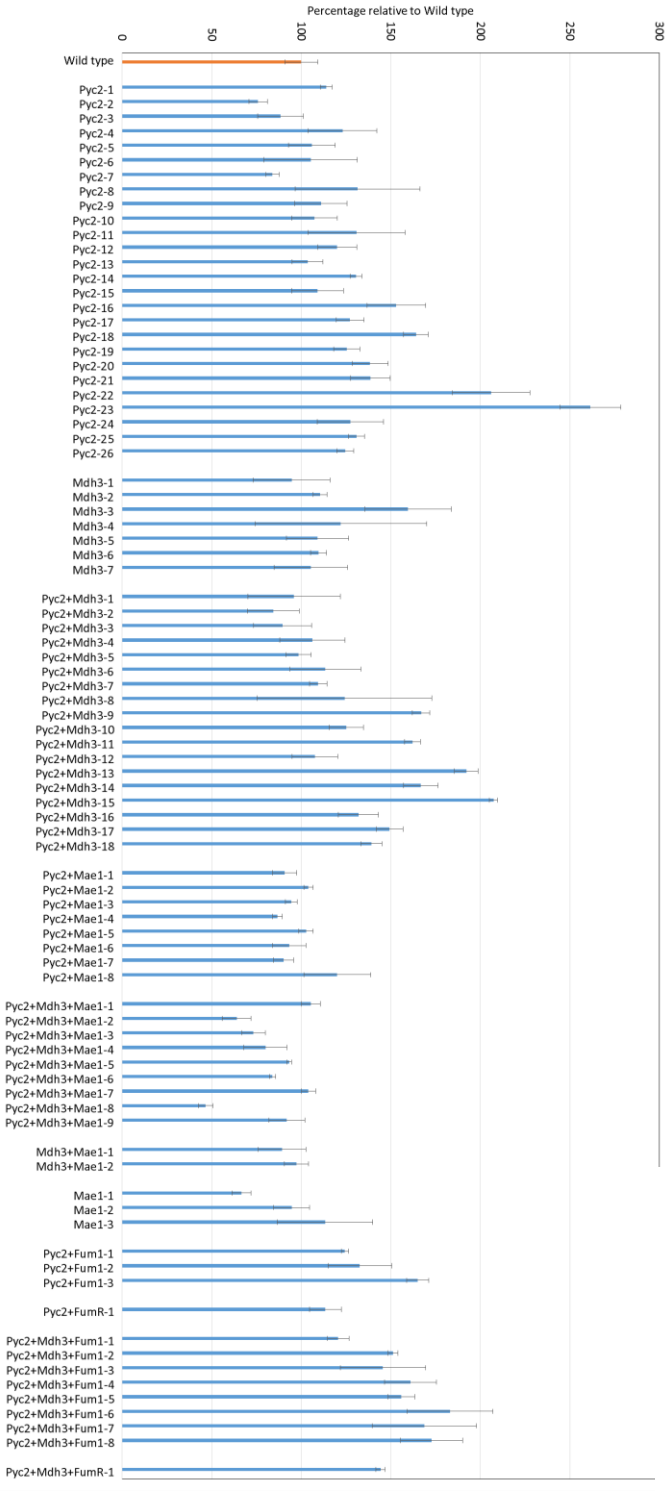

Figure 3.9 Malic acid accumulation relative to wild type. Each data point represent results from at least one and often from two or three repeated experiments with triplicate samples. Error bars represent standard deviation based on a sample.

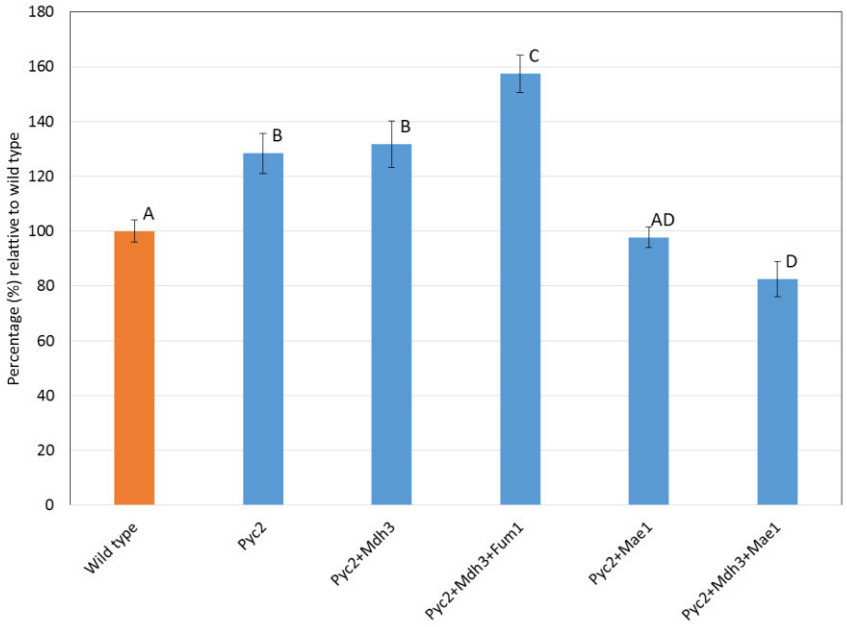

Figure 3.10 Average malic acid accumulation from all samples of five types of transformants and the wild type. Error bars represent standard error of the mean. Letters above the bars indicate homogenous groups within the experiment (T-test with significance level of $p<0.05$)

3.5. DISCUSSION

The co-transformation method successfully created 11 different genotypes in *A. carbonarius* with five different genes paired three at the time. Three of the 11 types did not include the *pyc2* gene. Although this might seem counter intuitive since the *pyc2* gene is carried by the selective vector, it is possible that selective marker was inserted in the genome without the full *pyc2* gene, thus enabling the co-plasmids to be incorporated nevertheless. The random insertion strategy used in this study is very efficient but also leaves uncertainties to the number of genes inserted, whether the inserted gene/genes are expressed correctly, and to what level. In future studies, this could be tested by southern blotting, Q-PCR or enzyme assays even though the sheer number of transformants will make this process cumbersome. A few of the transformants were tested in RT-PCR, which confirmed that the inserted genes were expressed. Nevertheless, due to the random insertion, transformants of the same type can behave differently because of several issues: The number of copies of genes inserted can vary, which can be even more pronounced when working with co-transformation; the expression level of one gene can vary due to localization of the inserted gene in the genome; inserted genes can disrupt native genes in the strain making new genotypes (Jeenes & Mackenzie 1991).

According to the hypothesis for the study, the accomplished genetic alterations of *A. carbonarius* should increase the accumulation of either malic acid or fumaric acid. However, a previous study in *A. niger* have shown that these types of genetic alterations increases the accumulation of citric acid instead (de Jongh & Nielsen 2008). Because of that, citric acid accumulation was followed closely as one of the primary result parameters in this study. When looking at the citric acid production, the results were in general not overwhelmingly significant and did not show any obvious trends different from wild type from the transformant genotypes. However, there were many outliers producing more citric acid than the wildtype, the best overproducer was Pyc2-2. The general trend between Pyc2-type transformants was that there were no significant differences compared to wild type, which raises questions about the reason behind why this single transformants increased production. It could be that the *Pyc2* gene was inserted in a good place in the genome and is well expressed, or that several copies of the gene were inserted, and thereby optimized the strains ability to produce citric acid. It is however not certain that this is the case, it could also be an artefact of the random insertion strategy where other native genes might have been disrupted thereby randomly making a different genotype that are better at producing citric acid. There were a few outliers producing significantly less citric acid than the wild type, four were confirmed outliers in successive fermentation experiments (Pyc2-17, Pyc2-23, Pyc2+Mdh3-9, Pyc2+Mdh3-17). It is difficult to explain this behavior; there is nothing in the literature that describes an effect like this from inserting these types of genes. The lack of significant increase in citric acid production from the different genotypes goes against what has been seen in a previous study by (de Jongh & Nielsen 2008). They find that *mdh2*, *fum1* and *fumR* all are able to enhance the citric acid production in *A. niger* either when expressed alone or in tandem.

It is difficult to make conclusions based on the citric acid production results alone. When looking at the malic acid results, differences that are more significant appear. Transformants with the genotype Pyc2, Pyc2+Mdh3 and Pyc2+Mdh3+Fum1 all produced significantly more malic acid than the wild type, and Pyc2+Mdh3+Fum1 produced significantly more malic acid than Pyc2 and Pyc2+Mdh3. These results support the hypothesis of combination two (see Introduction), that Pyc2, Mdh3 and Fum1 together introduces the reductive pathway in the cytosol of *A. carbonarius* and increases the production of malate. The malic acid production were increased with each succeeding gene inserted, indicating that these three genes are building on each other the reductive pathway in the cytosol. According to the results, the overexpression of *pyc2* had a greater effect than the introduction of *mdh3*. This is the opposite of what was seen in *S. cerevisiae* where *mdh3* had a much greater impact on malic acid production than that of *pyc2* (Zelle et al. 2008). Furthermore, according to the results the introduction of *fum1* had a greater effect than that of the introduction of *mdh3*. This is similar to what was seen in *A. niger* where *fum1* had a greater effect on citric acid production than that of *mdh2* (de Jongh & Nielsen 2008). The transformants with the genotype Pyc2+Mdh3+Mae1 produced significantly less

malic acid than the wild type rejecting the hypothesis of combination 1 (see Introduction). Furthermore, transformants with genotype Pyc2+Mae1 and Pyc2+Mdh3+Mae1 produced significantly less malic acid than Pyc2 and Pyc2+Mdh3 respectively. These results indicate that the *mae1* gene have a negative effect on malic acid production in this study which is the contrary of what has been seen in previous studies (Brown et al. 2013; Knuf et al. 2014; Zelle et al. 2008; Mcfarland & Fischer 2013). It has been explained in previous studies that increasing malate in the cytosol can trigger the onset of citric acid production via antiport to the mitochondria (Goldberg et al. 2006; Röhr & Kubicek 1981). One possibility in this present study is that the inserted malate transporter removes the initial malate from the cytosol before the malate can trigger the explained mechanism thereby slowing the metabolism down resulting in less malate accumulated at the sampling time. There were many outliers producing more malic acid than the wild type. Four transformants were tested in a second round of fermentation and they consistently produced more malic acid than the wild type. The best malic acid producer, Pyc2-23, was tested in a third round of fermentation where it produced more malic acid than the wild type, on average 161 % more malic acid. All the four transformants were of the genotypes that also in general produced more malic acid than the wild type: Pyc2, Pyc2+Mdh3 and Pyc2+Mdh3+Fum1, which furthermore implies that these three genes have the hypothesized effect on *A. carbonarius*. As mentioned earlier de Jongh and Nielsen (2008) found that *mdh2*, *fum1* and *fumR* had a positive effect on citric acid production in *A. niger*. In our study, the effect seems to be more pronounced on the malic acid production but these studies are not directly comparable because of the different species and the fact that we have included *pyc2* and *mae1* in the study. de Jongh and Nielsen (2008) did not measure malic acid in the fermentation broth, which furthermore makes it difficult to compare the two studies directly. In *S. cerevisiae* and *A. oryzae pyc2*, *mdh3* and *mae1* greatly increased the malic acid production which is more in line with the results that are shown in the present study (Zelle et al. 2008; Knuf et al. 2014).

When looking at the general effect of insertion of the five genes there is a clear picture to be seen. 41% of all the transformants constructed had an increased production of citric and malic acid compared to wild type, only 3% had a decreased production of citric and malic acid compared to wild type. This result implies that insertion of these genes in *A. carbonarius* has a positive effect on the acid production.

There was no success with the production of the pursued fumaric acid in the study, even though the *fumR* gene should enable a transformant to catalyze the dehydration of malate to fumarate. Following the hypothesis of combination 3 (see Introduction), Pyc2, Mdh3 and FumR should introduce the reductive pathway in the cytosol and increase the production of fumaric acid. Unfortunately, only two out of the 103 transformants had the *fumR* gene inserted and these two transformants did not produce any detectable fumaric acid. The follow-up strategy was to produce more

transformants with the *FumR* gene inserted but this could not be completed in time to be included in the present study.

3.6. ACKNOWLEDGEMENTS

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3.7. REFERENCES

Battat, E. et al., 1991. Optimization of L-malic acid production by *Aspergillus flavus* in a stirred fermentor. *Biotechnology and bioengineering*, 37(11), pp.1108–16. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18597343.

Brown, S.H. et al., 2013. Metabolic engineering of *Aspergillus oryzae* NRRL 3488 for increased production of L-malic acid. *Applied microbiology and biotechnology*, 97(20), pp.8903–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23925533 [Accessed August 16, 2014].

Buck, R. & Mottern, H., 1947. L-Malic Acid as by-product in Apple Sirup Manufactured by Ion Exchange. *Industrial & Engineering Chemistry*, 39(9), pp.1087–1090.

Ding, Y. et al., 2011. Production of fumaric acid by *Rhizopus oryzae*: role of carbon-nitrogen ratio. *Applied biochemistry and biotechnology*, 164(8), pp.1461–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21416336 [Accessed June 6, 2012].

Friedberg, D. et al., 1995. The fumR gene encoding fumarase in the filamentous fungus *Rhizopus oryzae*: cloning, structure and expression. *Gene*, 163(1), pp.139–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7557464.

Fu, Y.-Q. et al., 2010. Enhancement of fumaric acid production by *Rhizopus oryzae* using a two-stage dissolved oxygen control strategy. *Applied biochemistry and biotechnology*, 162, pp.1031–1038.

Goldberg, I. et al., 1983. Improved conversion of fumarate to succinate by *Escherichia coli* strains amplified for fumarate reductase. *Applied and environmental microbiology*, 45(6), pp.1838–1847.

Goldberg, I., Rokem, J.S. & Pines, O., 2006. Review Organic acids : old metabolites, new themes. *Journal of Chemical Technology and Biotechnology*, 1611(October 2005), pp.1601–1611.

Grobler, J. et al., 1995. The *mae1* gene of *Schizosaccharomyces pombe* encodes a permease for malate and other C4 dicarboxylic acids. *Yeast*, 11(15), pp.1485–

91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8750236.

Hansen, N.B., 2014. *Production of Malic acid by Aspergillus carbonarius*. Aalborg University Copenhagen Denmark.

Hansen, N.B., Lübeck, M. & Lübeck, P.S., 2014. Advancing USER cloning into simpleUSER and nicking cloning. *Journal of Microbiological methods*, 96, pp.42–49.

Jeenes, D. & Mackenzie, D., 1991. Heterologous protein production by filamentous fungi. *Biotechnology and Genetic Engineering Reviews*, 9, pp.327–334. Available at: http://www.tandfonline.com/doi/pdf/10.1080/02648725.1991.10647884.

de Jongh, W.A. & Nielsen, J., 2008. Enhanced citrate production through gene insertion in *Aspergillus niger*. *Metabolic engineering*, 10(2), pp.87–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18162426 [Accessed March 21, 2012].

Kajiwara, S. & Maeda, H., 1986. Coimmobilization of malate dehydrogenase and formate dehydrogenase in polyethyleneglycol (# 4000) diacrylate gel by droplet gel-entrapping method. *Biotechnology and bioengineering*, 28(12), pp.1794–1800.

Karaffa, L. & Kubicek, C.P., 2003. *Aspergillus niger* citric acid accumulation: do we understand this well working black box? *Applied microbiology and biotechnology*, 61(3), pp.189–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12698275 [Accessed March 21, 2012].

Knuf, C. et al., 2014. Physiological characterization of the high malic acid-producing *Aspergillus oryzae* strain 2103a-68. *Applied Microbiology and Biotechnology*, 98, pp.3517–3527.

Lee, S.B., Milgroom, M.G. & Taylor, J.W., 1988. A rapid, high yield mini-prep method for isolation of total genomic DNA from fungi. *Fungal Genet. Newsl.*, 35, pp.23–24.

Linde, T. et al., 2014. Fermentation in 24-well plates is an efficient screening platform for filamentous fungi. *Letters in applied microbiology*, 59(2), pp.224–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24725208 [Accessed September 5, 2014].

McAlister-Henn, L. et al., 1995. Expression and function of a mislocalized form of peroxisomal malate dehydrogenase (MDH3) in yeast. *Journal of Biological Chemistry*, 270(36), pp.21220–21225.

Mcfarland, S. & Fischer, A., 2013. C4 dicarboxylic acid production in filamentous fungi. United States Patent No. 20,130,089,902.

Peleg, Y., Stieglitz, B. & Goldberg, I., 1988. Malic acid accumulation by

Aspergillus flavus. *Applied Microbiology Biotechnology*, 28, pp.69–75.

Pines, O. et al., 1996. The cytosolic pathway of L-malic acid synthesis in *Saccharomyces cerevisiae*: the role of fumarase. *Applied microbiology and biotechnology*, 46(4), pp.393–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8987728.

Roa Engel, C.A. et al., 2008. Fumaric acid production by fermentation. *appl . microbiol. biotechnol*, 78, pp.379–389.

Roberts, I. et al., 1989. Expression of the *Escherichia coli* β-glucuronidase gene in industrial and phytopathogenic filamentous fungi. *Current genetics*, 15(3), pp.177–180.

Rossi, J. & Clementi, F., 1985. L-Malic acid production by polyacrylamide gel entrapped *Pichia membranaefaciens*. *Biotechnology Letters*, 7(5), pp.329–334.

Röhr, G. & Kubicek, C.P., 1981. Regulatory aspects of citric acid fermentation by *Aspergillus niger*. *Process Biochemistry*, 16(34).

Sambrook, J. & Russel, D.W., 2001. *Molecular Cloning: A Laboratory Manual*, CSHL Press.

Samson, R. a et al., 2007. Diagnostic tools to identify black aspergilli. *Studies in mycology*, 59, pp.129–45. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2275192&tool=pmcentrez&rendertype=abstract [Accessed March 26, 2012].

Shu, P. & Johnson, M.J., 1948. Production by Submerged Fermentation with *Aspergillus niger*. *Industrial & Engineering Chemistry*, 40(7), pp.1202–1205.

Stucka, R. et al., 1991. DNA sequences in chromosomes II and VII code for pyruvate carboxylase isoenzymes in *Saccharomyces cerevisiae*: analysis of pyruvate carboxylase-deficient strains. *Molecular & general genetics*, 229(2), pp.307–315.

Taing, O. & Taing, K., 2007. Production of malic and succinic acids by sugar-tolerant yeast *Zygosaccharomyces rouxii*. *European food research and technology*, 224(3), pp.343–347.

Wernars, K. et al., 1987. Cotransformation of *Aspergillus nidulans*: A tool for replacing fungal genes. *Molecular & general genetics*, 209(1), pp.71–77.

Werpy, T. et al., 2004. *Top Value Added Chemicals from Biomass Volume I — Results of Screening for Potential Candidates from Sugars and Synthesis Gas*, U.S. Department of Energy, Oak Ridge.

Weyda, I. et al., 2014. Point mutation of the xylose reductase (XR) gene reduces xylitol accumulation and increases citric acid production in *Aspergillus carbonarius*. *Journal of industrial microbiology & biotechnology*, 41(4),

pp.733–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3953602&tool=pmcentrez&rendertype=abstract [Accessed October 27, 2014].

Wu, Z.H. et al., 2001. A simplified method for chromosome DNA preparation from filamentous Fungi. *Mycosystema*, 20, pp.575–577.

Yang, L., Lübeck, M. & Lübeck, P.S., 2014. Deletion of glucose oxidase changes the pattern of organic acid production in *Aspergillus carbonarius*. *AMB Express*, 4(1), p.54. Available at: http://www.amb-express.com/content/4/1/54.

Yang, L., Lübeck, M. & Lübeck, P.S., 2015. Effects of heterologous expression of phosphoenolpyruvate carboxykinase and phosphoenolpyruvate carboxylase on organic acid production in *Aspergillus carbonarius*. *Journal of Industrial Microbiology & Biotechnology*. Available at: "http://dx.doi.org/10.1007/s10295-015-1688-4.

Zelle, R.M. et al., 2008. Malic acid production by *Saccharomyces cerevisiae*: engineering of pyruvate carboxylation, oxaloacetate reduction, and malate export. *Applied and environmental microbiology*, 74(9), pp.2766–77. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2394876&tool=pmcentrez&rendertype=abstract [Accessed March 6, 2012].

CHAPTER 4. MANUSCRIPT II – THE GLOBAL REGULATOR LAEA CONTROLS PRODUCTION OF CITRIC ACID AND ENDOGLUCANASE IN *ASPERGILLUS CARBONARIUS*

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4.1. ABSTRACT

The global regulatory protein methyltransferase LaeA is known for regulating the production of many different kinds of secondary metabolites such as gliotoxin, lovastatin, penicillin and endocrocin in *Aspergillus* species and in other ascomycetes. The regulatory effect also includes regulation of sexual and asexual reproduction as well as morphology. In *Aspergillus carbonarius,* it has been shown that LaeA regulates production of ochratoxin and conidiophore production. We have investigated the regulatory effect of LaeA on *A. carbonarius* cells by looking further into the effect of LaeA on citric acid production and activity of cellulolytic and hemicellulolytic enzymes. Two types of *A. carbonarius* strains, having *laeA* either knocked out or overexpressed, were constructed using USER cloning and were tested in fermentation for changed phenotypes. The knockout of *laeA* significantly decreased the production of citric acid and endoglucanases, but did not reduce the production of beta-glucosidases or xylanases. The citric acid accumulation in the transformants was reduced with 74-96% compared to the wildtype, after seven days of fermentation. The endoglucanase activity was reduced with 51-78%. Among transformants overexpressing *laeA*, one strain showed an increase of 113% in citric acid accumulation after 7 days of fermentation compared to the wildtype. However, *laeA* overexpression had no effect on endoglucanase, beta-glucosidase or xylanase activity. In conclusion, LaeA regulates production of citric acid and endoglucanases

in addition to the already known ochratoxin production in *A. carbonarius*. LaeA seems to have no regulatory effect on beta-glucosidases or xylanases in the strains.

4.2. INTRODUCTION

Recently, extensive efforts have been put into engineering of the regulatory network that controls expression and secretion of enzymes as well as organic acids in filamentous fungi (Dai & Baker 2015; Wang et al. 2013; Tamayo-Ramos & Orejas 2014). Simultaneous overproduction of enzymes and metabolites might be valuable for generation of strains for consolidated bioprocessing of biomass into organic acids, a process that will have a significant economic impact on the cost in a biorefinery concept. This implies a strain that saccharifies complex lignocellulosic biomass and metabolizes the resulting sugars into organic acids (Zoglowek et al. 2015). Engineering of the global regulatory gene, *laeA*, could be a potential target for improving the production of secondary metabolites and perhaps other products in *Aspergillus carbonarius* a closely related species to *Aspergillus niger*. The *laeA*, encoding a methyltransferase, was discovered in 2004 by Bok & Keller (2004) in *Aspergillus nidulans, Aspergillus terreus* and *Aspergillus fumigatus*. It was named as an abbreviation 'loss of aflatoxin-related (AflR) gene expression', after the mechanism, which was discovered to be regulated by the *laeA* gene. AflR is a transcription factor controlling production of sterigmatocystin and aflatoxin (Woloshuk et al. 1994; Fernandes et al. 1998). Regulation of AlfR has shown to constitute only a small part of LaeA's regulatory mechanism. Secondary metabolites such as gliotoxin, lovastatin, penicillin and endocrocin were also shown to be downregulated in *laeA* knockouts (Bok et al. 2005; Bok & Keller 2004; Lim et al. 2012). In 2008 it was shown that LaeA reacts with VeA and VelB in the Velvet complex, which has a global regulatory effect on secondary metabolism (Bayram et al. 2008). In filamentous ascomycetes the Velvet complex and its regulatory effect is conserved (Jain & Keller 2013). Since its first description in 2004 several hundred articles refer to LaeA (Jain & Keller 2013). It is clear from these articles that LaeA has a global regulatory effect not only on secondary metabolites but also on, for example asexual and sexual reproduction and morphology (Bayram & Braus 2012; Bayram et al. 2008). When *A. nidulans* is grown under conditions where light is present LaeA is needed for proper asexual development by controlling the levels of VeA and VelB. If LaeA is not present, VeA and VelB are not suppressed and sexual reproduction is favored. In *laeA* deletion strains of *A. nidulans* both sexual and asexual reproduction are impaired (Bayram et al. 2010). Many other examples of morphogenetic alterations in *laeA* deletion strains indicate that LaeA and the Velvet complex are involved in regulation which impacts morphology (Jain & Keller 2013). For example, altered sclerotial production in *Aspergillus flavus* (Kale et al. 2008); altered conidiophore production in *Penicillium chrysogenum* (Hoff et al. 2010); and reduced pigmentation in *Fusarium fujikuroi* (Wiemann et al. 2010) were observed. Bayram et al. (2010) suggests that the reason LaeA regulates secondary metabolites such as toxins at the same time as it regulates reproduction and morphology is that

LaeA is a global regulator of protection and nourishment for the next generation of fungi. In a recent patent in *A. niger* the overexpression of *laeA* alone or together with the disruption of *alg3* contributed to an increase in citric acid production indicating that it is not only secondary metabolites, reproduction and morphology that are regulated by LaeA (Dai & Baker 2015). A point mutation in the l*aeA* gene in *A. niger* was very recently shown to create a non-acidifying mutant with reduced production of citric and oxalic acid supporting the hypothesis that it is not only secondary metabolites LaeA regulates (Niu et al. 2016). It is not only in *Aspergillus* species that LaeA has a regulatory effect. The LaeA orthologous protein methyltransferase, LAE1, in *Trichoderma reesei* has a significant impact on the regulation of lignocellulose degrading enzymes (Seiboth et al. 2012). Deletion of *lae1* in *T. reesei* resulted in shutting down the expression of 9 out of 10 of the cellulase and cellulase enhancing proteins as well as decreasing the expression of beta-glucosidase and xylanase. On the other hand when *lae1* was overexpressed in *T. reesei* the cellulase gene transcription was enhanced (Seiboth et al. 2012). Furthermore, the lack of LAE1 in *T. reesei* also has caused phenotypic changes such as lack of yellow pigment and reduced sporulation. Seiboth et al. (2012) suggested that it is possible that the prime target for LAE1 regulation is sporulation.

The precise mode of regulation by the methyltransferase LaeA is still unclear. In Aspergilli, LaeA is proposed to carry out regulation at the level of histones by regulating clusters of genes encoding secondary metabolites, which are localized often near the telomeres of chromosomes (Bok & Keller 2004; Keller et al. 2005; McDonagh et al. 2008). Because of co-clustering of genes for secondary metabolites, LaeA has the potential to regulate a multitude of these genes (Bok & Keller 2004; Brakhage 2012; Häkkinen 2014). However, in *T. reesei* regulation through methylation of histones was not confirmed. It was suggested that the effect of LAE1 regulation is indirect due to the fact that methylation at specific cellulase and hemicellulase loci is unaffected (Seiboth et al. 2012). However, it was shown that the *T. reesei* LaeA ortholog was unable to complement a Δ*laeA* mutant of *A. nidulans* as it was unable to restore sterigmatocystin and sexual reproduction, which indicates that it could behave differently than other LaeA orthologs (Karimi-Aghcheh et al. 2013). Recently, an automethylation of LaeA was suggested which might indicate a completely novel mechanism of regulation of gene expression (Patananan et al. 2013).

Among all studies investigating the role of LaeA, only one has focused on *A. carbonarius* as research organism (Crespo-Sempere et al. 2013). When LaeA was deleted in *A. carbonarius* the production of ochratoxin, a potential human carcinogen, was decreased with 68 to 97% compared to wild type and the production of conidia in light and in dark was decreased as well (Crespo-Sempere et al. 2013). *A. carbonarius* is closely related to *A. niger* (Abarca et al. 2004) and similarly to *A. niger* it produces citric acid in high amounts when cultivated at optimal conditions (Linde et al. 2014; Yang et al. 2014).

In this study the global regulator LaeA and its effect on the production of citric acid and polysaccharide degrading enzymes was investigated in *A. carbonarius*. SimpleUSER cloning was used to create either gene deletion through targeted gene replacement or gene overexpression. Results from fermentation experiments and growth studies indicate that *laeA* is essential for citric acid and cellulase production in *A. carbonarius*.

4.3. MATERIALS AND METHODS

4.3.1. STRAINS AND GROWTH CONDITIONS

A. carbonarius ITEM 5010 was kindly supplied from Prof. Jens Frisvad, Danish Technical University, Lyngby (Department of Systems Biology, Center for Microbial Biotechnology). It was originally isolated from grapes in Italy (www.ispa.cnr.it/collection). *Escherichia coli* strain DH5α was used as host for plasmid propagation. Fungal cultivation was done on potato dextrose agar (PDA; Scharlau Chemie S.A. Barcelona, Spain) at 30°C in darkness. Spore suspension was stored as stock solution in 20% glycerol in -80°C.

4.3.2. GENE ANALYSIS

The target *laeA* gene in *A. carbonarius* was identified using the orthologous *laeA* gene from Asp*ergillus nidulans* as starting point (genbank ID CBF88745.1). The sequence was used as query in an amino acid vs. translated nucleotide blast (tblastn) in JGI's database for *A. carbonarius* (http://genome.jgi-psf.org/Aspca3/Aspca3.home.html). The blast returned several results; the best result had a significantly high similarity (66%) to the *laeA* gene from *A. nidulans*. The sequence was used in the further investigations. Gene prediction was done using the online gene prediction tool AUGUSTUS (http://bioinf.uni-greifswald.de/augustus/); validation was done using the Blast service from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.3.3. GENOMIC DNA EXTRACTION

Genomic DNA extraction was carried out with the established CTAB method (Lee et al. 1988; Wu et al. 2001). Briefly *A. carbonarius* spores were inoculated in YEPD (Sambrook & Russel 2001) and grown stationary for 24h at 30°C. The mycelia was grinded with a bead beater (Fastprep-24 MP Biomedicals) in CTAB buffer. The grinded mycelia were incubated at 60°C for 30 min and DNA was extracted with Phenol/Chloroform/isoamyl-alcohol 25:24:1 tris pH8 (Sigma-Aldrich). The DNA was precipitated with isopropanol and the pellet resuspended in 50µl TE buffer. DNA

quality and concentration was measured on spectrophotometer (Nanodrop 1000 Spectrophotometer, ThermoScientific).

4.3.4. PLASMID CONSTRUCTION

The high copy number plasmid pJET2.1 (ThermoFisher Scientific) was used as backbone plasmid for the plasmids constructed. The knockout plasmid backbone pSB4.1.1 (Figure 4.1A) and overexpression plasmid backbone pSBe1 (Figure 4.1C) was constructed in a previous study by Hansen et al. (2014). pSB4.1.1 contains the following genetic elements: RP27-*hph*-βT for hygromycin resistance, origin of replication and ampicillin resistance for growth in *E.coli* and a specific cassette facilitating simpleUSER cloning. pSBe1 contains the same genetic elements as pSB4.1.1 and includes a *gpdA* promoter and a *trpC* terminator for gene expression. Primers designed for plasmid construction and the rest of the primers used in this study were made with the primer software Primer3 (Olsen et al. 2011; Untergasser et al. 2000) (Table 4.1). All the plasmids were constructed using simpleUSER cloning (Hansen et al. 2014). For the knockout plasmid pSBkoLaeA (Figure 4.1B), upstream and downstream regions to the target genes were identified in the *A. carbonarius* genome provided by JGI. Approx. 1000bp 5' and 3' flanking regions were selected and primers with uracil tails were designed to amplify the regions (primers 1-4). The PCR reaction was carried out with *pfu* turbo cx polymerase (Agilent) and annealing at 65^{o}C. For the overexpression plasmid pSBeLaeA (Figure 4.1D), primers for amplifying the target gene *laeA* were designed with uracil tails (primer 5 and 6), and used in PCR as described above. The simpleUSER cassette in pSBe1 was activated with the restriction enzyme *AsiSI* and the nicking enzyme *Nb.BtsI*. Cloning, transformation and propagation was carried out as described above. Plasmid extraction was done using the GeneJET plasmid miniprep kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

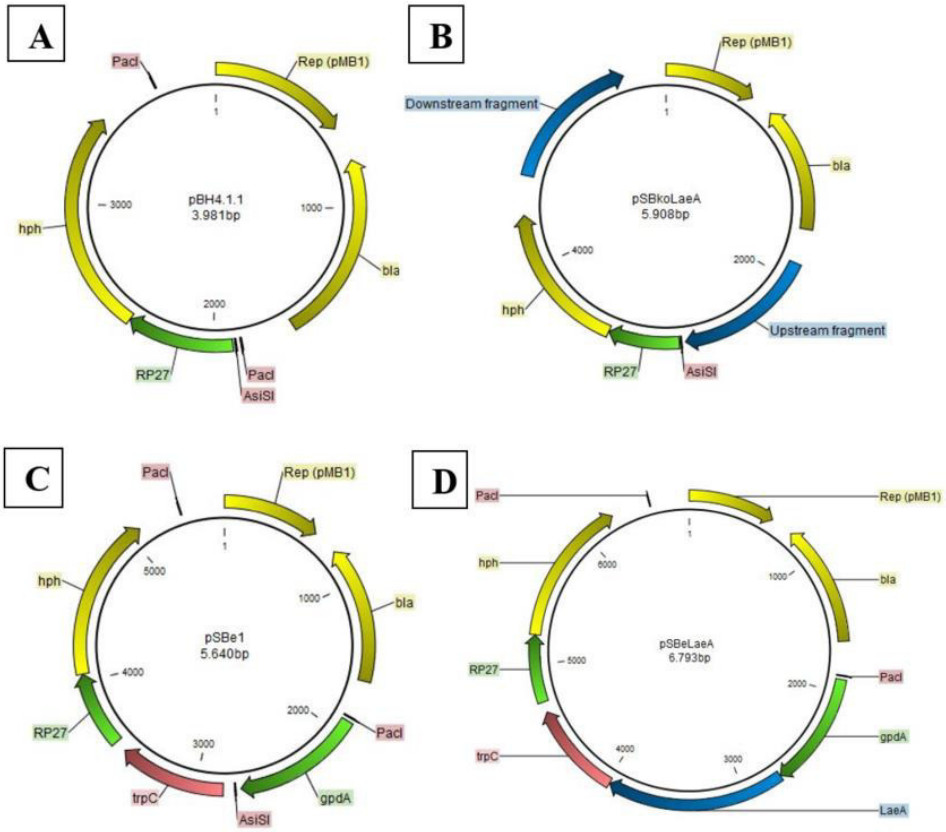

Figure 4.1 The four plasmids used in the experiments. A) Backbone plasmid pBH4.1.1 used as precursor for B) knockout plasmid pSBkoLaeA. C) Backbone plasmid PsBe1 used as precursor for D) overexpression plasmid pSBeLaeA.

4.3.5. PROTOPLAST TRANSFORMATION

Knockout of LaeA, was carried out using a bipartite method (Figure 4.2) (Frandsen et al. 2008). Bipartite PCR fragments were made from the knockout plasmids pSBkoLaeA and primer pair 11+12 and primer pair 13+14. These PCR fragments contains either the upstream part of the target gene plus the promoter and the first 2/3 of the hygromycin resistance gene; or the last 2/3 of the hygromycin resistance gene, the terminator and the downstream part of the target gene. The PCR reaction was carried with Phusion polymerase (Thermo Scientific) using an annealing temperature at 63ºC. These two types of PCR fragments were mixed 1:1 and used in transformation of 100 µl aliquots of protoplasts from *A. carbonarius* ITEM5010 as described by (Yang et al. 2014). The overexpression plasmid pSBeLaeA was used directly in transformation. Transformants able to grow on hygromycin were isolated

and transferred 3 times to new plates containing hygromycin in order to ensure that the DNA were stable inserted in the transformants. Validation of positive knockouts and overexpression transformants were carried out using PCR with transformant DNA as template. For knockout transformants of *laeA*, the primers 7 and 8 were used. For the overexpression transformants, the primers 9 and 10 were used. Spore-suspensions of each transformant in 20% glycerol were stored at -80°C for long-term storage.

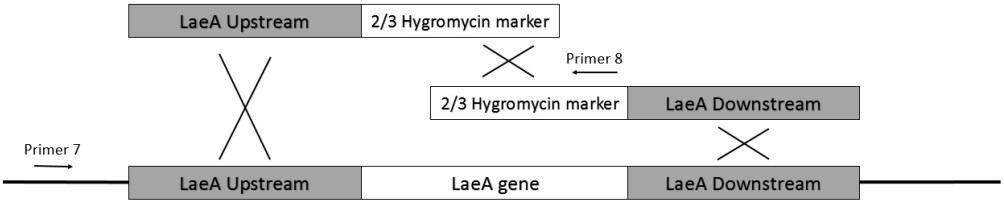

Figure 4.2 Bipartite knockout transformation. One PCR fragment contains the upstream region from the target *laeA* gene, the hygromycin promoter (RP27) and the first 2/3 of the hygromycin resistance gene. Another PCR fragment contains the last 2/3 of the hygromycin resistance gene, terminator (β-T) and the downstream region from the target *laeA* gene. The two PCR fragments are transformed into the wildtype *A. carbonarius* via protoplast transformation. Only when the two fragments join and cross in will the transformant gain hygromycin resistance. This method greatly increases the chances of positive knockout of the target gene. The positive knockouts are found using PCR and the primers 7 and 8. These primers will only give a PCR product (1450b) when the bipartite PCR fragments are crossed in at the right location in the genome.

4.3.6. FERMENTATION SETUP AND CONDITIONS

Fermentation setup in 24-well plates were made following the principles of Linde et al. (2014) and citric acid production media from Shu & Johnson (1948) was used (140 g/l sucrose, 2.5 g/l KH_2PO_4, 0.25 g/l $MgSO_4 \cdot 7H_2O$, 2.5 g/l NH_4NO_3, pH to 3.8, Cu^{2+} 0.06 mg/l, Zn^{2+} 0.25 mg/l, Fe^{2+} 1.3 mg/l). All fermentation experiments were made in biological triplicates. Fresh spore-suspension was used in a final concentration of $2*10^5$ spores/ml. Fermentation was carried out in 24-well plates (UNIPLATE, 24 wells, 10ml, Whatman). The plates were covered with silicone lids (BugStopper Venting Capmat, Whatman) and incubated for 7 days at 25°C and 420 rpm in a Glas-Col model 099A DPMINC24 (Glas-Col, Terre Haute, IN). The incubator was kept at a constant high humidity to avoid excess evaporation by having open boxes with distilled H_2O mounted inside the incubator. Samples were taken at day 7, filtered through 0.45 µm HPLC-grade regenerated cellulose membrane filters and analyzed on HPLC (Dionex Ultimate 3000-LC system, Dionex Corporation, Sunnyvale, CA) with an Aminex HPX-87H column coupled to an RI-detector. The eluent was 4 mmol/l H_2SO_4 with a flow rate of 0.6 ml/min at 60°C. All chromatograms were integrated using the Chromeleon software (Dionex Corporation). Flask fermentation was carried out with selected knockout transformants to test whether the well fermentation results were reproducible on a

bigger scale (*7). The fermentation was carried out in 250ml Erlenmeyer flasks with cotton stoppers and the same citric acid media was used. 20ml media was inoculated with $2*10^5$ spores/ml final concentration and the incubation was carried out in a shaker at 200 rpm with 2,5 cm amplitude and 25°C (KS 4000 I control, IKA). Samples were taken at day 3, 5 and 7. Biomass accumulation was measured in all fermentation experiments. The biomass was gathered, rinsed with plenty of water, then dried at 105°C for 48 h and weighed on a microscale.

4.3.7. CELLULOLYTIC ENZYME PRODUCTION

5 x 10^5 spores/ml were inoculated in 10 ml of pre-culture medium, potato dextrose broth (PDB, Sigma-Aldrich), in 50 ml Falcon tubes. The incubation was carried out at 30°C, 200 rpm for 2 days followed by collection of fungal mass on Miracloth (Merck), washed with sterile basic Czapek (Cz) medium (3 g/l $NaNO_3$, 1 g/l K_2HPO_4, 0.5 g/l KCl, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.01 g/l $FeSO_4 \cdot 7H_2O$, 0.01 g/l $ZnSO_4 \cdot 7H_2O$, 0.005 g/l $CuSO_4 \cdot 5H_2O$) (Samson, Hoekstra, & Frisvad, 2004), pH 4.8, in order to remove PDB carryover. Mycelia were aseptically transferred to 10 ml basic Cz medium containing either 2 % (w/v) wheat bran (WB), 2 % (w/v) Avicel PH-101 (Sigma-Aldrich) or 2 % (w/v) xylan (Sigma-Aldrich) in 50 ml Falcon tubes. Cultures were setup in triplicates and were incubated at 30°C, 200 rpm, for 5 days. The supernatant was filtered through Miracloth, centrifuged at 8000 x g for 10 min at room temperature for clarification and transferred to new Falcon tubes.

4.3.8. ENZYME ASSAYS

Endoglucanase activity was measured using Azo-CM (carboxymethyl)-Cellulose as a substrate, according to manufacturer's protocol (Megazyme) (Wood & Bhat, 1988) and carried out as described in Kolasa et al. (2014). Beta-glucosidase activity measurement was performed using 5 mM p-nitrophenyl β-D-glucopyranoside (pNPG) (Sigma-Aldrich) as a substrate and carried out in a microtiter format as described in Sørensen et al. (2011). Xylanase activity was tested using Azurine Crossed-Linked (AZCL) assay with dyed insoluble AZCL-Arabinoxylan (wheat) (Megazyme) as a substrate and carried out as described in Pedersen et al. (2009).

4.3.9. STATISTICAL ANALYSIS

Comparison of results were analyzed by One-way ANOVA followed by Tukey's honestly significant different test or Tukey Kramers test with a significance level of $p<0.05$. Error bars on figures are standard deviation based on a sample.

Table 4.1 Primers used in the study

Name	No.	Sequence (5' → 3')	Annotation
LaeA up-fw-U	1	GGGTTTAAUGGCTTGCTTGCTCCATCTAC	Amplifies upstream region of LaeA, contains uracil tail
LaeA up-rv-U	2	GGACTTAAUGAACTACTTGAGCCGGGAGA	Amplifies upstream region of LaeA, contains uracil tail
LaeA do-fw-U	3	GGCATTAAUACCTTTTTGCAACACCAAGC	Amplifies downstream region of LaeA, contains uracil tail
LaeA do-rv-U	4	GGTCTTAAUGCGTCGTCTAGACCAGGAAG	Amplifies downstream region of LaeA, contains uracil tail
LaeA fw-U	5	AGAGCGAUATGTTTGCCAACGGAAACGGACG	Amplifies LaeA, contains uracil tail
LaeA rv-U	6	TCTGCGAUTCAGTGGTCCACTGGCGG	Amplifies LaeA, contains uracil tail
LaeA ko-ch-fw	7	CAAGAGTCCAAGGCTCCAAC	Check for positive knockout of LaeA
LaeA ko-ch-rv	8	TATTGGAGAGCAAGGGATGG	Check for positive knockout of LaeA
LaeA ex-ch-fw	9	CTCTTTCTTTTCCCATCTTCAG	Check for positive overexpressing of LaeA
LaeA ex-ch-rv	10	CACCAACGATCTTATATCCAGA	Check for positive overexpressing of LaeA
Bipart-1 fw	11	GGCTTGCTTGCTCCATCTAC	Amplifies 1st bipartite fragment
Bipart-1 rv	12	GATGTTGGCGACCTCGTATT	Amplifies 1st bipartite fragment
Bipart-2 fw	13	GATGTAGGAGGGCGTGGATA	Amplifies 2nd bipartite fragment
Bipart-2 rv	14	GCGTCGTCTAGACCAGGAAG	Amplifies 2nd bipartite fragment

4.4. RESULTS

4.4.1. GENE ANALYSIS

The *laeA* gene was found on scaffold_8:1216435-1217693 in the *A. carbonarius* genome sequence assembly. The full *laeA* sequence was 1213bp including start and stop codon and one intron. The intron was located from base pair 191 to 378. The sequence was predicted to encode a sequence of 341 amino acids. For validation, the amino acid sequence was used as a query in a protein-protein blast that returned numerous hits with high similarity to the query, the majority was LaeA methyltransferases. The result with the highest similarity to the LaeA amino acid sequence from *A. carbonarius* was the methyltransferase LaeA from *Aspergillus parasiticus* with an identity of 78%, E-value of 0.0 and a query cover of 99%.

4.4.2. VALIDATION OF LAEA KNOCKOUT AND OVEREXPRESSING TRANSFORMANTS

Validation of transformants was carried out by PCR. For the knockout transformants, the PCR was designed to check if the hygromycin was correctly inserted inside the *laeA* gene disrupting the gene's transcription, resulting in a PCR product of 1.45kb in size. For the overexpression transformants, a PCR product of 1.4kb corresponded to a positive insertion of the LaeA gene. The knockout of *laeA* resulted in 30 transformants. Out of these 30 transformants, 17 were confirmed by PCR to have a disrupted *laeA* gene. Twenty-nine transformants were generated with the overexpressing plasmid. Out of the 29 transformants, 21 had the full *laeA* gene together with promoter and terminator incorporated into the genome as verified using PCR.

4.4.3. MORPHOLOGY AND OCHRATOXIN PRODUCTION

The morphology of the fungus during the acid production was pelleted growth. The pellets were on average 1mm in diameter and similar in size for the transformants and wildtype and throughout the fermentation experiments. No great change in sporulation was seen when grown in darkness or in light on PDA plates since both wild type, *laeA* knockouts and overexpression transformants produced the same amounts of spores (results not shown). However, the *laeA* knockouts had a tendency to produce smaller spores in size. When the fungus was grown in submerged fermentation, there was no sporulation during the acid production in either type of the strains. In a growth experiment conducted with ΔLaeA-1 the production of ochratoxin was diminished significantly compared to the wildtype (results not shown).

4.4.4. CITRIC ACID FERMENTATION OF LAEA KNOCKOUT AND OVEREXPRESSING TRANSFORMANTS

The 17 positive *laeA* knockouts were tested in biological triplicates in the 24-well system in citric acid fermentation experiments with the aim to investigate whether LaeA had a significant effect on citric acid production. All 17 knockout transformants had a significantly reduced citric acid production compared to the wildtype, with production ranging from 6 – 44 % of the wild type production. Four of the 17 transformants were selected for a repetition experiment. After 7 days of fermentation the wildtype accumulated 10.7 ± 0.4 g/l citric acid, the knockouts accumulated 0.6 ± 0.2 - 2.8 ± 0.2 g/l citric acid which corresponds to 6-26% of the wild type citric acid accumulation (Figure 4.3). The sugar consumption was approximately the same for all the transformants and the wildtype (results not shown). During the citric acid fermentation the knockout transformants instead directed the carbon flux towards biomass production. The wildtype produced 13.3 ± 1.4 g/ml biomass whereas the knockouts produced 20.9 ± 1.0 - 27.1 ± 2.9 g/l. On average approximately 80% more biomass was produced in the knockouts compared to the wildtype, this account well for the missing citric acid when looking at weight.

Two of the *laeA* knockouts (ΔLaeA-1 and ΔLaeA-2) were selected for a 7-fold scale-up experiment, to observe for any significant difference between 24-well and Erlenmeyer setup. The two selected knockouts were tested in triplicates and compared to wildtype *A. carbonarius*. Both knockouts still showed significantly reduced citric acid production compared to the wildtype (Figure 4.4). After 7 days of fermentation the wild type accumulated 22.7 ± 1.9 g/l, ΔLaeA-1 had accumulated 5.3 ± 1.1 g/l and ΔLaeA-2 0.8 ± 0.4 g/l giving a production of 23% and 4% of the wild type production respectively.

Twenty-one positive overexpression transformants were generated. Due to the nature of the random insertion strategy performed to produce the overexpression transformants, they were not necessarily true overexpression transformants even though the PCR validation showed positive results. RT-PCR, Q-PCR or enzyme assays could have given a more detailed description of which of the many transformants that were actually overexpressing LaeA as a functional protein, as opposed to having a sleeping gene incorporated. We took a different approach and used our 24-well screening setup to search directly after changes in citric acid production, which was the ultimate goal. The 21 positive overexpression transformants were screened for citric acid production and compared with wildtype *A. carbonarius*. As the result of this screening, one transformant, eLaeA-1, was identified to produce significantly higher level of citric acid as compared to the wild type (Figure 4.3). The wild type produced 10.7 ± 0.4 g/l citric acid and the transformant overexpressing *laeA* produced 22.7 ± 0.2 g/l, which is 212 % compared to the wild-type. The biomass accumulation was almost identical with the wild type at 13.3 ± 1.4 g/ml and eLaeA-1 at 13.0 ± 0.5 mg/ml.

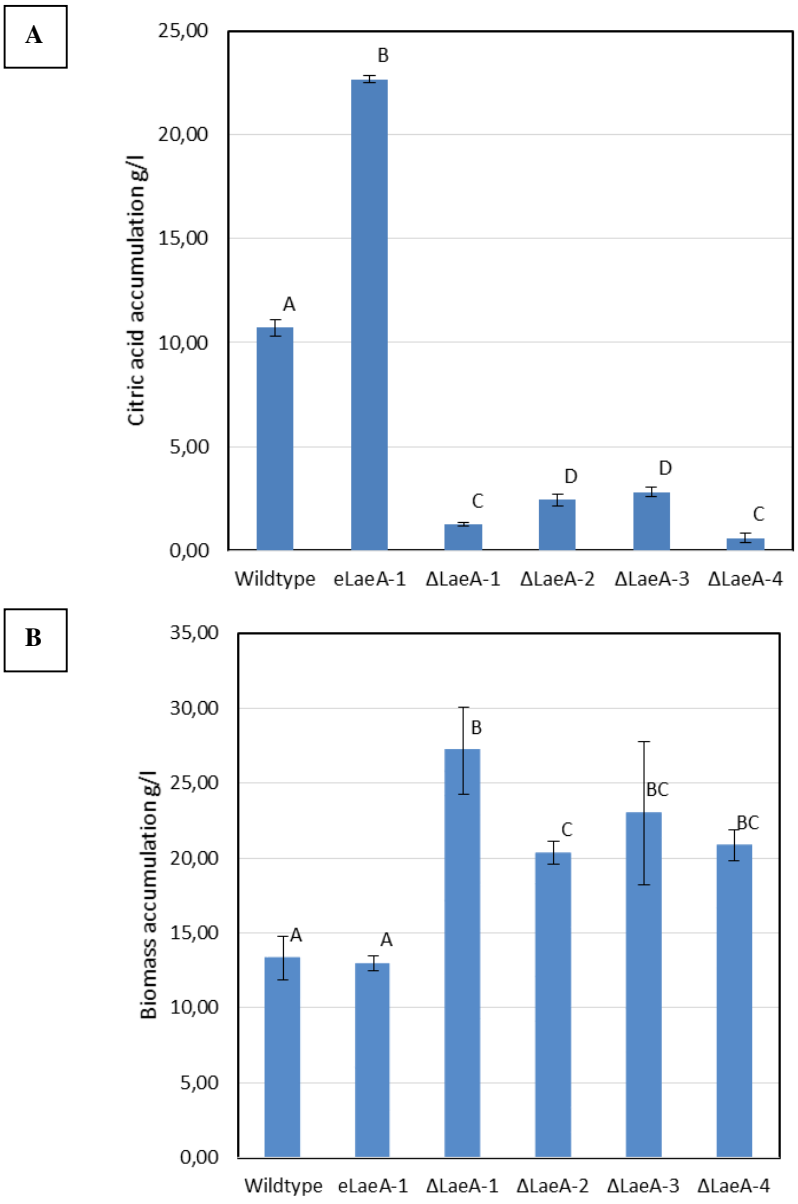

Figure 4.3 Citric acid (A) and biomass accumulation (B) in ΔLaeA transformants and overexpression transformant compared with wildtype after 7 days of fermentation. The most representative results are shown here. Error bars shows standard deviation based on a sample. Letters indicate homogenous groups within the experiment (Tukey's test $p<0.05$).

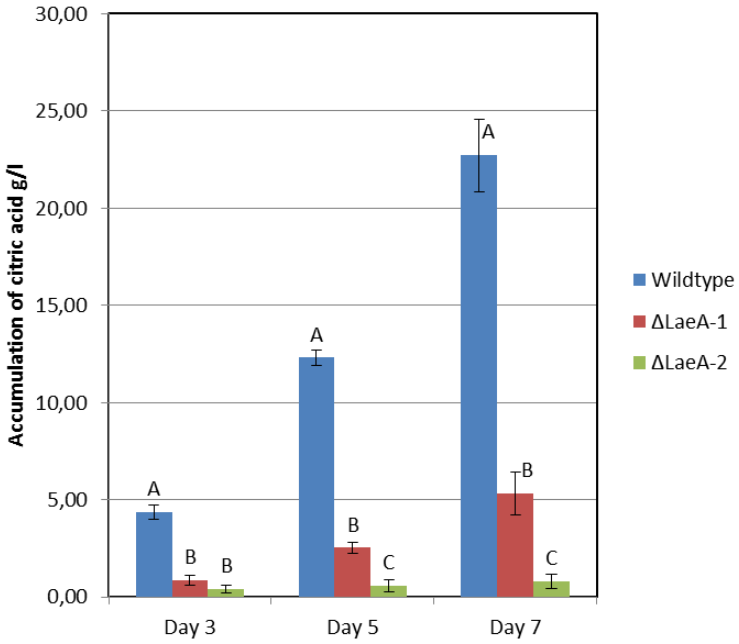

Figure 4.4 Time-course experiment for citric acid production in ΔLaeA transformants compared with wildtype *A. carbonarius*. Error bars shows standard deviation based on a sample. Letters indicate homogenous groups within one day (Tukey's test $p<0.05$).

4.4.5. ENZYME ACTIVITY OF LAEA KNOCK OUT AND OVEREXPRESSION TRANSFORMANTS

Three *laeA* knockouts, ΔLaeA-1, ΔLaeA-2 and ΔLaeA-4, were tested for endoglucanase, beta-glucosidase and xylanase activity and compared to wild type *A. carbonarius*. This was done in triplicates in growth experiments using different cellulolytic and hemicellulolytic carbon sources (wheat bran, avicel and xylan), followed by measurement of the enzyme activity. Significantly reduced endoglucanase activity was obtained in all tested transformants as compared to wild type (Figure 4.5). The reduction in enzyme activity was 22-37 %, 31-49 % and 45-48 % on wheat bran, avicel and xylan, respectively, compared to the activity in wild type. In contrast, no significant reduction of beta-glucosidase activity and xylanase activity was observed in the transformants (results not shown).

The overexpression transformants were also tested for changes in cellulolytic enzyme production in the same way as the knockout transformants. None of the transformants showed any difference in endoglucanase (Figure 4.5), beta-glucosidase or xylanase activity as compared to wildtype (results not shown).

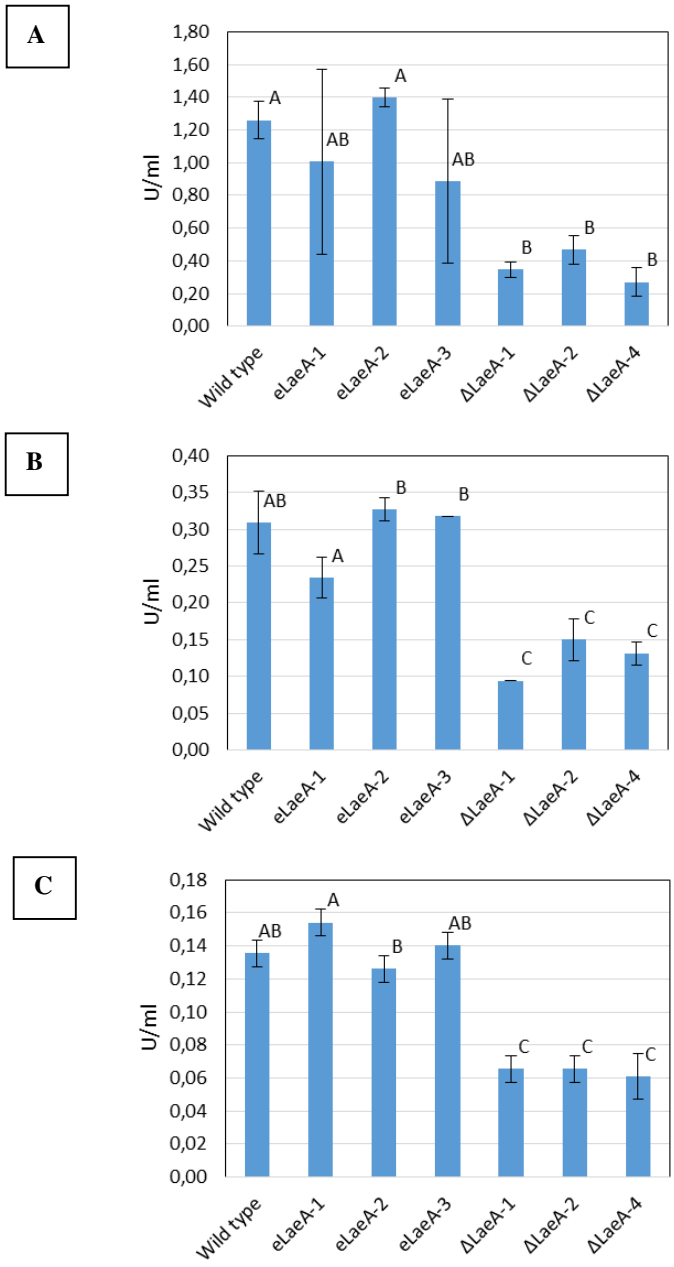

Figure 4.5 Endoglucanase activity in knockout transformants, overexpression transformants and wildtype grown on wheat bran (A), Avicel (B) and xylan (C). Error bars show standard deviation based on a sample. Letters indicate homogenous groups within one experiment (Tukey Kramer's test $p<0.05$).

4.5. DISCUSSION

The *laeA* gene that was identified and investigated in this study is an orthologue to the *A. nidulans laeA* gene. We have shown that LaeA in *A. carbonarius* has a regulatory effect on citric acid, endoglucanase and ochratoxin production. Regulatory effects of *laeA* orthologue on secretion of cellulases have been shown in *T. reesei* (Seiboth et al. 2012) and the effect on mycotoxins have also been shown previously in *A. carbonarius* (Crespo-Sempere et al. 2013). Findings of our study suggest that the regulatory effect extends even further to production of organic acids as it is also suggested by Niu et al. (2016) and Dai & Baker (2015). It was also shown that at the same time as LaeA regulates endoglucanase in *A. carbonarius*, it does not regulate two of the other important cell wall degrading enzymes, namely beta-glucosidases and xylanases. In *T. reesei* Lae1 was essential for production of both cellulases and hemicellulases, however the level of downregulation varied between different genes encoding cellulases, xylanases and beta-glucosidases; higher fold decrease in expression was generally observed for cellulases than beta-glucosidases (Seiboth, 2012). The small differences between the studies might be due to differences in gene clustering in *T. reesei* and *A. carbonarius* genomes, or general differences in regulatory mechanisms in those species, as it was shown that the *T. reesei* LaeA ortholog does not regulate in the same way as the *Aspergillus* ortholog does (Karimi-Aghcheh et al. 2013). The fact that the same gene controls production of three different types of compounds such as organic acid, enzyme and mycotoxin, suggests a global regulatory effect of LaeA in *A. carbonarius* which corresponds well with previous studies on LaeA in *Aspergilli* (Bok & Keller 2004; Bayram et al. 2008).

The precise function of how LaeA carry out its regulation is still unclear (Jain & Keller 2013). It has been suggested that regulation is epigenetic and gene expression is regulated at the histone level through modification of chromatin structure and therefore the regulation is associated with chromosomal location of particular gene clusters (Bok & Keller 2004; Bayram et al. 2008; Reyes-Dominguez et al. 2010). Following that hypothesis, one possibility of the simultaneous regulation of endoglucanase, mycotoxin and citric acid production in *A. carbonarius* could be that the genes responsible for these metabolites are localized in the same cluster (Brakhage 2012; Häkkinen 2014), or in different clusters all regulated by LaeA. Building on the thesis, the genes encoding beta-glucosidases and xylanases would then not be clustered together with the above-mentioned genes. This is however speculation and has not been investigated in the present study and many other conditions could be possible. It is noteworthy to mention that Seiboth et al. (2012) also found that not all genes in one cluster had to be up or down regulated by LaeA at the same time, indicating a more nuanced regulation than the one just described. Recently, automethylation of LaeA was proposed, which further indicates the complexity of the mechanisms of LaeA regulation (Patananan et al. 2013). One possible reason for the regulation by LaeA is suggested to be global regulation of protection and nourishment for the next generation of fungi (Bayram et al. 2010).

This could explain the simultaneous regulation of ochratoxin and citric acid production in *A. carbonarius* since these two compounds are used as protection of the fungus. The function of ochratoxin for the fungus is not fully investigated (Bayman & Baker 2006) but it has been shown to be very toxic towards different insect species (Dowd 1989) and is most likely used as a defense mechanism for the fungus. The function of the overproduction and excretion of citric acid by several *Aspergillus* species is believed to provide an ecological advantage by lowering the pH of the environment to a level where only a few organisms can proliferate thereby protecting the fungus from predation and competition (Karaffa et al. 2001).

Since, the global regulatory effect of *laeA* extents also to regulation of sexual and asexual reproduction (Bayram & Braus 2012; Bayram et al. 2008) as well as development of morphological features (Kale et al. 2008) (Hoff et al. 2010) (Wiemann et al. 2010), changes in growth, morphology and conidiation were investigated in the present study. Only differences in biomass accumulation between knockout transformants and wild type were observed during the fermentation experiments, no changes in spore amount or morphology were observed. A small difference in spore size was observed between transformants and wild type, where the *laeA* knockouts had slightly smaller spores. Hoff et al. (2010) and Crespo-Sempere et al. (2013) have specifically stated that the conidiation is changed during growth under light conditions in *P. chrysogenum* and *A. nidulans*; we did not observe the same distinguished features with *A. carbonarius*. Similarly to studies by Crespo-Sempere et al. (2013), we also found that when *laeA* has been disrupted in *A. carbonarius* the ochratoxin production diminishes significantly, close to a full shut down (personal communication J. Frisvad). (Dai & Baker 2015) found that in an un-specified *Aspergillus* species overexpressing the *laeA* gene enhanced the citric acid production. In the present study, a similar tendency was shown when overexpressing the *laeA* gene in *A.carbonarius* but only one out of our 21 transformants showed this enhanced production. The fact that only one of the 21 transformants showed significantly higher citric acid production could suggest that the site for gene insertion could be an important factor or that other environmental factors together with LaeA are responsible for the elevated citric acid production in eLaeA-1.

The *A. carbonarius* genome contained a second putative *laeA* gene. The gene had almost as high similarity to the *A. nidulans laeA* gene as the investigated *laeA* gene, and had 70% identity to the investigated *laeA* gene, but seemed to be missing both start and stop codon. This gene was also used in knockout studies but fermentation experiments showed no effect from knocking the gene out (see appendix D). The gene was most likely a non-functional gene.

4.6. CONCLUSION

In this study, we have shown that LaeA regulates the production of citric acid and cellulases in *A. carbonarius*. Knockout of *laeA* decreased the citric acid production to 4-26% and the endoglucanase production to 22-49% of the wild type *A. carbonarius* production. Overexpression of LaeA increased the citric acid production to 212% compared to wild type in one single transformant. Overexpression of *laeA* in *A. carbonarius* could potentially be used alone or in combination with other transcriptional regulators to generate strains for consolidated bioprocessing of biomass into organic acids.

4.7. ACKNOWLEDGEMENTS

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4.8. REFERENCES

Abarca, M.L. et al., 2004. Taxonomy and significance of black aspergilli. *Antonie van Leeuwenhoek*, 86, pp.33–49.

Bayman, P. & Baker, J.L., 2006. Ochratoxins: A global perspective. *Mycopathologia*, 162(3), pp.215–223.

Bayram, O. et al., 2008. VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. *Science (New York, N.Y.)*, 320(5882), pp.1504–1506.

Bayram, O.S. et al., 2010. LaeA control of velvet family regulatory proteins for light-dependent development and fungal cell-type specificity. *PLoS genetics*, 6(12), p.e1001226. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2996326&tool=pmcentrez&rendertype=abstract [Accessed October 28, 2014].

Bayram, Ö. & Braus, G.H., 2012. Coordination of secondary metabolism and development in fungi: The velvet family of regulatory proteins. *FEMS Microbiology Reviews*, 36(1), pp.1–24.

Bok, J.W. et al., 2005. LaeA, a regulator of morphogenic fungal virulence factors. *Eukaryot Cell*, 4(9), pp.1574–82.

Bok, J.W. & Keller, N.P., 2004. LaeA, a Regulator of Secondary Metabolism in *Aspergillus spp*. *Eukaryotic Cell*, 3(2), pp.527–535.

Brakhage, A.A., 2012. Regulation of fungal secondary metabolism. *Nature Reviews Microbiology*, 11(1), pp.21–32. Available at:

http://www.nature.com/doifinder/10.1038/nrmicro2916.

Crespo-Sempere, A. et al., 2013. VeA and LaeA transcriptional factors regulate ochratoxin A biosynthesis in [I]Aspergillus carbonarius. *International journal of food microbiology*, 166(3), pp.479–86. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24041999 [Accessed October 20, 2014].

Dai, Z. & Baker, S.E., 2015. Enhanced citric acid production in *Aspergillus* with inactivated asparagine-linked glycosylation protein 3 (Alg3), and/or increased LaeA expression. , p.US9023637 B2.

Dowd, P.F., 1989. Toxicity of Naturally Occurring Levels of the *Penicillium* Mycotoxins Citrinin., Ochratoxin A, and Penicillic Acid to the Corn Earworm, *Heliothis zea*, and the Fall Armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *Environ. Entomol.*, 18(1), pp.24–29.

Fernandes, M., Keller, N.P. & Adams, T.H., 1998. Sequence-specific binding by *Aspergillus nidulans* AflR, a C6 zinc cluster protein regulating mycotoxin biosynthesis. *Molecular Microbiology*, 28(6), pp.1355–1365.

Frandsen, R.J.N. et al., 2008. Efficient four fragment cloning for the construction of vectors for targeted gene replacement in filamentous fungi. *BMC molecular biology*, 9(70). Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2533011&tool=pmcentrez&rendertype=abstract [Accessed August 25, 2014].

Hansen, N.B., Lübeck, M. & Lübeck, P.S., 2014. Advancing USER cloning into simpleUSER and nicking cloning. *Journal of Microbiological methods*, 96, pp.42–49.

Hoff, B. et al., 2010. Two components of a *velvet*-like complex control hyphal morphogenesis, conidiophore development, and penicillin biosynthesis in *Penicillium chrysogenum*. *Eukaryotic Cell*, 9(8), pp.1236–1250.

Häkkinen, M., 2014. *Transcriptional analysis of Trichoderma reesei under conditions inducing cellulase and hemicellulase production, and identification of factors influencing protein production*. University of Helsinki, Finland.

Jain, S. & Keller, N., 2013. Insights to fungal biology through LaeA sleuthing. *Fungal Biology Reviews*, 27(2), pp.51–59. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1749461313000316 [Accessed November 28, 2014].

Kale, S. et al., 2008. Requirement of LaeA for secondary metabolism and sclerotial production in *Aspergillus flavus*. *Fungal genetics and biology*, 45, pp.1422–1429.

Karaffa, L. et al., 2001. The biochemistry of citric acid of accumulation by

Aspergillus niger (A review). *Acta microbiologica et Immunologica Hungarica*, 48, pp.429–440. Available at: http://www.akademiai.com/index/N312R5806J71503M.pdf.

Karimi-Aghcheh, R. et al., 2013. Functional analyses of *Trichoderma reesei* LAE1 reveal conserved and contrasting roles of this regulator. *G3 Genes Genomes Genetics*, 3(2), pp.369–378. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3564997&tool=pmcentrez&rendertype=abstract.

Keller, N.P., Turner, G. & Bennett, J.W., 2005. Fungal secondary metabolism - from biochemistry to genomics. *Nature reviews. Microbiology*, 3(12), pp.937–947.

Kolasa, M. et al., 2014. Co-cultivation of *Trichoderma reesei* RutC30 with three black *Aspergillus* strains facilitates efficient hydrolysis of pretreated wheat straw and shows promises for on-site enzyme production. *Bioresource technology*, 169, pp.143–148.

Lee, S.B., Milgroom, M.G. & Taylor, J.W., 1988. A rapid, high yield mini-prep method for isolation of total genomic DNA from fungi. *Fungal Genet. Newsl.*, 35, pp.23–24.

Lim, F.Y. et al., 2012. Genome-based cluster deletion reveals an endocrocin biosynthetic pathway in *Aspergillus fumigatus*. *Applied and Environmental Microbiology*, 78(12), pp.4117–4125.

Linde, T. et al., 2014. Fermentation in 24-well plates is an efficient screening platform for filamentous fungi. *Letters in applied microbiology*, 59(2), pp.224–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24725208 [Accessed September 5, 2014].

McDonagh, A. et al., 2008. Sub-telomere directed gene expression during initiation of invasive aspergillosis. *PLoS Pathogens*, 4(9), p.e1000154.

Niu, J. et al., 2016. Identification of a Classical Mutant in the Industrial Host *Aspergillus niger* by Systems Genetics: LaeA Is Required for Citric Acid Production and Regulates the Formation of Some Secondary Metabolites. *G3 Genes|Genomes|Genetics*, 6(January), pp.193–204. Available at: http://g3journal.org/cgi/doi/10.1534/g3.115.024067.

Olsen, L.R. et al., 2011. PHUSER (Primer Help for USER): a novel tool for USER fusion primer design. *Nucleic acids research*, 39(Web Server issue), pp.W61–W67. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3125786&tool=pmcentrez&rendertype=abstract [Accessed July 14, 2014].

Patananan, A.N. et al., 2013. A Novel Automethylation Reaction in the *Aspergillus nidulans* LaeA Protein Generates S-Methylmethionine. *Journal of Biological Chemistry*, 288(20), pp.14032–14045.

Pedersen, M. et al., 2009. Screening for cellulose and hemicellulose degrading enzymes from the fungal genus *Ulocladium*. *International Biodeterioation & biodegradation*, 63, pp.484–489.

Reyes-Dominguez, Y. et al., 2010. Heterochromatic marks are associated with the repression of secondary metabolism clusters in [I]Aspergillus nidulans. *Molecular Microbiology*, 76(6), pp.1376–1386.

Sambrook, J. & Russel, D.W., 2001. *Molecular Cloning: A Laboratory Manual*, CSHL Press.

Seiboth, B. et al., 2012. The putative protein methyltransferase LAE1 controls cellulase gene expression in *Trichoderma reesei*. *Molecular Microbiology*, 84(6), pp.1150–1164.

Shu, P. & Johnson, M.J., 1948. Production by Submerged Fermentation with *Aspergillus niger*. *Industrial & Engineering Chemistry*, 40(7), pp.1202–1205.

Sørensen, A. et al., 2011. b-Glucosidases from a new *Aspergillus* species can substitute commercial b-glucosidases for saccharification of lignocellulosic biomass. *Can. J. Microbiol.*, (57), pp.638–650.

Tamayo-Ramos, J.A. & Orejas, M., 2014. Enhanced glycosyl hydrolase production in *Aspergillus nidulans* using transcription factor engineering approaches. *Biotechnology for Biofuels*, 7(1), p.103. Available at: http://www.biotechnologyforbiofuels.com/content/7/1/103.

Untergasser, A. et al., 2000. Primer3 — new capabilities and interfaces. *Nucleic acids research*, 40(e115).

Wang, S. et al., 2013. Enhancing cellulase production in *Trichoderma reesei* RUT C30 through combined manipulation of activating and repressing genes. *Journal of Industrial Microbiology and Biotechnology*, 40(6), pp.633–641.

Wiemann, P. et al., 2010. FfVel1 and FfLae1, components of a *velvet*-like complex in *Fusarium fujikuroi*, affect differentiation, secondary metabolism and virulence. *Molecular Microbiology*, 77, pp.972–994.

Woloshuk, C.P. et al., 1994. Molecular characterization of aflR, a regulatory locus for aflatoxin biosynthesis. *Applied and environmental microbiology*, 60(7), pp.2408–2414.

Wu, Z.H. et al., 2001. A simplified method for chromosome DNA preparation from filamentous Fungi. *Mycosystema*, 20, pp.575–577.

Yang, L., Lübeck, M. & Lübeck, P.S., 2014. Deletion of glucose oxidase changes the pattern of organic acid production in *Aspergillus carbonarius*. *AMB Express*, 4(1), p.54. Available at: http://www.amb-express.com/content/4/1/54.

Zoglowek, M. et al., 2015. Fungal consortia for conversion of lignocellulose into

bioproducts. In *Mycology: Current and Future Developments, Vol. 1,(R.D. Silva, ed)*. pp. 329–365.

CHAPTER 5. MANUSCRIPT III - KNOCKOUT OF PUTATIVE CITRATE-MALATE ANTIPORTERS IN *ASPERGILLUS CARBONARIUS* AND THE EFFECT ON DI- AND TRI-CARBOXYLIC ACID PRODUCTION

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5.1. ABSTRACT

In filamentous fungi, transport of citrate across the mitochondrial membrane is believed to be facilitated by antiporters, which are integral membrane proteins involved in active transport across the phospholipid membranes. These antiporters transport citrate across the membrane while transferring other metabolites such as malate the opposite direction. In *Aspergillus niger*, a very efficient citric acid producer, citric acid production is believed to start by accumulation of malate in the cytosol, which in turn is transported to the mitochondria via malate-citrate antiporters. Citrate-malate antiporters have not yet been clearly identified; however, the mitochondrial citrate transport protein (CTP) from *Saccharomyces cerevisiae* is the most promising candidate resembling citrate-malate antiporters. So far, CTP has not been identified in filamentous fungi. In this study, seven putative antiporter genes were identified and knocked out of *Aspergillus carbonarius*, a close relative to *A. niger*, and the effect was investigated by fermentation experiments. This was done to study the role of the putative antiporters in di and tri-carboxylic acid production and to examine if it was possible to reroute the natural citric acid production to malic, fumaric or succinic acid. The knockout of one of the putative antiporters (*anti3*)

showed to decrease citric acid production and to increase malic acid production. The changes observed were minor and more extensive studies have to be conducted to conclude if *anti3* is in fact a fungal citrate-malate antiporter.

5.2. INTRODUCTION

By far the greatest part of the chemicals used in the world today is produced by the petro chemical industry from crude oil. An alternative to the production from fossil fuels is to make products from biomass conversion. The biorefinery concept offers a variety of building block chemicals that could potentially replace the products from the petro chemical industry but the biorefinery concept is not yet economically competitive (Gallezot 2012). One way to increase the revenue from a biorefinery is to produce more of high value products like e.g. malic, fumaric or succinic acid (Werpy et al. 2004); and to further increase productivity, well established fungal strains like the filamentous fungal species *Aspergillus niger* could be used as cell factories. *A. niger* is considered a very good citric acid producer because it produces citric acid naturally in great amounts; it can utilize a broad range of substrates and is very tolerant to stress conditions (de Jongh & Nielsen 2008; Papagianni 2007; Goldberg et al. 2006). *A. niger* is the work organism behind the vast majority of the citric acid produced in the world (Dhillon et al. 2010). A substantial amount of scientific research has been done in order to optimize and fine tune the citric acid production and it is now possible to produce titers above 200g/l (van der Straat & de Graaff 2014). Because *A. niger* is a very good acid producer a considerable research effort has been done to direct the production of citric acid towards other types of acids. Itaconic acids have been produced in genetically altered *A. niger* strains with some success (Li et al. 2012; Blumhoff et al. 2013). Examples of other types of acids that have been produced by *A. niger* are gluconic acid and oxalic acid (Mandal & Banerjee 2006; Ramachandran et al. 2008). In *A. niger,* citrate is produced in the mitochondria in the TCA cycle (de Jongh & Nielsen 2008). Citrate is transported out of the mitochondria into the cytosol by membrane bound citrate transporters (Ruijter et al. 2002). The common belief is that these transporters are antiporters, integral membrane proteins, that exchange citrate from the mitochondria with other acids from the cytosol and the primary export comes from antiport of cytosolic malate. (Ruijter et al. 2002; Röhr & Kubicek 1981; de Jongh & Nielsen 2008). It has been suggested that the onset of citric acid production is initiated by rising malate concentration in the cytosol that in turn is transported into the mitochondria in exchange for citrate. Röhr and Kubicek (1981) observed malate accumulation in the cytosol directly preceding start of production of citrate and Jongh and Nielsen (2008) showed that an *A. niger* strain transformed with malate dehydrogenase which in theory should produce elevated amounts of malate instead produced significantly higher amounts of citrate. Similar observations have been made in *Aspergillus carbonarius* a close relative to *A. niger* (Abarca et al. 2004). In several projects aiming to improve dicarboxylic acid production (fumaric, succinic and malic acid) an increased production of citric acid instead of the pursued dicarboxylic acid was

observed (Yang et al. 2014; Yang et al. 2015; Hansen 2014;). This supports the hypothesis that an increased concentration of malate or possible fumarate or succinate in the cytosol will result in an increased production of citric acid due to the antiporter mechanism. Even though malate is believed to be the main dicarboxylic acid that is transported in exchange of citrate, no detailed study have so far been reported on the affinity of the antiporters for different dicarboxylic acids in *A. niger* (de Jongh & Nielsen 2008). In *S. cerevisiae* it has been observed that the transport of citrate across the mitochondrial membrane is influenced by the concentration of malate, isocitrate, succinate, and phosphoenolpyruvate (Sandor et al. 1994). A clear identification of the citrate-malate antiporter protein or sequence has not been done. However, it is strongly suspected that the mitochondrial citrate transporter (CTP) is the primary transporter of citrate across the mitochondrial membrane in exchange of malate (Ruijter et al. 2002; Kaplan et al. 1993; Bisaccia et al. 1993). So far CTP has only been identified in very few species, primarily higher eukaryotes and yeast, though some attempts have been made to find the orthologous gene in *A. niger* (de Jongh & Nielsen 2008; Sandor et al. 1994; Kaplan et al. 1995; Kaplan et al. 1993). Two putative citrate/malate antiporter genes (An11g11230, An18g00070) have briefly been suggested in *A. niger* but so far no attempts to clone or disrupt them in vivo has been carried out (Pel et al. 2007). Studies have shown that the substrate affinity for dicarboxylic acids is high for CTP in higher eukaryotes, but in yeast this affinity is not as strong (Bisaccia et al. 1993; Kaplan et al. 1995). However, the structure and sequence similarities between cytosolic facing part of the yeast CTP and a yeast dicarboxylate transporter (DIC) suggests that malate is probably the main substrate for citrate antiport followed by other dicarboxylic acids e.g. succinate (de Jongh & Nielsen 2008).

The background hypothesis for this study is that increasing the carbon-flux through the glycolysis and guiding it towards e.g. malate via genetic alterations, results in accumulation of malate in the cytosol. When the accumulation occurs, the malate-citrate antiporter starts to transport malate into the mitochondria in exchange of citrate from the mitochondria. In that way, even though malic acid production is practically enhanced, the product exported out of the fungal cell is citric acid. The working hypothesis in this study is that a natural citric acid producer could potentially be changed into a malic acid producer by identification and alteration of the citrate-malate antiporter. The ability to reroute organic acid producers into more high-value acid would have great economic benefits (Werpy et al. 2004). The aim of the study was to identify antiporters in *A. carbonarius* and via a knockout approach investigate their role in the production of di- and tri-carboxylic acid. The main goal was to see if the antiporters had an influence on the citric acid production and if that citric acid production possibly could be rerouted to malic, fumaric or succinic acid. In the present study seven putative antiporters, here called a*nti1-7*, were identified in *A. carbonarius* ITEM5010 using bioinformatic approaches based on CTP from *S. cerevisiae* (Kaplan et al. 1995), An11g11230, An18g00070 from *A. niger* (Pel et al. 2007) and a 2-oxoglutarate/malate mitochondrial carrier protein from *Bos taurus*

(Runswick et al. 1990). The seven antiporter genes were knocked out of *A. carbonarius* using simpleUSER cloning (Hansen et al. 2014) and knockout strains were investigated in citric and malic acid fermentation experiments for changed phenotypes.

5.3. MATERIALS AND METHODS

5.3.1. STRAINS AND GROWTH CONDITIONS

A. carbonarius ITEM 5010 was kindly supplied from Prof. Jens Frisvad, Danish Technical University, Lyngby (Department of Systems Biology, Center for Microbial Biotechnology). It was originally isolated from grapes in Italy (www.ispa.cnr.it/collection). *Escherichia coli* strain DH5α was used as host for plasmid propagation. Fungal cultivation was done on potato dextrose agar (PDA; Scharlau Chemie S.A. Barcelona, Spain) at 30°C in darkness. Spore suspension was stored as stock solution in 20% glycerol at -80°C.

5.3.2. IDENTIFICATION OF THE PUTATIVE ANTIPORTER GENES IN THE A. CARBONARIUS GENOME

Seven putative mitochondrial transmembrane di- and tri- carboxylic acid carrier genes, here called *anti1-anti7*, were identified using different approaches and selected for the study. *anti1, anti2, anti3* and *anti4* were all identified using the same approach. A mitochondrial citrate transport protein (CTP) and a dicarboxylic acid transport protein (DIC) have been identified in *S. cerevisiae* and 3D structural comparison between the two proteins showed great similarities (de Jongh & Nielsen 2008). The CTP sequence from *S. cerevisiae* was used in a BLAST in JGI's database for *A. carbonarius* (http://genome.jgi-psf.org/Aspca3/Aspca3.home.html) and the hits with highest identity were further investigated by predicting their protein structure using the Swiss-Model tool (Arnold et al. 2006) and comparing them with the structure for the CTP and DIC from *S. cerevisiae*. The suggested putative antiporter An18g00070 from *A. niger* was also used as a blast query in JGI's database for *A. carbonarius* and returned *anti1* as the hit with highest similarity (51% identity). The suggested putative antiporter An11g11230 from *A. niger* was also used as blast query in JGI's database for *A. carbonarius* and returned *anti4* as the hit with highest similarity (94% identity). *anti5* was identified from a qualitative search among annotated genes in JGI's database for *A. carbonarius.* The gene resembles the dicarboxylic transport protein spMAE1 from *Schizosaccharomyces pombe* which is known to transport malate (Zelle et al. 2008; Grobler et al. 1995). *anti6* was identified from a BLAST in JGI's database for *A. carbonarius* using a 2-oxoglutarate/malate carrier as query (Runswick et al. 1990). *anti7* was identified from a qualitative search among annotated genes in JGI's database for *A. carbonarius.*

Putative antiporter genes were furthermore investigated with the gene prediction service from Augustus (http://bioinf.uni-greifswald.de/augustus/) (Stanke et al. 2004) and validated with NCBI's Blast service (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome). Phylogeny analysis was done with the online service from MABL (http://www.phylogeny.fr/index.cgi) using MUSCLE for alignment, PhyML for phylogeny and TreeDyn for tree rendering (Dereeper et al. 2008). Transmembrane prediction was carried out with the online prediction service from UCL Bioinformatics group (http://bioinf.cs.ucl.ac.uk/web_servers/) using MEMSAT and MEMSAT-SVM (Nugent & Jones 2012).

5.3.3. GENOMIC DNA EXTRACTION

Genomic DNA extraction was carried out with the established CTAB method (Lee et al. 1988; Wu et al. 2001). Briefly *A.carbonarius* spores were inoculated in YEPD (Sambrook & Russel 2001) and grown stationary for 24 h at 30°C. The mycelia was grinded with a beadbeater (Fastprep-24 MP Biomedicals) in CTAB buffer. The grinded mycelia were incubated at 60°C for 30 min and DNA was extracted with Phenol/Chloroform/isoamyl-alcohol 25:24:1 tris pH8 (Sigma-Aldrich). The DNA was precipitated with isopropanol and the pellet resuspended in 50 µl TE buffer. DNA quality and concentration was measured on spectrophotometer (Nanodrop 1000 Spectrophotometer, ThermoScientific).

5.3.4. PLASMID CONSTRUCTION

The high copy number plasmid pJET2.1 (ThermoFisher Scientific) was used as backbone plasmid for the plasmids constructed in this study. The knockout plasmid backbone pSB4.1.1 (Figure 5.1A) were constructed in a previous study by Hansen et al. (2014). pSB4.1.1 contains the following genetic elements: *RP27-hph-βT* for hygromycin resistance, origin of replication and ampicillin resistance for growth in *E.coli* and a specific cassette facilitating simpleUSER cloning. Primers designed for plasmid construction as well as the rest of the primers used in this study were made with the primer software Primer3 (Olsen et al. 2011; Untergasser et al. 2000) (see table 5.1). All plasmids were constructed using simpleUSER cloning (Hansen et al. 2014). For the knockout plasmids pSBkoAntiporter (Figure 5.1B) upstream and downstream regions to the target gene was identified in the *A. carbonarius* genome provided by JGI. Efficient homologous recombination can be achieved with 1000bp 5' and 3' flanking regions (Hynes 1996) so these were selected and primers with uracil tails were designed to amplify the regions (primers 1-28). Genomic DNA from *A. carbonarius* was used as template. The PCR reactions were done using *pfu* turbo cx polymerase (Agilent), using 65°C as annealing temperature. The simpleUSER cassette in pSB4.1.1 was activated with the restriction enzyme *PacI* and the nicking enzyme *Nb.BbvCI* that combined creates the complementary overhangs to the target PCR fragments. Through self-assembly the PCR fragments were cloned into the

plasmid followed by transformation of *E. coli* with the plasmid for further propagation; this was done using standard methods (Sambrook & Russel 2001). Plasmid extraction was done using the GeneJET plasmid miniprep kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. All plasmids were verified by Sanger sequencing using the sequencing service from StarSEQ (Mainz, Germany).

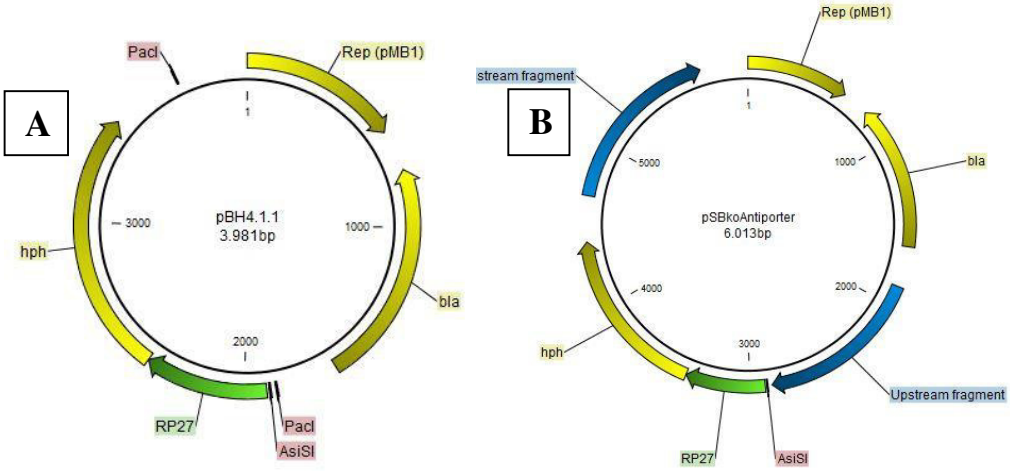

Figure 5.1 The two plasmid types used in the experiments. A) Backbone plasmid pBH4.1.1 used as precursor for B) knockout plasmid pSBkoAntiporter

5.3.5. PROTOPLAST TRANSFORMATION

Protoplasts were prepared from wild type *A. carbonarius* following the procedure described by Yang et al. (2014). Bipartite PCR fragments (Frandsen et al. 2008) were made from the knockout plasmids pSBkoAntiporter and primer pairs 29 + one of 1,5,9,13,17,21,25 without the uracil tail or primer pairs 30 + one of 4,8,12,16,20,24,28 without the uracil tail. These PCR fragments contain either the upstream part of the target gene plus the promoter and the first 2/3 of the hygromycin resistance gene; or the last 2/3 of the hygromycin resistance gene, the terminator and the downstream part of the target gene. The PCR reactions were carried out with Phusion polymerase (Thermo Scientific), with 63°C as annealing temperature. These two types of PCR fragments were mixed 1:1 (in total 4 ug DNA) and used in transformation of the protoplasts (Figure 5.2). Transformation was done in 100 µl aliquots of protoplasts according to the method described by Yang et al. (2014). Transformants able to grow on hygromycin were isolated and transferred 3 times to new plates containing hygromycin in order to ensure that the transformants were stable. Spore-suspensions of transformants with 20% glycerol were stored at -80°C for long-term storage. Validation of positive knockouts was carried out using PCR with transformant DNA as template. For knockout transformants, the primers 31 to 38 were used.

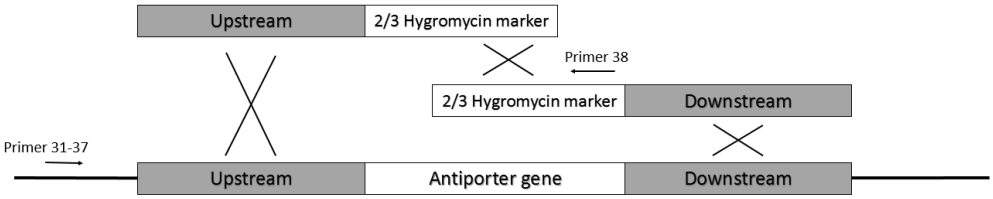

Figure 5.2 Bipartite knockout transformation. First PCR fragment contains the upstream region from the target antiporter gene, the hygromycin promoter (RP27) and the first 2/3 of the hygromycin resistance gene. The second PCR fragment contains the last 2/3 of the hygromycin resistance gene, terminator (β-T) and the downstream region from the target antiporter gene. The two PCR fragments are transformed into the wildtype *A. carbonarius* via protoplast transformation. Only when the two fragments join and cross in will the transformant gain hygromycin resistance. This method greatly increases the chances of positive knockout of the target gene. The positive knockouts are found using PCR and the primers 31-37 together with primer 38. These primers will only give a PCR product when the bipartite PCR fragments are crossed in at the right location in the genome.

5.3.6. FERMENTATION SETUP AND CONDITIONS

Citric acid fermentation was done in 24-well plates and the setup was made following the principles of Linde et al. (2014). Citric acid production media (Shu & Johnson 1948), 140 g/l sucrose, 2.5 g/l KH_2PO_4, 0.25 g/l $MgSO_4 \cdot 7H_2O$, 2.5 g/l NH_4NO_3, pH to 3.8, Cu^{2+} 0.06 mg/l, Zn^{2+} 0.25 mg/l, Fe^{2+} 1.3 mg/l. All fermentation experiments were made in biological triplicates. Fresh spore-suspension was used in a final concentration of $2*10^5$ spores/ml. 24-well plates (UNIPLATE, 24 wells, 10ml, Whatman) were used and covered with silicone lids (BugStopper Venting Capmat, Whatman) and incubated for 5 days at 25°C and 420rpm in a Glas-Col model 099A DPMINC24 (Glas-Col, Terre Haute, IN). Samples were taken at day 5 filtered through 0.45 µm HPLC-grade regenerated cellulose membrane filters and analyzed on HPLC. HPLC was a Dionex Ultimate 3000-LC system (Dionex Corporation, Sunnyvale, CA) with an Aminex HPX-87H column coupled to an RI- detector. The eluent was 4 mmol/l H2SO4 with a flow rate of 0.6 ml/min at 60°C. All chromatograms were integrated using the Chromeleon software (Dionex Corporation).

Malic acid fermentation was done in 50ml Erlenmeyer flasks in 10ml media. Fresh spore-suspension was added to 10ml preculture medium (Yeast extract 3.6g/l and peptone 10g/l) to a final concentration of $2*10^5$ spores/ml. The preculture was incubated at 25°C, 200 rpm and 2.5 cm amplitude for 48 h (KS 4000 I control, IKA). The mycelia pellets from the preculture were transferred to Erlenmeyer flasks with cotton stoppers and 10 ml production media (Peleg et al. 1988) containing 100 g/L glucose, 2 g/L $(NH_4)_2SO_4$, 0.15 g/L KH_2PO_4, 0.15 g/L K_2HPO_4, 0.1 g/L $MgSO_4$ x

$7H_2O$, 0.1g/L $CaCl_2$ x$2H_2O$, 0.005g/L NaCl, 0.1g/L $ZnSO_4$, 0.005g/L $FeSO_4 \cdot 7H_2O$ and 30 g/L $CaCO_3$. The supernatant obtained from each malic acid fermentation was prepared as described by (Goldberg et al. 1983). 1 ml samples were taken at day 5. 50 µl 50% H_2SO_4 was added and the mixture was heated to 80°C and incubated for 15 minutes. The mixture was cooled to room temperature and the supernatant was filtered through 0.45 µM HPLC-grade regenerated cellulose membrane filters. 250 µl of the filtrate were analyzed for the content of glucose, fructose and citric acid by HPLC. 50 µl of the filtrate was analyzed for the content of malic acid using the Megazyme™ L-Malic Acid Kit (Megazyme, Wicklow, Ireland) according to manufacturer's instructions.

Biomass measurements were carried out after termination of the fermentation experiments. The biomass was gathered, rinsed with plenty of water, dried at 105°C for 48h and weighed on a microscale.

5.3.7. STATISTICAL ANALYSIS

Comparison of results were analyzed by One-way ANOVA followed by Tukey Kramer's test with a significance level of $p<0.05$. Error bars on figures are standard error of the mean.

Table 5.1 Primers used in the study

Name	No.	Sequence (5' → 3')	Annotation
***anti1* up-fw-U**	1	GGGTTTAAUAAAGGTGGTGGGGTCGTATT	Amplifies upstream region of *anti1*
***anti1* up-rv-U**	2	GGACTTAAUCTCCGGACTGAGTGGATTGT	Amplifies upstream region of *anti1*
***anti1* do-fw-U**	3	GGCATTAAUCTTGGTCACGCCGATATCTT	Amplifies downstream region of *anti1*
***anti1* do-rv-U**	4	GGTCTTAAUTCCTCCACGAGATTCCTACG	Amplifies downstream region of *anti1*
***anti2* up-fw-U**	5	GGGTTTAAUGCTCTATTCCGTCCCTTTCC	Amplifies upstream region of *anti2*
***anti2* up-rv-U**	6	GGACTTAAUGCTCTTGTAAGCGGGAAGAA	Amplifies upstream region of *anti2*
***anti2* do-fw-U**	7	GGCATTAAUGTCTTGGCCTAGTCGTCTGC	Amplifies downstream region of *anti2*
***anti2* do-rv-U**	8	GGTCTTAAUACCTAACCCAACCCAAAAGC	Amplifies downstream region of *anti2*
***anti3* up-fw-U**	9	GGGTTTAAUAGACATACCGTCGACCTTGG	Amplifies upstream region of *anti3*
***anti3* up-rv-U**	10	GGACTTAAUGAGGGTGAGTCTGGCAGAAG	Amplifies upstream region of *anti3*
***anti3* do-fw-U**	11	GGCATTAAUTCAGTTTTGCATGGTTGAGC	Amplifies downstream region of *anti3*
***anti3* do-rv-U**	12	GGTCTTAAUGCGGGTGGTATTCTCTGTGT	Amplifies downstream region of *anti3*
***anti4* up-fw-U**	13	GGGTTTAAUCCTTCCCCGGTACTAGTTGG	Amplifies upstream region of *anti4*
***anti4* up-rv-U**	14	GGACTTAAUACCGCTAGACACTACTGACT	Amplifies upstream region of *anti4*
***anti4* do-fw-U**	15	GGCATTAAUGCCATGTCCGTACGCTATTC	Amplifies downstream region of *anti4*
***anti4* do-rv-U**	16	GGTCTTAAUCCAAATCGCGCCTGTTTCTA	Amplifies downstream region of *anti4*
***anti5* up-fw-U**	17	GGGTTTAAUGCAAGTTCTCTGCCTTGACC	Amplifies upstream region of *anti5*
***anti5* up-rv-U**	18	GGACTTAAUGTCAACTGCTCTCGGACCTC	Amplifies upstream region of *anti5*
***anti5* do-fw-U**	19	GGCATTAAUTCGAACACATCTGCCTCATC	Amplifies downstream region of *anti5*
***anti5* do-rv-U**	20	GGTCTTAAUAGACGAAGGTTACCGGTGTG	Amplifies downstream region of *anti5*
***anti6* up-fw-U**	21	GGGTTTAAUAGCTAATCCAGGCCAAGACC	Amplifies upstream region of *anti6*
***anti6* up-rv-U**	22	GGACTTAAUCTCGATGACAGCAGAGCAAG	Amplifies upstream region of *anti6*
***anti6* do-fw-U**	23	GGCATTAAUCCTCCACATTCACCACCTCT	Amplifies downstream region of *anti6*

***anti6* do-rv-U**	24	GGTCTTAAUACGCACAGGAACGACTTACC	Amplifies downstream region of *anti6*
***anti7* up-fw-U**	25	GGGTTTAAUTTGCCTTGCAGTATGGATCA	Amplifies upstream region of *anti7*
***anti7* up-rv-U**	26	GGACTTAAUAGCTTGTGCACCTGCTCTCT	Amplifies upstream region of *anti7*
***anti7* do-fw-U**	27	GGCATTAAUACCACCTCCACCAAGAACAC	Amplifies downstream region of *anti7*
***anti7* do-rv-U**	28	GGTCTTAAUGAGCCACGATCGAGAATGAT	Amplifies downstream region of *anti7*
Bipart-1 rv	29	GATGTTGGCGACCTCGTATT	Amplifies 1st bipartite fragment
Bipart-2 fw	30	GATGTAGGAGGGCGTGGATA	Amplifies 2nd bipartite fragment
***anti1* ko-ch-fw**	31	GACGACGGGCCAAAGATACC	Check for positive knockout of *anti1*
***anti2* ko-ch-fw**	32	CCCATTGCCTATCAATCCAC	Check for positive knockout of *anti2*
***anti3* ko-ch-fw**	33	GTCGCAAGCTTCAACTTTCC	Check for positive knockout of *anti3*
***anti4* ko-ch-fw**	34	CTCCTCGAGTGCACCAGTTT	Check for positive knockout of *anti4*
***anti5* ko-ch-fw**	35	TTGGCACCTCGTAGGTGAAT	Check for positive knockout of *anti5*
***anti6* ko-ch-fw**	36	AGATCCTCCGATGTGTACGG	Check for positive knockout of *anti6*
***anti7* ko-ch-fw**	37	CTGCGGGTCAGTACCATTCT	Check for positive knockout of *anti7*
All ko-ch-rv	38	TATTGGAGAGCAAGGGATGG	Check for positive knockout of All

5.4. RESULTS

5.4.1. VALIDATION OF PUTATIVE ANTIPORTER GENES

The di/tri-carboxylic mitochondrial antiporters are not well-characterized proteins in *Aspergillus* sp. The amount of candidate proteins is high, in yeast is known at least 34 different mitochondrial carrier proteins (El Moualij et al. 1997), which makes it a difficult task to identify antiporters in *A. carbonarius*. The seven putative antiporter genes investigated in the present study were identified using several different approaches, and a number of different bioinformatic tools were used to check if the genes were reliable in the context of being putative mitochondrial antiporters. Results from the bioinformatic investigation can be seen in table 5.2 together with the protein tag from JGI's database for *A. carbonarius* ITEM5010. Many of the putative antiporter genes does not share great resemblance with annotated genes from other organisms, and in most cases these genes are automatically annotated genes that have not been cloned in vivo. A phylogenetic analysis was made with the seven putative antiporters together with six reference proteins; DIC from *S. cerevisiae* (Kakhniashvili et al. 1997), and the five proteins that were described in other studies and found to bear resemblance to the putative antiporters (Figure 5.3). To further analyze the similarities and differences between the investigated proteins, a transmembrane prediction was carried out. All the seven putative antiporters and the six reference proteins were predicted to have transmembrane features (see appendix C for further information). In a phylogenetic study in yeast a mitochondrial carrier family protein structure were predicted (El Moualij et al. 1997). Of the investigated proteins in the present study, Anti1, Anti3, Anti4, An18g00070, An11g11230, 2-oxoglutarate/malate carrier and CTP were predicted to share the mitochondrial carrier family structure suggested by El Moualij et al. (1997) (Figure 5.4). Based on the transmembrane prediction an approximate similarity tree was created (Figure 5.5).

Table 5.2 The seven putative mitochondrial antiporter genes, protein tags from JGI's database for *A. carbonarius* ITEM5010, JGI prediction and similar annotated genes from other organisms.

Putative gene	Protein tag JGI	JGI prediction	Best NCBI Protein Blast hit	Resembles
anti1	ID:515063 scaffold6: 2091609-2093639	Mitochondrial substrate carrier with membrane association	Putative tricarboxylate transport protein from *Talaromyces marneffei* (60% identity)	An18g00070 (Pel et al. 2007)
anti2	ID:45724 scaffold5: 660628-662134	Mitochondrial substrate carrier with mitochondrial inner membrane association	ADP/ATP carrier protein from *A. niger* (98% identity)	
anti3	ID:209833 scaffold13: 961229-962928	Mitochondrial oxaloacetate carrier with membrane association	Mitochondrial oxaloacetate transport protein from *A. rambellii* (87% identity)	
anti4	ID:139563 scaffold3: 19646-22956	Mitochondrial substrate carrier with membrane association	Mitochondrial Tricarboxylate transporter from *A. kawachii* (95% identity)	AN11g11230 (Pel et al. 2007)
anti5	ID:172875 scaffold11: 955352-956640	Membrane bound C4-dicarboxylate/malate transport protein	Malic acid transport protein from *A. niger* (83% identity)	Dicarboxylic transport protein spMAE1 from *Schizosaccharomyces pombe* (Zelle et al. 2008; Grobler et al. 1995)
anti6	ID:204747 scaffold3: 1874073-1875424	Mitochondrial substrate carrier with membrane association	Oxoglutarate/malate carrier protein from *Aspergillus niger* (86% identity)	2-oxoglutarate/malate carrier (Runswick et al. 1990; Deng et al. 2008)
anti7	ID:206030 scaffold5: 879202-883364	Citrate transporter with transmembrane transporter activity	Plasma membrane phosphate transporter from *A. niger*. (72% identity)	

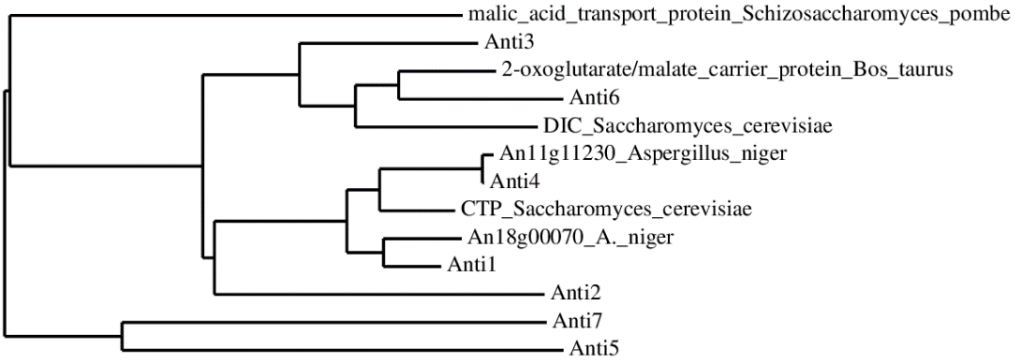

Figure 5.3 Phylogenetic tree analysis based on amino acid sequence alignment with the seven putative antiporters and the six reference proteins.

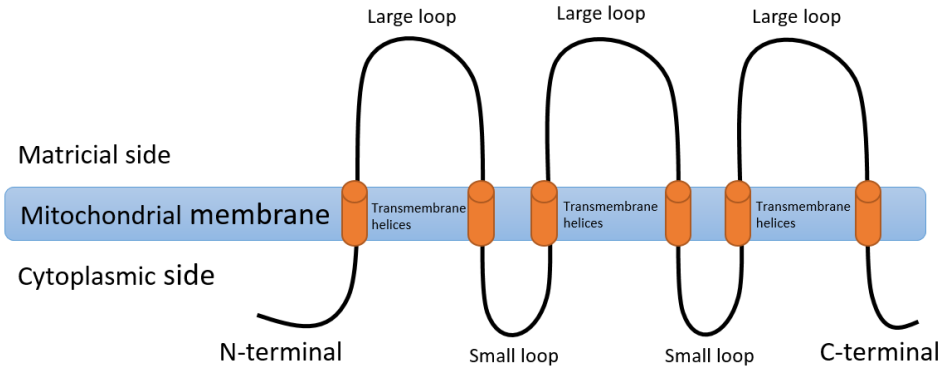

Figure 5.4 Simplified figure of the transmembrane structure for the mitochondrial carrier family proteins predicted by El Moualij et al. (1997), and shared by Anti1, Anti3, Anti4, An18g00070, An11g11230, 2-oxoglutarate/malate carrier and CTP.

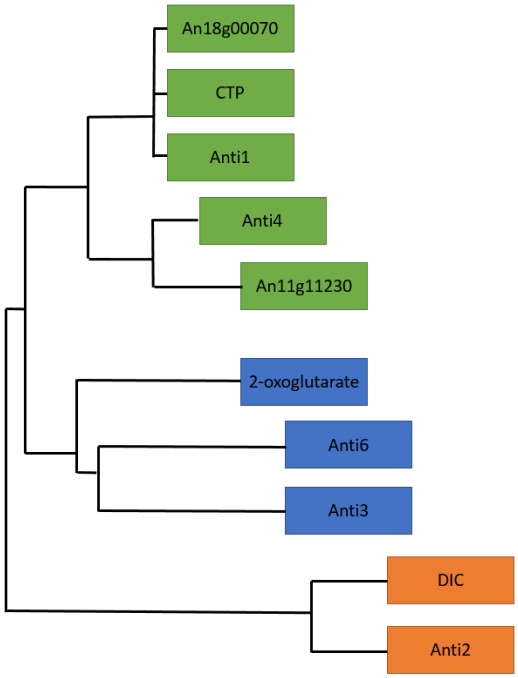

Figure 5.5 Similarity tree based on transmembrane prediction information. Anti5, Anti7 and the malate transporter are not depicted due to no apparent similarities between their transmembrane predictions.

5.4.2. GENERATION OF KNOCKOUT STRAINS

The seven putative antiporter genes: *anti1-7* were all knocked out of *A. carbonarius* wildtype in single knockout events creating seven different knockout strains. Positive transformants of each of the seven transformant types were verified by PCR with transformant DNA as template. The method used, was to check if the hygromycin was inserted correctly within the antiporter gene disrupting the transcription of the gene. This was done with a forward primer binding upstream from the gene in the genome paired with a reverse primer binding on the inserted hygromycin gene, giving a PCR product of a specific size (different between antiporter types). Generation of the predicted PCR product indicated that the hygromycin gene was inserted into the antiporter gene, thereby effectively disrupting it. The extensive knockout study with the seven putative antiporters produced in total 194 transformants. Of these 194 transformants, 45 were verified as positive knockouts by PCR giving a total transformation efficiency of 24%.

Table 5.3 Total knockout transformants and transformation efficiency

Putative Antiporter	Total transformants	Positive transformants	Transformation efficiency %
Anti1	35	4	11
Anti2	29	1	3
Anti3	32	7	22
Anti4	12	1	8
Anti5	26	9	35
Anti6	34	17	50
Anti7	26	6	23

All positive transformants (Table 5.3) were tested in citric acid fermentation experiments in triplicates in the 24-well screening system at least one time and most of them two times or more. The most promising transformants (in total 20 strains) were then selected and tested in malic acid fermentation experiments in Erlenmeyer flasks. All fermentation experiments were performed together with a wildtype sample to always be able to compare.

5.4.3. CITRIC ACID FERMENTATION RESULTS

As simple fermentation setups has a tendency to give great variances in the result outputs between experiments, comparison between experiments can be difficult. This was also the case in the present study with citric acid accumulation after 5 days of fermentation ranging between 1 and 14 g/l. In order to be able to compare the 150+ citric acid samples, the approach was to analyze all the data relative to wildtype instead of using numerical values. This makes sense in the current setup since the goal is not to optimize e.g. citric acid production to reach a specific value, but instead to look for changes in transformant performance compared to wildtype. For each experiment, the wildtype result was set to be equal to 100% and the transformant results were then calculated relative to the wildtype (Figure 5.6). Every data point represent one or more experiments in at least biological triplicates. To analyze for significant differences ANOVA and Tukey-Kramer test ($p<0.05$) were conducted with the antiporter-types that had more than three transformants (Anti1, Anti3, Anti5, Anti6, Anti7) (Figure 5.7). The test showed that none of the transformants were significantly different from wild type, however Anti3 showed a tendency to produce less citric acid than the wild type (70 % citric acid produced compared to wild type).

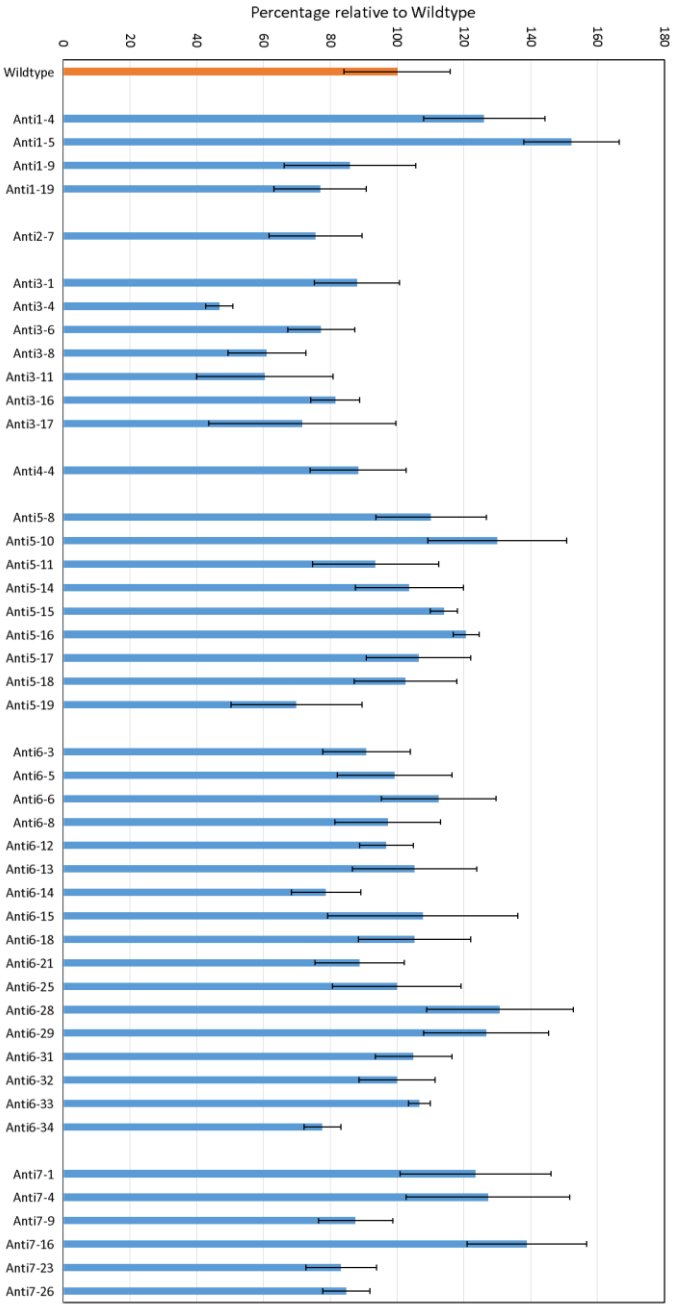

Figure 5.6 Average citric acid production relative to wildtype from antiporter knockout transformants. Error bars shows standard error of the mean.

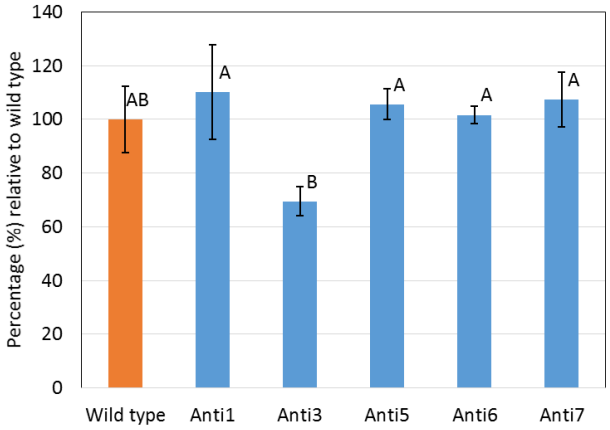

Figure 5.7 Average citric acid production relative to wild type from antiporter knockout transformant types. Error bars shows standard error based on a sample. Letters indicate homogenous groups within the experiment (Tukey Kramer's test $p<0.05$).

5.4.4. MALIC ACID FERMENTATION RESULTS

The amount of malic acid produced was very low ranging from 0 to 800 mg/l after 5 days of fermentation, which makes the comparison between wildtype and transformants difficult. No significant malic acid production differences between transformant and wild type was seen (results not shown). In figure 5.8, malic acid results from fermentation with Anti3 are shown. The malic acid production is slightly increased compared to wildtype, however not significant.

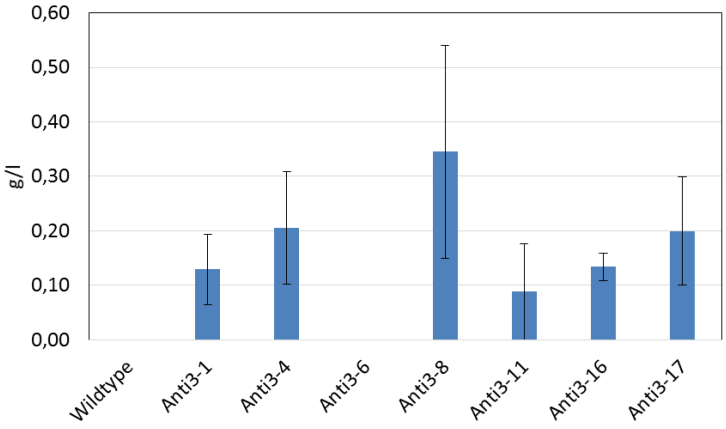

Figure 5.8 Malic acid production from *Anti3* antiporter knockout transformants. Error bars shows standard error of the mean.

5.5. DISCUSSION

Seven putative antiporters were identified in *A. carbonarius* and all were successfully knocked out and tested for production of citric acid and malic acid. The transformation efficiency with the applied bipartite method is generally approximately 10-50 % or even higher in some species (Nielsen et al. 2006). Very low transformation efficiency raises suspicion to whether something is not working correct and it could mean that the initial bioinformatic work of designing primers and constructing plasmids was sub-optimal or that the target gene is positioned in the genome in a way that makes it difficult to knock out. This might be the case for transformants a*nti2* and a*nti4* where only one positive transformant of each type is present, and the transformation efficiency was 3 and 8%. The low transformant numbers makes it difficult to draw conclusions from the fermentation results for these two types of transformants.

The transformants *Anti1*, *Anti3, Anti5, Anti6* and *Anti7* were produced in sufficient amount of copies to be able to make conclusions from the fermentation results. The transformants *Anti1, Anti5, Anti6* and *Anti7* all gave results that were so similar to wildtype that the conclusion was that the knockout of these putative antiporters one by one does not have an effect on citric and malic acid accumulation. One reason for the missing effect on acid accumulation could be that more than one copy of the putative antiporter genes exists in the genome (Cooper 2000). In *S. cerevisiae* it has been shown by southern blotting that only one copy of the mitochondrial citrate transport protein (CTP) gene exists (Kaplan et al. 1995). Southern blotting could be used in a similar way on *A. carbonarius* to test whether the seven putative antiporter exist in one or more copies in the genome. If more than one gene copy exists, the knockout design would have to be re-designed to ensure that all copies of one gene are disrupted. A very recent study done in zebrafish and published in Nature emphasized that knockout mutants does not always show an altered phenotype compared to e.g. morphants when the same gene is target, and the same observation has been seen in mouse and *Arabidopsis*. Other proteins and genes seems to be upregulated to cover for the disrupted gene function (Rossi et al. 2015). The same could be the case in filamentous fungi and this could be the reason for the missing effect from the knockout of the putative antiporters. Other reasons for the missing effect of the knockout of the putative antiporters could be that they are not in fact involved in transport of citrate across the mitochondrial membrane. It could also be that the transport system is so complex that the fungal cell manages to bypass the missing antiporter by using other mitochondrial antiporters with similar affinity for citrate and malate. To test the last hypothesis, knockouts of two or more putative antiporters in the same strain would be a possible strategy. In a study in *A. niger* the isocitrate lyase was overexpressed to activate the glyoxylate bypass to produce malate and succinate (Meijer et al. 2009). The outcome was that instead of changes in organic acid yield, the metabolic pathways through TCA-cycle, glyoxylate bypass and reductive pathway in the cytosol, cooperated to retain the balance among the

metabolites, energy and redox elements (Meijer et al. 2009). The same situation could be the case in this study; when one antiporter is disrupted thereby changing the flux of metabolites, other metabolic pathways will cover the gap generated, so that no differences in acid accumulation can be measured.

According to the phylogenetic analysis, the putative antiporters with the closest similarity to CTP from *S. cerevisiae* and the putative malate/citrate antiporters An11g11230 and An18g00070 from *A. niger* were Anti1 and Anti4 and this was backed up by the transmembrane analysis. The fermentation results with Anti1 and Anti4 did not show any differences from the wild type. Anti1 and Anti4 are very similar in protein sequence, which could mean that the two proteins share the same function in *A. carbonarius*. Because of this, it is a possibility that if one of these genes are knocked out, the other one take over the full functionality rendering a single knockout event inadequate. In further studies, a double knockout mutant with both *anti1* and *anti4* disrupted would be advantageous. Phylogeny furthermore showed that Anti2, Anti5 and Anti7 were all very distantly related to the other proteins investigated and they did not show any difference from wild type in fermentation experiments making conclusions on their roles difficult. However, the transmembrane analysis suggested relationship between Anti2 and DIC, but knockout of *anti2* did only produce one transformant making conclusion based on the fermentation experiments very hard. Anti6 was phylogenetically related to DIC and the 2-oxoglutarate/malate carrier, and seeing as DIC is responsible for transport of dicarboxylates (e.g. malonate, malate, succinate) in exchange for inorganic phosphate and certain sulfur containing compounds (Kakhniashvili et al. 1997) it is no surprise that there were no direct effect on citric acid production from Anti6 transformants.

The phylogenetic analysis showed that Anti3 had highest similarity to the 2-oxoglutarate/malate carrier and DIC however only distantly related, and transmembrane analysis suggested relations to Anti6 and 2-oxoglutarate/malate carrier. The NCBI blast showed highest similarity to a mitochondrial oxaloacetate transport protein from *A. kawachii*. The similarities were not very high thus no conclusions could be made from the bioinformatic investigation to which function Anti3 has. However, JGI predicted Anti3 to be a mitochondrial oxaloacetate carrier indicating that Anti3 might have this ability. The fermentation results did not show any changes in oxaloacetate accumulation (results not shown), but did show a minor reduction in citric acid production and a minor increase in malic acid production. The hypothesis of this research study was that the disruption of an antiporter could halt the secretion of citric acid and reroute it towards e.g. malic acid. Transformant Anti3 did fulfill this hypothesis, however, only with a minor effect. This could indicate that Anti3 might have an antiporter effect, but a certain conclusion will only be possible after more thorough investigations. These investigations could include fermentation experiments in different scales and under different optimized conditions; knockouts of *anti3* and one or more of the other putative antiporters in the same strain; and

overexpression of *anti3*. Other techniques that were not touched upon in this study could be of relevance for investigating the putative antiporters. Studies have been made with *S. cerevisiae* mitochondrial transport proteins that were purified and reconstituted into liposomes (Castegna et al. 2010; Kaplan et al. 1995). In these studies, the function of an antiporter was defined in the presence or absence of substrates or even radioactive labeled substrates and kinetic parameters and substrate affinities could be determined. Castegna et al. (2010) also reported that the inhibitor 1,2,3-benzenetricarboxylate is considered a powerful inhibitor of the citrate/malate carrier and thus could be used as substitute for knockout strategies.

5.6. CONCLUSION

Seven putative antiporter genes were successfully knocked out in *A. carbonarius*. Out of the seven types of knockout transformants, only one type (a*nti3*) had an effect on citric and malic acid production in fermentation experiments. This type of transformant produced less citric acid and more malic acid than the wild type. This was the pursued hypothesized effect but the effect was minor and therefore no definitive conclusion can be made whether *anti3* is in fact an *A. carbonarius* antiporter gene before more in-depth investigations are made.

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5.8. REFERENCES

Abarca, M.L. et al., 2004. Taxonomy and significance of black aspergilli. *Antonie van Leeuwenhoek*, 86, pp.33–49.

Arnold, K. et al., 2006. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics*, 22(2), pp.195–201. Available at: http://bioinformatics.oxfordjournals.org/cgi/doi/10.1093/bioinformatics/bti770.

Bisaccia, F. et al., 1993. Reaction mechanism of the reconstituted tricarboxylate carrier from rat liver mitochondria. *Biochimica et biophysica acta*, 1142(1-2), pp.139–45. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8457581.

Blumhoff, M.L. et al., 2013. Targeting enzymes to the right compartment:

metabolic engineering for itaconic acid production by *Aspergillus niger*. *Metabolic engineering*, 19, pp.26–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23727192 [Accessed October 23, 2014].

Castegna, A. et al., 2010. Identification and functional characterization of a novel mitochondrial carrier for citrate and oxoglutarate in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, 285(23), pp.17359–17370.

Cooper, G.M., 2000. *The Cell, A molecular approach* 2nd ed., Sunderland (MA): Sinauer Associates.

Deng, W. et al., 2008. Molecular cloning and characterization of a mitochondrial dicarboxylate/tricarboxylate transporter gene in *Citrus junos* response to aluminum stress. *Mitochondrial DNA*, 19(4), pp.376–384.

Dereeper, A. et al., 2008. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research*, 36(Web Server), pp.W465–W469. Available at: http://nar.oxfordjournals.org/lookup/doi/10.1093/nar/gkn180.

Dhillon, G.S. et al., 2010. Recent Advances in Citric Acid Bio-production and Recovery. *Food and Bioprocess Technology*, 4(4), pp.505–529. Available at: http://link.springer.com/10.1007/s11947-010-0399-0 [Accessed October 17, 2014].

Frandsen, R.J.N. et al., 2008. Efficient four fragment cloning for the construction of vectors for targeted gene replacement in filamentous fungi. *BMC molecular biology*, 9(70). Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2533011&tool=pmcentrez&rendertype=abstract [Accessed August 25, 2014].

Gallezot, P., 2012. Conversion of biomass to selected chemical products. *Chemical Society reviews*, 41(4), pp.1538–58. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21909591 [Accessed February 28, 2013].

Goldberg, I. et al., 1983. Improved conversion of fumarate to succinate by *Escherichia coli* strains amplified for fumarate reductase. *Applied and environmental microbiology*, 45(6), pp.1838–1847.

Goldberg, I., Rokem, J.S. & Pines, O., 2006. Review Organic acids : old metabolites, new themes. *Journal of Chemical Technology and Biotechnology*, 1611(October 2005), pp.1601–1611.

Grobler, J. et al., 1995. The *mae1* Gene of *Schizosaccharomyces pombe* encodes a Permease for Malate and other C4 Dicarboxylic Acids. *Yeast*, 11, pp.1485–1491.

Hansen, N.B., 2014. *Production of Malic acid by Aspergillus carbonarius*. Aalborg University Copenhagen Denmark.

Hansen, N.B., Lübeck, M. & Lübeck, P.S., 2014. Advancing USER cloning into simpleUSER and nicking cloning. *Journal of Microbiological methods*, 96, pp.42–49.

Hynes, M.J., 1996. Genetic transformation of filamentous fungi. *Genetics*, 75(3), pp.297–311.

de Jongh, W.A. & Nielsen, J., 2008. Enhanced citrate production through gene insertion in *Aspergillus niger*. *Metabolic engineering*, 10(2), pp.87–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18162426 [Accessed March 21, 2012].

Kakhniashvili, D. et al., 1997. Identification of a novel gene encoding the yeast mitochondrial dicarboxylate transport protein via overexpression, purification, and characterization of its protein product. *J Biol Chem*, 272(7), pp.4516–4521.

Kaplan, R.S. et al., 1995. High level expression and characterization of the mitochondrial citrate transport protein from the yeast *Saccharomyces cerevisiae*. *The Journal of biological chemistry*, 270(8), pp.4108–4114.

Kaplan, R.S., Mayor, J.A. & Woodv, D., 1993. The Mitochondrial Tricarboxylate Transport Protein. *Biochemistry*, 268(18), pp.13682–13690.

Lee, S.B., Milgroom, M.G. & Taylor, J.W., 1988. A rapid, high yield mini-prep method for isolation of total genomic DNA from fungi. *Fungal Genet. Newsl.*, 35, pp.23–24.

Li, A. et al., 2012. Enhanced itaconic acid production in *Aspergillus niger* using genetic modification and medium optimization. *BMC biotechnology*, 12, p.57. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3472327&tool=pmcentrez&rendertype=abstract [Accessed April 12, 2013].

Linde, T. et al., 2014. Fermentation in 24-well plates is an efficient screening platform for filamentous fungi. *Letters in applied microbiology*, 59(2), pp.224–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24725208 [Accessed September 5, 2014].

Mandal, S.K. & Banerjee, P.C., 2006. Oxalic acid production by *Aspergillus niger*: Influence of Hydrogen Ion Concentration and Nitrogen Source. *Research Journal of Microbiology*, 1(2), pp.190–197.

Meijer, S. et al., 2009. Overexpression of isocitrate lyase - Glyoxylate bypass influence on metabolism in *Aspergillus niger*. *Metabolic engineering*, 11, pp.107–116. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19558969 [Accessed April 27, 2012].

El Moualij, B. et al., 1997. Phylogenetic classification of the mitochondrial carrier family of *Saccharomyces cerevisiae*. *Yeast*, 13, pp.573–581.

Nielsen, M.L. et al., 2006. Efficient PCR-based gene targeting with a recyclable marker for *Aspergillus nidulans*. *Fungal Genetics and Biology*, 43(1), pp.54–64.

Nugent, T. & Jones, D.T., 2012. Detecting pore-lining regions in transmembrane protein sequences. *BMC Bioinformatics*, 13(169), pp.1471–2105. Available at: BMC Bioinformatics.

Olsen, L.R. et al., 2011. PHUSER (Primer Help for USER): a novel tool for USER fusion primer design. *Nucleic acids research*, 39(Web Server issue), pp.W61–W67. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3125786&tool=pmcentrez&rendertype=abstract [Accessed July 14, 2014].

Papagianni, M., 2007. Advances in citric acid fermentation by *Aspergillus niger*: biochemical aspects, membrane transport and modeling. *Biotechnology advances*, 25(3), pp.244–63. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17337335 [Accessed March 9, 2012].

Pel, H.J. et al., 2007. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nature biotechnology*, 25(2), pp.221–31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17259976 [Accessed September 26, 2014].

Peleg, Y., Stieglitz, B. & Goldberg, I., 1988. Malic acid accumulation by *Aspergillus flavus*. *Applied Microbiology Biotechnology*, 28, pp.69–75.

Ramachandran, S. et al., 2008. Fed-batch production of gluconic acid by terpene-treated *Aspergillus niger* spores. *Applied biochemistry and biotechnology*, 151(2-3), pp.413–23. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18427736 [Accessed October 23, 2014].

Rossi, A. et al., 2015. Genetic compensation induced by deleterious mutations but not gene knockdowns. *Nature*, 524, pp.230–235. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26168398.

Ruijter, G., Kubicek, C. & Visser, J., 2002. Production of organic acids by fungi. In H. Osiewacz, ed. *The Mycota X, Industrial Applications*. Berlin, Heidelberg: Springer, pp. 213–230.

Runswick, M.J. et al., 1990. Sequence of the bovine 2-oxoglutarate/malate carrier protein: structural relationship to other mitochondrial transport proteins. *Biochemistry*, 29(50), pp.11033–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2271695.

Röhr, G. & Kubicek, C.P., 1981. Regulatory aspects of citric acid fermentation by *Aspergillus niger*. *Process Biochemistry*, 16(34).

Sambrook, J. & Russel, D.W., 2001. *Molecular Cloning: A Laboratory Manual*,

CSHL Press.

Sandor, A., Johnson, J.H. & Srere, P.A., 1994. Cooperation between enzyme and transporter in the inner mitochondrial membrane of yeast. *The Journal of biological chemistry*, 269(47), pp.29609–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7961948.

Shu, P. & Johnson, M.J., 1948. Production by Submerged Fermentation with *Aspergillus niger*. *Industrial & Engineering Chemistry*, 40(7), pp.1202–1205.

Stanke, M. et al., 2004. AUGUSTUS: a web server for gene finding in eukaryotes. *Nucleic Acids Research*, 32(Web Server), pp.W309–W312. Available at: http://nar.oxfordjournals.org/lookup/doi/10.1093/nar/gkh379.

van der Straat, L. & de Graaff, L., 2014. Pathway transfer in fungi: Transporters are the key to success. *Bioengineered*, 5, pp.335–339. Available at: http://dx.doi.org/10.4161/bioe.29936.

Untergasser, A. et al., 2000. Primer3 — new capabilities and interfaces. *Nucleic acids research*, 40(e115).

Werpy, T. et al., 2004. *Top Value Added Chemicals from Biomass Volume I — Results of Screening for Potential Candidates from Sugars and Synthesis Gas*, U.S. Department of Energy, Oak Ridge.

Wu, Z.H. et al., 2001. A simplified method for chromosome DNA preparation from filamentous Fungi. *Mycosystema*, 20, pp.575–577.

Yang, L., Lübeck, M. & Lübeck, P.S., 2014. Deletion of glucose oxidase changes the pattern of organic acid production in *Aspergillus carbonarius*. *AMB Express*, 4(1), p.54. Available at: http://www.amb-express.com/content/4/1/54.

Yang, L., Lübeck, M. & Lübeck, P.S., 2015. Effects of heterologous expression of phosphoenolpyruvate carboxykinase and phosphoenolpyruvate carboxylase on organic acid production in *Aspergillus carbonarius*. *Journal of Industrial Microbiology & Biotechnology*. Available at: "http://dx.doi.org/10.1007/s10295-015-1688-4.

Zelle, R.M. et al., 2008. Malic acid production by *Saccharomyces cerevisiae*: engineering of pyruvate carboxylation, oxaloacetate reduction, and malate export. *Applied and environmental microbiology*, 74(9), pp.2766–77. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2394876&tool=pmcentrez&rendertype=abstract [Accessed March 6, 2012].

CHAPTER 6. MANUSCRIPT IV - FERMENTATION IN 24-WELL PLATES IS AN EFFICIENT SCREENING PLATFORM FOR FILAMENTOUS FUNGI

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ORIGINAL ARTICLE

Fermentation in 24-well plates is an efficient screening platform for filamentous fungi

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Significance and Impact of the Study: Fermentation studies with filamentous fungi and especially screening experiments often struggle with high inter-vessel variations in metabolite production. This study compares two different types of frequently used screening methods namely batch fermentation in Erlenmeyer flasks with batch fermentation in 24-well plates. The results demonstrate that the variance potentially can be reduced two and a half-fold using 24-well plates leading to improved resolution when testing the impact of varying fermentation parameters on product formation.

Keywords
bioprocessing, fermentation biotechnology, fungi, optimization.

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Abstract

Fermentation by filamentous fungi in Erlenmeyer flasks is a favoured method for comparing different fermentation conditions. However, significant inter-flask variation often occurs when using Erlenmeyer flasks, which makes the comparison of fermentation product levels less reliable. We have investigated the use of a 24-well plate method for citric acid, ethanol and glycerol batch fermentation using the filamentous fungi *Aspergillus carbonarius* and compared the relative standard deviation (RSD) from sextuplicates obtained using Erlenmeyer flasks and 24-well plates. The production levels using the Erlenmeyer flasks showed a combined average RSD of 29%, which is two and a half-fold higher than what was measured using the 24-well plates showing an average RSD of 12%. We conclude that fermentation in 24-well plates is a more reliable screening method for metabolite production by filamentous fungi and possibly for screening metabolites in general.

Introduction

Improvement of an organism-mediated process often involves the identification and optimization of several parameters, which may include temperature, media composition, shaking system and other factors (Sanchez-Marroquin *et al.* 1970; Xu *et al.* 1989), as for example growth or morphology of the production organism, or genetic changes (de Jongh and Nielsen 2008). Although each parameter modification might cause subtle improvements, they can be difficult to identify because of the variation associated with biological systems. The biological variation can be accounted for by setting up experiments in replica; however, the number of experiments needed to identify successful improvements is often high, thereby leading to an increase in resources allocated to each experiment. To overcome this, a high-throughput screening process is essential for the identification of multiple successful parameters (Nagy *et al.* 2007). Furthermore, the screening method has to be reliable, stable and able to generate reproducible results.

Traditionally, research on citric acid production by filamentous fungi has been performed by batch fermentation experiments. The basic strategy is as follows: an Erlenmeyer flask that contains a specific kind of media is inoculated with a filamentous fungus. The inoculated Erlenmeyer flask is then incubated at a specific temperature at shaking conditions for defined amount of time intervals at which a sample is withdrawn, measured and used to represent the production capacity of the filamentous fungi under these conditions (Shu and Johnson 1948).

Conventional batch screening in flasks or fermenters of newly developed strains is often time-consuming and laborious just because of the large number of strains that require testing. One approach to ease the workload is to scale down the fermentation process. Since 1951, multiwell plates have been used for downscaling fermentation processes with different organisms (Manns 1999). However, there are only few reports on successful experiments with filamentous organisms and very few on filamentous fungi (Bills *et al.* 2008; Alberto *et al.* 2009; Sohoni *et al.* 2012). Screening using 24-well plate (multiwell culture plates) batch fermentation methods for filamentous fungi would be beneficiary in experiments where a large number of replicas are needed to identify small yet significant changes. Fermentation in 24-well plates also offers reduced time and labour when the number of experiments is high. Also, the volume of medium used for each experiment is decreased, thus reducing resources allocated to each experiment.

To our understanding, it is not directly stated in any paper that conventional Erlenmeyer flask batch fermentation suffers from large variations. However, one study on citric acid batch fermentation reports a cut-off value for results yielding <75% of their maximum citric acid production result, to make sure that only high-yielding citric acid production results were taken into consideration (Peksel *et al.* 2002). Furthermore, the commercial citric acid producing company Chemtotal states 'Variations in results often occur even under apparently similar operating conditions. All plants prefer to rely upon their own experience rather than the so-called "proven" facts' (Chemtotal 2010). The mere fact that standard deviations are not reported in many batch production experiments emphasizes this issue (Shu and Johnson 1947; Tomlinson *et al.* 1950; Kitos *et al.* 1953; Noguchi and Johnson 1961; Kubicek-Pranz *et al.* 1990; Dhillon *et al.* 2011). The only way to account for the variation is by setting up experiments in a high number of replicas.

Aspergillus niger can produce high titres of citric acid (Shu and Johnson 1948). We have identified a strain of the closely related species *A. carbonarius*, which we have selected for different projects, involving optimization of growth conditions and pathway engineering. *Aspergillus carbonarius* has shown to be a very good producer of organic acids, especially citric acid and gluconic acid (Yang 2012). The aim of this study was to apply a 24-well plate method as a screening tool for batch production of citric acid with *A. carbonarius* and to study whether we could minimize the variation between repeated samples compared with the citric acid batch production in Erlenmeyer flasks. Hereby, we could attain more representative values for expressing the citric acid productivity of *A. carbonarius*. Therefore, our hypothesis was that fermentation in 24-well plates enables better homogeneity regarding aeration, water evaporation, temperature and surface structure in each well, than that obtained in Erlenmeyer flasks. Also included in the study were the production results of ethanol and glycerol in the two systems, to give a more profound picture of the variation problem.

Results and discussion

In several studies, researchers have shown that the microplate method is at least comparable to Erlenmeyer flask methods as batch fermentation in terms of reproducibility and variation between samples (Sohoni *et al.* 2012). In this study, we successfully scaled down the conventional batch fermentation from Erlenmeyer flasks to 24-well plates for *A. carbonarius* metabolite production while decreasing variability.

Sample-to-sample variation for citric acid production

In the initial experiment, citric acid titres were measured to assess the variation in fermentation. To test the difference in variation between Erlenmeyer flasks and wells of 24-well plates, the two set-ups were grown simultaneously and samples taken after 96 h. To test whether citric acid titres were affected by preinoculating conditions, two individual spore suspensions were prepared from two separate Petri dishes containing sporulating *A. carbonarius*. Each spore suspension was aliquoted in 24 wells (one plate) and six 500-ml Erlenmeyer flasks. The outcome of the citric acid fermentation is presented in Fig. 1.

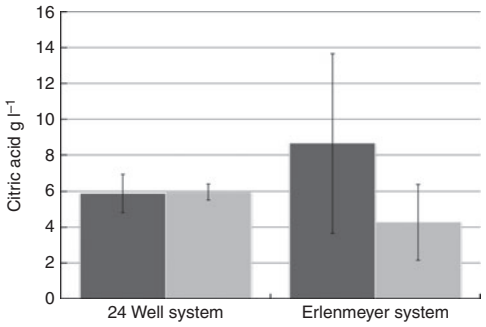

Figure 1 Comparison of the citric acid production after 96 h. Two citric acid batch productions were conducted in 24-well plates and Erlenmeyer flasks. Two different spore preparations were used to ensure that citric acid titre was unaffected by preinoculating conditions. The error bars represent the sample standard deviation obtained from six observations on one 24-well plate. Each Erlenmeyer flask result is obtained from six Erlenmeyer flasks. The light grey bars represent spore suspension 1; dark grey bars represent spore suspension 2.

The citric acid titre levels obtained after 96 h of fermentation in 24-well plates were 5·88 g l^{-1} ± 18% (mean ± relative standard deviation (RSD)) and 5·96 g l^{-1} ± 8%. In comparison, the titres obtained from the Erlenmeyer flasks were 8·66 g l^{-1} ± 58% and 4·27 g l^{-1} ± 50%. The very similar results from the two 24-well plates indicated that the preinoculation conditions did not have any significant effect on the outcome of the citric acid titre and variation.

Citric acid fermentation in Erlenmeyer flasks

To gain insight into the causes of the high RSD observed in Erlenmeyer flasks, a time-course experiment was carried out where *A. carbonarius* spores were added to six Erlenmeyer flasks in a final concentration of 10^5 spores per ml. Samples from the six replicates were taken for high-performance liquid chromatography (HPLC) analysis at 48, 96, 120 and 192 h after inoculation (Fig. 2).

The average RSD was very high in the flask experiment (48%). At 48 h, the citric acid production was almost identical in the different flasks. However, already at 96 h and beyond, the RSD increased in all analysed parameters. An exception to the variation observed was the pelleted morphology (Fig. 4) and also the amount of biomass accumulated at day eight (32·6 ± 0·6 g l^{-1}).

Comparison of metabolite production in 24-well plates and Erlenmeyer flasks

To make a clear comparison, fermentation was conducted in 24-well plates with the same set-up; the only differences were the vessel type and the incubator type. Samples were taken at 48, 96, 120 and 192 h. From the Erlenmeyer set-up, one ml was sampled from six individual flasks and the flasks reincubated. In contrast, six wells were emptied when sampling from the 24-well plate fermentation, thereby sampling in a noncontinuous manner. The resulting citric acid, glycerol and ethanol fermentation profiles over time are shown in Fig. 3.

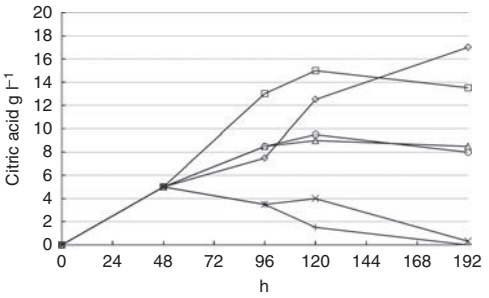

Figure 2 Citric acid fermentation in Erlenmeyer flasks. Six citric acid fermentations were performed in six Erlenmeyer flasks. Observations were made at 48, 96, 120 and 192 h after inoculation to determine citric acid titres. The figure shows the individual observation obtained from each Erlenmeyer flask. (□) Flask 1, (◊) Flask 2, (Δ) Flask 3, (○) Flask 4, (×) Flask 5 and (+) Flask 6.

The RSD was lower for all of the 24-well plate experiments compared to the RSD of the Erlenmeyer flask experiments except for citric acid production at 48 h. The mean RSD for Erlenmeyer production from 48, 96, 120 and 192 h in the citric acid, ethanol and glycerol production experiment was 48, 25 and 14%, respectively. In contrast, in the production in the 24-well plates, the mean RSD from all samples taken from 48 to 192 h was 11, 20 and 6%, respectively. The mean RSD for all the Erlenmeyer samples combined was 29%, while the mean RSD for all the well samples combined was 12% (Table 1).

Our studies with metabolite production, using *A. carbonarius* in batch fermentation in Erlenmeyer flasks with cotton stoppers, showed that this method in fact has large variations in production levels. Fermentation in 24-well plates largely reduced the RSD associated with citric acid production by *A. carbonarius*, making it possible to attain more representative citric acid productivity values. The highest citric acid titre level at 35·8 g l^{-1} was obtained after 192 h in 24-well plates, which also is the highest amount of citric acid concentration reported from *A. carbonarius*.

From a statistical point of view, the percentage needed to obtain a significant change from the mean value was determined. If for example a two-tailed *t* test was employed in each of the fermentation methods, the percentage needed to obtain a significant change from the mean value would need to be 46·9–48·1% (Student/Welch statistics) to show a significant change ($P < 0·05$). In contrast, the 24-well plate set-up showed an RSD at 12%, and only a 17% difference (independent on Student/Welch statistics) would show a significant change ($P < 0·05$). Results in 24-well plates more effectively resolve the effects of how different parameters affect the experimental outcomes.

As with conventional batch fermentation, fermentation in microwell plates has numerous parameters that have to be adjusted to obtain useful and reliable screening results (Sohoni *et al.* 2012). One very important parameter in aerobic fermentation and especially in citric acid fermentation is to ensure a sufficient oxygen transfer rate (OTR) for the organism to proliferate (Kubicek *et al.* 1985). One side effect of high OTRs is increased evaporation. It is very important to minimize evaporation from the production media, because the evaporation increases the concentrations of components in the media and thereby

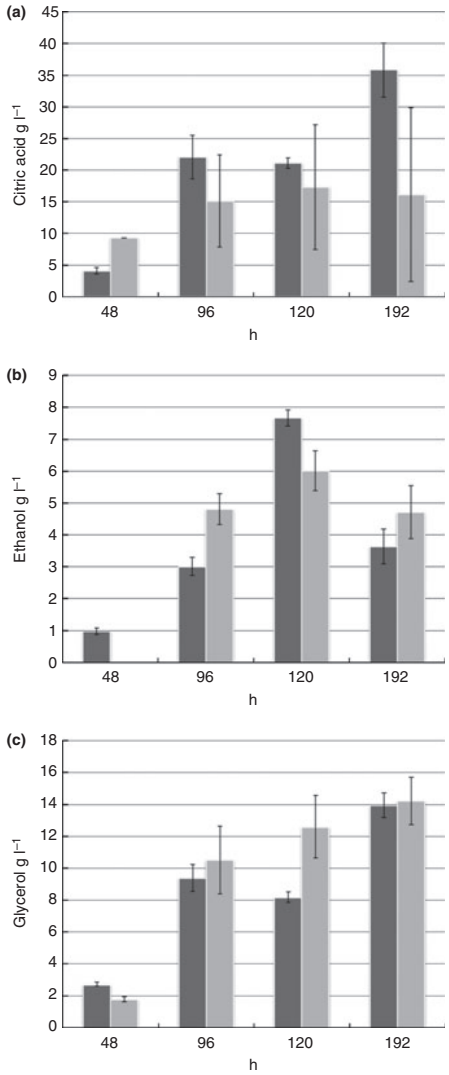

Figure 3 Fermentation profile for 24-well plates and Erlenmeyer flasks. Over a 192-h period, samples were taken and analysed. Each data entry represents the average titre for six samples. Each 24-well plate data entry represents the termination of six wells from the 24-well plate, whereas the Erlenmeyer flask fermentation is sampled continuously. The light grey bars represent the Erlenmeyer flasks; the dark grey bars represent the 24 wells. The three figures a, b and c show the results gathered for three different metabolites in the same experiment. (a) Citric acid production. The flask experiment values have been multiplied by two for easier visual comparison of the relative standard deviation. (b) Ethanol production. The well experiment values have been multiplied by two. (c) Glycerol production.

Table 1 The relative standard deviation (RSD) obtained in this study

Experiment	Time (h)	24-well RSD (%)	Erlenmeyer RSD (%)
Citric acid production	48	13	0
	96	16	48
	120	3	57
	192	12	85
Ethanol production	48	22	–
	96	19	20
	120	7	21
	192	30	35
Glycerol production	48	5	9
	96	9	20
	120	4	15
	192	6	10
Average RSD		12	29

From RSDs obtained in the metabolite production study, an average RSD was deduced from the 24-well plate method as well as the Erlenmeyer method. No RSD value could be obtained from ethanol production at 48 h because of lack of ethanol production.

gradually changes the foundation of the fermentation in an uncontrolled manner (Duetz and Witholt 2001). The OTR in batch experiments is directly related to what type of flask or microplate is used, what speed and diameter the shaking device is using and what amount of air is available through different lid types (Maier and Büchs 2001; Zhang *et al.* 2008). In the present study, the results suggest that the 24-well plates facilitate better homogeneity regarding OTR, water evaporation, temperature and surface structure compared to what can be achieved in Erlenmeyer flasks. Compared to Erlenmeyer flasks, 24-well plates are a better choice for screening of fungal strains for the production of at least citric acid because of high reproducibility. Moreover, the format facilitates screening large numbers of strains. Finally, Erlenmeyer flask could be omitted in the process of going from laboratory-scale production in 24-well plates to fermentation in 1–10-l fermentation vessels, because the production in 24-well plates is at least as good as in Erlenmeyer flasks and the reproducibility is much better.

Morphology and biomass production

The mycelia morphology during citric acid batch production with Erlenmeyer flasks and 24-well plates was similar. During both fermentation set-ups, *A. carbonarius* growth consists of pellets with a diameter varying between 50 and 200 μm. However, during the Erlenmeyer flask production, considerable wall growth occurred near the upper waterfront (Fig. 4).

Although the morphology of the micro-organism plays a role in controlling and augmenting citric acid formation

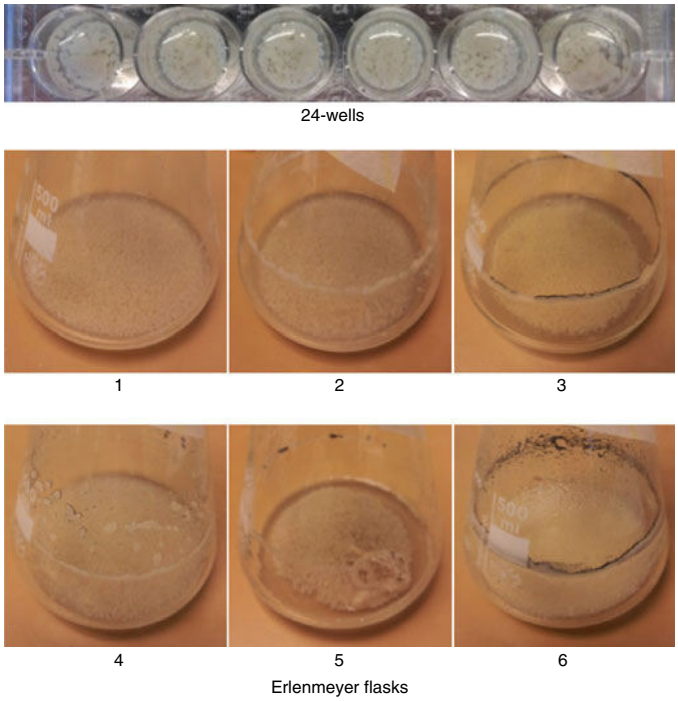

Figure 4 The morphology of *Aspergillus carbonarius* during citric acid batch production after 96 h of citric acid batch production in 24-well plates and Erlenmeyer flasks. First row shows the growth in 24-well plates. Second and 3rd rows show the growth in Erlenmeyer flasks.

(Max *et al.* 2010), biomass and pellet form did not differ significantly in our experiments and thus could not explain the variation in citric acid production.

Materials and methods

Fungal culture

The fungal strain *A. carbonarius* ITEM 5010 was originally isolated from grapes in Italy (http://server.ispa.cnr.it/ITEM/Collection/). It was kindly supplied from Jens Frisvad, Danish Technical University, Lyngby (Department of Systems Biology, Center for Microbial Biotechnology, Fungal Chemodiversity). The culture was stored in spore suspension at −80°C in 25% glycerol and was revived on potato dextrose agar (PDA) medium (Scharlau Chemie S.A. Barcelona, Spain).

Inoculum preparation

Ten microlitre spores from the culture stock was spread onto PDA plates and incubated 5 days at 30°C. Spores were harvested by adding 3 ml milliQ water and scraping the spores with a spatula. The concentrated spore suspension was defined by counting the spores in a sample under ×20 magnification (Olympus CX41, Olympus Europa SE & Co. KG, Hamburg, Germany) and a counting chamber (Fuchs Rosenthal, Hausser Scientific Company, Philadelphia, PA). The concentration of spores in the spore suspension was 10^7–10^8 spores per ml. The spore suspension was used directly as inoculum for the fermentation. The fermentation media was prepared, and the spore suspension was added to a final spore concentration of 10^5 spores per ml. Fermentation media and spores were prepared in one single flask for an experiment. The flask was then stirred using a magnetic stirrer to obtain a uniform solution, and afterwards, the replicate samples were distributed uniformly into the experiment vessel type.

Sample analysis

Eight hundred microlitre fermentation broth was filtered through 0·45-μm HPLC-grade regenerated cellulose membrane filters. One hundred and fifty microlitre of the filtrates was analysed for the content of cellobiose, glucose, xylose, glycerol, citric acid, ethanol, arabinose, lactate and acetate by HPLC on a Dionex Ultimate

3000-LC system (Dionex Corporation, Sunnyvale, CA) with an Aminex HPX-87H column coupled to an RI-detector. The eluent was 4 mmol l^{-1} H_2SO_4 with a flow rate of 0·6 ml min^{-1} at 60°C. All chromatograms were integrated using the Chromeleon software (Dionex Corporation).

Fungal biomass measurements

Biomass was measured at the end of an experiment. The remaining fermentation broth was filtered for biomass, and the liquid phase was discarded. The biomass was dried in an oven at 100°C for 48 h and weighted.

Media preparation

Potato dextrose agar plates for spore production were prepared using PDA mixed with milliQ water according to the manufactures instructions and autoclaved at 121°C for 20 min. Fifteen millilitre of the hot PDA solution was poured into Petri dishes with a diameter of 9 cm and was solidified in a sterile environment. The recipe for the fermentation media was obtained from (Shu and Johnson 1948), and no alteration was made to the original recipe. A total of 409 mmol l^{-1} sucrose, 18 mmol l^{-1} KH_2PO_4, 1 mmol l^{-1} $MgSO_4$ $7H_2O$, 31 mmol l^{-1} NH_4NO_3 and 1 mol l^{-1} HCl were used to adjust the pH to 3·8. The solution was autoclaved at 121°C for 20 min. After cooling, filter-sterilized trace metals were added to a final concentration of 0·94 μmol l^{-1} $CuSO_4$, 3·8 μmol l^{-1} $ZnSO_4$ and 20 μmol l^{-1} $FeSO_4$ (metal-based weight). The fermentation media were prepared and used fresh to avoid any precipitation.

Twenty-four-well plate and flask types

The strain was fermented in either Greiner CELLSTAR multiwell culture plates (24 wells) for suspension cultures (Cruinn, Dublin, Ireland) or 500-ml unbaffled Erlenmeyer flasks. The 24-well plates are capable of holding 3·3 ml media per well. However, 1·5 ml fermentation media including spore inoculum were aliquoted in each well. The plates were covered with plastic lids during the fermentation. The plastic lids were perforated with 15 1·0 mm holes to increase airflow to the set-up. In all Erlenmeyer flask experiments, volume of fermentation media was 50 ml (10% v/v). The flasks were sealed with cotton plugs for gas exchange and to prevent contamination.

Incubator set-up parameters

The incubator type used for the 24-well plates was an incubator shaker from Glas-Col model 099A DPMINC24 (Glas-Col, Terre Haute, IN). The incubator was shaking at 450 rev min^{-1}, 25°C. The shaking amplitude was 0·7 cm in diameter. The humidity in the incubator was controlled by mounting plastic boxes with open milliQ water inside the closed incubator, thereby creating a very humid environment to avoid excess evaporation from the fermentation broth. The incubator type used for the Erlenmeyer flasks was an incubator rotator from IKA model KS 4000-ic-control (IKA-Werke GmbH & Co. KG, Staufen, Germany). The incubator was rotating at 270 rev min^{-1} with 2·5 cm amplitude in diameter. Both incubators were run at 25°C in the same incubator room. All incubations were run with a comparable flask or plate containing milliQ water to account for water loss due to evaporation.

Statistical analysis

The mean value, standard deviation based on sample and RSD were used as comparison factors throughout the research. Mean values are separated from their sample standard deviation with the plus–minus sign ($\pm$). The RSD was calculated as the coefficient of variation. The sample coefficient of variation is defined as the ratio of the sample standard deviation to the sample mean. All samples consist of six observations to be able to give a higher statistical impact than the conventional triplicates. Levene's test verified that both Student's *t* test and Welch's *t* test, both with a two-tailed distribution, should be used to give a ubiquitous and comparable P ($P < 0{\cdot}05$). All calculations and modelling were performed in R-Development (2008).

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Conflict of Interest

No conflict of interest declared.

References

Alberto, F., Navarro, D., De Vries, R., Asther, M. and Record, E. (2009) Technical advance in fungal biotechnology: development of a miniaturized culture method and an automated high-throughput screening. *Lett Appl Microbiol* **49**, 278–282.

Bills, G.F., Platas, G., Fillola, A., Jiménez, M.R., Collado, J., Vicente, F., Martín, J., González, A. *et al.* (2008) Enhancement of antibiotic and secondary metabolite detection from filamentous fungi by growth on nutritional arrays. *J Appl Microbiol* **104**, 1644–1658.

Chemtotal (2010) Profile—Citric Acid. [Online] Available from: http://www.chemtotal.com/citric-acid-a-profile.html. [Accessed: June 19, 2013].

Dhillon, G.S., Brar, S.K., Verma, M. and Tyagi, R.D. (2011) Apple pomace ultrafiltration sludge: a novel substrate for fungal bioproduction of citric acid: optimisation studies. *Food Chem* **128**, 864–871.

Duetz, W.A. and Witholt, B. (2001) Effectiveness of orbital shaking for the aeration of suspended bacterial cultures in square-deepwell microtiter plates. *Biochem Eng J* **7**, 113–115.

de Jongh, W.A. and Nielsen, J. (2008) Enhanced citrate production through gene insertion in *Aspergillus niger*. *Metab Eng* **10**, 87–96.

Kitos, P.A., Campbell, J.J.R. and Tomlinson, N. (1953) Influence of temperature on the trace element requirements for citric acid production by *Aspergillus niger*. *Appl Environ Microbiol* **1**, 156–159.

Kubicek, C.P., Röhr, M. and Rehm, H. (1985) Citric acid fermentation. *Crit Rev Biotechnol* **3**, 331–373.

Kubicek-Pranz, E.M., Mozelt, M., Röhr, M. and Kubicek, C.P. (1990) Changes in the concentration of fructose 2, 6-bisphosphate in *Aspergillus niger* during stimulation of acidogenesis by elevated sucrose concentration. *Biochim Biophys Acta* **1033**, 250–255.

Maier, U. and Büchs, J. (2001) Characterisation of the gas-liquid mass transfer in shaking bioreactors. *Biochem Eng J* **7**, 99–106.

Manns, R. (1999) Microplate History. [Online] Available from: http://www.plastiquarian.com/index.php?id=13&subid=179. [Accessed: May 18, 2013].

Max, B., Salgado, J.M., Rodríguez, N., Cortés, S., Converti, A. and Domínguez, J.M. (2010) Biotechnological production of citric acid. *Braz J Microbiol* **41**, 862–875.

Nagy, V., Seidl, V., Szakacs, G., Komoń-Zelazowska, M., Kubicek, C.P. and Druzhinina, I.S. (2007) Application of DNA bar codes for screening of industrially important fungi: the haplotype of *Trichoderma harzianum sensu stricto* indicates superior chitinase formation. *Appl Environ Microbiol* **73**, 7048–7058.

Noguchi, Y. and Johnson, M.J. (1961) Citric acid fermentation of sugars purified with chelating resin. *J Bacteriol* **82**, 538–541.

Peksel, A., Torres, N., Liu, J., Juneau, G. and Kubicek, C.P. (2002) 13C-NMR analysis of glucose metabolism during citric acid production by *Aspergillus niger*. *Appl Microbiol Biotechnol* **58**, 157–163.

R-Development (2008). *R: A Language and Environment for Statistical Computing*. [Open source] ISBN 3-900051-07-0. Vienna, Austria: R Foundation for Statistical Computing.

Sanchez-Marroquin, A., Carreno, R. and Ledezma, M. (1970) Effect of trace elements on citric acid fermentation by *Aspergillus niger*. *Appl Environ Microbiol* **20**, 888–892.

Shu, P. and Johnson, M.J. (1947) Effect of the composition of the sporulation medium on citric acid production by *Aspergillus niger* in submerged culture. *J Bacteriol* **54**, 161–167.

Shu, P. and Johnson, M.J. (1948) Citric acid. *Ind Eng Chem* **40**, 1202–1205.

Sohoni, S.V., Bapat, P.M., and Lantz, A.E. (2012) Robust, small-scale cultivation platform for *Streptomyces coelicolor*. *Microb Cell Fact* doi: 10.1186/1475-2859-11-9.

Tomlinson, N., Campbell, J.J.R. and Trussell, P.C. (1950) The influence of zinc, iron, copper, and manganese on the production of citric acid by *Aspergillus niger*. *J Bacteriol* **59**, 217.

Xu, D.B., Madrid, C.P., Röhr, M. and Kubicek, C.P. (1989) The influence of type and concentration of the carbon source on production of citric acid by *Aspergillus niger*. *Appl Microbiol Biotechnol* **30**, 553–558.

Yang, L. (2012) Metabolic Engineering of *Aspergillus carbonarius* for Organic Acids. A Thesis Submitted in partial fulfilment of the Requirements of Aalborg University for the Degree of Master of Science. Copenhagen, Denmark: Aalborg University Denmark.

Zhang, H., Lamping, S.R., Pickering, S.C., Lye, G.J. and Shamlou, P.A. (2008) Engineering characterisation of a single well from 24-well and 96-well microtitre plates. *Biochem Eng J* **40**, 138–149.

CHAPTER 7. DISCUSSION

7.1. THE MANUSCRIPTS

The overall objective of this study was to enhance the organic acid production from *A. carbonarius* using a metabolic engineering approach. Three different approaches were investigated; in manuscript I, a direct intuitive alteration of the cytosolic pathway from pyruvate to malate and fumarate was accomplished. The following two approaches were alteration of the genome in a more indirect manner that could alter the organic acid production via global regulation (manuscript II) and via transport mechanisms (manuscript III). All three approaches changed the organic acid production in the desired direction. Finally, in manuscript IV, an optimized method for fermentation screening was investigated and the method successfully minimized the variation between experimental samples.

The aim of the study in manuscript I was to enhance the production of malic and fumaric acid from *A. carbonarius* by introducing and enhancing the pathway through the cytosolic reductive TCA branch from pyruvate to malate and fumarate. Co-transformation of *pyc2*, *mdh3*, *mae1*, *fumR*, and *fum1* successfully created 11 different genotypes with up to three genes inserted per transformant. These 11 different types of transformants were investigated in fermentation experiments to analyze for altered phenotypic patterns in organic acid production. In contrast to an earlier study (de Jongh & Nielsen 2008) we did not see any increase in citric acid production from the transformants. However, we did see significant changes in malic acid production instead, which correlates well with other studies (Zelle et al. 2008; Knuf et al. 2014). The insertion of *pyc2* alone increased malic acid production with 28% compared to wild type, insertion of *pyc2* and *mdh3* increased malic acid production with 32% and the insertion of *pyc2*, *mdh3* and *fum1* increased the malic acid production with 57%. The highest malic acid production increase was found in a single transformant with *pyc2*, *mdh3* and *fum1* inserted that produced on average 161% more malic acid than the wild type. To our puzzlement, the insertion of the malic acid transporter gene *mae1* significantly decreased the production of malic acid. This was the opposite of what has been shown before in other studies (Brown et al. 2013; Knuf et al. 2014; Zelle et al. 2008; Mcfarland & Fischer 2013), and no obvious explanation for this result was found. The results from this manuscript indicate that the insertion of *pyc2*, *mdh3* and *fum1* introduce and enhance the cytosolic reductive TCA branch in *A. carbonarius* and thereby significantly increases the accumulation of malic acid.

In manuscript II the global regulator LaeA was the target of the investigation. It was hypothesized that LaeA acts as a regulator of citric acid production as was seen before in *A. niger* (Dai & Baker 2015). Separately knockout and overexpression of *laeA* in

A. carbonarius gave two phenotypes different from the wild type strain. Knockout of *laeA* significantly decreased the production of citric acid, the transformant strains only produced 4-26% citric acid compared to wild type. Only one overexpression transformant successfully showed a different phenotype than the wild type. This single transformant produced 213% citric acid compared to the wild type, which was a significant increase. In *T. reesei* it has been shown that LaeA both up- and down-regulates the production of lignocellulose degrading enzymes (Seiboth et al. 2012). We investigated our knockout and overexpression transformants for the same traits. Similar to what Seiboth et al. (2012) saw in *T. reesei*, the knockout of *laeA* in *A. carbonarius* significantly decreased the production of endoglucanase (51-78% reduction). However, we did not see any differences with the other lignocellulose degrading enzymes investigated, nor did we see any changes in phenotype in the overexpression transformants. The results from manuscript II shows that the global regulator LaeA have a regulatory effect on both citric acid production and endoglucanase production in *A. carbonarius*.

In manuscript III the citrate-malate mitochondrial antiporter was the target of the investigation. It is generally accepted that the transport of citrate across the mitochondrial membrane primarily comes from antiport exchange of citrate for malate or other cytosolic acids (Ruijter et al. 2002; Röhr & Kubicek 1981; de Jongh & Nielsen 2008). However, these antiporters have not been identified in filamentous fungi. The hypothesis of our study was that if the correct antiporter could be disrupted it would be possible to make a shift in organic acid production from citric acid to malic acid. Seven putative antiporters were successfully knocked out and fermentation experiments were conducted with the transformants to screen for altered organic acid production phenotypes. One of the transformant types (Anti3) showed an altered organic acid production profile. The citric acid production was decreased (30 %) compared to wild type, and the malic acid production was increased. However, the two results were not statistically significant so further investigations will have to be conducted before a clear conclusion can be made.

Concerning manuscript I, II and III Torres et al. (1996) conducted research of the metabolic pathways involved in the citric acid biosynthesis of *A. niger*, which verifies many of the features discovered in this study. It was a study based on a mathematical model of the carbohydrate metabolism during citric acid accumulation. The main conclusion from the study was that it was necessary to change the enzyme activity of at least seven out of the ten enzymes to achieve a change in the citric acid production that was significant (2.48-fold), if six enzyme activities were changed only a 1.22-fold increase was achieved. Four activities were in particular important to achieve the increase in citric acid production: hexokinase-substrate transport, pyruvate carboxylase, malate transport and ATPases (Torres et al. 1996). The study indicate two issues: 1) citric acid production by *A. niger* is already very close to be optimal so only small improvements can be achieved by molecular engineering; 2) changing of one or two enzyme activities in carbohydrate metabolism will most likely not have

an effect. The study was updated in 2000 where the model was expanded significantly. Now it was shown that 13 out of 22 enzyme activities would have to be changed for a significant increase in citric acid production (3.07-fold). The most important steps in the model were the transport steps, specifically the substrate uptake system, the mitochondrial carriers of pyruvate and citrate and the citrate excretion system (Alvarez-Vasquez et al. 2000). These two modelling studies support and explain many of the features discovered in manuscript I, II and III. The models find that pyruvate carboxylase and the mitochondrial citrate carrier are important steps in the system. These two steps are also the target of the metabolic engineering described in manuscript I and III respectively, and they both show a positive effect when altered. In manuscript I, there are no significant change on citric acid production from inserting the four genes *pyc2*, *mdh3*, *fum1* and *mae1*. This result is supported by the model, which states that many enzyme activities have to be changed before a significant effect can be observed. This is also seen in manuscript III where only one enzyme activity is changed namely the disruption of an antiporter and the effect is minor, however, important. The significant change in citric acid production from only changing one enzyme (LaeA) as described in manuscript II does not go against the two described models since this enzyme is not included in the models. LaeA most likely act as a regulator on several enzymes by regulating clusters of genes (Bok & Keller 2004; Keller et al. 2005; McDonagh et al. 2008), which in terms of the models could be translated in to changing the activity of many enzymes in one go, thereby achieving a significant change in citric acid production.

Screening for phenotypic changes in transformant and mutant strains of filamentous fungi is very often done in simple batch fermentation experiments with shaking (Büchs 2001). However, a common problem with this method in research as well as in industrial production is the unwanted variation between identical samples (Max et al. 2010; Büchs 2001). In manuscript IV we investigated an optimized method for fermentation screening in 24-well culture plates. *A. carbonarius* wild type was used in citric acid fermentation in 24-well plates and in Erlenmeyer flasks and the relative standard deviation (RSD) was used as comparison factor. The average RSD from the Erlenmeyer experiment was 29 % whereas the average RSD from the comparable 24-well experiment was 12 %, which is a 2.4 fold reduction in variance. The results from manuscript IV shows that 24-well plates can be used as an efficient screening platform for filamentous fungi, both because the variance is lower than with the classical Erlenmeyer setup, and because the experiments can be scaled down thus many more samples can be investigated at the same time.

7.2. PERSPECTIVE

As described in the introduction of the thesis, it is possible to partially substitute oil-derived products with biomass-derived alternatives. In this thesis, *A. carbonarius* was genetically altered with the aim of producing an industrial capable cell factory strain with enhanced organic acid productivity. The long-term aim of this approach is

indeed to contribute to minimizing the use of fossil fuel in the future. However, for the present research to have a direct impact, the productivity of either malic or fumaric acid have to reach both economically and technically efficiencies comparable to that of the citric acid production from *A. niger*. Unfortunately, sufficient efficiency was not accomplished. Citric acid production titers from *A. niger* can reach almost 200 g/l and the conversion efficiency can be as high as 77 % of the theoretical possible (van der Straat & de Graaff 2014; Verhoff 2005). If we imagine a perfect cell factory strain of *A. carbonarius* with the combined malic acid producing effect from all the alterations described in manuscript I, II and III, the transformant hypothetically could increase production of malic acid by 200-300 % than wild type. The wild type strain on average accumulated approximately 0.5g/l malic acid, so the hypothetical cell factory strain would be able to accumulate 1.5-2.0 g/l malic acid. However, this is far from the target of 200 g/l citric acid from *A. niger*. Unfortunately also less than achievements in other studies attempting to produce malic acid, e.g. in *S. cerevisiae* where final malic acid titers reached 59 g/l (Zelle et al. 2008), and in *A. oryzae* where final malic acid titers reached 66.3 g/l (Knuf et al. 2014). In this context, the malic acid production titers we achieved are not interesting; yet, the knowledge accumulated from the changes in phenotypes due to the genetic alterations could be interesting for further studies.

In manuscript I, we described a successful increase in malic acid production due to the introduction of a cytosolic reductive TCA branch from pyruvate to malate by inserting the three genes *pyc2*, *mdh3* and *fum1*. The study was comparable to other studies investigating the same genes, however, with a different combination of genes and a different microorganism (Zelle et al. 2008; de Jongh & Nielsen 2008; Knuf et al. 2014). Our findings support these studies and when looking forward, the combined knowledge from the studies would be beneficial for further metabolic engineering studies where the cytosolic rTCA branch is the target region and organic acid production is the main goal. In manuscript II, we describe a regulation of citric acid production and endoglucanase production carried out by the methyltransferase LaeA. The fact that one regulator gene acts on many different pathways (Jain & Keller 2013), and among these are both end-products like citric acid and cellulose degrading enzymes like endoglucanase, indicates that the enzyme would be a good target for further investigations aimed at producing strains for consolidated bioprocessing. One strain able to both saccharify complex lignocellulosic biomass and metabolize the resulting sugars into organic acids would have a significant economic impact on the cost in a biorefinery concept (Zoglowek et al. 2015). In manuscript III, putative mitochondrial citrate-malate antiporters were investigated for their role in organic acid production. One putative antiporter showed to have an impact on the ratio between citric and malic acid produced. If this putative antiporter is in fact a true citrate-malate antiporter there are several possibilities for further investigations and implementations. In manuscript III, the similarities and differences between the putative antiporters were analyzed and it was shown that many of the putative antiporters were very much alike. In further studies of these putative antiporters, it

would be beneficial to e.g. knock out groups of similar antiporter genes. This would decrease the possibility of the cell to bypass the missing antiporter by using other mitochondrial antiporters with similar affinity for citrate and malate. It would furthermore be an obvious idea to try to disrupt the citrate-malate antiporter in other strains, e.g. *A. niger* to see what effect on organic acid production that would accomplish. In general, artificial control of the mitochondrial membrane transporters would give the possibility of partly bypassing the flux to and from the TCA cycle, and instead increase the flux through other metabolic pathways situated in the cytosol, be that artificial or natural, like the one described in manuscript I.

When looking at the possibilities of implementing the alterations described in manuscript I, II and III in an industrial production strain there is a specific problem that needs to be solved. As described in the introduction *A. carbonarius* is notoriously known for its capability of producing ochratoxin a mycotoxin that are considered to be nephrotoxic, immunosuppressive, teratogenic and carcinogenic (Cabañes et al. 2002; Nielsen et al. 2009). This trait is not beneficial in industrial production strain; however, there are possibilities to circumvent this problem. A simple solution is to use *A. carbonarius* as it is and only use the products for non-foods and non-food-grade goods. With this approach it will not be necessary to stay below ochratoxin A threshold values set by authorities (Schuster et al. 2002). Another approach is to seek to eliminate the production of ochratoxin A from *A. carbonarius*, which can be achieved by inactivating a specific gene coding for a non-ribosomal peptide synthase (Gallo et al. 2012). With this alteration, *A. carbonarius* could be used in the production of food additives. A third option is to use the knowledge obtained in the manuscripts and implement the alterations in *A. niger* instead of *A. carbonarius*. *A. niger* has GRAS status and are so similar to *A. carbonarius* that it is valid to believe that the alterations will have the same effect in *A. niger* as they had in *A. carbonarius* thus making the same phenotype without the ochratoxin A production (Nielsen et al. 2009; Schuster et al. 2002).

7.3. SUPPLEMENT TO CHAPTER 6, MANUSCRIPT IV

The experiments leading to the definition of the problem investigated in manuscript IV were conducted over a 2-year period. These experiments were conducted as a part of a screening process for different transformed strains. In the beginning, biomass measurements were carried out following every experiment, but we soon came to realize that the production of biomass did not change significantly between samples in an experiment and the small differences did not explain the great variance in e.g. citric acid production that we were seeing. For that reason, biomass accumulation did not figure as a primary parameter in the manuscript and instead the emphasis of the screening work were on the production of citric acid, ethanol and glycerol and the consumption of sugar. Hence, the biomass production are merely mentioned in the manuscript with an average amount and an explanation that the difference between samples are negligible. A more thorough explanation for this approach is given in

chapter 3, manuscript I in this thesis, were it is shown that the biomass production is close to similar between strains where both the sucrose consumption and the metabolite production is very varied.

The many screening experiments that lay ground for the article were conducted in a comparable way each time. Sampling was generally only done one time at 96h instead of several times during the experiment. This is not ideal, however, when screening a large number of samples, sampling several times on each experiment would be very comprehensive and cumbersome. The 96h mark was chosen from a time-course experiment (see chapter 3, manuscript I in this thesis) were it was shown that at 96h, sufficient amount of sugar was consumed and sufficient amount of citric acid malic acid was produced. As experiments were discontinued after 96h the sugar was not completely consumed thus the experiments was not allowed to run to completion. It was chosen not to run experiments to completion, as each experiments would be very time consuming compared to the expected gain. Our goal with the fermentation experiments were in a screening setup to be able to compare transformant strains with wild type strains and to compare 24-well fermentation with Erlenmeyer flasks fermentation. For these two goals to be fulfilled, the fermentations did not need to run the full time nor needed many times of sampling during one experiment. If the goal had been to achieve a maximum amount of product or a specific production rate, the experiment setup would have had to be different.

The fungal morphology during the screening fermentations were very similar between the many experiments. It was always pellet morphology with white fungal mass. Pellets started out very small, forming around clumps of spores including both growing and none-growing spores. At the end of the fermentation, the pellets were approximately 1½-3mm in diameter (correction to the pellet size described in the manuscript). The morphology of the pellets in the flasks fermentation was slightly different from the well fermentation, but between flasks, and between wells, the morphology was similar. The morphology has a big impact on citric acid production in *A. niger* (Paul et al. 1999; Papagianni & Mattey 2006), and since *A. carbonarius* is very closely related to *A. niger* (Nielsen et al. 2009) it is also assumed that the morphology also has a big impact on citric acid production in *A. carbonarius*. We state in the manuscript that the morphology did not differ significantly and thus could not explain the differences in citric acid production. However, it is possible that the morphology could explain some of the differences that we see, but it is very unlikely that the very small differences in morphologies seen can explain the large differences seen in citric acid production. Even though the morphology could explain some of the differences seen in citric acid production it does not renter the conclusion of the manuscript invalid since it still support the hypothesis that the 24-well plates enables better homogeneity regarding aeration, water evaporation, temperature and surface structure in each well, than that obtained in Erlenmeyer flask. The outcome of this is a less varied production result that to some extent might come from a less varied

morphology, but the less varied morphology comes from the above-mentioned parameters.

7.4. REFERENCES

Alvarez-Vasquez, F., González-Alcón, C. & Torres, N. V, 2000. Metabolism of citric acid production by *Aspergillus niger*: model definition, steady-state analysis and constrained optimization of citric acid production rate. *Biotechnology and bioengineering*, 70(1), pp.82–108.

Bok, J.W. & Keller, N.P., 2004. LaeA, a Regulator of Secondary Metabolism in *Aspergillus spp*. *Eukaryotic Cell*, 3(2), pp.527–535.

Brown, S.H. et al., 2013. Metabolic engineering of *Aspergillus oryzae* NRRL 3488 for increased production of L-malic acid. *Applied microbiology and biotechnology*, 97(20), pp.8903–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23925533 [Accessed August 16, 2014].

Büchs, J., 2001. Introduction to advantages and problems of shaken cultures. *Biochemical Engineering Journal*, 7(2), pp.91–98.

Cabañes, F.J. et al., 2002. What is the source of ochratoxin A in wine? *International journal of food microbiology*, 79(3), pp.213–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12371656.

Dai, Z. & Baker, S.E., 2015. Enhanced citric acid production in *Aspergillus* with inactivated asparagine-linked glycosylation protein 3 (Alg3), and/or increased LaeA expression. , p.US9023637 B2.

Gallo, A. et al., 2012. New insight into the ochratoxin a biosynthetic pathway through deletion of a nonribosomal peptide synthetase gene in *Aspergillus carbonarius*. *Applied and Environmental Microbiology*, 78(23), pp.8208–8218.

Jain, S. & Keller, N., 2013. Insights to fungal biology through LaeA sleuthing. *Fungal Biology Reviews*, 27(2), pp.51–59. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1749461313000316 [Accessed November 28, 2014].

de Jongh, W.A. & Nielsen, J., 2008. Enhanced citrate production through gene insertion in *Aspergillus niger*. *Metabolic engineering*, 10(2), pp.87–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18162426 [Accessed March 21, 2012].

Keller, N.P., Turner, G. & Bennett, J.W., 2005. Fungal secondary metabolism - from biochemistry to genomics. *Nature reviews. Microbiology*, 3(12), pp.937–947.

Knuf, C. et al., 2014. Physiological characterization of the high malic acid-producing *Aspergillus oryzae* strain 2103a-68. *Applied Microbiology and Biotechnology*, 98, pp.3517–3527.

Max, B. et al., 2010. Biotechnological production of citric acid. *Brazilian journal of microbiology*, 41(4), pp.862–75. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3769771&tool=pmcentrez&rendertype=abstract.

McDonagh, A. et al., 2008. Sub-telomere directed gene expression during initiation of invasive aspergillosis. *PLoS Pathogens*, 4(9), p.e1000154.

Mcfarland, S. & Fischer, A., 2013. C4 dicarboxylic acid production in filamentous fungi. United States Patent No. 20,130,089,902.

Nielsen, K.F. et al., 2009. Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. *Analytical and Bioanalytical Chemistry*, 395(5), pp.1225–1242.

Papagianni, M. & Mattey, M., 2006. Morphological development of *Aspergillus niger* in submerged citric acid fermentation as a function of the spore inoculum level. Application of neural network and cluster analysis for characterization of mycelial morphology. *Microbial cell factories*, 5, p.3. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1386700&tool=pmcentrez&rendertype=abstract [Accessed March 16, 2012].

Paul, G.., Priede, M.. & Thomas, C.., 1999. Relationship between morphology and citric acid production in submerged *Aspergillus niger* fermentations. *Biochemical Engineering Journal*, 3(2), pp.121–129. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1369703X99000121.

Ruijter, G., Kubicek, C. & Visser, J., 2002. Production of organic acids by fungi. In H. Osiewacz, ed. *The Mycota X, Industrial Applications*. Berlin, Heidelberg: Springer, pp. 213–230.

Röhr, G. & Kubicek, C.P., 1981. Regulatory aspects of citric acid fermentation by *Aspergillus niger*. *Process Biochemistry*, 16(34).

Schuster, E. et al., 2002. On the safety of *Aspergillus niger* - a review. *Applied Microbiology and Biotechnology*, 59(4-5), pp.426–435. Available at: http://link.springer.com/10.1007/s00253-002-1032-6.

Seiboth, B. et al., 2012. The putative protein methyltransferase LAE1 controls cellulase gene expression in *Trichoderma reesei*. *Molecular Microbiology*, 84(6), pp.1150–1164.

van der Straat, L. & de Graaff, L., 2014. Pathway transfer in fungi: Transporters are the key to success. *Bioengineered*, 5, pp.335–339. Available at: http://dx.doi.org/10.4161/bioe.29936.

Torres, N. V, Voit, E.O. & González-Alcón, C., 1996. Optimization of nonlinear biotechnological processes with linear programming: Application to citric acid production by *Aspergillus niger*. *Biotechnology and bioengineering*, 49, pp.247–258.

Verhoff, F.H., 2005. Citric Acid. In *Ullmann's Encyclopedia of Industrial Chemistry*. Weinheim: Wiley-VCH Verlag GmbH & CO, pp. 1–6.

Zelle, R.M. et al., 2008. Malic acid production by *Saccharomyces cerevisiae*: engineering of pyruvate carboxylation, oxaloacetate reduction, and malate export. *Applied and environmental microbiology*, 74(9), pp.2766–77. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2394876&tool=pmcentrez&rendertype=abstract [Accessed March 6, 2012].

Zoglowek, M. et al., 2015. Fungal consortia for conversion of lignocellulose into bioproducts. In *Mycology: Current and Future Developments, Vol. 1,(R.D. Silva, ed)*. pp. 329–365.

CHAPTER 8. CONCLUSION

Three different metabolic engineering approaches were carried out with the aim of enhancing the organic acid production by *A. carbonarius*. Furthermore, an optimization of the fermentation screening platform used in the thesis was successfully conducted.

Five genes encoding for enzymes responsible for introducing a cytosolic rTCA branch were successfully inserted in to *A. carbonarius* genome, *pyc2*, *mdh3*, *fum1*, *fumR* and *mae1*. Three of the genes, *pyc2*, *mdh3* and *fum1* introduced the cytosolic rTCA branch from pyruvate to malate, which increased the malic acid production from the transformants significantly. *pyc2* alone increased the malic acid production with 28 % compared to wild type, *pyc2* and *mdh3* increased the malic acid production with 32 %, and *pyc2*, *mdh3* and *fum1* increased the malic acid production with 57 %.

The global regulator LaeA was successfully disrupted as well as overexpressed in *A. carbonarius* with the aim of investigating the regulators role on citric acid production and cellulose degrading enzymes. In the transformants with the disrupted LaeA the citric acid production was decreased with 74-96 % compared to wild type and the production of endoglucanase was decreased with 51-78%. In the transformant with the overexpressed LaeA the citric acid production was increased with 113% compared to wild type, however, the endoglucanase production was unchanged.

Seven putative citrate-malate antiporters were singularly knocked out of *A. carbonarius* with the aim of redirecting the natural citric acid production to malic acid. One of the antiporters showed an effect on citric and malic acid production. The citric acid production was decreased and the malic acid production was increased, however, the results were not statistically significant and further investigations will have to be conducted before a conclusion can be made.

A 24-well plate fermentation screening method was investigated with the aim of reducing variation between identical samples in fermentation experiments. The baseline experiment was conducted with the conventional Erlenmeyer flask system and gave a relative standard deviation (RSD) of 29 %. The comparable 24-well plate system gave a RSD of 12 %, which was a 2.4-fold reduction.

APPENDICES

Appendix A. Variation in fermentation due to water source and light/darkness

Variation between identical samples in fermentation batch experiments are well known, however, not that many studies address the problem (Max et al. 2010; Büchs 2001; Linde et al. 2014). There are many factors that can influence the fermentation performance; however, much of the problem is understood to be because of aeration (Max et al. 2010; Büchs 2001). Other seemingly negligible factors like amount and type of lighting do actually also have an impact on fermentation performance (Tisch & Schmoll 2010).

In this small study, two seemingly insignificant factors were investigated for their impact on fermentation performance. Firstly, fermentation with wild type *Aspergillus carbonarius* in light was compared to fermentation in darkness, secondly two different pure water sources were compared in fermentation. These experiments were initiated as a search for a cause for problems with high variations in fermentation results between identical samples in the laboratory.

Materials and methods

A. carbonarius ITEM 5010 was kindly supplied from Prof. Jens Frisvad, Danish Technical University, Lyngby (Department of Systems Biology, Center for Microbial Biotechnology). It was originally isolated from grapes in Italy (www.ispa.cnr.it/collection).

Fermentation setup in 24-well plates were made following the principles of Linde et al. (2014) and citric acid production media from (Shu & Johnson 1948) was used (140g/l sucrose, 2.5g/l KH_2PO_4, 0.25g/l $MgSO_4 \cdot 7xH_2O$, 2.5g/l NH_4NO_3, pH to 3.8, Cu^{2+} 0.06mg/l, Zn^{2+} 0.25mg/l, Fe^{2+} 1.3mg/l). The fermentation experiments were made with six biological replicates. Fresh spore-suspension was added to 20 ml fermentation media to a final concentration of $2*10^5$ spores/ml. The fermentation media was distributed with 3ml each in to six wells in the 24-well plate (UNIPLATE, 24 wells, 10ml, Whatman). The plates were covered with silicone lids (BugStopper Venting Capmat, Whatman) and incubated for 5 days at 25°C and 420rpm in a Glas-Col model 099A DPMINC24 (Glas-Col, Terre Haute, IN). The incubator was kept at a constant high humidity to avoid excess evaporation by having open boxes with distilled H_2O mounted inside the incubator. Samples were taken at day 5, filtered through 0.45-µm HPLC-grade regenerated cellulose membrane filters and analyzed on HPLC (Dionex Ultimate 3000-LC system,Dionex Corporation, Sunnyvale, CA) with an Aminex HPX-87H column coupled to an RI- detector. The eluent was 4 mmol/l H2SO4 with a flow rate of 0.6ml/min at 60°C. All chromatograms were integrated using the Chromeleon software (Dionex Corporation).

Light and dark samples were run at the same time in the same incubator in a dark room. The light source was red, blue and white light which simulated day-time light by being turned on from 7 to 20. The dark samples were completely covered in no see through tape that only had small holes for the aeration.

The two water sources that were compared were milliQ-water from Aalborg University Copenhagen (AAU-water), and milliQ-water from Copenhagen University (KU-water). The citric acid media was prepared exactly similar except that the two different water sources were used.

Statistical comparison of transformant results against wild type samples were done by independent (unpaired) two-sample *t*-test with a significant level of $p<0.05$. Letters on the figures represent statistically significant different groups. Error bars on figures are standard deviation based on a sample.

Results

Comparison of fermentation performance with light/darkness as the changing parameter showed differences in glucose consumption, citric acid production and variation between replicas; however, the variation between replicas was so high that the applied statistical method (T-test) was unable to show a significant difference between any of the samples. The light samples consumed on average 23 % more glucose than the dark samples and produced on average 100 % more citric acid; the variation between replicas were higher in the light samples than the dark (Figure A.1).

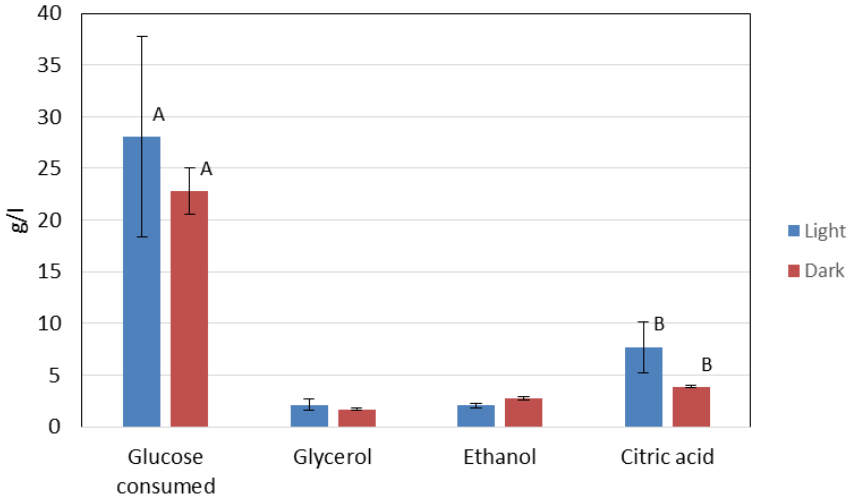

Figure A.1 Comparison of fermentation performance with light/darkness as the only altered parameter

Comparison of fermentation performance with water source as the changing parameter showed statistical significant differences in glucose consumption and citric acid production. The KU-water samples consumed on average 44 % more glucose than the AAU-water samples and produced on average 83 % more citric acid (Figure A.2).

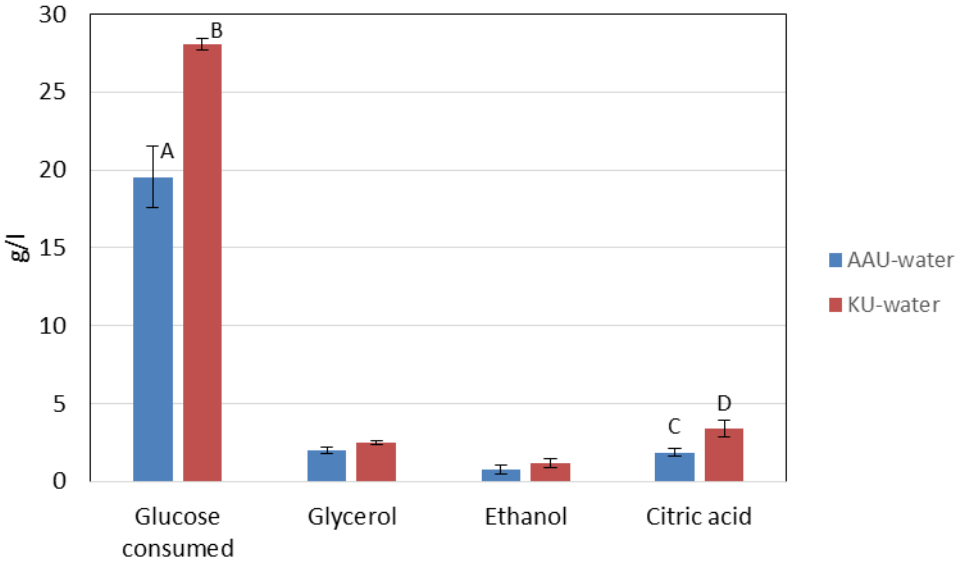

Figure A.2 Comparison of fermentation performance with water source as the only altered parameter

Discussion

The results from the two experiments showed that both light/darkness and water source in the media had an impact on citric acid fermentation performance with *A. carbonarius*. The impact was rather high on citric acid production with 100 and 83 % differences from light/darkness and water-source respectively, and the water-source experiment even proved to have statistically significant different results. This small study shows that even factors that by some scientist are considered unimportant can have an impact on fermentation performance.

References

Büchs, J., 2001. Introduction to advantages and problems of shaken cultures. *Biochemical Engineering Journal*, 7(2), pp.91–98.

Linde, T. et al., 2014. Fermentation in 24-well plates is an efficient screening

platform for filamentous fungi. *Letters in applied microbiology*, 59(2), pp.224–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24725208 [Accessed September 5, 2014].

Max, B. et al., 2010. Biotechnological production of citric acid. *Brazilian journal of microbiology*, 41(4), pp.862–75. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3769771&tool=pmcentrez&rendertype=abstract.

Shu, P. & Johnson, M.J., 1948. Production by Submerged Fermentation with *Aspergillus niger*. *Industrial & Engineering Chemistry*, 40(7), pp.1202–1205.

Tisch, D. & Schmoll, M., 2010. Light regulation of metabolic pathways in fungi. *Applied microbiology and biotechnology*, 85(5), pp.1259–77. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2807966&tool=pmcentrez&rendertype=abstract [Accessed August 14, 2014].

Appendix B. DNA and protein sequences

Sequences used in Manuscript I

>pyc2

ATGGCTGCCCCCCCGTCAGCCCGAGGAGGCGGTCGATGACGTCGAGTTCA
TCGATGACCACCATGACCACCACCGGGACTCCGTCCACACCCGTCTGCG
TGCCAACTCGGCCATCATGAAATTCCAGAAGATCCTCGTCGCCAACCGT
GGTGAAATCCCCATTCGTATTTTCCGGACGGCCCACGAGCTGTCCCTGC
AGACCGTTGCCGTCTACTCGCATGAGGACCGTCTCTCCATGCACCGTCA
GAAGGCCGATGAGGCCTACATGATTGGCCACCGTGGCCAATATACCCCG
GTCGGAGCCTACCTGGCCAGTGATGAGATCGTCAAGATTGCTCTCGAGC
ATGGCGTGCACCTGATCCACCCCGGTTATGGTTTCCTGTCCGAGAACGC
CGACTTCGCCCGCAAGGTCGAGGCGGCCGGCATGGTCTTCGTCGGCCCC
ACCCCCCACACTATCGATAGCCTCGGTGACAAGGTCTCCGCCCGTCAGC
TGGCCATGCGCTGCGACGTGCCCGTCGTGCCCGGTACCCCCGGCCCCGT
CGAGCGTTTTGAGGAGGTCAAGGCCTTCACCGACACCTACGGCTTCCCC
ATCATTATCAAGGCCGCCTTTGGAGGTGGTGGCCGTGGTATGCGGGTCG
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GGCCCGCTCTGCCTTTGGCAACGGCACCGTCTTCGTCGAGCGGTTCCTCG
ACCGCCCCAAGCACATTGAAGTCCAGCTCCTGGGTGACAACCACGGCAA
CGTCGTGCACCTGTTCGAGCGTGACTGCTCCGTTCAGCGTCGCCACCAG
AAGGTCGTTGAGATTGCGCCGGCCAAGGACCTGCCTACTGATGTCCGTG
ACAGCATCCTGGCTGATGCCGTCAAGCTGGCCAAGTCCGTCAACTACCG
CAACGCCGGTACCGCAGAGTTCCTGGTGGACCAGCAGAACCGCTACTAC
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>*mdh3*

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>*fum1*

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>*mae1*

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Sequences used in Manuscript II

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>laeA2

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Sequences used in Manuscript III

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>CTP *Saccharomyces cerevisiae*
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>malic acid transport protein *Schizosaccharomyces pombe*
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>Dic1 Saccharomyces cerevisiae
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Appendix C. Transmembrane prediction from Manuscript III

Transmembrane prediction was carried out with the online prediction service from UCL Bioinformatics group (http://bioinf.cs.ucl.ac.uk/web_servers/) using MEMSAT and MEMSAT-SVM (Nugent & Jones 2012). First line is the prediction using MEMSAT-SVM, the second line is the prediction using MEMSAT. Both prediction types were used to make the comparison tree of transmembrane structures between the sequences.

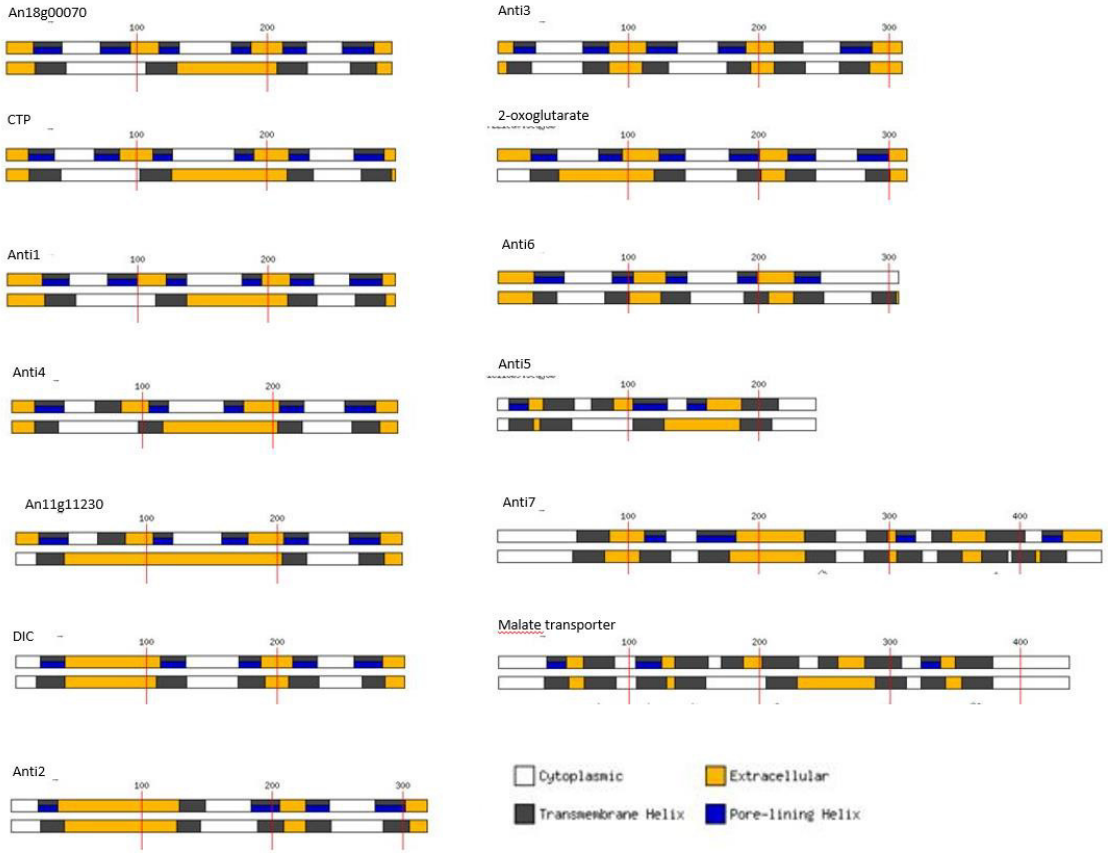

Figure C.1 Transmembrane prediction using MEMSAT-SVM and MEMSAT

Appendix D. Manuscript II – Supplementary experiment

As described briefly in the thesis a second putative *laeA* gene was identified in the *Aspergillus carbonarius* genome (*laeA2*). This gene was investigated in a similar way as *laeA1* (chapter 4), however, the results were inconclusive and was therefore excluded from the thesis. Here is a brief description of the experiments and the results.

Methods and materials

Strains and growth conditions
See chapter 4

Gene analysis
See chapter 4. *laeA2* seemed to be missing the first 20% of the gene compared to *laeA1*. No knowledge where obtainable whether the missing part of the gene was because of an error in the genome sequence in JGI or if it was a true disrupted gene in the genome, thus work was continued with this gene as well. LaeA2 was only used in knockout attempts since overexpression of a partial gene in this instance would make no sense.

Genomic DNA extraction
See chapter 4

Plasmid construction
pSBKoLaeA2 was made with *laeA2* as target gene, in the same way as pSBKoLaeA was made with *laeA1* as target (described in chapter 4).

Protoplast transformation
See chapter 4

Fermentation setup and conditions
See chapter 4

Table D.1 Primers used in the study

Name	No.	Sequence (5' → 3')	Annotation
LaeA2 up-fw-U	1	GGGTTTAAUAGCTCTGCCGTCTTGGTAAA	Amplifies upstream region of LaeA2, contains uracil tail
LaeA2 up-rv-U	2	GGACTTAAUAAGGCGAAATGATCACAAGG	Amplifies upstream region of LaeA2, contains uracil tail
LaeA2 do-fw-U	3	GGCATTAAUGGCGGGAGAAAGGGTATTTA	Amplifies downstream region of LaeA2, contains uracil tail
LaeA2 do-rv-U	4	GGTCTTAAUCCTAGGCTTATGGCCAGCTT	Amplifies downstream region of LaeA2, contains uracil tail
LaeA2 ko-ch-fw	5	GTGCGTTCGCGACTGTAATA	Check for positive knockout of LaeA2
LaeA ko-ch-rv	6	TATTGGAGAGCAAGGGATGG	Check for positive knockout of LaeA

Results and discussion

Knockout of *laeA2* gave 31 transformants. Of these 31, only two were confirmed by PCR to have a truly disrupted *laeA2* gene. It was already established early on that the *laeA2* gene seemed to be missing the first 20% to be a functional gene, and due to the very low success rate of *laeA2* knockouts, question arises whether JGI's genome assembly was completely correct regarding *laeA2*. If it is a true partial gene in the genome it should still be possible to knockout it out with a normal success rate, it seemed not to be the case with *laeA2*. The two positive *laeA2* knockouts did not show any difference from the wildtype in fermentation experiments (results not shown). Most probably, the *laeA2* gene identified in the database is an artefact resulting from the nature of genome-assembly algorithms. Another reason could be that a partial non-functioning gene in the genome is present. In both cases knockout of this gene has no effect for the organism.

Appendix E. Manuscript III - Supplementary experiment

As briefly described in the thesis, the antiporter study initially included overexpression of two of the seven putative antiporters. Due to lack of time to fully finalize the experimental procedures and to inconclusive results, this part of the experiment was excluded from the thesis. Here is a brief description of the experimental setup and the results.

Materials and methods

Strains and growth conditions
See chapter 5

Gene analysis of the putative antiporter genes
anti1 and *anti4* were selected for overexpression analysis. See chapter 5 for further information.

Genomic DNA extraction
See chapter 5

Plasmid construction
The high copy number plasmid pJET2.1 (ThermoFisher Scientific) was used as backbone. The overexpression plasmid backbone pSBe1 (Figure E.1A) was constructed prior as described by Hansen, Lübeck, & Lübeck, 2014. pSBe1 contains the following genetic elements: *RP27-hph-βT* for hygromycin resistance, origin of replication and ampicillin resistance for growth in *E.coli*, a specific cassette facilitating simpleUSER cloning, a *gpdA* promoter and a *trpC* terminator for gene expression. Two overexpression plasmids (pSBeAntiporter Figure E.1B) were constructed with *anti1* and *anti4* respectively. Primers were designed with uracil tails; forward primer was designed to start at the start codon of the gene; reverse primer was designed to start at the stop codon of the gene (primer 29, 30, 31 and 32). PCR setup for the amplification of *Anti1* and *Anti4* was done in the same way as described for the knockout plasmids (see chapter 5). The simpleUSER cassette in pSBe1 was activated with the restriction enzyme *AsiSI* and the nicking enzyme *Nb.BtsI*. Cloning, transformation and propagation was done as described in chapter 5. Plasmid extraction was done using the GeneJET plasmid miniprep kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. All plasmids were verified by Sanger sequencing using the sequencing service from StarSEQ (Mainz, Germany).

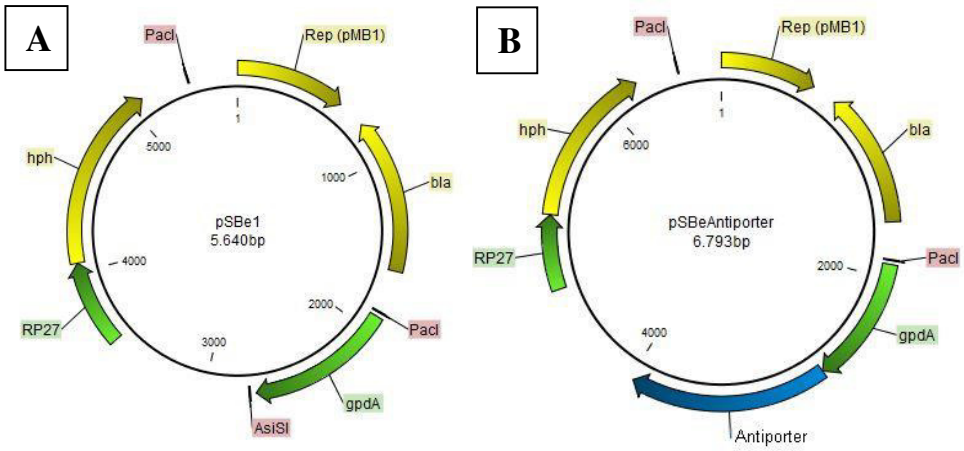

Figure E.1 The two plasmids used in the experiments. A) Backbone plasmid PsBe1 used as precursor for B) overexpression plasmid pSBeAntiporter.

Protoplast transformation

See chapter 5. Validation of positive knockouts and overexpression transformants were done using PCR with transformant DNA as template. Primers binding on the plasmid backbone right before (primer 41) and right after (primer 42) the antiporter gene in the plasmid was used which should give a PCR product of 1.2kb for *anti1* and 1.5kb for a*nti4*. This should ensure that only a reading from the inserted gene would be seen in cells transformed with the plasmid, thus being a positive transformant as it can be seen in figure E.2.

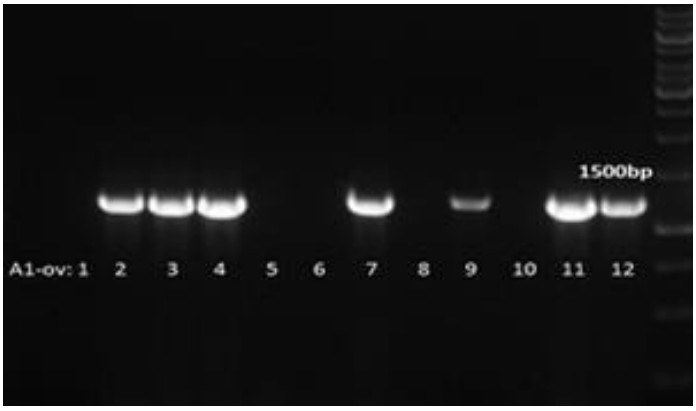

Figure E.2 Verification of the *Anti1* overexpression transformants. Overexpression transformants were identified from PCR products at 1200bp.The figure shows the first seven positive overexpression transformants.

Fermentation setup and conditions

See chapter 5

Table E.1 Primers used in the study

Name	No.	Sequence (5' → 3')	Annotation
***Anti1* fw-U**	29	AGAGCGAUATGGCCACA AAGCAACTCG	Amplifies *Anti1*, contains uracil tail
***Anti1* rv-U**	30	TCTGCGAUCTACATCGC ACTGGACAGGA	Amplifies *Anti1*, contains uracil tail
***Anti4* fw-U**	31	AGAGCGAUGTCCGCAGA AACCACTACATTA	Amplifies *Anti4*, contains uracil tail
***Anti4* rv-U**	32	TCTGCGAUACCGTTTGCA ACCATCAGAT	Amplifies *Anti4*, contains uracil tail
***Anti1*+4 ex-ch-fw**	41	CTCTTTCTTTTCCCATCTT CAG	Check for positive overexpressing of *Anti1* and *Anti4*
***Anti1*+4 ex-ch-rv**	42	CACCAACGATCTTATATC CAGA	Check for positive overexpressing of *Anti1* and *Anti4*

Results

anti1 and a*nti4* was overexpressed in *A. carbonarius* wildtype creating two different types of overexpression transformants. Positive transformants of the transformant types were verified by PCR. All positive transformants were subsequently tested in citric acid fermentation in the 24-well screening system. The selection of the two candidates were done prior to any knockout results were seen (see chapter 5) therefore these were not taken into consideration. The two putative antiporters selected were *Anti1* and *Anti4*. 34 transformants were produced in total, 17 of these were verified by PCR to be positive for the target gene giving a total transformation efficiency of 50% (Table E.2). The normal transformation efficiency with the applied method in our lab is approximately 50%.

Table E.2 Total overexpression transformants and transformation efficiency

Putative Antiporter	Total transformants	Positive transformants	Transformation efficiency %
anti1	22	14	64
anti4	12	3	25

The 17 positive transformants were tested in one round of citric acid fermentation; the results can be seen in figure E.3. The results are relative to wildtype. Unfortunately, the production of citric acid was very low in this experiment and at the same time, the variance between triplicates of the samples including the wildtype was very high, making it impossible to make a conclusion from these results.

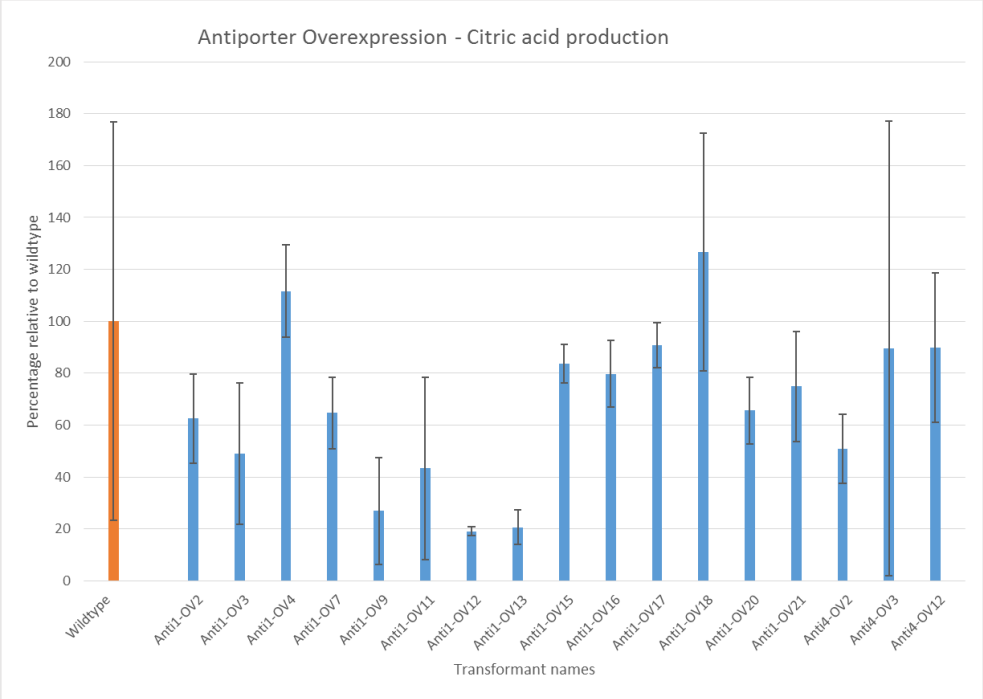

Figure E.3 Citric acid production relative to wildtype from antiporter overexpression transformants. Error bars show standard deviation of triplicate samples.

Discussion

The overexpression study with the two selected antiporters unfortunately came to a stop before it was possible to draw any conclusions from the results. If this part of the study should be continued, it would be obvious to repeat both the citric acid and malic acid fermentations with *anti1* and *anti4* to get enough results to make a conclusion. Furthermore, the knockout of a*nti3* showed some promising results; therefore it would make good sense to try to overexpress the gene as well to see what effect that would have on the acid production. A final approach in the overexpression scheme would be to overexpress more than one putative antiporter gene simultaneously in a transformant to see the effect on acid production. One problem with comparing overexpression transformants with each other and wildtype is that an inserted gene can be expressed differentially depending on its inserted location, and furthermore the number of copies of the gene introduced into the genome does not always correlate well with the expression level (Lubertozzi & Keasling 2009). To ensure that the overexpression in different transformants can be compared, an approach similar to what have been developed for *S. cerevisiae* (Jensen et al. 2014) could be used.

References

Hansen, N.B., Lübeck, M. & Lübeck, P.S., 2014. Advancing USER cloning into simpleUSER and nicking cloning. *Journal of Microbiological methods*, 96, pp.42–49.

Jensen, N.B. et al., 2014. EasyClone: Method for iterative chromosomal integration of multiple genes in *Saccharomyces cerevisiae*. *FEMS Yeast Research*, 14(2), pp.238–248.

Lubertozzi, D. & Keasling, J.D., 2009. Developing *Aspergillus* as a host for heterologous expression. *Biotechnology Advances*, 27(1), pp.53–75. Available at: http://dx.doi.org/10.1016/j.biotechadv.2008.09.001.

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