Development of new Aspergillus fungal cell factories for organic acid production

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BY
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DENMARK

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ENGLISH SUMMARY

Organic acid production has an important status in industrial production of commodity chemicals. Due to the increasing demand of organic acids in the global market, integration of biorefinery concepts with current manufacturing processes becomes inevitable trends to strengthen the sustainability of future organic acid production using renewable biomass as feedstocks.

In this PhD project, two *Aspergillus* strains, *Aspergillus carbonarius* ITEM5010 and *Aspergillus saccharolyticus* IBT28231, were selected for their great potential as cell factories for industrial production of succinic acid. *A. carbonarius* produced high amounts of citric acid and gluconic acid in different pH conditions but no succinic acid was detected. In contrast, *A. saccharolyticus* produced high amounts of malic acid and succinic acid in pH buffered conditions. Based on their organic acid profiles, different strain improvement strategies using metabolic engineering were made individually to *A. carbonarius* and *A. saccharolyticus* for enhanced succinic acid production.

For *A. carbonarius*, strain improvement was carried out to reduce the formation of by-products (gluconic acid) and to increase the carbon flux towards the reductive tricarboxylic acid branch. Elimination of gluconic acid production was achieved by identifying and deleting the *gox* gene encoding glucose oxidase that was responsible for conversion of glucose to gluconic acid in *A. carbonarius*. This genetic modification also increased production of oxalic acid, citric acid and malic acid. Another strategy was to insert a cytosolic bypass in *A. carbonarius* by expressing two heterologous genes, *pepck* and *ppc*, which encode phosphoenolpyruvate carboxykinase in *Actinobacillus succinogenes* and phosphoenolpyruvate carboxylase in *Escherichia coli*, individually and in combination in *A. carbonarius*. Insertion of the cytosolic bypass increased citric acid production by *A. carbonarius* in both glucose and xylose based media but this impact was dependent on ambient pH.

For *A. saccharolyticus*, the *frd* gene encoding a non-membrane bound NADH dependent fumarate reductase from *Trypanosoma brucei* was cloned and successfully expressed in *A. saccharolyticus*. The impacts of expression of *frd* gene on succinic acid production were examined and the results showed that succinic acid production increased significantly in the *frd* transformants compared with the wild type strain. In addition, the impacts on the general organic acid production were also elucidated in the comparative study of the transformants in pH buffered and pH non-buffered conditions.

In addition, *A. carbonarius* was cultivated in a wheat straw hydrolysate to demonstrate the feasibility of using lignocellulosic biomass as substrate for biotechnological production of organic acid. Several advantages of *A. carbonarius*, including an efficient co-utilization of glucose and xylose, a promising tolerance to inhibitors and the varieties of organic acid products, were verified for its future use as cell factories for organic acid production from lignocellulosic biomass. Furthermore, the Δ*gox* mutant was also cultivated in the wheat straw hydrolysate and the results showed a different impact of the same genetic modification on organic acid production in the defined media and in the wheat straw hydrolysate.

DANSK RESUME

Organiske syrer har en vigtig status indenfor industriel produktion af råvare kemikalier. På grund af stigende global efterspørgsel af organiske syrer vil en integration af bioraffinaderi koncepter med nye fremstillingsprocesser være en vigtig bidrag med henblik på at styrke bæredygtigheden af fremtidig produktion af organisk syrer ud fra vedvarende biomasser som råmateriale.

I nærværende ph.d.-projekt blev to *Aspergillus* stammer, *Aspergillus carbonarius* ITEM 5010 og *Aspergillus saccharolyticus* IBT 28231, udvalgt på grund af deres store potentiale som cellefabrikker til industriel produktion af ravsyre. *A. carbonarius* producerer store mængder af citronsyre og gluconsyre under forskellige pH-betingelser, men uden detekterbar produktion af ravsyre. I modsætning hertil producerede *A. saccharolyticus* store mængder af æblesyre og ravsyre under pH kontrollerede betingelser. Baseret på deres profiler af organiske syrer blev der udviklet forskellige stammeforbedringsstrategier til henholdsvis *A. carbonarius* og *A. saccharolyticus* for at øge deres ravsyre produktion.

Stammeforbedringen for *A. carbonarius* blev udført med henblik på at reducere dannelsen af biprodukter (gluconsyre) og øge kulfstofstrømmen til den reduktive tricarboxylsyre syntesevej. Eliminering af gluconsyre produktion blev opnået ved at identificere og deletere glucose oxidase genet (*gox*), der omdanner glucose til gluconsyre i *A. carbonarius*. Denne genetiske modifikation medvirkede også til øget produktion af oxalsyre, citronsyre og æblesyre. En anden strategi var at indsætte en cytosolisk genvej i *A. carbonarius* ved at udtrykke to heterologe gener, PEPCK og PPC, som koder for henholdsvis phosphoenolpyruvatcarboxykinase i *Actinobacillus succinogenes* og phosphoenolpyruvatcarboxylase i *Escherichia coli*, enkeltvis og i kombination i *A. carbonarius*. Indsættelse af den cytosoliske genvej resulterede i øget citronsyreproduktion i både glucose og xylose baserede medier, men denne påvirkning var afhængig af den omgivende pH i mediet.

Med hensyn til *A. saccharolyticus* blev frd-genet, der koder for en ikke-membran bundet NADH afhængig fumarat reduktase fra *Trypanosoma brucei*, klonet og succesfuldt udtrykt. Virkningerne af ekspressionen af FRD til ravsyre produktion blev undersøgt, og resultaterne viste, at ravsyre produktionen steg betydeligt i FRD transformanterne sammenlignet med vildtype stamme. Desuden blev virkningerne på den generelle produktion af organiske syrer også belyst i den sammenlignende undersøgelse af transformanterne i både pH kontrollerede og pH ikke-kontrollerede betingelser.

Endeligt blev *A. carbonarius* dyrket i et hydrolysat fra hvedehalm med henblik på at undersøge muligheden for at bruge lignocellulose-holdigt biomasse som substrat

til bioteknologisk produktion af organisk syre. Adskillige fordele ved *A. carbonarius* blev verificeret for den fremtidige anvendelse som cellefabrikker til produktion af organiske syrer fra lignocellulose-holdigt biomasse, herunder en effektiv samtidig anvendelse af glucose og xylose, en lovende tolerance over for inhibitorer og produktion af forskellige typer af organiske syrer. Endvidere blev GOX mutanten også dyrket i hvedehalm hydrolysatet, og resultaterne viste en forskellig virkning af den samme genetiske modifikation på organisk syre produktion i henholdsvis det definerede medium og i hvedehalm hydrolysatet.

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This PhD project has been a wonderful learning experience in my life and a strong impetus for my future career. As I am finishing my PhD thesis at this moment, I am also approaching to the end of this fantastic journey as a PhD student. I would like to use this opportunity to express my gratitude to the people who have supported me for the past few years.

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INTRODUCTION

Biorefineries and MycofuelChem project

Biorefineries have received extensive research interest for the past decades due to rapid depletion of crude oil and environmental problems caused by the petroleum industry. The current mode of economic growth, which is excessively dependent on use of nonrenewable crude oil, has shown its disadvantage in supporting the development of human society in a sustainable way. Biorefining of renewable biomass is considered as a feasible substitution for oil refinery to fulfill the increasing demands of liquid fuels and various commodity chemicals for the manufacturing industries. With those considerations, the MycoFuelChem project was created and designed to develop bio-based processes for varieties of value added chemicals by integrating and inventing the most advanced technologies for fungal transformation, biomass degradation, enzyme screening and strain development. The ultimate goal of the MycoFuelChem project was to develop consolidated bioprocesses by integrating the hydrolysis and fermentation steps in the fungal biocatalysts into a single process, thereby significantly reducing the amount of steps in the biorefinery.

Organic acids, especially low-molecular weight carboxylic acids, have a wide range of applications in food, pharmaceuticals, and detergents etc. Certain groups of organic acids, such as C4-dicarboxylic acids (malic acid, fumaric acid and succinic acid), have great potential as building blocks for the chemical industry and can be converted into varieties of chemicals [1]. Production of these organic acids is mainly via chemical processes using feedstocks obtained from crude oil. However, organic acids currently produced via biotechnological processes, such as citric acid, using sugar based substrates are seeking new resources to fulfill the increasing demand of cheap feedstocks in manufacturing processes [2]. Those facts are driving organic acid production to be integrated with Biorefineries, from which the carbohydrates existing in the renewable biomass can be used as alternative resources to support production of organic acids in a more sustainable way.

Aim of the PhD project

As part of the MycoFuelChem project, this PhD project aims to develop fungal cell factories for production of organic acids from lignocellulosic biomass. Two proposed production strains were obtained from screening tests carried out in the early phase of MycoFuelChem project. Continued from the previous work, the present PhD project made a number of attempts to increase the carbon flux towards the organic acids of interests in the selected strains via genetic engineering and to demonstrate the feasibility of using lignocellulosic biomass for fungal organic acid production.

Strain development via genetic engineering

As the core technology of biotechnological processes, production strains are of great importance for consolidated bioprocessing of renewable biomass into desired organic acids. Aspergilli have a long history of industrial application of production of organic acids and are well-known for their capabilities of secreting high amounts of organic acids and utilizing a wide range of carbon sources. In this project, *Aspergillus carbonarius* and *Aspergillus saccharolyticus* were selected and exploited for their future use in production of organic acids. *A. carbonarius* is a black *Aspergillus* that has been extensively studied as a food contaminant for its production of mycotoxin ochratoxin *A.* It was briefly described for its capabilities of producing citric acid from different types of carbon sources before this study [3]. In this PhD project, the main focus of the research effort was on *A. carbonarius* due to the following considerations: 1. The fully sequenced genome of *A. carbonarius* has been published; 2. It is amendable to genetic manipulation; 3. The biosynthetic pathway of ochratoxin A is elucidated in *A. carbonarius* and elimination of OTA can be achieved via genetic modification; and 4. It is able to produce various organic acids from the sugars commonly presented in renewable biomass, such as glucose and xylose. *A. saccharolyticus* is a newly identified *Aspergillus* species previously reported for its enzyme production, especially with regards to beta-glucosidases. It was selected in the later stages of this project mainly due to its abilities to secrete high amounts of malic acid and succinic acid in glucose-containing media. Genetic manipulation of *A. saccharolyticus* could be achieved with the transformation systems and genetic tools that were set up for *A. carbonarius*. However, compared with *A. carbonarius*, limited amount of information regarding the genetics and physiology of *A. saccharolyticus* is available.

For the past decades, genetic engineering of Aspergilli has been greatly powered by newly developed DNA recombinant technologies and next-generation DNA sequencing methods. Availability of different fungal transformation systems and genetic tools enables the genetic manipulation in *Aspergillus* strains, and meanwhile increasing amount of available information regarding fungal genetics and physiology facilitates designing the strategies for strain improvement (Chapter 1). In this project, strategies for genetic engineering of *A. carbonarius* and *A. saccharolyticus* were designed to improve succinic acid production. The preliminary analysis on organic acid profiles of *A. carbonarius* and *A. saccharolyticus* provided basic information for designing the strategies. *A. carbonarius* could produce high amount of citric acid in low pH condition. When it was cultivated in buffered pH condition, citric acid production decreased significantly but high amount of gluconic acid was produced instead. Under both pH conditions, *A. carbonarius* produced low amounts of malic acid but no succinic acid. Therefore, genetic engineering of *A. carbonarius* was carried out in two directions: 1. Elimination of unwanted organic acids (Chapter 3); 2. Increase the carbon flux towards pathways in relation to succinic acid production (Chapter 2). In addition, *A. carbonarius* was cultivated in

the hydrolysate of pretreated wheat straw in order to check the feasibility of using lignocellulosic biomass as feedstock for organic acid production by the selected strains (Chapter 4). Sugar consumption, inhibitor resistance and organic acid production by *A. carbonarius* were examined from the hydrolysate at different pH values. *A. saccharolyticus* can naturally secrete high amounts of malic acid and succinic acid, and the strategy was therefore made to continue the carbon flux from malic acid to succinic acid in the rTCA branch. For this purpose, a gene encoding a NADH-dependent fumarate reductase was cloned and expressed in *A. saccharolyticus* and the impacts of genetic modification on succinic acid as well as other organic acids were examined in glucose-containing media under different pH conditions (Chapter 5).

References

1. Holladay J, Bozell J, White J, Johnson D. Top value-added chemicals from biomass. DOE Report PNNL-16983 2007.

2. Angumeenal AR, Venkappayya D. An overview of citric acid production. LWT - Food Science and Technology 2013;50(2):367-370.

3. Ghareib M. Assimilation of galacturonate by *Aspergillus carbonarius*. Folia Microbiol (Praha) 1987;32(3):211-215.

CHAPTER 1. ASPERGILLUS AS A VERSATILE CELL FACTORY FOR ORGANIC ACID PRODUCTION

Aspergillus as a versatile cell factory for organic acid production (mini-review)

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Abstract

Aspergilli are of great importance for fungal biotechnology. Metabolic versatility of Aspergilli indicates huge potential of using Aspergilli as cell factories for production of various organic acids. So far, a number of *Aspergillus* strains belonging to phylogentically quite distantly related species have been successfully applied in industrial organic acid production due to their excellent capabilities of secreting high amounts of desired organic acids. For the past decades, tremendous efforts have been made to reveal the mechanisms of organic acid biosynthesis in Aspergilli and to improve the production of desired organic acids via genetic engineering. This review summarizes the recent breakthroughs in the fundamental understanding in physiological aspects of organic acid accumulation by production strains and highlights the progresses in genetic engineering of Aspergilli for organic acid production. The challenges for the future applications of Aspergilli as commercial biocatalysts for organic acid production are also discussed.

Keywords: *Aspergillus*; genetic engineering; organic acid; cell factory

Introduction

Studies on *Aspergillus* species have a long historic standing in biological research. The broad diversity of *Aspergillus* genus leads to a wide spectrum of valuable metabolites from Aspergilli for industrial applications such as food, pharmaceuticals and detergents. Outstanding abilities of Aspergilli to naturally accumulate high amounts of organic acids and to utilize a wide range of carbon sources have been widely reported. In addition, production of various secondary metabolites with biological activities and efficient secretion of a series of enzymes and proteins warrant the value of exploring Aspergilli for possible future uses in industry [1]. So far, a number of industrial processes have been developed employing isolates of *Aspergillus* for production of organic acids (e.g. citric acid, gluconic acid and kojic acid), enzymes (e.g. amylases, cellulases and hemicellulases) and food (e.g. soy sauce) [2-8].

Despite the successful industrial applications of Aspergilli, there are several barriers hindering comprehensive exploitations of rapidly expanding *Aspergillus* genus: 1. potential detrimental impacts of harmful species on human society due to mycotoxin production (e.g. aflatoxins and ochratoxins), food contamination and infection to human and livestock (e.g. Aspergillosis) [9]; 2. limited amount of genetic information regarding fungal genomes and annotated genes in identified *Aspergillus* species; 3. difficulty in genetic manipulation including the lack of efficient transformation systems and limited availability of genetic tools and selectable markers. For the past decades, emergence of next-generation DNA sequencing technologies and high-throughput analysis of cell metabolites have substantially removed some technical constraints in fungal research and have also opened a new era for efficiently obtaining rich amount of information regarding the genetics and physiology of Aspergilli. A research process starting from isolation of a new fungal strain to acquisition of a set of its genetic information, including genomics, transcriptomics and proteomics, has been dramatically shortened and becomes economically acceptable, which, to a large extent, facilitates the rational design for strain development and boosts the applications of bioinformatics tools and genome-scale metabolic models [10]. In addition, the implementation of genetic manipulation in Aspergilli can be achieved via more diversified systems.

The specific focus of this review concerns research on organic acid production by Aspergilli. By summarizing the major progresses in fundamental understanding of biosynthesis of organic acids in *Aspergillus* strains as well as in the recent strain improvement for organic acid production, this review aims at providing valuable information for future development of Aspergilli as cell factories for industrial production of desired organic acids.

Genetic manipulation of Aspergilli

Methods for gene cloning and plasmid construction have evolved from classic restriction enzyme based approaches to seamless, fast and more flexible approaches like uracil-specific excision reagent (USER) cloning, Gibson assembly and Golden gate assembly[11-13]. Several fungal transformation systems have been successfully applied in Aspergilli (Table1), and more selectable markers based on antibiotics resistance and nutritional defects have become available for fungal transformation. Multiple genetic modifications in one strain can now be achieved by using the different markers or recycling a counter-selectable marker [14]. However, low frequency of DNA homologous recombination greatly restricts efficiency of gene targeting due to the dominating non-homologous end joining (NHEJ) events (random integration) of transformed DNA into the genome of the *Aspergillus* strains. In recent years, tremendous efforts have been made to improve the efficiency of gene targeting in filamentous fungi and so far, several technologies and strategies have been successfully implemented in Aspergilli showing promising results (Table 2). Those newly emerging technologies have greatly promoted the progresses of genetic engineering of Aspergilli for various value-added products.

Table 1 Transformation strategies for *Aspergillus* strains

Approach	Starting materials	Species (example)
Protoplast transformation	Protoplast	*A. nidulans* [15], *A. niger* [16], *A. cabonarius* [17], *A. oryzae* [18], *A. saccharolyticus* [19]
Agrobacterium-mediated transformation	Conidia, protoplast, fungal mycelia	*A. niger* [20], *A. carbonarius* [21], *A. awamori* [22]
Electroporation	Germinating conidia	*A. nidulans*[23], *A. niger*[24]
Biolistic transformation	Conidia	*A. nidulans*[25]
Shock waves	Conidia	*A. niger* [26]

Please note this list is not exhaustive.

Table 2 Strategies for gene targeting in Aspergilli

Strategy	Advantages/Disadvantages	Species (example)
Vectors with homologous flanking regions via protoplast transformation	Fast implementation, high transformation efficiency. / Low gene targeting frequency, ectopic integration of transformed DNA in the knock-out tranformants, intensive screening work.	*A. niger* [27], *A. nidulans* [28]
Vectors with homologous flanking regions via *Agrobacterium*-mediated transformation	Fast implementation, high number of single copy transformants. / Relatively low gene targeting frequency, transformation efficiency varies among different species, intensive screening work.	*A. awamori*[29], *A. aculeatus* [30]
Bipartite gene-targeting substrate	Increased frequency of gene targeting, easy implementation. / Lower amount of transformants.	*A. nidulans* [31], *A. niger* [32], *A. carbonarius* [33]
Ku deficient strain	High frequency of gene targeting, low possibility of random insertion. / Construction of ku deficient strain before implementation, potential risk of mutagenesis in transformants under stressed conditions.	*A. niger* [34], *A. carbonarius* [35, 36], *A. nidulans*[37], *A. oryzae* [38], *A. sojae* [38]
CRISPR (clustered regularly - interspaced short palindromic repeats) /Cas9 system	Fast implementation, high transformation efficiency, multi-gene targeting via a vector. / Possible off-target effects, efficiency of gene targeting varies among different fungal species	*A. nidulans* [39], *A. niger* [39], *A. carbonarius* [39], *A. brasiliensis* [39], *A. oryzae* [40]
RNA interference	Silencing of multi-copy gene, alteration of gene expression instead of complete knock-out. / Variation of gene silencing on different genes, incomplete gene siliencing	*A. nidulans*[41], *A. niger*[42], *A. oryzae*[43], *A. flavus*[44]

Please note this list is not exhaustive

Organic acid production and applications

Organic acid production by Aspergilli, especially for low molecular weight carboxylic acids, has an important status in biotechnological production of commodity chemicals. A number of organic acids are currently produced via biotechnological processes employing *Aspergillus* strains, such as citric acid production by *Aspergillus niger* and itaconic acid production by *Aspergillus terreus*. Other organic acids with huge market potential like succinic acid and malic acid are under research development for their production with fungal biotechnology. The applications of organic acids have been extending from traditional uses such as food additives, chelators, acidulants to newly exploited applications in nanomedicine and as building-blocks for chemical industries over a period of decades [45, 46], which leads to an increasing demand of organic acids in global market (Table 3). Due to the consideration of environment protection, food security and rapid depletion of crude oil, integration of organic acid production with biorefineries using renewable lignocellulosic biomass as feedstocks becomes an inevitable trend, which also significantly elevates the status of biotechnological processes in organic acid production. As production strains are among the core technologies, they have significant impacts on the economic feasibility of the biotechnological processes of organic acids.

Selection and development of production strains are two critical phases in a project aiming at industrial production of organic acids via biotechnological processes [47]. There are several performance indices normally employed in the evaluation of suitable production strains: 1. Titers, yields and productivities of the desired products by candidate strains; 2. Tolerance of the strains to the desired products and to other potential inhibitors presented in the production process; 3. Availability of genetic tools to modify candidate strains; 4. Abundance of information regarding the aspects of physiology and genetics in candidate strains; 5. Safety consideration in industrial application of candidate strains; 6. Compatibility of candidate strains with existing technologies in industrial-scale processes. The criteria of suitable production strains may vary depending on the added-value of desired products, for instance, low product titer by a production strain can still fulfill the economic demand for production processes if the products have high added-value. Organic acids that are produced on a large scale normally have much lower added-value than fine chemicals, e.g. pharmaceuticals, and high efficiency in the production processes is crucial to lower the cost of production. Certain strains of *Aspergillus* are well-known industrial workhorses for microbial organic acid production, so these fungi have received extensive research interest as fungal cell factories for production of various organic acids. Some newly identified species have also shown potential for future uses as biocatalysts for industrial organic acid production. To date, a large number of organic acids with high market value can be naturally produced by different *Aspergillus* species and efforts have also been made to improve the efficiency of organic acid production by Aspergilli via genetic

engineering. These data have become valuable resources for a better understanding of the mechanism of organic acid accumulation by Aspergilli and also for future development of tailor-made fungal cell factories for desired organic acid products.

Table 3 Organic acids with high market potential

Organic acid	Annual Production (t)	Production process	Applications (example)
Oxalic acid	124000	Oxidation of carbohydrate	Textile treatment, bleaching agents[48]
Lactic acid	130000 - 150000	Catalytic synthesis from acetaldehyde / microbial fermentation	Food, polymer precursor, detergent [49]
Malic acid	12000	Hydration of maleic anhydride	Food, cosmetics, building block chemicals [46]
Fumaric acid	10000	Catalytic isomerization of maleic acid	Food, pharmaceuticals, building block chemicals [46]
Succinic acid	16000	Hydrogenation of maleic acid/microbial fermentation	Food, polymer precursor, building block chemicals [46]
Itaconic acid	15000	Microbial fermentation	Polymer precursor[50]
Citric acid	1600000	Microbial fermentation	Food and beverage, pharmaceutical, detergent [51]
Gluconic acid	87000	Microbial fermentation/ enzymatic biocatalysis	Food, phamarceuticals[52]

Annual production of organic acids in the list is cited from the review by Sauer, M. et al [53]. Please note this list is not exhaustive.

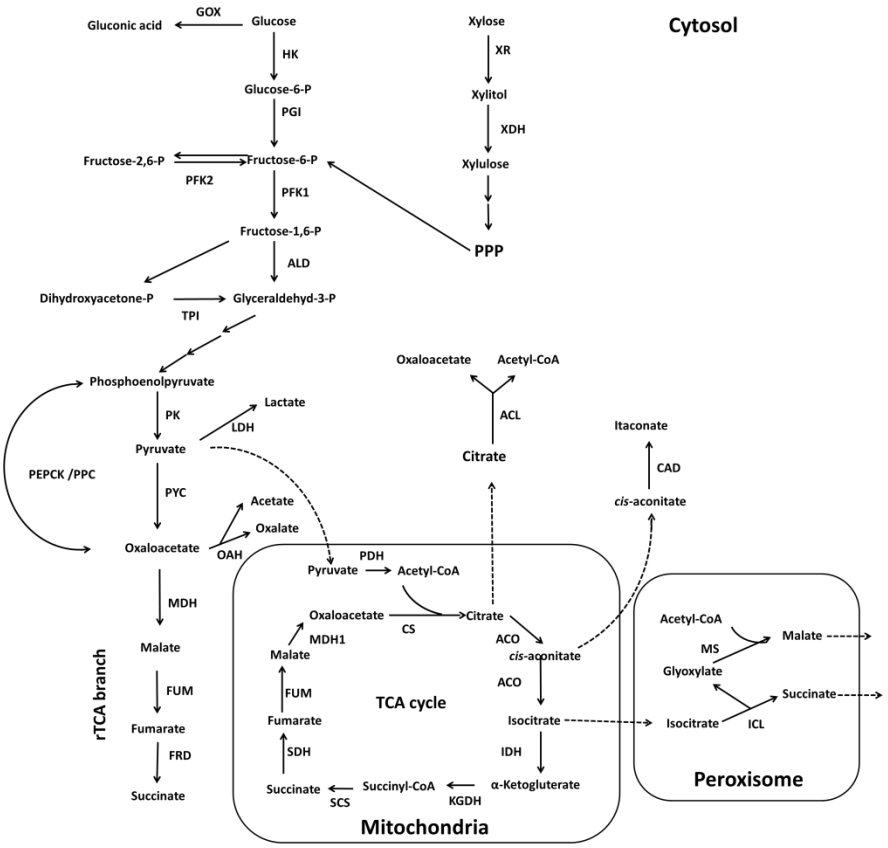

Fig. 1 Metabolic pathway for organic acid production by Aspergilli. Abbreviations refer to the proteins/Enzymes listed in Table 4. Abbreviations of pathways: PPP, Pentose phosphate pathway; TCA cycle, Tricarboxylic acid cycle, rTCA branch, reductive tricarboxylic acid cycle.

Table 4 Enzymes and regulatory proteins involved in the pathways relevant to organic acid production

Abbreviation	Enzymes/regulatory proteins	Species (example)
GOX	Glucose oxidase	*A. niger* [54] *A. carbonarius* [36]
HK/GK	Hexokinase/glucokinase	*A. niger* [55, 56], *A. nidulans* [57], *A. oryzae* [58]
PGI	Phosphoglucose isomerase	*A. nidulans* [59], *A. nidulans* [60], *A. oryzae* [58]
PFK1	Phosphofructokinase 1	*A. niger* [61], *A. terrus*[62]
PFK2	Phosphofructokinase 2	*A. niger* [63], *A. oryzae* [58]
ADL	Aldolase	*A. nidulans* [64], *A. niger* [65],
TPI	Triose phosphate isomerase	*A. nidulans* [66], *A. oryzae* [58]
PK	Pyruvate kinase	*A. nidulans* [67], *A. niger*[68]
PYC	Pyruvate carboxylase	*A. nidulans* [69], *A. niger* [70], *A. oryzae* [71], *A. flavus* [72], *A. terreus* [73]
MDH	Malate dehydrogenase*	*A. niger* [74], *A. oryzae* [75], *A. flavus* [76]
FUM	Fumarase*	*A. niger* [74], *A. flavus* [77]
PDH	Pyruvate dehydrogenase	*A. nidulans* [78]
CS	Citrate synthase	*A. niger* [79] *A. terreus* [62]
ACO	Aconitase	*A. niger* [80]
IDH	Isocitrate dehydrogenase	*A. niger* [81]
KGDH	α-ketoglutarate dehydrogenase	*A. niger* [82]
SCS	Succinyl-CoA synthetase	*A. oryzae* [83]

(continued)

SDH/FRD	Succinate dehydrogenase /Fumarate reductase*	*A. niger* [74] *A. saccharolyticus* [19]
MS	Malate synthase	*A. niger* [84]
ICL	Isocitrate lyase	*A. niger* [84]
ACL	ATP citrate lyase	*A. niger* [32]
CAD	*Cis*-aconitate decarboxylase	*A. niger* [85], *A. terreus* [62]
LDH	Lactate dehydrogenase*	*A. niger* [86], *A. brasiliensis* [87]
PPC	Phosphoenolpyruvate carboxylase*	*A. carbonarius*[88]
PEPCK	Phosphoenolpyruvate carboxykinase*	*A. carbonarius*[88]
XR	D-xylose reductase	*A. carbonarius* [33]
XDH	Xylitol dehydrogenase	*A. niger* [89]
Regulatory protein		
LaeA	Global regulator	*A. niger* [90], *A. nidulans* [91]
CreA	Carbon catabolite repressor	*A. niger* [92], *A. nidulans* [93]
AreA	Nitorgen regulatory protein	*A. flavus* [94], *A. nidulans* [95]
AcuM/K	Transcription factor	*A. nidulans* [96]
PacC	pH-dependent regulator	*A. niger*, *A. nidulans* [97]
AcuB/FacB	Transcription factor	*A. niger* [98], *A. nidulans* [99]
OafA	Oxalic acid repression factor	*A. niger* [100]

Note "*" indicates the heterologous expression of enzymes/proteins from other microorganisms into *Aspergillus* host strains. Please note this list is not exhaustive.

Industrial workhorses for organic acid production

Aspergillus niger

Due to its successful application for citric acid production since 1923, the black *A. niger* is a well-known industrial workhorse for organic acid production [7]. *A. niger* was firstly discovered for its capabilities of producing large quantities of citric acid in a high sugar containing medium by Currie in 1917 [51]. Moreover, several virtues that *A. niger* possesses rapidly paved its way to commercial production of citric acid, including excellent tolerance to low pH (2-3.5), efficient utilization of varieties of cheap substrates and its status as generally recognized as safe (GRAS). In spite of the great success in application of *A. niger* for citric acid production, the biochemical mechanism of citric acid accumulation by *A. niger* has yet been fully elucidated. Although enzymes located in the pathways relevant to citric acid production have been identified and investigated in *A. niger*, and plenty of information has been obtained regarding the correlation between important parameters in fermentation conditions and the derived changes in cell metabolism of *A. niger* [101], the biochemical and physiological mechanism of citric acid accumulation by *A. niger* is only partially revealed and some parts still remain unknown [102]. In addition to citric acid, *A. niger* is able to produce high amounts of gluconic acid and oxalic acid under certain conditions. The biosynthetic pathways for production of oxalic acid and gluconic acid have also been studied in *A. niger*. For gluconic acid production, the key enzyme responsible for extracellular conversion of glucose to gluconic acid, glucose oxidase, has been identified in *A. niger* [103], and moreover, a derived biotechnological process has been developed for production of gluconic acid employing glucose oxidase as a biocatalyst instead of microbial fermentation [104]. Oxalic acid is produced by *A. niger* exclusively via the hydrolysis of oxaloacetate into oxalate and acetate, which is carried out by oxaloacetate hydrolase in the cytosol [105]. In *A. niger*, disruption of genes encoding glucose oxidase and oxaloacetate hydrolase can lead to an elimination of production of gluconic acid and oxalic acid [106].

Presently, *A. niger* is studied as an excellent paradigm for fungal regulatory aspects of the central carbon metabolism in relation to efficient organic acid production. Meanwhile, due to its successes in industrial application, *A. niger* is also used as cell factories for production of varieties of organic acids, such as lactic acid and itaconic acid. There are several regulatory steps that are assumed to control carbon flux in the central carbon metabolic pathways in *A. niger* (Fig.1). In the glycolytic pathway, phosphofructokinase-1 (PFK1) is a major regulatory enzyme that is considered to play a crucial role in metabolic flux controlling during production of citric acid by *A. niger*. The early studies on PFK1 have shown that its activities are affected by a series of intracellular compounds like ATP, cAMP, ammonia, trace metals (Mn, Zn and Mg) and most importantly citrate [107, 108], and further investigations have revealed that a posttranslational modification of PFK1 produces

a shorter protein of the native PFK1, which is not inhibited by intracellular citrate [61, 109]. Based on this discovery, overexpression of PFK1 with a truncated mutation in *A. niger* led to an enhanced production of citric acid, which was not observed when the native PFK1 was overexpressed [110, 111]. Moreover, the elevated metabolic flux in the glycolytic pathway can also provide a good basis for improving production of other organic acids such as itaconic acid production by *A. niger* using a combination of different genetic modifications [112]. In addition to PFK1, there are two other irreversible steps in the glycolytic pathway, phosphorylation of glucose carried by hexokinase/glucokinase and phosphate transfer from phosphoenolpyruvate to ADP by pyruvate kinase, which were assumed to regulate the metabolic flux in glycolysis. However, overexpression of the pyruvate kinase did not show any significant impact on citric acid production [111]. For hexokinase/ glucokinase, disruption of the gene encoding trehalose 6-phosphate synthase, which produces trehalose 6-phosphate that is a strong inhibitor of hexokinase, showed a limited impact on the productivity of citric acid only in the early phase of fermentation [113]. A branch point of great importance in citric acid producing pathway is the carboxylation and decarboxylation of pyruvate, which provides an entrance for the carbon flux flowing into the TCA cycle in the mitochondria and the anaplerotic pathway rTCA branch in the cytosol. However, besides early studies on the properties and localization of pyruvate carboxylase [70, 72, 114, 115], to the best of our knowledge, there is yet no attempt to control the metabolic flux by manipulating the expression of the two enzymes. In contrast, a series of genetic modifications have been made in the rTCA branch, TCA cycle and glyoxylate shunt to examine the impacts of the involved enzymes on citric acid accumulation by *A. niger* and also to exploit the potential of using *A. niger* as cell factories to produce other organic acids such as C4- dicarboxylic acids and itaconic acid (Table 4). Overexpression of malate dehydrogenase, fumarase and insertion of fumarate reductase in the rTCA branch led to enhanced citric acid production but had limited impacts on the production of C4-dicarboxylic acids (malate, fumarate and succinate) by *A. niger* [74]. In the glyoxylate pathway, overexpression of isocitrate lyase resulted in elevated production of fumarate and citrate instead of the two end products in this pathway, succinate and malate [84]. In the TCA cycle, overexpression of citrate synthase did not increase the citric acid production; on the other hand, disruption of the gene encoding a methylcitrate synthase that also exhibited citrate synthase activities had no impact on citric acid production. This indicates that citrate synthase, as the key enzyme for biosynthesis of citric acid, is mainly responsible for citric acid production by *A. niger* but not for the regulation of the metabolic flux [79, 116].

In recent years, metabolic engineering of *A. niger* for itaconic acid production has received increasing research attention. Citric acid produced in the TCA cycle can be theoretically used as precursor in the biosynthesis pathway of itaconate. Indeed, expression of genes encoding cis-aconitate decarboxylase and a mitochondrial transporter from *A. terreus* enabled *A. niger* to produce itaconic acid. Further

elevated production of itaconic acid can be obtained using a combination of media optimization and other genetic modifications including overexpression of an endogenous *A. niger* mitochondrial transporter and elimination of the biosynthetic pathways of gluconic acid and oxalic acid [85, 117-119]. In addition to itaconic acid, a recent study on genetic engineering of *A. niger* for lactic acid production by expressing lactate dehydrogenase from mouse has opened a new window of opportunity to produce lactic acid under aerobic conditions [86]. However, despite the progresses in applying *A. niger* as cell factories in production of various organic acids, one of the main issues remains unaddressed that the metabolic flux flowing towards citric acid cannot be efficiently controlled via any of reported genetic modification, unlike the situation with gluconic acid and oxalic acid. Citric acid accumulation by *A. niger* seems to be under control by a series of regulatory steps. Application of powerful system biology approaches may reveal useful information regarding the gene regulation in *A. niger*. The comparative analysis of genomics and transcriptomics in *A. niger* in different pH conditions implies that transcriptional regulation of pH responses is closely related to organic acid production [120]. Meanwhile, several transcription factors have been studied individually for their impacts on organic acid production. Disruption of the *acuB* gene encoding acetate regulatory DNA binding protein had significant impacts on organic acid production [98]. Deletion of the *oafA* gene encoding the oxalic acid repression factor, led to elevated production of oxalic acid and different expression of 241 genes in *A. niger* [100]. Recently, a gene *laeA* encoding the global regulatory protein methyltransferase previously studied for its regulatory role on fungal secondary metabolites has also been shown to have impacts on citric acid production. Increased expression of *laeA*, could enhance citric acid production by *A. niger*, whereas, disruption of the *laeA* gene led to elimination of citric acid production [90].

From many perspectives, *A. niger* is considered as the most competitive fungal cell factory for organic acid production. A platform for metabolic engineering of *A. niger* for organic acid production, especially via rational design can be easily constructed due to the abundant knowledge on physiological and biochemical aspects of *A. niger*, the quantity of genetics information regarding genomics and transcriptomics, and the diversified tools for genetic manipulation. In addition, its successful applications in industrial production of organic acids provide possibilities to adopt the existing fermentation processes, of which parameters have been optimized for production of citric acid and gluconic acid, into the production of other organic acids. However, few disadvantages of *A. niger* cannot be ignored when it is used as cell factories for organic acid production. The facts that *A. niger* prefers to produce citric acid under most of the acid production conditions and the lack of understanding on the regulation of the metabolic flux towards citric acid can lead to great difficulties in rerouting carbon flux towards other desired organic acid products. Moreover, *A. niger* is a broad and diverse species, containing strains with different physiological characteristics. The comparative analysis of genomes of two

A. niger strains, the high citric acid producing strain ATCC 1015 and the efficient enzyme producing strain CBS 513.88, has shown the diversity within this species group [121]. This requires additional considerations when information obtained from different *A. niger* strains is applied into the genetic engineering of a certain strain.

Aspergillus oryzae

A. oryzae is a species that include several fermenting strains with a long history of extensive use in Asian food industries for producing soy sauce, rice wine (Sake) and vinegar, etc. *A. oryzae* is phylogenetically close to *A. flavus* and shares very high similarity with *A. flavus* in genome size and in amino acid sequences [122]. It even has similar gene clusters responsible for production of aflatoxins by *A. flavus*, but expression of the relevant genes for aflatoxins biosynthesis was not detected in *A. oryzae* under different fermentation conditions [123]. Like *A. niger*, *A. oryzae* is also included in the GRAS lists of microorganisms [124], which leads to its widely applications in the food industry. For the past century, *A. oryzae* has received extensive research interest for production of kojic acid and also for its highly efficient secretion of enzymes including proteases, α-amylases and hydrolases. It is also used as efficient cell factories for large-scale industrial enzyme production. *A. oryzae* can naturally produce high amount of kojic acid during the fermentation as a major secondary metabolite. Through optimizing the fermentation condition, *A. oryzae* could accumulate over 100 g/L kojic acid in a membrane-surface liquid culture with glucose as carbon source [5]. It has been reported that the kojic acid biosynthesis is regulated by a zinc finger transcriptional activator *kojR*, and deletion of *kojR* gene completely eliminated kojic acid production in the fermentation [125], whereas, overexpression of kojR gene in *A. oryzae* resulted in enhanced production of the kojic acid [126]. On the other hand, *A. oryzae* is also able to produce high amount of malic acid under specific fermentation conditions, indicating the possibilities to develop an efficient malic acid producing strain through genetic engineering. In *A. oryzae* mutant 2103a-68, overexpression of three genes encoding pyruvate carboxylase, malate dehydrogenase and malate transporter in combination could significantly increase the titer of malic acid up to 154 g/L in glucose based media [75]. Furthermore, a comparative study on enzyme activities was made between *A. oryzae* strain 2103a-68 and its parental strain NRRL3488 and intracellular metabolic flux distribution confirmed the increase in enzyme activities of PYC and MDH and significantly enhanced carbon flux towards rTCA branch in the mutant 2103a-68 strain [127]. This work also proves the feasibility of improving production of malic acid by Aspergilli via pathway engineering of the rTCA branch from which malic acid production were increased in bacteria and yeast with similar genetic modifications [128, 129]. In addition to kojic acid and malic acid, *A. oryzae* has been recently reported for its gluconic acid production after random mutagenesis [130]. Different from *A. niger*, *A. oryzae* produces gluconic acid from glucose mainly using glucose dehydrogenase instead of glucose

oxidase. Although the production of gluconic acid by *A. oryzae* (72 g/L) was inferior to that obtained from *A. niger* [130], the potential of *A. oryzae* has been demonstrated for industrial production of gluconic acid.

A. oryzae, as another biotechnological workhorse, resembles *A. niger* in many aspects when it is considered as cell factories for industrial production of organic acids. In lab-scale research, the genetic manipulation of *A. oryzae* can be achieved via different well established DNA recombinant technologies, and as one of the earliest fully genome sequenced *Aspergillus* species, the genetic information of *A. oryzae* can be easily accessed. *A. oryzae* has advantages of integrating its current fermentation technologies into the future production of desired organic acids for large industrial-scale production. However, although biosynthesis of kojic acid is well studied in *A. oryzae*, the information regarding biochemical aspects of enzymes in primary metabolic pathways (e.g. TCA cycle) in relation to production of other organic acids is relatively limited compared with *A. niger*. The lack of knowledge on fundamental biology of *A. oryzae* might hinder its strain improvement for potential applications for organic acid production.

Aspergillus terreus

A. terreus is a brownish *Aspergillus*, also known as an industrial workhorse for production of itaconic acid. Presently, the global production of itaconic acid is mainly via biotechnological processes using *A. terreus* as production strain. Over a period of decades, extensive efforts have been made to identify the biosynthetic pathway of itaconic acid in *A. terreus*, and a major progress has been obtained in the basic understanding of itaconic acid biosynthesis due to isolation of a key enzyme cis-aconitate decarboxylase (CAD), which carries out conversion of cis-aconitate to itaconate [131]. The CAD encoding gene has been cloned and studied for its function. Moreover, an itaconic acid gene cluster, comprising genes encoding a putative mitochondrial tricarboxylate family transporter, a putative major facilitator super-family transporter, a putative regulator protein and the CAD, has also been identified in *A. terreus* via a clone-based transcriptomics approach [132, 133]. The identification of key genes in the itaconic acid biosynthetic pathway forms a solid basis for further improvement of itaconic acid production by *A. terreus*.

In recent years, several attempts have been made to improve itaconic acid production by *A. terreus* via genetic engineering. The similarities between the biosynthetic pathways for itaconic acid production and citric acid production imply that the successful strategies applied in improvement of citric acid production by *A. niger* may also work on *A. terreus* for itaconic acid production. Indeed, expression of the mutated truncated *pfkA* gene from *A. niger* in *A. terreus* increased itaconic acid production [62, 134]. Overexpression of other enzymes involved in the glycolytic pathway and in the TCA cycle, glyceraldehyde-3-dehydrogenase, citrate

synthase and aconitase, decreased the itaconic acid production [62]. Enhanced itaconic acid production was found in the mutant with overexpression of CAD and a major facilitator super-family transproter, but not in the mutant with overexpression of the putative regulator protein and mitochondrial tricarboxylate family transporter [62]. On the other hand, since itaconic acid production by *A. terreus* is significantly affected by aeration, itaconic acid production can be increased by stabilizing intracellular oxygen supply. Expression of hemoglobin from *Vitreoscilla* improved itaconic acid production by alleviating oxygen limitation in *A. terreus* during the aeration interruption [135].

A. terreus is therefore another *Aspergillus* species of biotechnological importance for organic acid production. Although most of the research studies carried out with respect to organic acid production by *A. terreus* have mainly focused on itaconic acid, *A. terreus* also shows its potential for production of other organic acids, such as gluconic acid [136]. Rich amount of valuable information have been obtained regarding physiological and biochemical aspects of *A. terreus* through the constant attempts to elucidate the mechanism of itaconic acid accumulation and to optimize fermentation conditions. However, compared with *A. niger*, there are still some safety concerns about the industrial use of *A. terreus*. Although the microbial process of itaconic acid production using *A. terreus* has received GRAS status, *A. terreus* is not itself a GRAS microorganism due to the rapidly increasing incidence of its infection to human [137], which, to some extent, limits its industrial applications. On the other hand, although extensive efforts have been made to improve itaconic acid production by *A. terreus*, the titers of itaconic acid only increased at a very limited level (up to ~80-90 g/L), which is relatively low compared with the titer of citric acid by *A. niger* (~200 g/L) [50, 51]. This raises the concern regarding the limited capacity of *A. terreus* for accumulating high titers of organic acids due to its physiological and genetics constraints and also triggers research interest in exploiting other microorganisms for itaconic acid production, such as *A. niger* and *Yarrowia lipolytica* [117, 138].

Other *Aspergillus* species with potential for organic acid production

Aspergillus flavus

A. flavus, firstly known as plant pathogenic fungus responsible for the contamination of different important agriculture crops including wheat, maize, soybean and nuts, has been studied extensively for its organic acid production. It is shown that *A. flavus* has a very similar organic acid profile with *A. oryzae*, and both of them have been studied as excellent producers for malic acid and kojic acid [76, 139]. The investigations on malic acid production by *A. flavus* have provided quantity of useful information regarding fermentation conditions and biochemical mechanism of malic acid accumulation by filamentous fungi. By measuring activities of several key enzymes, a significant increase in activities of malate

dehydrogenase was observed in cytosol compared with fumarase, isocitrate lyase and succinate dehydrogenase [76], which implied that the accumulation of malic acid mainly resulted from the cytosolic reductive TCA branch in which pyruvate is carboxylated to produce oxaloacetate and eventually converted to malate through oxaloacetate reduction (Fig. 1). This conclusion was later supported by metabolic flux analysis using C13 labeled glucose and the analysis of nuclear magnetic resonance (NMR) showed that the C13 labeled carbon in glucose was only detected in C-3 carbon in the produced malic acid [77]. Although the biochemical mechanism of malic acid accumulation was well explained in *A. flavus*, the effort towards improving malic acid production was mainly made in the fermentation optimization. The effects of agitation speed, aeration rate, nutrients and morphology on malic acid production were examined in the fermentation with *A. flavus* [140, 141]. The highest concentration of malic acid could be achieved at 113 g/L from 120 g/L glucose with all the above mentioned factors at optimal levels [141].

The main barrier in industrial applications of *A. flavus* is the safety issue raised by the produced aflatoxins and infection of *A. flavus* to human and animals [142]. Aflatoxins are lethal mycotoxins that can cause the disease aflatoxicosis. Acute aflatoxicosis leads to death, and chronic aflaxoicosis can cause cancer [143]. Due to those concerns, *A. flavus* is not considered as a possible production host strain for industrial processes. However, the research work on malic acid production by *A. flavus* is still inspiring and valuable to strain development of other *Aspergillus* species for organic acid production. For instance, the investigation on malic acid accumulation by *A. flavus* elucidated the importance of the rTCA branch for malic acid production, which provides a theoretical foundation for genetic engineering of *Aspergillus* strains (e.g. *A. oryzae*) for malic acid production via this pathway. Recently, a naturally aflatoxin free *A. flavus* strain AF36 has been reported for its application in bio-control on crops to reduce aflatoxin exposure [144]. The future study of this strain may reveal the mechanism of aflatoxin biosynthesis in *A. flavus* leading to elimination of aflatoxins production in malic acid producing *A. flavus* strains and might also open a window of opportunity for biotechnological applications of *A. flavus* for in organic acid production.

Aspergillus carbonarius

A. carbonarius is a black *Aspergillus* exhibiting potentials to be cell factories for organic acid production. Its excellent capabilities of producing citric acid have been reported using glucose, galacturonate and molasses as carbon sources [145, 146]. Recent studies on *A. carbonarius* for organic acid production have provided deep insights on its organic acid production under different fermentation conditions. The pattern of organic acid production by *A. carbonarius* at uncontrolled pH and neutral pH has high similarities with that reported from *A. niger*. Both of the species can accumulate high amounts of gluconic acid and relatively low amounts of oxalic acid and citric acid at neutral pH, whereas, they mainly produce citric acid after

cultivation pH decreases to lower than 3. Indeed, *A. carbonarius* is phylogenetically close to *A. niger*, and they resemble in many features including morphology and physiology. Several attempts have been made in *A. carbonarius* to explore its potential for organic acid production via genetic engineering. Heterologous expression of phosphoenolpyruvate carboxylase from *Escherichia coli* and phosphoenolpyruvate carboxykinase from *Actinocbacillus succinogenes*, aiming to enhance the carbon flux towards the rTCA branch, increased the citric acid production by *A. carbonarius* [88]. Deletion of glucose oxidase, which is mainly responsible for converting glucose to gluconic acid in *A. carbonarius*, also led to enhanced production of citric acid and oxalic acid in the cultivation in pH buffered conditions [36]. For improvement of xylose utilization, expression of a mutated xylose reductase, of which the cofactor preference was assumed to change from NADPH to NADH, reduced the accumulation of xylitol as intermediate in pentose catabolic pathway and increased thereby the yield of citric acid by *A. carbonarius* [33]. In addition, the abilities of *A. carbonarius* to utilize lignocellulosic biomass as substrates for hydrocarbon production have been recently reported [147]. Similarly in order to seek cheaper substrates in industrial processes, lignocellulosic hydrolysate was also found valuable for organic acid production by *A. carbonarius* [148].

A. carbonarius has a number of virtues for fungal organic acid production, but it has only received very limited research attention mainly due to its ochratoxin A production. *A. carbonarius* is well known as the primary source of ochratoxin A (OTA) contamination in food commodities including dried fruit, grape, wine and coffee [149-152]. OTA is documented as a nephrotoxic, carcinogenic and teratogenic mycotoxin which can have the detrimental health effects on human and livestocks [153]. Although several species from genus *Aspergillus* are known to produce OTA, *A. carbonarius* appeared to have a very high reported percentage of ochratoxigenic strains and the ability to consistently produce OTA compared with other species such as *A. niger* [154-156]. For the last decade, extensive attempts have been made to reveal the mechanism of OTA production by *A. carbonarius* from both biochemical aspects and growth conditions. The effects of growth conditions (e.g. temperature, incubation time and pH) on OTA production have been studied in *A. carbonarius* [157-159]. Moreover, the biosynthetic pathway of OTA was proposed and disruption of the polyketide synthase involved in the biosynthesis of the polyketide part of ochratoxins led to an absence of OTA and ochratoxin α in *A. carbonarius* [17, 35]. Recently, three atoxigenic strains that are naturally unable to produce OTA have been identified in *A. carbonarius* [160]. The efficient elimination of OTA production via genetic engineering and newly identified atoxigenic strains may offer excellent prospects for biotechnological applications of *A. carbonarius*.

Aspergillus saccharolyticus and *Aspergillus brasiliensis*

A. saccharolyticus and *A. brasiliensis* are two relatively newly described *Aspergillus* species, belonging to the balck Aspergilli, with great potential for production of organic acids [161, 162]. The early studies on *A. saccharolyticus* focused on its enzyme production, especially Beta-glucosidase [163, 164]. Recently, *A. saccharolyticus* has also received research interest for its organic acid production. The organic acid profiles of *A. saccharoyticus* were obtained in different pH conditions showing that it could secrete high amounts of malic acid and succinic acid in the buffered pH condition (pH 6.5). Furthermore, heterologous expression of a NADH dependent fumarate reductase that converts fumarate into succinate significantly increased the production of succinic acid (up to 16.2 g/L) by *A. saccharolyticus* [19]. *A. brasiliensis* is also reported for the production of different types of organic acids from glucose based media, including malic acid, citric acid and oxalic acid [165]. Based on a study on the mycotoxin production by *Aspergillus* strains, *A. brasiliensis* has been recommended for biotechnological applications due to absence of carcinogenic compounds, such as fumoisins and ochratoxin, during the cultivation [155]. Recently, an attempt has been made to use *A. brasiliensis* as fungal cell factory for lactic acid production. Expression of the *ldhA* gene encoding lactate dehydrogenase from *Rhizopus oryzae* enabled *A. brasiliensis* to produce high amounts of lactic acid from varieties of carbon sources including glucose, xylose and starch [87]. This result gives promising perspectives for improving the lactic acid production in low pH processes via genetic engineering of fungal cells.

The research works carried out on *A. saccharolyticus* and *A. brasilinesis* have indicated the potential of these two *Aspergillus* species as cell factories for organic acid production, and at the same time, have also shown the simplified procedure of setting up platforms for genetic manipulation on newly identified fungal species nowadays. Certainly, there are still some constraints in developing *A. saccharolyticus* and *A. brasiliensis* into efficient biocatalysts for organic acid production: First, there is very limited amount of information available regarding the physiological features of the two species for organic acid production. Second, the fully sequenced genomes of *A. saccharolyticus* and *A. brasiliensis* are still not released yet, which restricts the metabolic engineering of the two species via rational design.

Aspergillus nidulans and other *Aspergillus* species

A. nidulans is one of the most important model organisms for fungal research. It is of great industrial significance not due to its direct applications in industrial production but its impacts on the research of fungal genetics and physiology. Although *A. nidulans* is not engineered for organic acid production, a series of key enzymes located in the primary metabolic pathways in relation to fungal organic acid production have been identified and characterized in *A. nidulans*. The regulatory properties of several key enzymes, such as pyruvate kinase, hexokinase,

pyruvate carboxylase and citrate synthase, which were considered to regulate the carbon flux towards production of organic acids, have been well elucidated in *A. nidulans*. Furthermore, since the advent of applying system biological approaches in strain development, there is increasing information regarding the impacts of regulatory proteins on fungal metabolism. *A. nidulans* accounts for the main part of progresses in the research of regulatory genes in *Aspergillus*. So far, several transcription factors and global regulators that may affect fungal organic acid production, such as CreA, FacB, AcuM/K, AreA, PacC and LaeA, have been identified and studied for their functions in metabolism regulation in *A. nidulans* (Table 4). AreA and CreA, which are responsible for nitrogen metabolite repression and carbon catabolite repression respectively, are two of the earliest transcription factors studied in filamentous fungi. The effects of their regulation on the primary metabolism of different carbon and nitrogen sources have been elucidated in great details in *A. nidulans*. Deletion of *creA* gene in *A. nidulans* results in increased levels of polyols and also a 20% decrease in the metabolic flux through the oxidative part of the pentose phosphate pathway in xylose and glucose mixture [93]. AreA, and a recently identified regulator AreB have been studied for their negatively regulation on utilization of different nitrogen sources in presence of ammonia or glutamine [95, 166], and the mutation in *areA* gene could lead to the inhibition of the growth on ammonia at low pH in *A. nidulans* [167]. Although it has not been reported that those two transcription factors are directly involved in the regulation of any organic acid producing pathway, the fact that concentrations of carbon and nitrogen sources play very important roles in organic acid production by Aspergilli implies their potential impacts on organic acid production. Compared with CreA and AreA, PacC that is involved in pH regulation of gene expression seems more relevant to organic acid production since the pattern of organic acid production by Aspergilli are significantly influenced by ambient pH [97]. PacC acts positively on the genes expressed in alkaline condition, but becomes inactive at acidic pH [168]. Although the proteolytic processing of PacC in response to different pH has been described in details, its role in transcriptional regulation on the genes potentially involved in organic acid production remains unknown. The regulatory genes *laeA* and *facB* had also been studied in *A. nidulans* before their impacts on organic acid production were reported in other *Aspergillus* species [99]. Recently, two transcription factors *acuK/M* have been identified and reported for their regulations on expression of the gluconeogenic genes *acuF* and *acuG* encoding phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase. The induction of *acuK/M* regulated genes by accumulation of intracellular malate reveals a new insight to mechanism of controlling carbon flux in the primary metabolic pathways in fungi, which may counteract the increased carbon flux that results from introduced genetic modifications [96]. *A. nidulans* is closely related to several other *Aspergillus* species of industrial importance for organic acid production, e.g. *A. niger* and *A. oryzae*. The knowledge regarding the regulatory system of *A. nidulans* can be valuable resource for genetic engineering of other *Aspergillus* species.

In addition to *A. nidulans*, a number of *Aspergillus* species that are used in industrial production of enzymes and food are of potential interest for organic acid production. Although their organic acid production have been previously studied or briefly described, no further efforts have been made to develop them as cell factories for varieties of organic acids. *Aspergillus awamori*, *Aspergillus sojae* and *Aspergillus wentii* are employed in the industrial production of enzymes and fermented food, and they all have exhibited abilities of producing organic acids including citric acid, malic acid or gluconic acid. Compared with the high efficient organic acid producing strains (e.g. *A. niger*), the titers and productivities of organic acids by those species are relatively low, which may require more efforts in strain development, as well as more information regarding physiological features and genetics of those species. However, it is not deniable that, for a specific organic acid, they might be suitable production strains after intense improvements with regard to optimizing fermentation conditions and metabolic engineering.

Future perspectives

Bio-production of organic acids has received increasing attention over a period of decades. As alternatives for chemical processes, biotechnological processes aim at incorporating renewable feedstock into organic acid production and providing long-term sustainable solutions to organic acid manufacturers. Especially for biotechnological processes aiming to utilize lignocellulosic biomass as feedstocks, microbial cell factories are required to efficiently utilize different types of carbohydrates present in the feedstocks. Moreover, optimization of upstream processes (e.g. pretreatment and saccharification of biomass) also needs to consider its potential impacts on microbial fermentation, such as inhibitory compounds generated from different pretreatment conditions. Aspergilli, compared with bacterial or yeast strains, can provide more flexibilities to the optimization of biotechnological processes for organic acid production due to their excellent abilities to utilize varieties of carbon sources and to tolerate stressed cultivation conditions (e.g. a broad range of pH, temperature and salinity) and various inhibitors.

Currently, genetic engineering has been widely applied in the improvement of *Aspergillus* strains for organic acid production. As physiological traits of production strains can orient the direction of strategy designing for genetic engineering, the selection of suitable production strain is a crucial step to influence the efficiency of a project aiming to develop cell factories for desired products. In general, there are two strategies for choosing a production strain in a project: either directly applying a well-studied strain or comprehensive screening of all the accessible strains. The advantages of developing well-studied strains to become a versatile cell factory for desired organic acid products have been elucidated previously in the case with *A. niger*. The complexity of fungal metabolism empowers the filamentous fungi with excellent abilities to utilize varieties of carbon

sources and with high resistance to wide spectra of inhibitors, but their metabolic traits and regulatory systems that have been evolved for certain types of organic acids in *Aspergillus* strains may stand as barriers in strain development for other products. For example, when *A. niger* is engineered for production of itaconic acid and lactic acid, citric acid becomes the byproduct that needs to be eliminated. Therefore, understanding the mechanism of citric acid accumulation by *A. niger* in order to further manipulate carbon flux towards desired products becomes an important step, which may require tremendous efforts. Alternatively, screening of potential suitable strains might provide new perspectives for this dilemma. To date, it is evident that the successful applications of *Aspergillus* strain for organic acid production partially result from naturally high accumulation of desired products, e.g. *A. niger* for citric acid production and *A. terreus* for itaconic acid production. Strains that can already accumulate high amounts of desired products provide opportunities to utilize their existing regulatory systems and metabolic traits to facillitate further strain development. This is reflected in the case that pathway engineering of the rTCA branch in *A. niger* and *A. oryzae* with similar strategies leads to distinct impacts on their organic acid production. In *A. oryzae*, malic acid production was significantly improved, whereas, in *A. niger*, enhanced citric acid production was obtained. A drawback of using this strategy is that a high-throughput screening might be required in the early phase of project, and if new species or less studied species have been selected, it also needs to take upfront time and effort to acquire necessary information for strain improvement. However, with current analytical technologies, a metabolite profile of a selected strain including organic acids, enzymes and mycotoxins can be easily obtained. This information can be used in evaluation of the selected strain for industrial applications and for optimization of the fermentation conditions for desired products. Furthermore, basic genetic information can be acquired for genetic engineering via fungal genome sequencing and transcriptomic analysis, and genetic manipulation can be achieved via versatile genetic tools. The progresses that have been obtained in genetic engineering *A. saccharolyticus* and *A. brasiliensis* for production of succinic acid and lactic acid reflect the values of exploring new *Aspergillus* species for organic acid production.

References

1. Frisvad JC, Larsen TO. Chemodiversity in the genus *Aspergillus*. Appl Microbiol Biotechnol 2015;99(19):7859-7877.

2. Botella C, Diaz A, De Ory I, Webb C, Blandino A. Xylanase and pectinase production by *Aspergillus awamori* on grape pomace in solid state fermentation. Process Biochemistry 2007;42(1):98-101.

3. Finkelstein DB. Improvement of enzyme production in *Aspergillus*. Antonie Van Leeuwenhoek 1987;53(5):349-352.

4. Francis F, Sabu A, Nampoothiri KM, Ramachandran S, Ghosh S, Szakacs G, Pandey A. Use of response surface methodology for optimizing process parameters for the production of α-amylase by *Aspergillus oryzae*. Biochem Eng J 2003;15(2):107-115.

5. Ogawa A, Wakisaka Y, Tanaka T, Sakiyama T, Nakanishi K. Production of kojic acid by membrane-surface liquid culture of *Aspergillus oryzae* NRRL484. J Ferment Bioeng 1995;80(1):41-45.

6. Sugiyama S. Selection of micro-organisms for use in the fermentation of soy sauce. Food Microbiol 1984;1(4):339-347.

7. Vandenberghe LP, Soccol CR, Pandey A, Lebeault J. Microbial production of citric acid. Brazilian Archives of Biology and Technology 1999;42(3):263-276.

8. Znad H, Markoš J, Baleš V. Production of gluconic acid from glucose by *Aspergillus niger*: growth and non-growth conditions. Process Biochemistry 2004; 39(11):1341-1345.

9. Sweeney MJ, Dobson AD. Mycotoxin production by *Aspergillus, Fusarium* and *Penicillium* species. Int J Food Microbiol 1998;43(3):141-158.

10. Meyer V, Wu B, Ram AF. *Aspergillus* as a multi-purpose cell factory: Current status and perspectives. Biotechnol Lett 2011;33(3):469-476.

11. Engler C, Kandzia R, Marillonnet S. A one pot, one step, precision cloning method with high throughput capability. PLoS ONE 2008;3(11).

12. Gibson DG, Young L, Chuang R-, Venter JC, Hutchison CA, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods 2009;6(5):343-345.

13. Nour-Eldin HH, Geu-Flores F, Halkier BA. USER cloning and USER fusion: the ideal cloning techniques for small and big laboratories. Plant Secondary Metabolism Engineering 2010;3:185-200.

14. Lakshmi Prabha V, Punekar NS. Genetic transformation in Aspergilli: Tools of the trade. Indian J Biochem Biophys 2004;41(5):205-215.

15. Koukaki M, Giannoutsou E, Karagouni A, Diallinas G. A novel improved method for *Aspergillus nidulans* transformation. J Microbiol Methods 2003;55(3):687-695.

16. Unkles SE, Campbell EI, Carrez D, Grieve C, Contreras R, Fiers W, Van den Hondel CAMJJ, Kinghorn JR. Transformation of *Aspergillus niger* with the homologous nitrate reductase gene. Gene 1989;78(1):157-166.

17. Gallo A, Bruno KS, Solfrizzo M, Perrone G, Mulè G, Visconti A, Baker SE. New insight into the ochratoxin a biosynthetic pathway through deletion of a nonribosomal peptide synthetase gene in *Aspergillus carbonarius*. Appl Environ Microbiol 2012;78(23):8208-8218.

18. Mattern IE, Unkles S, Kinghorn JR, Pouwels PH, van den Hondel, Cees AMJJ. Transformation of *Aspergillus oryzae* using the *A. niger* pyrG gene. Molecular and General Genetics MGG 1987;210(3):460-461.

19. Yang L, Lübeck M, Ahring BK, Lübeck PS. Enhanced succinic acid production in *Aspergillus saccharolyticus* by heterologous expression of fumarate reductase from *Trypanosoma brucei*. Appl Microbiol Biotechnol 2016;100(12):1799-1809 .

20. Li M, Zhou L, Liu M, Huang Y, Sun X, Lu F. Construction of an Engineering Strain Producing High Yields of α-Transglucosidase via Agrobacterium tumefaciens-Mediated Transformation of *Asperillus niger*. Biosci Biotechnol Biochem 2013;77(9):1860-1866.

21. Morioka LRI, Furlaneto MC, Bogas AC, Pompermayer P, Duarte RT, Vieira MLC, Watanabe MAE, Fungaro MHP. Efficient genetic transformation system for the ochratoxigenic fungus *Aspergillus carbonarius*. Curr Microbiol 2006;52(6):469-472.

22. Gouka RJ, Gerk C, Hooykaas PJJ, Bundock P, Musters W, Verrips CT, De Groot MJA. Transformation of *Aspergillus awamori* by Agrobacterium tumefaciens- mediated homologous recombination. Nat Biotechnol 1999;17(6):598-601.

23. Chakraborty BN, Patterson NA, Kapoor M. An electroporation-based system for high-efficiency transformation of germinated conidia of filamentous fungi. Can J Microbiol 1991;37(11):858-863.

24. Ozeki K, Kyoya (Fujii) F, Hizume K, Kanda A, Hamachi M, Nunokawa Y. Transformation of intact *Aspergillus niger* by electroporation. Biosci Biotechnol Biochem 1994;58(12):2224-2227.

25. Fungaro MHP, Rech E, Muhlen GS, Vainstein MH, Pascon RC, de Queiroz MV, Pizzirani-Kleiner AA, de Azevedo JL. Transformation of *Aspergillus nidulans* by microprojectile bombardment on intact conidia. FEMS Microbiol Lett 1995;125(2-3):293-297.

26. Magaña-Ortíz D, Coconi-Linares N, Ortiz-Vazquez E, Fernández F, Loske AM, Gómez-Lim MA. A novel and highly efficient method for genetic transformation of fungi employing shock waves. Fungal Genetics and Biology 2013;56:9-16.

27. Pedersen H, Christensen B, Hjort C, Nielsen J. Construction and Characterization of an Oxalic Acid Nonproducing Strain of *Aspergillus niger*. Metab Eng 2000;2(1):34-41.

28. Tilburn J, Scazzocchio C, Taylor GG, Zabicky-Zissman JH, Lockington RA, Davies RW. Transformation by integration in *Aspergillus nidulans*. Gene 1983;26(2):205-221.

29. Michielse CB, Hooykaas PJ, van den Hondel, Cees AMJJ, Ram AF. Agrobacterium-mediated transformation of the filamentous fungus *Aspergillus awamori*. Nature protocols 2008;3(10):1671-1678.

30. Kunitake E, Tani S, Sumitani J-, Kawaguchi T. *Agrobacterium tumefaciens*-mediated transformation of *Aspergillus aculeatus* for insertional mutagenesis. AMB Express 2011;1(1):1-11.

31. Nielsen ML, Albertsen L, Lettier G, Nielsen JB, Mortensen UH. Efficient PCR-based gene targeting with a recyclable marker for *Aspergillus nidulans*. Fungal Genet Biol 2006;43(1):54-64.

32. Meijer S, Nielsen ML, Olsson L, Nielsen J. Gene deletion of cytosolic ATP: citrate lyase leads to altered organic acid production in *Aspergillus niger*. J Ind Microbiol Biotechnol 2009;36(10):1275-1280.

33. Weyda I, Lübeck M, Ahring BK, Lübeck PS. Point mutation of the xylose reductase (XR) gene reduces xylitol accumulation and increases citric acid production in *Aspergillus carbonarius*. Journal of Industrial Microbiology and Biotechnology 2014;41(4):733-739.

34. Meyer V, Arentshorst M, El-Ghezal A, Drews A-, Kooistra R, van den Hondel CAMJJ, Ram AFJ. Highly efficient gene targeting in the *Aspergillus niger kusA* mutant. J Biotechnol 2007;128(4):770-775.

35. Gallo A, Knox BP, Bruno KS, Solfrizzo M, Baker SE, Perrone G. Identification and characterization of the polyketide synthase involved in ochratoxin A biosynthesis in *Aspergillus carbonarius*. Int J Food Microbiol 2014;179:10-17.

36. Yang L, Lübeck M, Lübeck PS. Deletion of glucose oxidase changes the pattern of organic acid production in *Aspergillus carbonarius*. AMB Express 2014;4(1).

37. Nayak T, Szewczyk E, Oakley CE, Osmani A, Ukil L, Murray SL, Hynes MJ, Osmani SA, Oakley BR. A versatile and efficient gene-targeting system for *Aspergillus nidulans*. Genetics 2006;172(3):1557-1566.

38. Takahashi T, Feng JJ, Sunagawa M, Machida M, Koyama Y. Generation of large chromosomal deletions in koji molds *Aspergillus oryzae* and *Aspergillus sojae* via a loop-out recombination. Appl Environ Microbiol 2008;74(24):7684-7693.

39. Nødvig CS, Nielsen JB, Kogle ME, Mortensen UH. A CRISPR-Cas9 system for genetic engineering of filamentous fungi. PLoS ONE 2015;10(7).

40. Katayama T, Tanaka Y, Okabe T, Nakamura H, Fujii W, Kitamoto K, Maruyama J: Development of a genome editing technique using the CRISPR/Cas9 system in the industrial filamentous fungus *Aspergillus oryzae*. Biotechnol Lett 2015;1-6.

41. Hammond TM, Keller NP. RNA silencing in *Aspergillus nidulans* is independent of RNA-dependent RNA polymerases. Genetics 2005;169(2):607-617.

42. Brody H, Maiyuran S. RNAi-mediated gene silencing of highly expressed genes in the industrial fungi Trichoderma reesei and *Aspergillus niger*. Ind Biotechnol 2009;5(1):53-60.

43. Fernandez EQ, Moyer DL, Maiyuran S, Labaro A, Brody H. Vector-initiated transitive RNA interference in the filamentous fungus *Aspergillus oryzae*. Fungal Genetics and Biology 2012;49(4):294-301.

44. McDonald T, Brown D, Keller NP, Hammond TM. RNA silencing of mycotoxin production in *Aspergillus* and *Fusarium* species. Mol Plant-Microbe Interact 2005;18(6):539-545.

45. Naeini AT, Adeli M, Vossoughi M. Poly(citric acid)-block-poly(ethylene glycol) copolymers—new biocompatible hybrid materials for nanomedicine. Nanomedicine: Nanotechnology, Biology and Medicine 2010;6(4):556-562.

46. Holladay J, Bozell J, White J, Johnson D. Top value-added chemicals from biomass. DOE Report PNNL-16983 2007.

47. Lee SY, Kim HU. Systems strategies for developing industrial microbial strains. Nat Biotechnol 2015;33(10):1061-1072.

48. Riemenschneider W, Tanifuji M. Oxalic Acid. Ullmann's Encyclopedia of Industrial Chemistry. 2000.

49. Castillo Martinez FA, Balciunas EM, Salgado JM, Domínguez González JM, Converti A, Oliveira RPdS. Lactic acid properties, applications and production: A review. Trends Food Sci Technol 2013;30(1):70-83.

50. Okabe M, Lies D, Kanamasa S, Park EY. Biotechnological production of itaconic acid and its biosynthesis in *Aspergillus terreus*. Appl Microbiol Biotechnol 2009;84(4):597-606.

51. Angumeenal AR, Venkappayya D. An overview of citric acid production. LWT - Food Science and Technology 2013;50(2):367-370.

52. Ramachandran S, Fontanille P, Pandey A, Larroche C. Gluconic acid: Properties, applications and microbial production. Food Technology and Biotechnology 2006;44(2):185-195.

53. Sauer M, Porro D, Mattanovich D, Branduardi P. Microbial production of organic acids: expanding the markets. Trends Biotechnol 2008;26(2):100-108.

54. Kriechbaum M, Heilmann HJ, Wientjes FJ, Hahn M, Jany K, Gassen HG, Sharif F, Alaeddinoglu G. Cloning and DNA sequence analysis of the glucose oxidase gene from *Aspergillus niger* NRRL-3. FEBS Lett 1989;255(1):63-66.

55. Panneman H, Ruijter GJG, Van Den Broeck HC, Driever ETM, Visser J. Cloning and biochemical characterisation of an *Aspergillus niger* glucokinase. Evidence for the presence of separate glucokinase and hexokinase enzymes. Eur J Biochem 1996;240(3):518-525.

56. Panneman H, Ruijter GJG, Van Den Broeck HC, Visser J. Cloning and biochemical characterisation of *Aspergillus niger* hexokinase the enzyme is strongly inhibited by physiological concentrations of trehalose 6-phosphate. Eur J Biochem 1998;258(1):223-232.

57. Dunn-Coleman NS, Pateman JA. The regulation of hexokinase and phosphoglucomutase activity in *Aspergillus nidulans*. Molec Gen Genet 1979;171(1):69-73.

58. Nakajima K, Kunihiro S, Sano M, Zhang Y, Eto S, Chang Y, Suzuki T, Jigami Y, Machida M. Comprehensive cloning and expression analysis of glycolytic genes from the filamentous fungus, *Aspergillus oryzae*. Curr Genet 2000;37(5):322-327.

59. Ruijter GJ, Visser J. Characterization of *Aspergillus niger* phosphoglucose isomerase. Use for quantitative determination of erythrose 4-phosphate. Biochimie 1999;81(3):267-272.

60. Upadhyay S, Shaw BD. A phosphoglucose isomerase mutant in *Aspergillus nidulans* is defective in hyphal polarity and conidiation. Fungal Genetics and Biology 2006;43(11):739-751.

61. Mlakar T., Legiša M. Citrate inhibition-resistant form of 6-phosphofructo-1-kinase from *Aspergillus niger*. Appl Environ Microbiol 2006;72(7):4515-4521.

62. Huang X, Lu X, Li Y, Li X, Li J. Improving itaconic acid production through genetic engineering of an industrial *Aspergillus terreus* strain. Microb Cell Fact 2014;13(1).

63. Harmsen HJM, Kubicek-Pranz EM, Röhr M, Visser J, Kubicek CP. Regulation of 6-phosphofructo-2-kinase from the citric-acid-accumulating fungus *Aspergillus niger*. Appl Microbiol Biotechnol 1992;37(6):784-788.

64. JAGANNATHAN V, SINGH K, DAMODARAN M. Carbohydrate metabolism in citric acid fermentation. 4. Purification and properties of aldolase from *Aspergillus niger*. Biochem J 1956;63(1):94-105.

65. Roumelioti K, Vangelatos I, Sophianopoulou V. A cryptic role of a glycolytic–gluconeogenic enzyme (aldolase) in amino acid transporter turnover in *Aspergillus nidulans*. Fungal Genetics and Biology 2010;47(3):254-267.

66. McKnight GL, O'Hara PJ, Parker ML. Nucleotide sequence of the triosephosphate isomerase gene from *Aspergillus nidulans*: implications for a differential loss of introns. Cell 1986;46(1):143-147.

67. de Graaff L, van den Broek H, Visser J. Isolation and transformation of the pyruvate kinase gene of *Aspergillus nidulans*. Curr Genet 1988;13(4):315-321.

68. de Graaff L, van den Broeck H, Visser J. Isolation and characterization of the *Aspergillus niger* pyruvate kinase gene. Curr Genet 1992;22(1):21-27.

69. OSMANI SA, MAYER F, MARSTON FA, SELMES IP, SCRUTTON MC. Pyruvate carboxylase from *Aspergillus nidulans*. European journal of biochemistry 1984;139(3):509-518.

70. Feir HA, Suzuki I. Pyruvate carboxylase of *Aspergillus niger*: kinetic study of a biotin-containing carboxylase. Can J Biochem 1969;47(7):697-710.

71. Knuf C, Nookaew I, Brown SH, McCulloch M, Berry A, Nielsen J. Investigation of malic acid production in *aspergillus oryzae* under nitrogen starvation conditions. Appl Environ Microbiol 2013;79(19):6050-6058.

72. Bercovitz A, Peleg Y, Battat E, Rokem JS, Goldberg I. Localization of pyruvate carboxylase in organic acid-producing *Aspergillus* strains. Appl Environ Microbiol 1990;56(6):1594-1597.

73. Songserm P, Thitiprasert S, Tolieng V, Piluk J, Tanasupawat S, Assabumrungrat S, Yang S, Karnchanatat A, Thongchul N. Regulating Pyruvate Carboxylase in the Living Culture of *Aspergillus Terreus* Nrrl 1960 by l-Aspartate for Enhanced Itaconic Acid Production. Appl Biochem Biotechnol 2015;177(3):595-609.

74. de Jongh WA, Nielsen J. Enhanced citrate production through gene insertion in *Aspergillus niger*. Metab Eng 2008;10(2):87-96.

75. Brown SH, Bashkirova L, Berka R, Chandler T, Doty T, McCall K, McCulloch M, McFarland S, Thompson S, Yaver D, Berry A. Metabolic engineering of *Aspergillus oryzae* NRRL 3488 for increased production of l-malic acid. Appl Microbiol Biotechnol 2013;97(20):8903-8912.

76. Peleg Y, Stieglitz B, Goldberg I. Malic acid accumulation by *Aspergillus flavus* - I. Biochemical aspects of acid biosynthesis. Appl Microbiol Biotechnol 1988;28(1):69-75.

77. Peleg Y, Barak A, Scrutton MC, Goldberg I. Malic acid accumulation by *Aspergillus flavus* - III. 13C NMR and isoenzyme analyses. Appl Microbiol Biotechnol 1989;30(2):176-183.

78. Bos CJ, Slakhorst M, Visser J, Roberts CF. A third unlinked gene controlling the pyruvate dehydrogenase complex in *Aspergillus nidulans*. J Bacteriol 1981;148(2):594-599.

79. Ruijter GJG, Panneman H, Xu D-, Visser J. Properties of *Aspergillus niger* citrate synthase and effects of citA overexpression on citric acid production. FEMS Microbiol Lett 2000;184(1):35-40.

80. La Nauze JM. Aconitase and isocitric dehydrogenases of *Aspergillus niger* in relation to citric acid production. J Gen Microbiol 1966;44(1):73-81.

81. Meixner-Monori B, Kubicek CP, Harrer W, Schreferl G, Rohr M. NADP-specific isocitrate dehydrogenase from the citric acid-accumulating fungus *Aspergillus niger*. Biochem J 1986;236(2):549-557.

82. Meixner-Monori B, Kubicek CP, Habison A, Kubicek-Pranz EM, Rohr M. Presence and regulation of the alpha-ketoglutarate dehydrogenase multienzyme

complex in the filamentous fungus *Aspergillus niger*. J Bacteriol 1985;161(1):265-271.

83. Zhao G, Hou L, Yao Y, Wang C, Cao X. Comparative proteome analysis of *Aspergillus oryzae* 3.042 and *A. oryzae* 100–8 strains: towards the production of different soy sauce flavors. Journal of proteomics 2012;75(13):3914-3924.

84. Meijer S, Otero J, Olivares R, Andersen MR, Olsson L, Nielsen J. Overexpression of isocitrate lyase—glyoxylate bypass influence on metabolism in *Aspergillus niger*. Metab Eng 2009;11(2):107-116.

85. van der Straat L, Vernooij M, Lammers M, van den Berg W, Schonewille T, Cordewener J, van der Meer I, Koops A, De Graaff LH. Expression of the *Aspergillus terreus* itaconic acid biosynthesis cluster in *Aspergillus niger*. Microb Cell Fact 2014;13(1).

86. Dave KK, Punekar NS. Expression of Lactate Dehydrogenase in *Aspergillus niger* for L-Lactic Acid Production. PLoS One 2015;10(12):e0145459.

87. Liaud N, Rosso M-, Fabre N, Crapart S, Herpoël-Gimbert I, Sigoillot J-, Raouche S, Levasseur A. L-lactic acid production by *Aspergillus brasiliensis* overexpressing the heterologous ldha gene from *Rhizopus oryzae*. Microb Cell Fact 2015;14(1).

88. Yang L, Lübeck M, Lübeck PS. Effects of heterologous expression of phosphoenolpyruvate carboxykinase and phosphoenolpyruvate carboxylase on organic acid production in *Aspergillus carbonarius*. J Ind Microbiol Biotechnol 2015;42(11):1533-1545.

89. WITTEVEEN CF, WEBER F, BUSINK R, VISSER J. Isolation and characterization of two xylitol dehydrogenases from *Aspergillus niger*. Microbiology 1994;140(7):1679-1685.

90. Niu J, Arentshorst M, Nair PD, Dai Z, Baker SE, Frisvad JC, Nielsen KF, Punt PJ, Ram AF. 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 (Bethesda) 2015; 6(1):193-204.

91. Bok JW, Keller NP. LaeA, a regulator of secondary metabolism in *Aspergillus spp*. Eukaryot Cell 2004;3(2):527-535.

92. Ruijter GJ, Vanhanen SA, Gielkens MM, van de Vondervoort PJ, Visser J. Isolation of A*spergillus niger creA* mutants and effects of the mutations on

expression of arabinases and L-arabinose catabolic enzymes. Microbiology 1997;143 (9):2991-2998.

93. David H, Krogh AM, Roca C, Åkesson M, Nielsen J. CreA influences the metabolic fluxes of *Aspergillus nidulas* during growth on glucose and xylose. Microbiology 2005;151(7):2209-2221.

94. Christensen T, Hynes MJ, Davis MA. Role of the regulatory gene *areA* of *Aspergillus oryzae* in nitrogen metabolism. Appl Environ Microbiol 1998;64(9):3232-3237.

95. Macios M, Caddick MX, Weglenski P, Scazzocchio C, Dzikowska A. The GATA factors AREA and AREB together with the co-repressor NMRA, negatively regulate arginine catabolism in *Aspergillus nidulans* in response to nitrogen and carbon source. Fungal Genetics and Biology 2012;49(3):189-198.

96. Suzuki Y, Murray SL, Wong KH, Davis MA, Hynes MJ. Reprogramming of carbon metabolism by the transcriptional activators AcuK and AcuM in *Aspergillus nidulans*. Mol Microbiol 2012;84(5):942-964.

97. Arst Jr HN, Peñalva MA. pH regulation in *Aspergillus* and parallels with higher eukaryotic regulatory systems. Trends in Genetics 2003;19(4):224-231.

98. Meijer S, De Jongh WA, Olsson L, Nielsen J. Physiological characterisation of acuB deletion in *Aspergillus niger*. Appl Microbiol Biotechnol 2009;84(1):157-167.

99. Papadopoulou S, Sealy-Lewis HM.The *Aspergillus niger acuA* and *acuB* genes correspond to the *facA* and *facB* genes in *Aspergillus nidulans*. FEMS Microbiol Lett 1999;178(1):35-37.

100. Poulsen L, Andersen MR, Lantz AE, Thykaer J. Identification of a Transcription Factor Controlling pH-Dependent Organic Acid Response in *Aspergillus niger*. PLoS ONE 2012;7(12).

101. Papagianni M. Advances in citric acid fermentation by *Aspergillus niger*: Biochemical aspects, membrane transport and modeling. Biotechnol Adv 2007;25(3):244-263.

102. Karaffa L, Kubicek CP. *Aspergillus niger* citric acid accumulation: Do we understand this well working black box? Appl Microbiol Biotechnol 2003;61(3):189-196.

103. Witteveen CF, Veenhuis M, Visser J. Localization of Glucose Oxidase and Catalase Activities in *Aspergillus niger*. Appl Environ Microbiol 1992;58(4):1190-1194.

104. Das Neves LCM, Vitolo M. Use of glucose oxidase in a membrane reactor for gluconic acid production. Appl Biochem Biotechnol 2007;137-140(1-12):161-170.

105. CLELAND WW, JOHNSON MJ. Studies on the formation of oxalic acid by *Aspergillus niger*. The Journal of biological chemistry 1956;220(2):595-606.

106. Ruijter GJG, Van De Vondervoort PJI, Visser J. Oxalic acid production by *Aspergillus niger*: An oxalate-non-producing mutant produces citric acid at pH 5 and in the presence of manganese. Microbiology 1999;145(9):2569-2576.

107. Arts E, Kubicek CP, Röhr M. Regulation of phosphofructokinase from *Aspergillus niger*: effect of fructose 2, 6-bisphosphate on the action of citrate, ammonium ions and AMP. J Gen Microbiol 1987;133(5):1195-1199.

108. Habison A, Kubicek CP, Rohr M. Partial purification and regulatory properties of phosphofructokinase from *Aspergillus niger*. Biochem J 1983;209:669-676.

109. Mesojednik S., Legiša M. Posttranslational modification of 6-phosphofructo-1-kinase in *Aspergillus niger*. Appl Environ Microbiol 2005;71(3):1425-1432.

110. Capuder M, Šolar T, Benčina M, Legiša M. Highly active, citrate inhibition resistant form of *Aspergillus niger* 6-phosphofructo-1-kinase encoded by a modified pfkA gene. J Biotechnol 2009;144(1):51-57.

111. Ruijter GJG, Panneman H, Visser J. Overexpression of phosphofructokinase and pyruvate kinase in citric acid-producing *Aspergillus niger*. Biochimica et Biophysica Acta (BBA) - General Subjects 1997;1334(2–3):317-326.

112. van der Straat L, Tamayo-Ramos JA, Schonewille T, de Graaff LH. Overexpression of a modified 6-phosphofructo-1-kinase results in an increased itaconic acid productivity in *Aspergillus niger*. AMB Express 2013;3:1-19.

113. Arisan-Atac I, Wolschek MF, Kubicek CP. Trehalose-6-phosphate synthase A affects citrate accumulation by *Aspergillus niger* under conditions of high glycolytic flux. FEMS Microbiol Lett 1996;140(1):77-83.

114. BLOOM SJ, JOHNSON MJ. The pyruvate carboxylase of *Aspergillus niger*. J Biol Chem 1962;237:2718-2720.

115. Pel HJ, De Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, Turner G, De Vries RP, Albang R, Albermann K, Andersen MR, Bendtsen JD, Benen JAE, Van Den Berg M, Breestraat S, Caddick MX, Contreras R, Cornell M, Coutinho PM, Danchin EGJ, Debets AJM, Dekker P, Van Dijck PWM, Van Dijk A, Dijkhuizen L, Driessen AJM, D'Enfert C, Geysens S, Goosen C, Groot GSP, De Groot PWJ, Guillemette T, Henrissat B, Herweijer M, Van Den Hombergh JPTW, Van Den Hondel CAMJJ, Van Der Heijden RTJM, Van Der Kaaij RM, Klis FM, Kools HJ, Kubicek CP, Van Kuyk PA, Lauber J, Lu X, Van Der Maarel MJEC, Meulenberg R, Menke H, Mortimer MA, Nielsen J, Oliver SG, Olsthoorn M, Pal K, Van Peij NNME, Ram AFJ, Rinas U, Roubos JA, Sagt CMJ, Schmoll M, Sun J, Ussery D, Varga J, Vervecken W, Van De Vondervoort PJJ, Wedler H, Wösten HAB, Zeng A-, Van Ooyen AJJ, Visser J, Stam H. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nat Biotechnol 2007;25(2):221-231.

116. Kobayashi K, Hattori T, Honda Y, Kirimura K. Gene identification and functional analysis of methylcitrate synthase in citric acid-producing *Aspergillus niger* WU-2223L. Biosci Biotechnol Biochem 2013;77(7):1492-1498.

117. Li A, Pfelzer N, Zuijderwijk R, Punt P. Enhanced itaconic acid production in *Aspergillus niger* using genetic modification and medium optimization. BMC Biotechnol 2012;12(8).

118. Li A, Pfelzer N, Zuijderwijk R, Brickwedde A, Van Zeijl C, Punt P. Reduced by-product formation and modified oxygen availability improve itaconic acid production in *Aspergillus niger*. Appl Microbiol Biotechnol 2013;97(9):3901-3911.

119. Blumhoff ML, Steiger MG, Mattanovich D, Sauer M. Targeting enzymes to the right compartment: Metabolic engineering for itaconic acid production by *Aspergillus niger*. Metab Eng 2013;19:26-32.

120. Andersen MR, Lehmann L, Nielsen J. Systemic analysis of the response of *Aspergillus niger* to ambient pH. Genome Biol 2009;10(5).

121. Andersen MR, Salazar MP, Schaap PJ, Van De Vondervoort PJI, Culley D, Thykaer J, Frisvad JC, Nielsen KF, Albang R, Albermann K, Berka RM, Braus GH, Braus-Stromeyer SA, Corrochano LM, Dai Z, Van Dijck PWM, Hofmann G, Lasure LL, Magnuson JK, Menke H, Meijer M, Meijer SL, Nielsen JB, Nielsen ML, Van Ooyen AJJ, Pel HJ, Poulsen L, Samson RA, Stam H, Tsang A, Van Den Brink JM, Atkins A, Aerts A, Shapiro H, Pangilinan J, Salamov A, Lou Y, Lindquist E, Lucas S, Grimwood J, Grigoriev IV, Kubicek CP, Martinez D, Van Peij NNME, Roubos JA, Nielsen J, Baker SE. Comparative genomics of citric-acid-producing *Aspergillus niger* ATCC 1015 versus enzyme-producing CBS 513.88. Genome Res 2011;21(6):885-897.

122. Payne GA, Nierman WC, Wortman JR, Pritchard BL, Brown D, Dean RA, Bhatnagar D, Cleveland TE, Machida M, Yu J. Whole genome comparison of *Aspergillus flavus* and *A. oryzae*. Medical Mycology 2006;44(SUPPL. 1):9-11.

123. Zhang Y, Wilkinson H, Keller N, Tsitsigiannis D, An Z. Secondary metabolite gene clusters. Handbook of Industrial Mycology.New York: Marcel Dekker 2005;355-386.

124. Barbesgaard P, Heldt-Hansen HP, Diderichsen B. On the safety of *Aspergillus oryzae*: a review. Appl Microbiol Biotechnol 1992;36(5):569-572.

125. Marui J, Yamane N, Ohashi-Kunihiro S, Ando T, Terabayashi Y, Sano M, Ohashi S, Ohshima E, Tachibana K, Higa Y, Nishimura M, Koike H, Machida M. Kojic acid biosynthesis in *Aspergillus oryzae* is regulated by a Zn(II)2Cys6 transcriptional activator and induced by kojic acid at the transcriptional level. Journal of Bioscience and Bioengineering 2011;112(1):40-43.

126. Yamada R, Yoshie T, Wakai S, Asai-Nakashima N, Okazaki F, Ogino C, Hisada H, Tsutsumi H, Hata Y, Kondo A. *Aspergillus oryzae*-based cell factory for direct kojic acid production from cellulose. Microbial Cell Factories 2014;13(1).

127. Knuf C, Nookaew I, Remmers I, Khoomrung S, Brown S, Berry A, Nielsen J. Physiological characterization of the high malic acid-producing *Aspergillus oryzae* strain 2103a-68. Appl Microbiol Biotechnol 2014;98(8):3517-3527.

128. Zelle RM, De Hulster E, Van Winden WA, De Waard P, Dijkema C, Winkler AA, Geertman J-A, Van Dijken JP, Pronk JT, Van Maris AJA. Malic acid production by *Saccharomyces cerevisiae*: Engineering of pyruvate carboxylation, oxaloacetate reduction, and malate export. Appl Environ Microbiol 2008;74(9):2766-2777.

129. Moon SY, Hong SH, Kim TY, Lee SY. Metabolic engineering of *Escherichia coli* for the production of malic acid. Biochem Eng J 2008;40(2):312-320.

130. Raksha S, Srinivasan S, Prasant G, Prabu R. Over-expression of gluconic acid in *Aspergillus oryzae* RP-21 mutants generated by a random mutagenesis approach. 3 Biotech 2012;2(3):219-223.

131. Dwiarti L, Yamane K, Yamatani H, Kahar P, Okabe M. Purification and characterization of cis-Aconitic acid decarboxylase from *Aspergillus terreus* TN484-M1. J Biosci Bioeng 2002;94(1):29-33.

132. Li A, van Luijk N, ter Beek M, Caspers M, Punt P, van der Werf M. A clone-based transcriptomics approach for the identification of genes relevant for itaconic acid production in *Aspergillus*. Fungal Genet Biol 2011;48(6):602-611.

133. Kanamasa S, Dwiarti L, Okabe M, Park EY. Cloning and functional characterization of the cis-aconitic acid decarboxylase (CAD) gene from *Aspergillus terreus.* Appl Microbiol Biotechnol 2008;80(2):223-229.

134. Tevž G, Bencina M, Legiša M. Enhancing itaconic acid production by *Aspergillus terreus*. Appl Microbiol Biotechnol 2010;87(5):1657-1664.

135. Lin Y, Li Y, Huang M, Tsai Y. Intracellular expression of Vitreoscilla hemoglobin in *Aspergillus terreus* to alleviate the effect of a short break in aeration during culture. Biotechnol Lett 2004;26(13):1067-1067-1072.

136. Dowdells C, Jones RL, Mattey M, Benina M, Legia M, Mousdale DM. Gluconic acid production by *Aspergillus terreus*. Lett Appl Microbiol 2010;51(3):252-257.

137. Fernández MS, Rojas FD, Cattana ME, De los Ángeles Sosa M, Mangiaterra ML, Giusiano GE. *Aspergillus terreus* complex: An emergent opportunistic agent of Onychomycosis. Mycoses 2013;56(4):477-481.

138. Wang J-, Tsai S-, Teng K. Producing Itaconic Acid in Yeast Using Glycerol as the Substrate. Industrial Technology Research Institute (ITRI) 2011.

139. Zhang J, Han L, Yan S, Liu C. The non-metabolizable glucose analog D-glucal inhibits aflatoxin biosynthesis and promotes kojic acid production in *Aspergillus flavus*. BMC Microbiology 2014;14(1):95-107.

140. Peleg Y, Rahamim E, Kessel M, Goldberg I. Malic acid accumulation by *Aspergillus flavus* - II. Crystals and hair-like processes formed by *A. flavus* in a l-malic acid production medium. Appl Microbiol Biotechnol 1988;28(1):76-79.

141. Battat E, Peleg Y, Bercovitz A, Rokem JS, Goldberg I. Optimization of L-malic acid production by *Aspergillus flavus* in a stirred fermentor. Biotechnol Bioeng 1991;37(11):1108-1116.

142. Yu J. Current understanding on aflatoxin biosynthesis and future perspective in reducing aflatoxin contamination. Toxins 2012;4(11):1024-1057.

143. KLICH MA. *Aspergillus flavus*: the major producer of aflatoxin. Molecular Plant Pathology 2007;8(6):713-722.

144. Doster MA, Cotty PJ, Michailides TJ. Evaluation of the atoxigenic *Aspergillus flavus* strain AF36 in pistachio orchards. Plant Dis 2014;98(7):948-956.

145. Ghareib M. Assimilation of galacturonate by *Aspergillus carbonarius*. Folia Microbiol (Praha) 1987;32(3):211-215.

146. El Dein Selim M. Production of citric acid from molasses in presence of potassium ferrocyanide by *Aspergillus aculeatus* and *A. carbonarius.* Short communication. Indian J Exp Biol 1979;17(1):105-106.

147. Sinha M, Sørensen A, Ahamed A, Ahring BK. Production of hydrocarbons by *Aspergillus carbonarius* ITEM 5010. Fungal Biology 2015;119(4):274-282.

148. Yang L, Lübeck M, Souroullas K, Lübeck PS. Co-consumption of glucose and xylose for organic acid production by *Aspergillus carbonarius* cultivated in wheat straw hydrolysate. World J Microbiol Biotechnol 2016, *In Press*.

149. Battilani P, Pietri A, Bertuzzi T, Languasco L, Giorni P, Kozakiewicz Z. Occurrence of ochratoxin A-producing fungi in grapes grown in Italy. J Food Prot 2003;66(4):633-636.

150. Cabañes FJ, Accensi F, Bragulat MR, Abarca ML, Castellá G, Minguez S, Pons A. What is the source of ochratoxin A in wine? Int J Food Microbiol 2002;79(3):213-215.

151. Battilani P, Magan N, Logrieco A. European research on ochratoxin A in grapes and wine. Int J Food Microbiol 2006;111:S2-S4.

152. Abarca ML, Accensi F, Bragulat MR, Castellá G, Cabañes FJ. *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. J Food Prot 2003;66(3):504-506.

153. Pfohl-Leszkowicz A, Manderville RA. Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. Molecular Nutrition and Food Research 2007;51(1):61-99.

154. Palumbo JD, O'Keeffe TL, Vasquez SJ, Mahoney NE. Isolation and identification of ochratoxin A-producing *Aspergillus* section Nigri strains from California raisins. Lett Appl Microbiol 2011;52(4):330-336.

155. Frisvad JC, Larsen TO, Thrane U, Meijer M, Varga J, Samson RA, Nielsen KF. Fumonisin and ochratoxin production in industrial *Aspergillus niger* strains. PLoS ONE 2011;6(8).

156. Valero A, Begum M, Hocking AD, Marín S, Ramos AJ, Sanchis V. Mycelial growth and ochratoxin A production by *Aspergillus* section Nigri on simulated grape medium in modified atmospheres. J Appl Microbiol 2008;105(2):372-379.

157. Esteban A, Abarca ML, Bragulat MR, Cabañes FJ. Effects of temperature and incubation time on production of ochratoxin a by black aspergilli. Res Microbiol 2004;155(10):861-866.

158. Esteban A, Abarca ML, Bragulat MR, Cabañes FJ. Influence of pH and incubation time on ochratoxin A production by *Aspergillus carbonarius* in culture media. J Food Prot 2005;68(7):1435-1440.

159. Mitchell D, Parra R, Aldred D, Magan N. Water and temperature relations of growth and ochratoxin A production by *Aspergillus carbonarius* strains from grapes in Europe and Israel. J Appl Microbiol 2004;97(2):439-445.

160. Cabañes FJ, Bragulat MR, Castellá G. Characterization of nonochratoxigenic strains of *Aspergillus carbonarius* from grapes. Food Microbiol 2013;36(2):135-141.

161. SØrensen A, Lübeck PS, Lübeck M, Nielsen KF, Ahring BK, Teller PJ, Frisvad JC. Aspergillus saccharolyticus sp. nov., a black *Aspergillus* species isolated in Denmark. Int J Syst Evol Microbiol 2011;61(12):3077-3083.

162. Varga J, Kocsubé S, Tóth B, Frisvad JC, Perrone G, Susca A, Meijer M, Samson RA. *Aspergillus brasiliensis* sp. nov., a biseriate black *Aspergillus* species with world-wide distribution. Int J Syst Evol Microbiol 2007;57(8):1925-1932.

163. Sørensen A, Lübeck PS, Lübeck M, Teller PJ, Ahring BK. ß-Glucosidases from a new *Aspergillus* species can substitute commercial ß-glucosidases for saccharification of lignocellulosic biomass. Can J Microbiol 2011;57(8):638-650.

164. Rana V, Eckard AD, Teller P, Ahring BK. On-site enzymes produced from *Trichoderma reesei* RUT-C30 and *Aspergillus saccharolyticus* for hydrolysis of wet exploded corn stover and loblolly pine. Bioresour Technol 2014;154:282-289.

165. Liaud N, Liaud C, Giniés D, Navarro N, Fabre S, Herpoël Crapart I, Gimbert A, Levasseur S, Raouche J, Sigoillot. Exploring fungal biodiversity: organic acid production by 66 strains of filamentous fungi. Fungal Biology and Biotechnology 2014;1(1).

166. Wong KH, Hynes MJ, Todd RB, Davis MA. Deletion and overexpression of the *Aspergillus nidulans* GATA factor AreB reveals unexpected pleiotropy. Microbiology 2009;155(12):3868-3880.

167. Pereira M, Martinez-Rossi NM. The *areA*(r) mutations of *Aspergillus nidulans* confer low pH sensitivity in the presence of ammonium as the only nitrogen source. Lett Appl Microbiol 1998;27(1):54-56.

168. Orejas M, Espeso EA, Tilburn J, Sarkar S, Arst Jr. HN, Penalva MA. Activation of the *Aspergillus* PacC transcription factor in response to alkaline ambient pH requires proteolysis of the carboxy-terminal moiety. Genes and Development 1995;9(13):1622-1632.

CHAPTER 2. EFFECTS OF HETEROLOGOUS EXPRESSION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE AND PHOSPHOENOLPYRUVATE CARBOXYLASE ON ORGANIC ACID PRODUCTION IN ASPERGILLUS CARBONARIUS

Effects of heterologous expression of phosphoenolpyruvate carboxykinase and phosphoenolpyruvate carboxylase on organic acid production in *Aspergillus carbonarius*

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Abstract

Aspergillus carbonarius has a potential as a cell factory for production of various organic acids. In this study, the organic acid profile of *A. carbonarius* was investigated under different cultivation conditions. Moreover, two heterologous genes, *pepck* and *ppc*, which encode phosphoenolpyruvate carboxykinase in *Actinobacillus succinogenes* and phosphoenolpyruvate carboxylase in *Escherichia coli*, were inserted individually and in combination in *A. carbonarius* to enhance the carbon flux towards the reductive TCA branch. Results of transcription analysis and measurement of enzyme activities of phosphoenolpyruvate carboxykinase and phosphoenolpyruvate carboxylase in the corresponding single and double transformants demonstrated that the two heterologous genes were successfully expressed in *A. carbonarius.* The production of citric acid increased in all the transformants in both glucose and xylose based media at pH higher than 3 but did not increase in the pH non-buffered cultivation compared with the wild type.

Keywords *Aspergillus carbonarius*; phosphoenolpyruvate carboxykinase; phosphoenolpyruvate carboxylase; citric acid; organic acids

Introduction

Organic acid production by filamentous fungi has been investigated for decades. The applications of organic acids, especially carboxylic acids, can be found in a wide range of industries including food, beverage, cosmetics, pharmaceuticals and detergents [1]. Presently, some organic acids, such as citric acid, gluconic acid and lactic acid, can be produced via biological processes using one-step fermentation [2-4]. Certain strains of *Aspergillus niger* are used commercially for the production of citric acid due to high titer of citric acid (>150 g/L) combined with utilization of cheap substrates [5, 6]. However, some other organic acids with huge marketing potential, like malic acid and fumaric acid, are still produced via chemical processes at relatively low cost compared with biological processes [7-9]. Suitable producing strains are considered as the key factors influencing the economic feasibility of the entire biological processes for production of organic acids. The black filamentous fungus *Aspergillus carbonarius* can naturally produce high amount of citric acid in response to stress conditions using a variety of substrates including hexoses and pentoses [10, 11]. For the last decade, research carried out on *A. carbonarius* has mainly focused on the food contamination caused by its mycotoxin ochratoxin A (OTA), which is normally considered as an important issue for microorganisms to be applied in industrial processes. However, a recent report published on *A. carbonarius* ITEM 5010 revealed a new insight into the OTA biosynthetic pathway and demonstrated an efficient way to eliminate the OTA production through genetic engineering [12]. The progress in inactivation of OTA biosynthetic pathway paves the way to industrial applications of *A. carbonarius*. In addition, it is reported that *A. carbonarius* is able to produce a series of hydrocarbons from different types of lignocellulosic materials [13]. The abilities of *A. carbonarius* to produce various types of organic acids and utilize a wide range of carbon sources indicate its potential as a cell factory for industrial production of organic acids using renewable biomass (e.g. lignocellulosic materials) [13, 14]. *A. carbonarius* is phylogenetically close to *A. niger*, resembling many features in the morphology and physiology [15], which provides the possibility to use *A. niger* as a reference strain for investigating acid producing pathways in *A. carbonarius*. However, the differences in fungal metabolism between two fungal species are not negligible.

In filamentous fungi, the reductive tricarboxylic acid (rTCA) branch is highly involved in the synthesis of several organic acids including malic acid, fumaric acid and citric acid [16-18]. In *Aspergillus flavus*, a sharply increased enzyme activity of pyruvate carboxylase, which carries out the conversion of pyruvate to oxaloacetate (OAA), was detected while malic acid was accumulated during cultivation [19]. In *Rhizopus oryzae*, fumaric acid is mainly produced through the rTCA branch involving the conversion of oxaloacetate to malate catalyzed by malate dehydrogenase and dehydration of malate to fumarate catalyzed by fumarase [20]. For *A. niger*, it has been shown that genetic modifications in the rTCA branch exhibited effects on citric acid production [18]. Citric acid production was initiated

in *A. niger* in the early phase of cultivation when intracellular concentration of malate was increased by overexpressing cytosolic malate dehydrogenase and enhanced citric acid production was observed when fumarase was overexpressed to increase the concentration of fumarate in the cytosol. Those effects on citric acid production in *A. niger* might result from an anti-port between dicarboxylic acids and citric acid across the mitochondrial membrane [18, 21], similar to what was elucidated in *Saccharomyces cerevisiae* [22].

In this study, an alternative cytosolic pathway carrying out one-step conversion of phosphoenolpyruvate (PEP) to oxaloacetate was inserted into *A. carbonarius* to enhance the carbon flux towards oxaloacetate as the onset of the rTCA branch (Fig. 1). Two exogenous genes *pepck* and *ppc* encoding phosphoenolpyruvate carboxykinase (AsPEPCK from *Actinobacillus succinogenes*) and phosphoenolpyruvate carboxylase (EcPPC from *Escherichia coli*), which can convert PEP to oxaloacetate with a fixation of CO_2, were expressed in *A. carbonarius.* The EcPPC carries out an irreversible conversion of PEP to oxaloacetate and one phosphate group [23]. The AsPEPCK carries out a reversible conversion of PEP to oxaloacetate with the generation of one ATP. Commonly in microorganisms, PEPCK is responsible for converting oxaloacetate to PEP in the gluconeogenic pathway, but there is no phosphoenolpyruvate carboxylase activity exhibited in *A. succinogenes*, and PEPCK is used to produce oxaloacetate directly from PEP in this species [24, 25]. It has been reported that overexpression of EcPPC in *R. oryzae* resulted in elevated fumaric acid production indicating its impact on organic acid production via the rTCA branch [17], however, there is yet no report regarding expression of AsPEPCK in filamentous fungi. In this study, the aims were to express both genes in *A. carbonarius*, individually, and in combination, and to compare the impacts of the genetic modifications on organic acid production in cultivations using different conditions. Besides, a preliminary analysis on the organic acid profile of *A. carbonarius* wild type was conducted at different pH to investigate the impact of pH change on organic acid production.

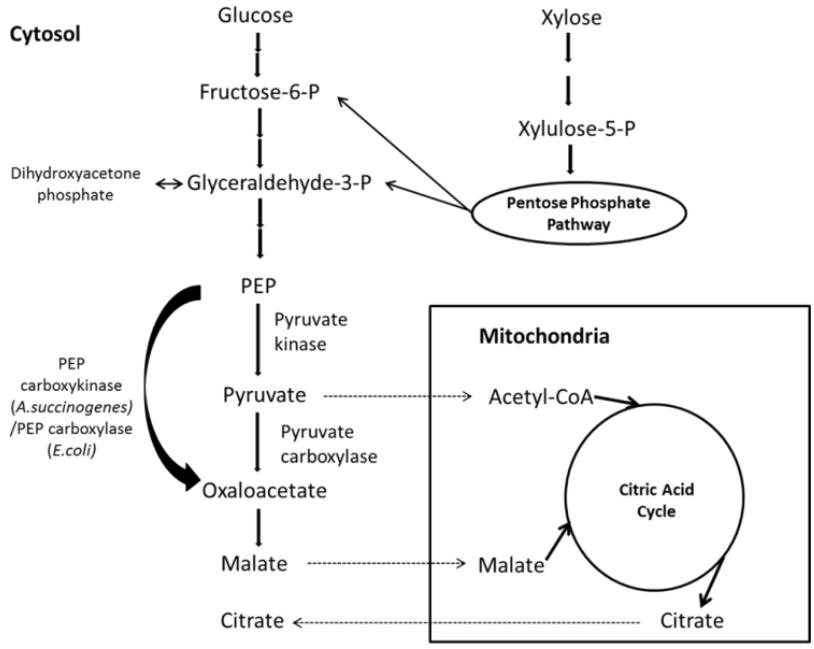

Fig. 1 Proposed metabolic pathway for organic acid production in *A. carbonarius* the bold arrow indicates the introduced pathway; the dash arrow indicates transport across mitochondrial membrane; and multiple arrows indicate the multiple reactions omitted in the pathway map.

Materials and methods

Strains

A. carbonarius ITEM5010 (ATCC® MYA-4641™) was selected to construct new strains. *E. coli* K-12 (ATCC® 10798™) and *A. succinogenes* 130Z (ATCC® 55618™) were used to obtain the genes *ppc* and *pepck*, respectively, for the genetic modifications.

Culture media

Fungal strains were cultured in potato dextrose agar (PDA) medium at 30 ^{o}C for harvesting of spores. For genomic DNA extraction, the strains were cultured in yeast extract peptone dextrose (YPD) medium containing: (g/L) yeast extract, 10; peptone, 20 and glucose, 20, at 30 ^{o}C for 2 days after which mycelia were collected for DNA isolation. *E. coli* K-12 was cultivated in LB medium composed of (g/L) tryptone, 10, yeast extract 5 and NaCl 10 at 37 ^{o}C and *A. succinogenes* 130Z was

grown in BHI (Brain Heart infusion) medium containing 15 g/L brain heart infusion powder (Sigma) at 37 ^{o}C. The pre-culture was carried out in the medium containing (g/L): yeast extract, 3.6 and peptone, 10. The cultivation was carried out in the three different production media for organic acid production. For buffered pH conditions, the glucose production medium consisted of (g/L): glucose, 100; $(NH_4)_2SO_4$, 2; KH_2PO_4, 0.15; K_2HPO_4, 0.15; $MgSO_4$ $7H_2O$, 0.1; $CaCl_2$ $2H_2O$, 0.1; NaCl, 0.005; ZnSO4, 0.1g/L; $FeSO_4$ $7H_2O$, 0.005 and $CaCO_3$, 60 [26]. For pH non-buffered conditions, the glucose production medium was made using the above mentioned recipe but omitting calcium carbonate. The xylose production medium contains (g/L): D-xylose, 100; NH_4NO_3, 2.5; K_2HPO_4, 0.1; $MgSO_4{\cdot}7H_2O$, 1; $CaCl_2{\cdot}2H_2O$, 0.168; KCl, 0.43; $ZnSO_4{\cdot}7H_2O$, 4.5 × 10−3; $FeSO_4{\cdot}$7H2O, 0.75 × 10^{-3} [11].

Construction of plasmids expressing *ppc* and *pepck*

Genomic DNA isolated from *E. coli* K-12 and *A. succinogenes* 130Z was used to amplify the genes *ppc* and *pepck* respectively with primers containing uracil overhangs compatible with the USER cloning based plasmid pSBe1 [27]. The PCR reactions amplifying *ppc* (~2.6 kb) and *pepck* (~1.6 kb) were set up in 50 µL reaction volumes containing: 5 µL 10× Pfu turbox buffer; 1 µL 10 µM dNTP; 2.5 µL 10 µM forward primer and 2.5 µL 10 µM reverse primer (Table 1); 1 µL Pfu turbo cx polymerase (Agilent); appropriate amount of DNA template and water added up to 50 µL. The cycling parameters in the PCR program was: initial denature step at 95 ^{o}C for 3 min; 25-30 cycles of denature step at 94 ^{o}C for 30 s; annealing step at 55-65 ^{o}C for 30 s; elongation step at 72 ^{o}C for a specific amount of time calculated by the size of desired fragments (1 min/kb); followed by a final elongation step at 72 ^{o}C for 5 min. The *ppc* and *pepck* genes were then cloned separately into the vector pSBe1 between the *gpdA* promoter and the *TrpC* terminator as described previously [27] followed by transformation of *E. coli* DH5α which was grown in LB medium with 100 µg/ml ampicillin at 37 ^{o}C (adding 15 g/L agar for solid LB plate). The plasmids pSBe1ppc and pSBe1pepck were verified in colony PCR and used for fungal transformation. To construct double transformants with two genes inserted (*pepck* and *ppc*), the two plasmids were used together in co-transformation of protoplasts. In order to increase the frequency of co-transformation, the hygromycin resistance gene *hph* was inactivated in the plasmid pSBe1pepck by linearizing the plasmid through *NcoI* restriction site which solely exists in the region of *hph* gene in the plasmid (Appendix A-2). The sequences, design of primers and plasmid maps were handled using the CLC workbench (CLC Bio).

Protoplast transformation

Protoplasts of *A. carbonarius* were made from young mycelia as previously described [28]. The final concentration of protoplasts for aliquots was adjusted to 2 x10^{7} /mL and the fresh protoplasts were preserved at -80^{o}C with the addition of

40% PEG4000 and 7% DMSO. The procedure for protoplast transformation was described in [14]. 5 μg plasmids in 10 μL were added into 100 μL protoplast suspension. For co-transformation, 4 μg linearized plasmid pSBe1pepck and 1 μg circular plasmid pSBe1ppc in total volume of 10 μL were added into protoplast suspension. Transformants were selected after sporulation. Single colonies of individual transformants were obtained by streaking out the spores on PDA medium followed by overnight incubation at 30 ^{o}C. To prepare genomic DNA, the spores of the selected transformants were inoculated into YPD medium and grown at 30 ^{o}C for 24 h, and young mycelia were used directly in genomic DNA extraction the following day. The inserted target genes in the transformants were verified by examining the amplified fragments with expected sizes in PCR. The verified transformants were then preserved for further steps.

RNA extraction and Transcription analysis of inserted genes

The mycelia of transformants were harvested after 3 days cultivation in YPD medium and directly used for RNA extraction. RNA was extracted using total RNA isolation kit (A&A Biotechnology) according to the procedure described by the manufacturer, and the purified RNA samples were treated with DNase to remove potential contaminating DNA by incubating approx. 1 μg RNA with 1 unit DNaseI (Fermentas) and 1 μL 10× reaction buffer in total 10 μL reaction volume at 37 ^{o}C for at least 30 min. The reaction was terminated by adding 1 μL 50 mM EDTA and incubated at 65 ^{o}C for 10 min. The treated RNA samples were used directly to make cDNA without any further purification.

The cDNA was generated using the reverse transcription kit (Bio-rad). The reaction setup was made as follows: 1 μg of RNA template was mixed with 1 μL transcriptase and 4 μL reaction mix containing the random hexamers in total 20 μL reaction volume. The reaction mix was then incubated at 25 ^{o}C for 5 min; 42 ^{o}C for 30 min and 85 ^{o}C for 5 min. The cDNA product was applied to PCR without clean-up. The transcription of the inserted gene in the transformants was verified by amplifying a partial sequence of the inserted genes (150-200 bp) from cDNA in PCR. A housekeeping gene, *beta-actin*, was used as control to confirm the quality of cDNA and preliminarily analyze the transcription levels of the inserted gene by comparing the intensity of amplified bands in the gel electrophoresis. The PCR reactions were set up in 50 μL reaction volumes containing: 5 μL 10× RUN buffer; 1 μL 10 μM dNTP; 2.5 μL 10 μM forward primer and 2.5 μL 10 μM reverse primer (Table 1); 1 μL RUN polymerase (A&A Biotechnology); cDNA template and water added up to 50 μL. The cycling parameters in the PCR program was: Initial denature step at 95 ^{o}C for 3 min; 25 cycles of denature step at 94 ^{o}C for 30 s; annealing step at 55-65 ^{o}C for 30 s; elongation step at 72 ^{o}C for a specific amount of time calculated by the size of desired fragments (1min/kb); followed by a final elongation step at 72 ^{o}C for 5 min.

Table 1 Primers used in this research

Name	Sequence (5'→3')	Annotation
Pck uFw1	AGAGCGAUATGACTGACTTAA ACAAACTCGTT	USER cloning of pepck
Pck uRv1	TCTGCGAUTTATGCTTTTGGAC CGGCGCCA	USER cloning of pepck
Ppc uFw1	AGAGCGAU ATGAACGAACAATATTCCGCA	USER cloning of ppc
Ppc uRv1	TCTGCGAU AGATTAGCCGGTATTACGCAT	USER cloning of ppc
Pck Fw1	GCTTAAGAATGCCGCACCGAA	PCR of pepck on cDNA
Pck Rv1	TTATGCTTTTGGACCGGCGCCA	PCR of pepck on cDNA
Ppc Fw1	CGATTGCCAACGATTCCCAT	PCR of ppc on cDNA
Ppc Rv1	AACTCGGCCTGCAATACGTTC	PCR of ppc on cDNA
Actin Fw	AGAGCGGTGGTATCCATGAG	PCR of beta-actin on cDNA
Actin Rv	TGGAAGAGGGAGCAAGAGCG	PCR of beta-actin on cDNA

Enzyme activity assay

A. carbonarius wild type and transformants were cultivated in YPD medium for 24 h at 30 ^{o}C. The mycelium were then harvested and washed with 0.01M Tris-HCl buffer before suspension in 0.01 M Tris-HCl buffer supplemented with 0.2 M KCl. The cell extract was prepared by treating the mycelia suspension with glass bead beaters for 2 min in a Fast Prep®-24 instrument (MP Biomedicals) followed by centrifugation at 20.000 x g for 10 min. The supernatant was used directly for enzyme assay [29].

All the enzymes were assayed spectrophotometrically using the Spectrophotometer DR3800 (Hach company) at room temperature in 1.5 mL cuvettes (1.0 cm light path). The PPC activity was measured in a reaction solution containing 0.1 M Tris-HCl buffer at pH 8.0, 0.01 M $MgCl_2$, 2.5 mM phosphoenolpyruvic acid, 0.2 mM NADH, 0.01 M $NaHCO_3$ and 5 units of malate dehydrogenase [17]. The reaction was started by adding cell extracts containing 0.4 mg protein and the rate of decrease in absorbance of NADH at 340 nm was measured. The extinction coefficient was 6.22 mM^{-} cm^{-} . The specific enzyme activity was calculated as the oxidation of 1 µmol NADH based on the amount of protein per minute at 30 ^{o}C and pH 8.0. For measuring enzyme activity of AsPEPCK towards carboxylation of PEP,

2.5 mM ADP was added into the reaction mixture with the same assay procedure as described above for EcPPC. The enzyme activity of AsPEPCK in double transformants was obtained by subtracting the enzyme activity of EcPPC from the total enzyme activity measured with PEPCK enzyme assay in the same sample. The concentration of protein in cell extract was measured by using BCA protein assay reagent kit (Thermo Scientific). The increased fold of total enzyme activities of PEP carboxylation in the selected transformants was calculated by summing up measured activities of PEPCK and PPC in the individual strains and comparing the activity of selected transformants to the wild type.

Organic acid production

Spores of fungal strains were harvested from PDA medium after 5-7 days of cultivation at 30 ^{o}C. The spores were collected by filtering through sterilized Miracloth (Fisher Scientific) to remove the mycelium and counted in a haemacytometer followed by inoculation into 50 mL falcon tubes containing 10 mL pre-culture medium. The final concentration of spores in the pre-culture medium was approximately 1 x 10^{5} /mL. The pre-cultivation was carried out at 30 ^{o}C with agitation at 250 rpm for 2 days. Pellets formed in the pre-cultivation were filtered through sterile miracloth and added to 20 mL production media in 100 mL flasks and incubated 30 ^{o}C with agitation of 180 rpm for 7 days. The initial pH of the production media were adjusted to 5.5 before inoculation. All the cultivations were carried out in triplicates.

Analysis of extracellular metabolites

The samples taken from the cultivation broth were acidified with 72% sulfuric acid to precipitate the calcium ion in form of calcium sulfate and exchange the organic acid back to liquid phase. The acidified samples were incubated at 80 ^{o}C for at least 15 min to complete the reaction followed by centrifugation at 14000 rpm for 1 min. HPLC analysis of the supernatants for sugar (glucose and xylose) and organic acids were carried out in an Aminex 87H column (Biorad) at 60 ^{o}C by using HPLC mobile phase at a flow rate of 0.6 mL/min. The HPLC samples were kept at 4 ^{o}C in the machine during the analysis process and then stored at -20 ^{o}C. Beside HPLC analysis, measurements of L-malic acid and D-gluconate in the samples were carried out using L-malate (L-malic acid) kit and D-gluconate kit (Megazyme).

Fungal biomass measurement

To measure the dry weight of fungal biomass obtained in non-buffered pH conditions, the fungal culture were filtered through the filter paper (Whatman) and then washed thoroughly with distilled water until pH reached 6.0. The washed fungal cells on the filter paper were dried at 100 ^{o}C for 48 h before weighing. The measurement of fungal biomass obtained in pH buffered condition was carried out

in the same procedure as described before except an additional acidification step. The fungal culture was acidified with 1N HCl solution to dissolve calcium carbonate before the washing step with distilled water.

Statistical analysis

Unless specified, all the experiments were carried out in triplicates and the average value with standard deviation are reported. T-test analysis was performed to evaluate the significant difference in the reported results ($p=0.05$ as the threshold of significant difference).

Results

Analysis of extracellular organic acids produced by wild type *A. carbonarius* under non-buffered and buffered pH conditions

In order to analyze organic acid production by *A. carbonarius* wild type strain, cultivation was carried out in shake flasks in non-buffered pH and buffered pH 5.5 conditions in glucose based media. At non-buffered pH, *A. carbonarius* produced approx. 40 g/L citric acid (Fig. 2A) as the main extracellular organic acid and low amount of oxalic acid (1.8 g/L). During the cultivation, the pH decreased to 2-2.5 within 3 days and stayed at this level for the rest of the period of cultivation (Appendix A-1). During cultivation at pH 5.5, which was maintained by addition of $CaCO_3$, the pattern of extracellular organic acids was different. Instead of accumulating citric acid as the main organic acid, high amount of gluconic acid was produced due to the direct conversion of glucose catalyzed by glucose oxidase, which also led to a rapid depletion of glucose as carbon substrate after the first 7 days (Fig. 2B). A shift of carbon source from glucose to gluconate occurred in the later phase of cultivation, and this shift resulted in a continued production of citric acid. In addition, malic acid was present at a concentration of 0.06 g/L in these conditions on day 7, which indicated that *A. carbonarius* was capable of transiently producing malate during the cultivation (data not shown). However, malic acid was not detected in measurable quantity in non-buffered pH cultivation.

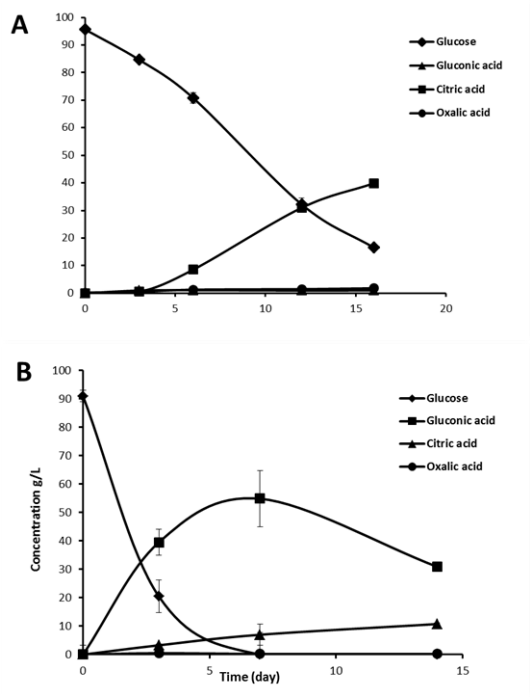

Fig. 2 Glucose consumption and production of major extracellular organic acids **(A)** concentration of measured organic acids (g/L) at non-buffered pH (error bars are presented in the curves but may be invisible due to the size); **(B)** concentration of measured organic acids (g/L) at buffered pH 5.5.(in shake flasks, 180 rpm, at 30 ^{o}C)

Insertion of gene *pepck* and *ppc* in *A. carbonarius* by protoplast transformation

The *ppc* and *pepck* genes were amplified from *E. coli* K-*12* and *A. succinogenes* 130Z respectively, and then cloned into vector pSBe1 for heterologous expression in *A. carbonarius* wild type strain. After inserting the target genes into the vector, the resulting plasmids, pSBe1ppc and pSBe1pepck (Appendix A-2), were used in protoplast transformation of *A. carbonarius*. For co-transformation, the plasmid pSBe1pepck was linearized at the *NcoI* restriction site to inactivate the *hph* gene and was used for transformation of *A. carbonarius* together with plasmid pSBe1ppc. Transformants with inserted *hph* gene were able to grow on minimal medium with 100 µg/mL hygromycin. Nine stable transformants with the *pepck* gene, ten transformants with the *ppc* gene and three double transformants with both genes inserted were obtained. All transformants were screened for organic acid production in glucose based medium at pH 5.5 (data not shown). The best organic

acid producing transformants of each type of genetic modification were selected for further analysis.

Transcription analysis of gene *pepck* and *ppc*

In order to verify the expression of the inserted genes in the derived strains, cDNA was synthesized from purified RNA of the three selected transformants and then used as templates in PCR to amplify partial sequences of target genes. As shown in Fig. 3, expression of *pepck* and *ppc* was verified by PCR. As a control for the quality of the cDNA, PCR amplification of beta-actin on the cDNA was carried out.

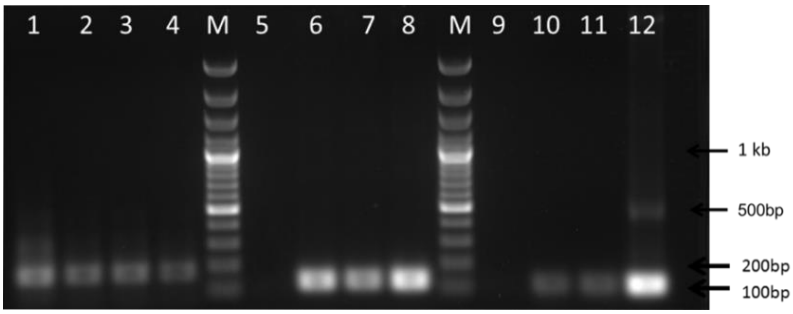

Fig. 3 PCR verification of the transcription of ppc and pepck genes using cDNA as template Amplification of beta-actin DNA (approx. 180 bp): lane 1, wild type; lane 2, *pepck* transformant; 3, *ppc* transformant; 4, double transformant; Amplification of *pepck* gene (approx. 150 bp): lane 5, wild type; lane 6, *pepck* transformant; lane 7, double transformant; lane 8, positive control (plasmid pSBe1pepck); Amplification of *ppc* gene (approx. 140 bp): lane 9, wild type; lane 10, *ppc* transformant; lane 11, double transformant; lane 12, positive control (plasmid pSBe1ppc). The appearance of a smear band might be caused by overloaded DNA templates

Enzyme assays

The enzyme activities of PEP carboxylase (EcPPC) and PEP carboxykinase (AsPEPCK) were measured in *A. carbonarius* wild type and in the selected transformants. As shown in Fig. 4A, the EcPPC activity was detected in cell extract of single and double transformants containing the *ppc* gene but not in the wild type. Since no PPC activity has been reported to occur in filamentous fungi, the absence of PPC activity in the wild type of *A. carbonarius* was expected. However, the specific enzyme activity of AsPEPCK in the transformants could not be compared in a similar way as activity of native PEPCK in *A. carbonarius* was also present. The enzyme activity of the inserted AsPEPCK was measured by comparing the increase of PEPCK activity of the transformants compared to the wild type. The

activity of AsPEPCK in the double transformant was obtained by subtracting the measured EcPPC activity from total enzyme activity since the enzyme activity of PEPCK and EcPPC could not be distinguished in the PEPCK enzyme assay. As shown in Fig. 4B, a significant increase in PEPCK activity was measured in both single and double transformants compared with the wild type (0.0029 U/mg in *pepck* transformant and 0.0023 U/mg in double transformant, vs. 0.0011 U/mg in the wild type, $p < 0.05$), which is an indication of expression of AsPEPCK in the transformants. Due to the insertion of the two genes, the enzyme activity of PEP carboxylation was increased approx. 1.7 times, 2.5 times and 3.2 times in *pepck* transformant, *ppc* transformant and double transformant respectively compared to the wild type.

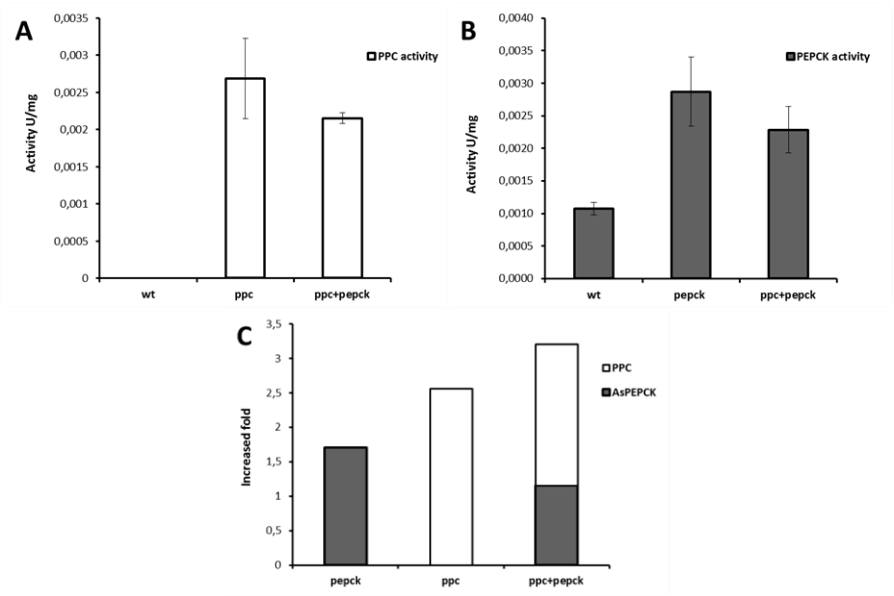

Fig. 4 Enzyme activity measurements in the wild type and the selected transformants **(A)** PPC activities (U/mg) in the wild type, *ppc* transformant and double transformants; **(B)** PEPCK activities (U/mg) in the wild type, *pepck* transformant and double transformants; **(C)** Estimated increased fold of enzyme activity contributed by the two genes *pepck* and *ppc* towards PEP carboxylation in transformants compared to the wild type (calculated results without standard deviations).

Effects of inserting *ppc* and *pepck* on organic acid production at non-buffered pH in glucose medium

In order to investigate the effects of the inserted *ppc* and *pepck* genes on organic acid production, especially citric acid production, the two single transformants were tested in shake flasks in glucose medium without pH control. During the cultivation, pH decreased gradually along with the organic acid production by the two transformants and the wild type, and reached a pH at 2-2.5 within 3 days (Appendix A-3). As shown in Fig. 5A-B, on both day 3 and 7, there was no significant difference in citric acid yield and glucose consumption rate by any of the strains. A minor increase in oxalic acid yield was observed in the derived strains compared to the wild type (Fig. 5C). All the strains produced a similar dry weight after 7 days cultivation (Fig. 5D)

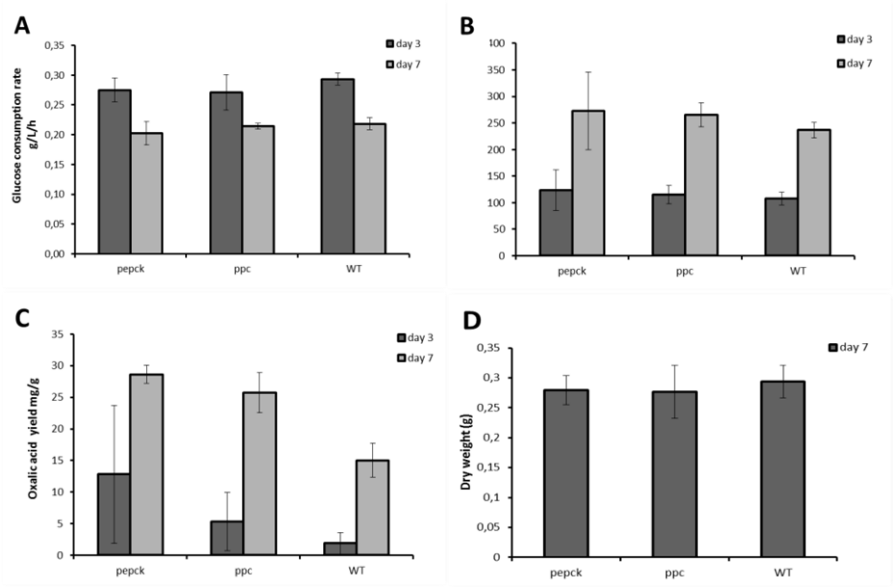

Fig. 5 Glucose consumption rate and major extracellular organic acids in non-buffered pH glucose medium **(A)** glucose consumption rate (g/L/h) by the wild type and selected transformants; **(B)** citric acid yield (mg/g glucose) in the wild type strain and selected transformants; **(C)** oxalic acid yield (mg/g glucose) in the wild type-strain and selected transformants; **(D)** dry weight (g) of fungal biomass after 7 days cultivation. (In shake flasks, 180 rpm, at 30 ^{o}C)

Effects of inserting *ppc* and *pepck* on organic acid production at buffered pH in glucose medium

In order to investigate the effects of the inserted *ppc* and *pepck* genes on organic acid production, especially citric acid production, the single and the double transformants were tested in shake flasks in glucose medium for 7 days at pH 5.5.

At this pH, the yield of citric acid was significantly increased in all the selected transformants compared with the wild type (Fig. 6C). After 7 days of cultivation, the citric acid yield of the wild type was 58 mg/g (mg citric acid/g glucose), whereas the *pepck* and *ppc* transformants with the single genetic modifications and the double transformant with two genetic modifications produced 109 mg/g, 138 mg/g and 118 mg/g citric acid, respectively ($p < 0.05$). A slight increase of malic acid production was also observed in the derived strains (Fig. 6D). However, the yield of gluconic acid, biomass growth and glucose consumption rate were not affected by the genetic modifications in any of the transformants compared with the wild type after 7 days of cultivation (Fig. 6A-B, E).

Effect of inserting *ppc* and *pepck* on organic acid production in xylose medium

In order to further investigate the impact of the alternative pathway on organic acid production, the three selected transformants were also tested in xylose based production medium. As shown in Fig. 7A and 7C, the genetic modifications did not significantly affect the xylose consumption rate and biomass growth in the transformants compared with the wild type. The yield of citric acid was significantly increased in all the selected transformants compared with the wild type after 7 days of cultivation (80 mg/g in *pepck* transformant, 91 mg/g in *ppc* transformant and 93 mg/g in double transformant vs 56 mg/g in the wild type, $p < 0.05$), although no increase in the yield of citric acid was observed on day 3 (Fig. 7B). It is also worth mentioning that, although no neutralizer was added to maintain the pH during cultivation, pH was still 3-3.5 in all the cultivation samples after 7 days, compared with pH 2-2.5 in glucose medium (Appendix A-3).

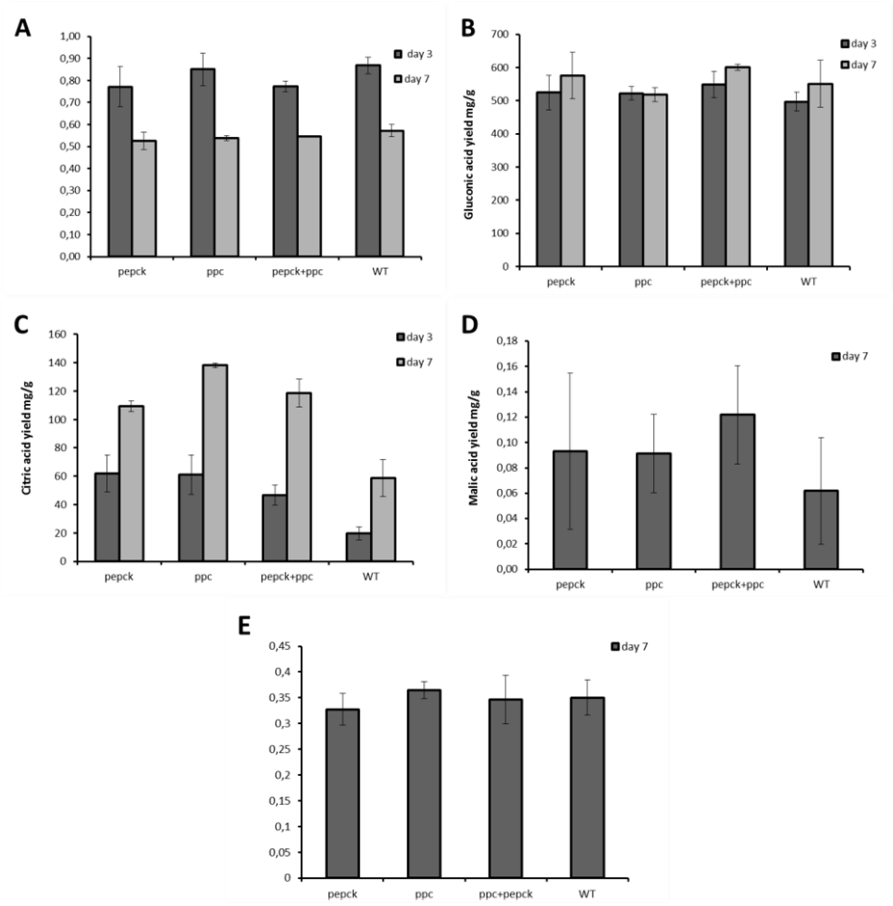

Fig. 6 Glucose consumption rate and major extracellular organic acids in buffered pH (5.5) glucose medium **(A)** glucose consumption rate (g/L/h) by the wild type and selected transformants; **(B)** gluconic acid yield (mg/g glucose) in the wild type strain and selected transformants; **(C)** citric acid yield (mg/g glucose) in the wild type strain and selected transformants; **(D)** malic acid yield (mg/g glucose) in the wild type strain and selected transformants; **(E)** dry weight (g) of fungal biomass after 7 days cultivation. (In shake flasks, 180 rpm, at 30 ^{o}C)

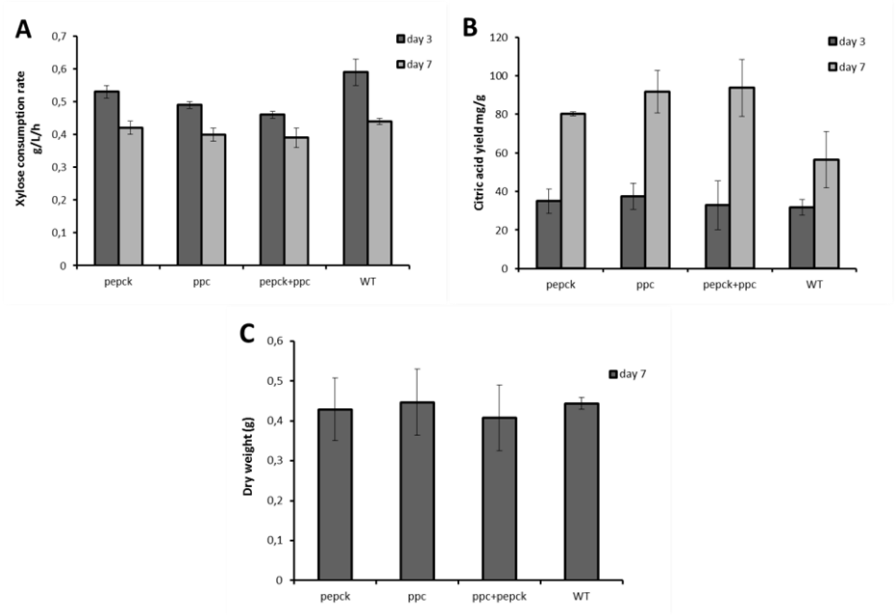

Fig.7 Xylose consumption rate and major extracellular organic acids in non-buffered pH xylose medium **(A)** xylose consumption rate (g/L/h) by the wild type and selected transformants; **(B)** citric acid yield (mg/g) in the wild type strain and selected transformants; **(C)** dry weight (g) of fungal biomass after 7 days cultivation. (In shake flasks, 180 rpm, at 30 ^{o}C)

Discussion

In this study, two different bacterial genes (*pepck* and *ppc*) that convert phosphoenolpyruvate directly to oxaloacetate were inserted in the filamentous fungus *A. carbonarius* ITEM 5010 in order to investigate the impact on organic acid production. The derived strains were tested under different cultivation conditions to exploit their potentials for the production of different organic acids. The profile analysis of the organic acids produced by *A. carbonarius* wild type strain was conducted and a comparison of the pattern of the different organic acids produced in non-buffered pH (2-2.5) and at buffered pH 5.5 conditions revealed that the acid producing pathways in *A. carbonarius* are influenced by ambient pH. Like *A. niger* [5, 30], *A. carbonarius* is able to produce high amount of citric acid at low pH under similar cultivation conditions, whereas at pH 5.5 which is usually adopted for producing malic acid or fumaric acid by filamentous fungi, the production of citric acid was almost inhibited and the resulting response by *A. carbonarius* was to accumulate high amount of gluconic acid using high glucose concentration as carbon source similar to what is reported in *A. niger* [4]. It was

observed that glucose was rapidly converted into gluconate by *A. carbonarius* leading to a rapid depletion of glucose in the early phase of cultivation (Fig. 2B). Although the gluconate could also be utilized by *A. carbonarius* as a carbon source, the uncertain effect of shifting carbon sources on cell metabolism might affect the productivity and amount of organic acid production.

In filamentous fungi, it has been shown that the rTCA branch is highly involved in production of different organic acids [17-20, 31]. In order to investigate the impact of increasing carbon flux towards the rTCA branch for organic acid production, a new cytosolic bypass was constructed by expressing the genes *pepck* from *A. succinogenes* and *ppc* from *E. coli* in *A. carbonarius*. Two derived strains with individual insertion of the *pp*c and *pepck* genes, respectively, were first tested in non-buffered pH condition. Oxalic acid increased slightly in the culture broth of the transformants compared with the wild type but there was no significant increase in the yield of citric acid. It seems that the impact of this cytosolic bypass is very limited on organic acid production in *A. carbonarius* in this condition. However, at pH 5.5 the yield of citric acid increased significantly in the engineered strains compared with the wild type. This result implies that the effects of the heterologous expressed genes on organic acid production are dependent on cultivation conditions. In addition to the different ambient pH, the carbon dioxide released from the neutralizer $CaCO_3$ may also facilitate the PEP carboxylation since it is required as the co-substrate in the reaction.

To explain the enhanced citric acid production observed in the engineered strains, it is likely that the alternative cytosolic pathway enhanced the carbon flux towards OAA, and then further increased the intracellular concentration of cytosolic C4-dicarboxylic acids which in *A. niger* has been demonstrated to positively correlate to citric acid production [18]. The competition between the inserted pathway and the original glycolytic pathway on the substrate PEP could lead to an increased carbon flux towards the reductive TCA pathway by reducing the generation of pyruvate. In eukaryotic cells, pyruvate carboxylase is normally regulated by acetyl-CoA, which may result in the strict allocation of carbon flux between OAA and acetyl-CoA [32, 33]. By introducing this alternative pathway, it provides the possibility to establish a new balance on those two key intermediates in the metabolic pathway in *A. carbonarius*. In an attempt to further enhance the carbon flux via the inserted bypass, a strain with two different genes (*ppc* and *pepck*) was constructed by transforming the wild type with two individual plasmids containing the *ppc* and *pepck* genes simultaneously. This double transformant was constructed to investigate eventual combined effects of the two genetic modifications on organic acid production. However, no further increase of citric acid production or any significant change in other organic acids was observed compared with the *ppc* and *pepck* single transformants. A similar result has been reported in *E. coli*, when AsPEPCK was heterologously expressed in *E. coli* containing the *ppc* gene. No significant increase in succinic acid production was observed in this strain [24]. In

another study, a positive synergistic effect of inserting the two genes in combination was observed on succinic acid production in *E. coli* when the EcPPC activity could be controlled below a certain level since AsPEPCK has lower affinity to PEP than EcPPC and it carries out a reversible reaction of PEP to OAA [34-36]. In addition, in *A. carbonarius*, the potential competition between the inserted bypass and the original glycolytic pathway on carbon flux may also limit the impact of expressing two enzymes in combination on organic acid production. Therefore, simply expressing these two genes in combination seems not to be a suitable strategy to reroute more carbon flow towards the new cytosolic bypass from glycolysis.

The three engineered strains were also tested in a xylose medium. In a previous study carried out on *A. carbonarius* wild type, it was shown that *A. carbonarius* was able to produce citric acid from xylose [11]. As xylose is utilized through the pentose phosphate pathway in the cell and the resulting carbon flux join into the glycolytic pathway (Fig. 1), it may also provide a similar platform to evaluate the effect of the inserted bypass on organic acid production. Under these conditions, the expression of the *ppc* and *pepck* genes also led to an enhanced citric acid production in the engineered strains, which is similar to the result obtained in the glucose medium at pH 5.5 but opposite to the results from non-buffered cultivation. Compared with the pH change in the non-buffered cultivation using glucose as substrate, it was shown that pH decreased slower in the xylose medium (Appendix A-3). After 7 days of cultivation, the final pH measured in the culture was 3-3.5. The slow pH decrease during cultivation may influence the productivity through the inserted bypass and the relevant metabolic pathways. In *A. niger*, oxalic acid was dramatically inhibited in cultivation at pH lower than 3 compared to pH 5. It was assumed that the oxaloacetate hydrolyase was inhibited by lowering the ambient pH below 3 [37]. Therefore, the enhanced citric acid production in the xylose medium also indicates that the impact of this new bypass on organic acid production by *A. carbonarius* exists in a certain pH range.

Finally, it seems that there is no clear difference between the bypasses carried out by AsPEPCK and EcPPC. The main difference in the reactions catalyzed by PPC and PEPCK is ATP yield. In the reaction carried out by PEPCK, one ATP is generated together with one molecule OAA. However, the reaction carried out by PPC is ATP neutral, as only one phosphate group is generated together with OAA. Therefore, compared with the original cytosolic reductive pathway, the inserted cytosolic bypass with AsPEPCK is supposed to influence both the carbon flux and energy balance in *A. carbonarius*. It has been reported in genetic engineered *E. coli* that higher succinic acid production was observed in transformants overexpressing AsPEPCK than overexpressing EcPPC [38]. The differences of those two genes were also observed in their impacts on cell growth when they were expressed in a *S. cerevisiae* mutant devoid of pyruvate carboxylases. Overexpression of EcPPC in *S. cerevisiae* Δ*pyc* mutant can restore the cell growth in glucose medium but it was not

observed in a strain overexpressing AsPEPCK [33, 39]. However, in this study, insertions of two genes individually into the wild type lead to a similar change in organic acid production in *A. carbonarius*. On the other hand, inserting this cytosolic bypass did not result in any overflow of malic acid or fumaric acid via the rTCA branch. The low amount of malic acid production observed in the transformants demonstrates that one genetic modification targeting in the onset of rTCA branch is not enough to reroute the carbon flux from the production citric acid to other organic acids like malic acid or fumaric acid.

Conclusions

In this study, a profile analysis of organic acid production was carried out in *A. carbonarius* wild type cultivated at non-buffered pH and at pH 5.5. The pattern of organic acids as well as the amount of organic acid production was influenced by pH. An alternative cytosolic pathway has been constructed by inserting two heterologous genes *pepck* and *ppc* in *A. carbonarius*. The derived strains carrying individual and combined gene insertions were tested to verify the impact of corresponding genetic modifications on organic acid production. An enhanced production of citric acid was obtained in all the derived strains in both glucose and xylose media when cultivation pH was above 3 but not in glucose cultivation at lower pH. This study demonstrates that insertion of *ppc* and *pepck* in *A. carbonarius* increases carbon flux towards the rTCA branch resulting in increased citric acid production and it also implies that the cultivation conditions, like ambient pH, affect the acid producing pathway. The further consideration on production process and conditions is necessary when designing strategies of metabolic engineering for specific products. A series of genetic modifications are required for improving the capability of *A. carbonarius* as a new cell factory for the production of different organic acids.

Competing interests and Ethical approval

The authors declare that they have no competing interests. This study does not contain any experiment with human participants or animals performed by any of the authors.

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References

1. Sauer M, Porro D, Mattanovich D, Branduardi P. Microbial production of organic acids: expanding the markets. Trends Biotechnol 2008;26(2):100-108.

2. Wee Y-, Kim J-, Ryu H. Biotechnological production of lactic acid and its recent applications. Food Technology and Biotechnology 2006;44(2):163-172.

3. Max B, Salgado JM, Rodríguez N, Cortés S, Converti A, Domínguez JM. Biotechnological production of citric acid. Brazilian J Microbiol 2010;41(4):862-875.

4. Ramachandran S, Fontanille P, Pandey A, Larroche C: Gluconic acid. Properties, applications and microbial production. Food Technology and Biotechnology 2006;44(2):185-195.

5. Papagianni M. Advances in citric acid fermentation by *Aspergillus niger*: Biochemical aspects, membrane transport and modeling. Biotechnol Adv 2007;25(3):244-263.

6. Wang L, Zhang J, Cao Z, Wang Y, Gao Q, Zhang J, Wang D. Inhibition of oxidative phosphorylation for enhancing citric acid production by *Aspergillus niger*. Microbial Cell Factories 2015;14(7).

7. Goldberg I, Peleg Y, Rokem JS. Citric, fumaric and malic acids. Biotechnology and Food Ingredients 1991;10(5):349-374.

8. Holladay J, Bozell J, White J, Johnson D. Top value-added chemicals from biomass. DOE Report PNNL-16983 2007.

9. Goldberg I, Rokem JS, Pines O. Organic acids: Old metabolites, new themes. Journal of Chemical Technology and Biotechnology 2006;81(10):1601-1611.

10. Ghareib M. Assimilation of galacturonate by *Aspergillus carbonarius*. Folia Microbiol (Praha) 1987;32(3):211-215.

11. Weyda I, Lübeck M, Ahring BK, Lübeck PS. Point mutation of the xylose reductase (XR) gene reduces xylitol accumulation and increases citric acid production in *Aspergillus carbonarius*. Journal of Industrial Microbiology and Biotechnology 2014;41(4):733-739.

12. Gallo A, Bruno KS, Solfrizzo M, Perrone G, Mulè G, Visconti A, Baker SE. New insight into the ochratoxin a biosynthetic pathway through deletion of a nonribosomal peptide synthetase gene in *Aspergillus carbonarius*. Appl Environ Microbiol 2012;78(23):8208-8218.

13. Sinha M, Sørensen A, Ahamed A, Ahring BK. Production of hydrocarbons by *Aspergillus carbonarius* ITEM 5010. Fungal Biology 2015;119(4):274-282.

14. Yang L, Lübeck M, Lübeck PS. Deletion of glucose oxidase changes the pattern of organic acid production in *Aspergillus carbonarius.* AMB Express 2014;4(54).

15. Abarca ML, Accensi F, Cano J, Cabañes FJ. Taxonomy and significance of black *aspergilli.* Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology 2004; 86(1):33-49.

16. Knuf C, Nookaew I, Remmers I, Khoomrung S, Brown S, Berry A, Nielsen J. Physiological characterization of the high malic acid-producing *Aspergillus oryzae* strain 2103a-68. Appl Microbiol Biotechnol 2014;98(8):3517-3527.

17. Zhang B, Skory CD, Yang S. Metabolic engineering of Rhizopus oryzae: Effects of overexpressing *pyc* and *pepc* genes on fumaric acid biosynthesis from glucose. Metab Eng 2012;14(5):512-520.

18. de Jongh WA, Nielsen J. Enhanced citrate production through gene insertion in *Aspergillus niger*. Metab Eng 2008;10(2):87-96.

19. Peleg Y, Stieglitz B, Goldberg I. Malic acid accumulation by *Aspergillus flavus* - I. Biochemical aspects of acid biosynthesis. Appl Microbiol Biotechnol 1988; 28(1):69-75.

20. Kenealy W, Zaady E, Du Preez JC. Biochemical aspects of fumaric acid accumulation by *Rhizopus arrhizus*. Appl Environ Microbiol 1986;52(1):128-133.

21. Karaffa L, Kubicek CP. *Aspergillus niger* citric acid accumulation: Do we understand this well working black box? Appl Microbiol Biotechnol 2003;61(3):189-196.

22. Sandor A, Johnson JH, Srere PA. Cooperation between enzyme and transporter in the inner mitochondrial membrane of yeast: Requirement for mitochondrial citrate synthase for citrate and malate transport in *Saccharomyces cerevisiae*. J Biol Chem 1994;269(47):29609-29612.

23. Gokarn RR, Eiteman MA, Altman E. Metabolic analysis of *Escherichia coli* in the presence and absence of the carboxylating enzymes phosphoenolpyruvate carboxylase and pyruvate carboxylase. Appl Environ Microbiol 2000;66(5):1844-1850.

24. Kim P, Laivenieks M, Vieille C, Zeikus JG. Effect of Overexpression of *Actinobacillus succinogenes* Phosphoenolpyruvate Carboxykinase on Succinate Production in *Escherichia coli*. Appl Environ Microbiol 2004;70(2):1238-1241.

25. McKinlay JB, Shachar-Hill Y, Zeikus JG, Vieille C. Determining *Actinobacillus succinogenes* metabolic pathways and fluxes by NMR and GC-MS analyses of 13C-labeled metabolic product isotopomers. Metab Eng 2007;9(2):177-192.

26. Peleg Y, Rahamim E, Kessel M, Goldberg I. Malic acid accumulation by *Aspergillus flavus* - II. Crystals and hair-like processes formed by *A. flavus* in a l-malic acid production medium. Appl Microbiol Biotechnol 1988;28(1):76-79.

27. Hansen NB, Lübeck M, Lübeck PS. Advancing USER cloning into simpleUSER and nicking cloning. J Microbiol Methods 2014; 96(1):42-49.

28. Gruber F, Visser J, Kubicek CP, De Graaff LH. The development of a heterologous transformation system for the cellulolytic fungus *Trichoderma reesei* based on a pyrG-negative mutant strain. Curr Genet 1990;18(1):71-76.

29. Payne J, Morris JG. Pyruvate carboxylase in *Rhodopseudomonas spheroides*. J Gen Microbiol 1969;59(1):97-101.

30. Andersen MR, Lehmann L, Nielsen J. Systemic analysis of the response of *Aspergillus niger* to ambient pH. Genome Biol 2009;10(5).

31. Gallmetzer M, Meraner J, Burgstaller W. Succinate synthesis and excretion by *Penicillium simplicissimum* under aerobic and anaerobic conditions. FEMS Microbiol Lett 2002;210(2):221-225.

32. Jitrapakdee S, Wallace JC. Structure, function and regulation of pyruvate carboxylase. Biochem J 1999;340(1):1-16.

33. Flores C, Gancedo C. Expression of PEP carboxylase from *Escherichia coli* complements the phenotypic effects of pyruvate carboxylase mutations in *Saccharomyces cerevisiae*. FEBS Lett 1997;412(3):531-534.

34. Kai Y, Matsumura H, Inoue T, Terada K, Nagara Y, Yoshinaga T, Kihara A, Tsumura K, Izui K. Three-dimensional structure of phosphoenolpyruvate carboxylase: A proposed mechanism for allosteric inhibition. Proc Natl Acad Sci U S A 1999;96(3):823-828.

35. Krebs W, Bridger WA. The kinetic properties of phosphoenolpyruvate carboxykinase of *Escherichia coli*. Can J Biochem 1980;58(4):309-318.

36. Tan Z, Zhu X, Chen J, Li Q, Zhanga X. Activating phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase in combination for improvement of succinate production. Appl Environ Microbiol 2013;79(16):4838-4844.

37. Ruijter GJG, Van De Vondervoort PJI, Visser J. Oxalic acid production by *Aspergillus niger*: An oxalate-non-producing mutant produces citric acid at pH 5 and in the presence of manganese. Microbiology 1999;145(9):2569-2576.

38. Moon SY, Hong SH, Kim TY, Lee SY. Metabolic engineering of *Escherichia coli* for the production of malic acid. Biochem Eng J 2008;40(2):312-320.

39. Zelle RM, Trueheart J, Harrison JC, Pronk JT, Van Maris AJA. Phosphoenolpyruvate carboxykinase as the sole anaplerotic enzyme in *Saccharomyces cerevisiae*. Appl Environ Microbiol 2010;76(16):5383-5389.

CHAPTER 3. DELETION OF GLUCOSE OXIDASE CHANGES THE PATTERN OF ORGANIC ACID PRODUCTION BY ASPERGILLUS CARBONARIUS

Deletion of Glucose Oxidase changes the pattern of organic acid production in *Aspergillus carbonarius*

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Abstract

Aspergillus carbonarius has potential as a cell factory for the production of different organic acids. At pH 5.5, *A. carbonarius* accumulates high amounts of gluconic acid when it grows on glucose based medium whereas at low pH, it produces citric acid. The conversion of glucose to gluconic acid is carried out by secretion of the enzyme, glucose oxidase. In this work, the gene encoding glucose oxidase was identified and deleted from *A. carbonarius* with the aim of changing the carbon flux towards other organic acids. The effect of genetic engineering was examined by testing glucose oxidase deficient (*Δgox*) mutants for the production of different organic acids in a defined production medium. The results obtained showed that the gluconic acid accumulation was completely inhibited and increased amounts of citric acid, oxalic acid and malic acid were observed in the *Δgox* mutants.

Keywords: *Aspergillus carbonarius*; citric acid; glucose oxidase; gluconic acid; malic acid

Introduction

With depletion of crude oil and increased environmental concern, biologically based processes for producing organic acids that can be used as building blocks in the chemical industries have begun to raise attention in recent years [1]. It is widely accepted that a suitable industrial strain for organic acid production is one of the key factors affecting the feasibility of the entire production process. Filamentous fungi have been well studied as cell factories for different production of organic acids for decades because they exhibit excellent abilities to utilize a variety of carbon sources and naturally accumulate high amount of specific organic acids under stressed conditions, like citric acid production by *Aspergillus niger* and malic acid production by *Aspergillus flavus* [2, 3]. Although the mechanisms of organic acid accumulation by different fungi have not been fully understood, many successful attempts have been made to improve organic acid production in filamentous fungi by using genetic modification and production optimization. In this study, *Aspergillus carbonarius*, which has a close phylogenetic relationship to *A. niger* [4] was selected to exploit its potential as a new cell factory for production of different organic acids. It resembles *A. niger* in many features, including morphology and high capacity of producing citric acid [5-7], which creates the possibility to apply the knowledge accumulated for *A. niger* directly to metabolic engineering of *A. carbonarius* for production of organic acids.

In organic acid production using filamentous fungi, pH is an important parameter. It has been reported that low pH is necessary for obtaining high yields of citric acid using *A. niger*, and reversely pH around 5~6 is preferred for producing malic acid using *A. flavus* and fumaric acid using *Rhizopus oryzae* [8, 9]. The production pattern of organic acids can also be changed dramatically in filamentous fungi by adjusting pH during cultivation. In *A. niger*, production of citric acid was dramatically suppressed at near neutral pH as gluconic acid started accumulating in high amount [10, 11] . In this study we investigated the effect of deleting the glucose oxidase in *Aspergillus carbonarius* for the purpose of organic acid production at pH 5-6. At this pH range, the fungus accumulates high amounts of gluconic acid, presumably due to secretion of glucose oxidase, whereby the fungus quickly converts glucose into gluconic acid outside the cell thus preventing further metabolism of glucose [12]. The aim of this study was therefore to eliminate the gluconic acid production in order to increase the carbon flux towards other organic acids. The hypothesis was: by eliminating gluconic acid production, *A. carbonarius* would increase 1,4-dicarboxylic acid production. Deletion of the gene encoding the glucose oxidase (*gox*) was conducted in *A. carbonarius* to suppress the conversion of glucose to gluconic acid. However, low frequency of homologous recombination in filamentous fungi often leads to very limited gene targeting efficiency in fungal transformation. Therefore, a Ku complex deficient strain of *A. carbonarius* [13], which is supposed to dramatically increase the homologous recombination frequency due to the inactivation of Ku complex, was selected for this work.

Materials and Methods

Strains and culture medium

A Ku deficient strain, KB1039 (*Δkus*A, obtained from K. Bruno, PNNL, US), which is also uracil auxotrophic (*ΔpyrG)*, of the wild type *A. carbonarius* ITEM5010 (ATCC® MYA-4641™) [13], was used as the parental strain to construct the *Δgox* mutants. Both the *A. carbonarius* KB1039 (*Δkus*A) and the wild type ITEM5010 was cultured at PDA (potato dextrose agar) medium at 30℃ and for the Ku deficient strain, the medium was supplemented with uracil and uridine, each at the final concentration of 2 mM.

Identification of the glucose oxidase gene in *A. carbonarius*

Due to lack of information about glucose oxidase (GOX) genes in *A. carbonarius*, the sequence of the *gox* gene in *A. niger* (accession no. X16061.1) was selected to identify the orthologous *gox* gene in *A. carbonarius* based on the close phylogenetic relationship between *A. niger* and *A. carbonarius.* One sequence with high identity was identified in the alignment hit as a putative *gox* gene in *A. carbonarius.* The sequence containing extension of 1000 bp from both 5' and 3' flanking regions of the *gox* encoding gene sequence was also identified with the purpose of using these 2 kb sequence flanking regions surrounding the putative *gox* gene for deletion of the gene. The sequence was submitted to GenBank with the accession no. KF741791.

Plasmid construction for gene knock-out

The genomic DNA was isolated from wild type *A. carbonarius* ITEM5010 by using phenol-chloroform extraction [14] and used as template to amplify 5' and 3' flanking regions of the *gox* gene with primers containing uracil overhang (Tab 1). Since the gene knock-out was carried out in a Ku deficient strain, it was not necessary to have very long fragments to increase the frequency of homologous recombination. It is reported that fragments about 1 kb both upstream and downstream to a target gene could provide efficient homologous recombination in *A. niger* [15]. Therefore, the size of fragments amplified from *A. carbonarius* was between 900 and 1000 bp.The PCR reaction was set up in 50 μL reaction volume: 5 μL 10X *pfu* turbox buffer; 1μL 10μM dNTP; 2.5 μL 10uM forward and reverse primer; 1 μL pfu turbo cx polymerase (Agilent); appropriate amount of DNA template and water added up to 50 μL. The PCR program was as follows: Initial denaturing step at 95℃ for 3 min; 25-30 cycles of denaturing step at 94℃ for 30s; annealing step at 55-65℃ for 30s; elongation step at 72℃ for specific amount of time calculated by the size of desired fragments (1min/kb); final elongation step at 72℃ for 5 minutes. The *pfu* turbocx polymerase was only used to amplify the DNA fragment with primers with uracil overhang in simpleUSER cloning [16], whereas

all other PCR reactions were carried out using RUN (taq) polymerase (A&A biotechnology). The plasmid pSB414 was designed and constructed for simpleUSER cloning [16] and contains the following genetic elements: *gpdA* promoter, *trpC* terminator and *pyrG* gene, including a specific cassette facilitating simpleUSER cloning. The cassette was activated by the restriction enzyme *Pac*I and the nicking enzyme *Nb.BbvCI* to generate the complementary overhang to the target fragments. The target fragments were cloned into the plasmid through self-assembly followed by transformation of *E. coli* with the plasmid for further propagation using standard procedures.

Protoplast transformation

Protoplasts of *A. carbonarius* were made from young mycelium harvested after overnight growth in YPD medium. The cell walls were degraded by 60 mg/ml of the commercial product Vino Taste Pro (Novozymes A/S) in protoplasting buffer (1.2 M $MgSO_4$, 50mM Phosphate Buffer, pH 5.0) for approx. 4 hours. Protoplasts were filtered and purified from the mixture, suspended in STC buffer (1.0 M sorbitol, 50 mM Tris, 50 mM $CaCl_2$ pH 8.0) and counted with appropriate dilution folds in a haemacytometer. The final concentration of protoplasts for aliquots was adjusted to $2x10^7$/mL. Protoplast transformation was carried out by adding 5 µg plasmids in 100 µl protoplast suspension and incubated on ice for 15 minutes followed by incubation for 15 minutes at room temperature after adding 1 mL of 40% PEG. The mixture was transferred into 10 mL minimum medium [13] with 1 M sorbitol at 30℃ for 1 hour with agitation at 80 rpm in the incubator shaker (KS 4000 I control, IKA). Then the cells were concentrated by centrifugation for 5 minutes at 800 x g, re-suspended in minimal medium containing 1M sorbitol and 0.8% agar and poured into petri-dishes. Next day, a second layer of the same medium was poured on the top. The plates with potential transformants were incubated at 30℃ for at least 3 days until transformants appeared.

Sporulating transformants were inoculated by streaking out on PDA medium and incubated at 30℃ overnight. Single colonies were identified and picked out to verify the deletion of the target gene by extracting the genomic DNA from the transformants and amplifying the fragments with expected size in PCR. The transformants were further transferred to normal PDA plates and preserved for further steps.

Growth conditions

Spores of fungal transformants were harvested from PDA plates after 5-7 days of cultivation at 30℃, and collected through sterilized Miracloth (EMD Millipore, USA) in sterile 0.05M phosphate buffer pH 6.8 in a 15 mL falcon tube. The spores were counted in a haemacytometer and then inoculated into 50 mL falcon tubes containing 10 mL pre-culture medium (3.6 g/L yeast extract and 10 g/L peptone).

The final concentration of spores in the pre-culture medium was approximately $1x10^5$/mL. Pre-cultivation was carried out at 30℃ with agitation of 250 rpm for 2 days. Pellets formed in the pre-culture medium were then transferred into production medium by filtering the pre-culture medium through Miracloth, and all the pellets on the top were collected and transferred into the production medium, which was modified from the production medium C described by Peleg et al., 1988: Glucose, 100 (g/L); $(NH_4)_2SO_4$, 2 (g/L); KH_2PO_4, 0.15 (g/L); K_2HPO_4, 0.15 (g/L); $MgSO_4$ $7H_2O$, 0.1 (g/L); $CaCl_2$ $2H_2O$, 0.1 (g/L); NaCl, 0.005 (g/L); $FeSO_4$ $7H_2O$, 0.005 (g/L), 0.1 g/L $ZnSO_4$and $CaCO_3$, 60 (g/L) [8]. Cultivation was carried out in 100 mL flasks containing 20 mL production medium at 30℃ with agitation of 180 rpm. The cultivation time varied from 7 to 10 days. The pre-culture and acid production was carried out in triplicates and pH was kept at 5.5 for the entire procedure.

Analysis of extracellular metabolites

Samples taken from organic acid fermentation were acidified with 72% sulfuric acid to a final concentration of 5% in order to precipitate the calcium ion in form of calcium sulfate and exchange the organic acids back to liquid phase. The acidified samples were incubated at 80℃ for at least 15 minutes to complete the reaction. After incubation, pH of the samples should be lower than 2 and was checked by pH indicator paper. The acidified samples were then centrifuged at 10,000 rpm for 1 minute. , and the supernatant was used for HPLC analysis. The analysis for sugar and organic acids were carried out in Aminex 87H column (Biorad) at 60℃ by using HPLC mobile phase at a flow rate of 0.6 mL/minute. The HPLC samples were kept at 4℃ in the machine during the analysis process and then stored at -20℃. The measurements of L-malic acid and D- gluconic acid in the samples were carried out respectively with L-malate (L-malic acid) kit and D-gluconate kit as described by the manufacturer (Megazyme).

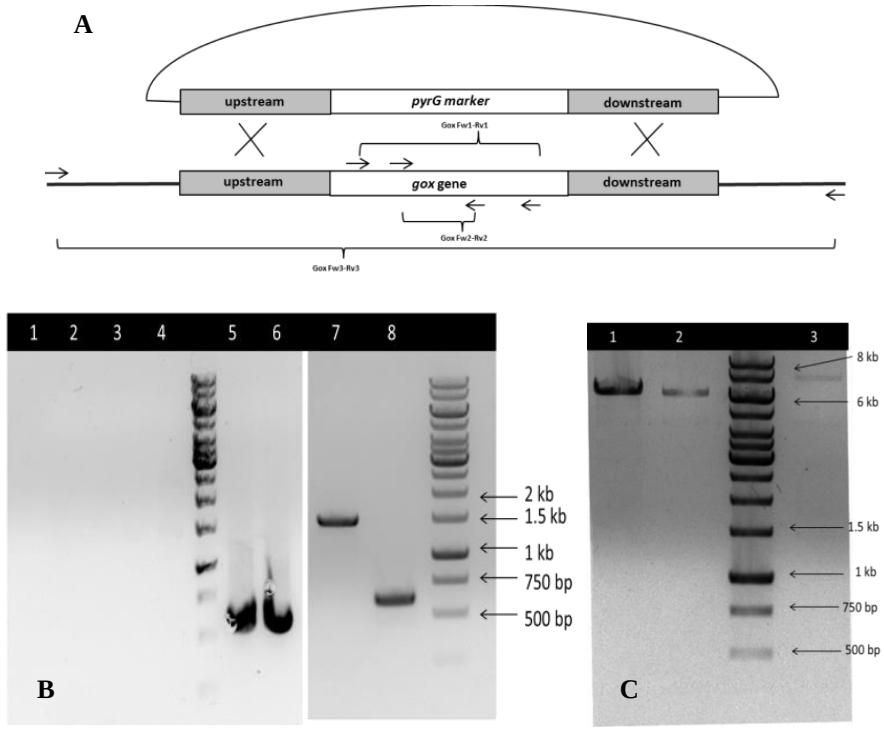

Figure 1 Verification of deletion of the *gox* gene in transformant 4 and 5 **(a)** Disruption of *gox* gene and primer binding sites **(b)** Amplification of internal sequence with primer Gox Fw1-Rv1 and Fw2-Rv2. Lane 1-2, *Δgox* transformant 4; lane 3-4, *Δgox* transformant 5; lane 5-6, ITS sequence amplified from *Δgox* transformant 4 and 5; lane 7-8, internal fragments of the gox gene from the wildtype strain (~1,5 kb and 0,6 kb) **(c)** Amplification of the *gox* gene containing region with the external primers Gox Fw3 and Rv3. Lane 1-2, amplified fragments from gox transformants 4 and 5. (~6.2 kb). Lane 3, amplified fragment from wild type (~7.7kb)

Results

Protoplast transformation and deletion of the *gox* gene from *A. carbonarius*

Protoplast transformation was carried out with circular pSB414*gox* plasmids. Two *Δgox* transformants were obtained and checked for the deletion of the putative *gox* gene by 3 different pairs of primers (Table 1). Two pairs of primers (Gox Fw1-Rv1 and Fw2-Rv2) amplified the internal sequence of the putative *gox* gene, and the third pair of primers (Gox Fw3-Rv3) was designed to amplify the putative *gox* gene together with the flanking regions in order to check the replacement of the *gox* gene

(Fig 1a). As shown in Figure 1b, no fragments could be amplified by the two pairs of internal primers in PCR, indicating that the *gox* gene was deleted. In PCR with external primers, a fragment at approx. 6.2 kb was amplified from both of the transformants, which indicated that the putative *gox* gene had been successfully replaced by the marker gene, since the length of the original sequence containing the *gox* gene was approx.7.7 kb (Fig 1c). A PCR amplifying the ITS region [17] confirmed the quality of the genomic DNA (Fig 1b). The size of all the amplified DNA fragments were estimated by comparing with 1 kb DNA ladder (Fig 1b)

Effect of deletion of the *gox* gene on gluconic acid production by *A. carbonarius*

The *gox* gene deleted from *A. carbonarius* is supposed to play a key role in the formation of gluconic acid. In order to evaluate the effect of this genetic modification on gluconic acid production, growth experiments were carried out with the *Δgox* mutants for 7 days at pH 5.5 by employing the *A. carbonarius* ITEM5010 and KB1039 (*Δkus*A) as control. As shown in Fig 2, the KB1039 (*Δkus*A) and wild type strain (ITEM5010) produced high amount of gluconic acid (72 g/L and 53 g/L respectively) in the production medium after 7 days, and both of the *Δgox* transformants only produced 0.1 g/L gluconic acid by the end of the growth experiment. However, in order to confirm that the low gluconic acid production was not caused by different growth rates of the *Δgox* mutants, the biomass of the *Δgox* mutants was determined after the growth experiment. As shown in Fig 3, the *Δgox* mutants produced similar amount of biomass as the parent strain, which indicated that they grew equally well under the same conditions. The result confirmed that deletion of the *gox* gene can effectively inhibit the formation of gluconic acid by *A. carbonarius*.

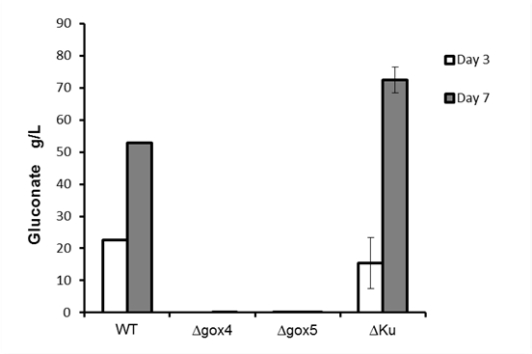

Fig 2 The results of fermentation test on *Δgox* mutants in malic acid production medium. Only one sample from wild type strain was measured for gluconic acid production, whereas all others were tested in triplicates

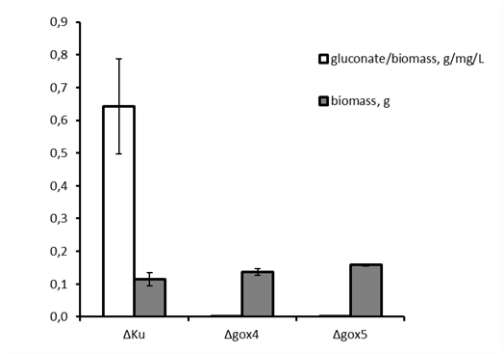

Fig 3 The yield of gluconic acid production based on the biomass growth. The yield was calculated based on the results from the samples taken on day 7

Analysis of extracellular metabolites of the *Δgox* mutants in the acid production media

Cultivation with the selected *Δgox* mutants was carried out in shaking flasks at pH 5.5 to investigate the effect of the *gox* gene deletion on production of organic acids in *A. carbonarius.* However, due to the accumulation and consumption of gluconic acid by wild type *A. carbonarius*, it is difficult to use glucose as the sole carbon source to compare the performance of wild type strain with the *Δgox* mutant during growth. The evaluation of organic acid production was carried out based on the concentration of extracellular acid products in the cultivation broth. As shown in Figure 4a, the concentration of citric acid was dramatically increased after 7 days cultivation in the *Δgox* mutants compared with the wild type ITEM5010 and the parent KB1039 (*Δkus*A) strains. In addition, an accumulation of oxalic acid was also observed in the *Δgox* mutants during the cultivation (Fig 4b) and the production of malic acid also increased 2.4 and 1.8 folds, respectively, compared with the parent strain and the wild type strain (Fig 4c).

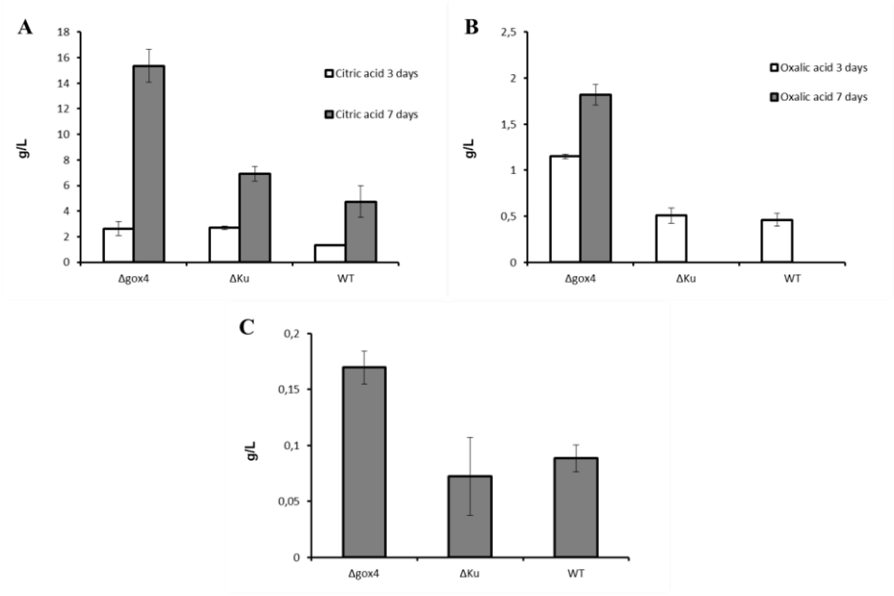

Fig 4 (a) The concentration of citric acid in the fermentation medium after day 3 and 7 **(b)** The concentration of oxalic acid in the fermentation medium after day 3 and 7 **(c)** The concentration of malic acid in the fermentation medium after day 7

Discussion

In the present work, a glucose oxidase gene (*gox*) in *A. carbonarius* involved in gluconic acid production was identified and deleted. In order to achieve a high gene targeting efficiency to facilitate the process of deleting the *gox* gene, a Ku deficient strain KB1039 (*ΔkusA*) was selected to construct *Δgox* transformants. The Ku complex including Ku70 and Ku80 has been reported to play an essential role in the non-homologous end joining pathway [18]. Deletion of the Ku encoding gene can inactivate the non-homologous recombination mechanism and increase the frequency of homologous recombination [15, 19, 20]. In this study it was shown that the transformed DNA solely was integrated via homologous recombination in all the obtained transformants which proved that Ku deficient strain could be used as an efficient tool in gene targeting and study of gene function in *A. carbonarius.* By deleting the *gox* gene in a *Ku* deficient strain, it was also shown that the production of gluconic acid was dramatically reduced in *Δgox* mutants. Evaluation of growth experiments after 7 days of cultivation showed that the mutants produced increased amounts of malic acid and citric acid as well as an accumulation of oxalic acid (Fig 4).

Since the aim of this work was to investigate the effect of deleting glucose oxidase in *A. carbonarius* on the production of organic acids at a pH where the glucose oxidase is expressed, the cultivation was carried out at pH 5.5. Under this condition, *A. carbonarius* was capable of producing high amount of gluconic acid in media containing high concentration of glucose, resulting in a fast depletion of glucose, and it was shown that the elimination of glucose oxidase had an impact on production of other organic acids. Sugar concentration is considered as an important factor in organic acid production for filamentous fungi and has been used e.g. for citric acid production by *A. niger*, malic acid by *A. flavus*, and fumaric acid and lactic acid by *R. oryzae* [21-23]. The effect of high sugar concentration on organic acid production has been investigated in *A. niger* for citric acid production. These studies indicate that high sugar concentration seems to repress the α-keto-glutarate dehydrogenase in *A. niger* and increases the intracellular concentration of fructose-2,6-bisphosphate which leads to high yield of citric acid production by activating phosphofructokinase (PFK1)[24, 25]. Reversely, the conversion of glucose to gluconic acid will decrease the sugar concentration very fast during cultivation and further eliminate the effect of high sugar concentration. This assumption was supported in this study by comparing the pattern of organic acid production in the *Δgox* mutants with the parental strain and the wild type strain after 3 days of cultivation. In the early phase of acid production, the glucose concentration still remained high although part of the glucose was already converted to gluconic acid. Therefore, the pattern of organic acids was similar among the *Δgox* mutants and the parental and wild type strains. However, when the glucose concentration in the media became much lower for the parental and wild type strains than for the *Δgox* mutants in the later phase of cultivation due to the accumulation of gluconic acid, the pattern of production of organic acid in the parental and wild type strains started varying from the *Δgox* mutants. The *Δgox* mutants produced higher amount of malic acid and citric acid compared with the parental and wild type strains, and after 7 days of cultivation, production of oxalic acid was also observed in the *Δgox* mutants.

The enhanced production of citric acid and malic acid as well as accumulation of oxalic acid in the *Δgox* mutants must be a result of a higher carbon flux through the glycolysis. Citric acid is solely produced through the TCA cycle, and the enhanced production indicates an improvement of the carbon flux into relevant pathways in *A. carbonarius* including glycolysis and TCA cycle. Moreover, the accumulation of oxalic acid might imply an elevated carbon flux towards cytosolic oxaloacetate reduction pathway which converted oxaloacetate to malate in the cytosol. Although, the oxalic acid producing pathway has not been well studied in *A. carbonarius*, it has been shown in *A. niger*, that oxalic acid is mainly produced by hydrolyzing oxaloacetate into oxalate and acetate by the enzyme oxaloacetase located in the cytosol [26]. Due to the close phylogenetic relationship between *A. niger* and *A. carbonarius*, it could be concluded that the accumulation of oxalic acid was most probably attributed to an increase of oxaloacetate in the cytosol, which was also in

accordance with the increased production of malic acid during cultivation. Therefore, the high sugar concentration and improved carbon flux towards acid producing pathway might result in the different organic acid pattern in *Δ*gox mutant.

It is very likely that malic acid in *A. carbonarius*is transiently produced but not capable of accumulating at high concentration, especially in the *Δgox* mutant, the enhanced carbon flux towards the cytosolic oxaloacetate reduction pathway was supposed to result in a much higher concentration of malic acid during cultivation, but this was not achieved in our experiments. Malate, as an important intermediate of the TCA cycle, plays multiple roles in cell metabolism. A dramatically increase of intracellular malate concentration might influence the cell function and therefore result in immediately reduction via other pathways. The transiently produced malate may not be exported and accumulated during cultivation since *A. carbonarius* is able to produce high amount of citric acid and the malate instead may be directly used in intracellular anti-port of citrate and malate across the membrane of mitochondria as it is suggested for *A. niger* [27]. Therefore, it may suggest that a simple change in cultivation condition and single genetic modification may not be enough to completely reroute the carbon flux from citric acid production to other organic acids. A series of genetic modifications may be required in the future work on metabolic engineering of *A. carbonarius* for production of valuable organic acids.

Competing interests

The authors declare that they have no competing interests.

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References

1. Holladay J, Bozell J, White J, Johnson D. Top value-added chemicals from biomass. DOE Report PNNL-16983, 2007.

2. Battat E, Peleg Y, Bercovitz A, Rokem JS, Goldberg I. Optimization of L-malic acid production by *Aspergillus flavus* in a stirred fermentor. Biotechnol Bioeng 1991;37(11):1108-1116.

3. Papagianni M, Mattey M, Berovĭ M, Kristiansen B. Aspergillus niger Morphology and Citric Acid Production in Submerged Batch Fermentation: Effects of Culture pH, Phosphate and Manganese Levels. Food Technology and Biotechnology 1999;37(3):165-171.

4. Thom C, Currie JN. *Aspergillus niger* group. Jour.Agr.Research 1916;7:1-15.

5. Ghareib M. Assimilation of galacturonate by *Aspergillus carbonarius*. Folia Microbiol (Praha) 1987;32(3):211-215.

6. Weyda I, Lübeck M, Ahring BK, Lübeck PS. Point mutation of the xylose reductase (XR) gene reduces xylitol accumulation and increases citric acid production in *Aspergillus carbonarius*. Journal of Industrial Microbiology and Biotechnology 2014;41(4):733-739.

7. Joosten HMLJ, Goetz J, Pittet A, Schellenberg M, Bucheli P. Production of ochratoxin A by *Aspergillus carbonarius* on coffee cherries. Int J Food Microbiol 2001;65(1-2):39-44.

8. Peleg Y, Rahamim E, Kessel M, Goldberg I. Malic acid accumulation by *Aspergillus flavus* - II. Crystals and hair-like processes formed by *A. flavu*s in a l-malic acid production medium. Appl Microbiol Biotechnol 1988;28(1):76-79.

9. Xu Q, Li S, Huang H, Wen J. Key technologies for the industrial production of fumaric acid by fermentation. Biotechnol Adv 2012;30(6):1685-1696.

10. Bercovitz A, Peleg Y, Battat E, Rokem JS, Goldberg I. Localization of pyruvate carboxylase in organic acid-producing *Aspergillus* strains. Appl Environ Microbiol 1990;56(6):1594-1597.

11. Goldberg I, Rokem JS, Pines O. Organic acids: Old metabolites, new themes. Journal of Chemical Technology and Biotechnology 2006;81(10):1601-1611.

12. Mischak H, Kubicek CP, Rohr M. Formation and location of glucose oxidase in citric acid producing mycelia of *Aspergillus niger*. Appl Microbiol Biotechnol 1985;21(1-2):27-31.

13. Gallo A, Bruno KS, Solfrizzo M, Perrone G, Mulè G, Visconti A, Baker SE. New insight into the ochratoxin a biosynthetic pathway through deletion of a nonribosomal peptide synthetase gene in *Aspergillus carbonarius.* Appl Environ Microbiol 2012;78(23):8208-8218.

14. Andreou L. Preparation of genomic DNA from bacteria. In *Methods in Enzymology* 2013;529:143-151.

15. Meyer V, Arentshorst M, El-Ghezal A, Drews A-, Kooistra R, van den Hondel CAMJJ, Ram AFJ. Highly efficient gene targeting in the *Aspergillus niger kusA* mutant. J Biotechnol 2007;128(4):770-775.

16. Hansen NB, Lübeck M, Lübeck PS. Advancing USER cloning into simpleUSER and nicking cloning. J Microbiol Methods 2014;96(1):42-49.

17. Bulat SA, Lubeck M, Alekhina IA, Jensen DF, Knudsen IMB, Lubeck PS. Identification of a universally primed-PCR-derived sequence-characterized amplified region marker for an antagonistic strain of Clonostachys rosea and development of a strain-specific PCR detection assay. Appl Environ Microbiol 2000;66(11):4758-4763.

18. Dudášová Z, Dudáš A, Chovanec M. Non-homologous end-joining factors of *Saccharomyces cerevisiae*. FEMS Microbiol Rev 2004;28(5):581-601.

19. Kooistra R, Hooykaas PJJ, Steensma HY. Efficient gene targeting in *Kluyveromyces lactis*. Yeast 2004;21(9):781-792.

20. Ninomiya Y, Suzuki K, Ishii C, Inoue H. Highly efficient gene replacements in *Neurospora* strains deficient for nonhomologous end-joining. Proc Natl Acad Sci U S A 2004;101(33):12248-12253.

21. Alvarez-Vasquez F, González-Alcón C, Torres NV. Metabolism of citric acid production by *Aspergillus niger*: Model definition, steady-state analysis and constrained optimization of citric acid production rate. Biotechnol Bioeng 2000;70(1):82-108.

22. Peleg Y, Stieglitz B, Goldberg I. Malic acid accumulation by *Aspergillus flavus* - I. Biochemical aspects of acid biosynthesis. Appl Microbiol Biotechnol 1988;28(1):69-75.

23. Ding Y, Li S, Dou C, Yu Y, He H. Production of fumaric acid by *Rhizopus oryzae*: Role of carbon-nitrogen ratio. Appl Biochem Biotechnol 2011;164(8):1461-1467.

24. Kubicek-Pranz EM, Mozelt M, Rohr M, Kubicek CP. Changes in the concentration of fructose 2,6-bisphosphate in *Aspergillus niger* during stimulation of acidogenesis by elevated sucrose concentration. Biochimica et Biophysica Acta - General Subjects 1990;1033(3):250-255.

25. Papagianni M. Advances in citric acid fermentation by *Aspergillus niger*: Biochemical aspects, membrane transport and modeling. Biotechnol Adv 2007;25(3):244-263.

26. Ruijter GJG, Van De Vondervoort PJI, Visser J. Oxalic acid production by *Aspergillus niger*: An oxalate-non-producing mutant produces citric acid at pH 5 and in the presence of manganese. Microbiology 1999;145(9):2569-2576.

27. de Jongh WA, Nielsen J. Enhanced citrate production through gene insertion in *Aspergillus niger*. Metab Eng 2008;10(2):87-96.

CHAPTER 4. CO-CONSUMPTION OF GLUCOSE AND XYLOSE FOR ORGANIC ACID PRODUCTION BY ASPERGILLUS CARBONARIUS CULTIVATED IN WHEAT STRAW HYDROLYSATE

Co-consumption of glucose and xylose for organic acid production by *Aspergillus carbonarius* cultivated in wheat straw hydrolysate

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Abstract

Aspergillus carbonarius exhibits excellent abilities to utilize a wide range of carbon sources and to produce various organic acids. In this study, wheat straw hydrolysate containing high concentrations of glucose and xylose was used for organic acid production by *A. carbonarius.* The results indicated that *A. carbonarius* efficiently co-consumed glucose and xylose and produced various types of organic acids in hydrolysate adjusted to pH 7. The inhibitor tolerance of *A. carbonarius* to the hydrolysate at different pH values was investigated and compared using spores and recycled mycelia. This comparison showed a slight difference in the inhibitor tolerance of the spores and the recycled mycelia based on their growth patterns. Moreover, the wild-type and a glucose oxidase deficient (Δ*gox)* mutant were compared for their abilities to produce organic acids using the hydrolysate and a defined medium. The two strains showed a different pattern of organic acid production in the hydrolysate where the Δ*gox* mutant produced more oxalic acid but less citric acid than the wild-type, which was different from the results obtained in the defined medium This study demonstrates the feasibility of using lignocellulosic biomass for the organic acid production by *A. carbonarius*.

Keywords

Aspergillus carbonarius; filamentous fungi; lignocellulosic biomass; organic acids; wheat straw hydrolysate

Introduction

For the past decades, lignocellulosic biomass has received extensive research attention as renewable raw materials for the production of varieties of fuels and chemicals. The abundance of lignocellulosic biomass on the earth reveals the potential as industrial precursors for the production of commodity chemicals. The advantages of using lignocellulosic biomass, compared with other raw materials obtained from crude oil or edible food crops, are demonstrated from different points of view including environmental sustainability, low greenhouse gas emission, food security and planting cost [1]. With the developments in the technologies of pretreatment and saccharification, the sugars originally existing in the lignocellulosic biomass in the form of polysaccharides can be degraded and eventually released as fermentable sugars for downstream processes. So far, a significant amount of efforts have been focused on utilizing the lignocellulosic biomass in the bioconversion processes based on microbial systems for varieties of products, especially biofuels [2-5]. However, the inhibitory effects resulting from the inhibitory compounds (e.g. acetic acid, furfural and 5-hydroxymethylfurfural), which are mainly generated by the side reactions during the biomass pretreatment, remain an issue for microbial growth [6-8]. Besides, the efficiency of utilizing both hexoses and pentoses in the cultivation is considered as another important criterion for selecting appropriate industrial strains, for example, the commonly used yeast for bioethanol production, *Saccharomyces cerevisiae*, is incapable of metabolizing pentoses unless genetically engineered. Among the studied microorganisms, filamentous fungi have raised research interest due to their abilities to utilize a wide range of carbon sources and to naturally excrete various products, especially organic acids [9, 10]. Fungal organic acid production has an important status in the commodity chemicals of industrial biotechnology. A number of organic acids are currently produced via biological processes employing filamentous fungi, such as the production of citric acid and gluconic acid by *Aspergillus niger*, itaconic acid production by *Aspergillus terrus* and kojic acid production by *Aspergillus oryzae* [11-14]. The expanding market of organic acids, which arises from their wide applications as food additives, pharmaceuticals, detergents and so on, leads to an increasing demand of raw materials in the industry. Therefore, the industrial production of organic acids in conjunction with utilization of lignocellulosic biomass becomes an inevitable trend in the future.

Aspergilli, as the well-known industrial workhorses for organic acid production, have been the most widely-studied fungal genus over a period of decades. Many attempts have been made on the bioconversion of different types of raw materials to organic acids in *Aspergillus* species. Various lignocellulosic biomass, especially agro-industrial residues like cassava bagasse, coffee husk, sugar cane bagasse and wheat straw, have been investigated for their potentials as substrates for organic acid production by Aspergilli [12, 15-17]. Theoretically, the most economically feasible process of using lignocellulosic biomass for fungal organic acid production

is to grow fungi in the culture where fungi can secrete the enzymes to degrade the biomass and use the released sugars simultaneously for organic acid production. However, the slow degradation of lignocellulosic biomass cannot fulfill the sugar demand by fungi during the production phase. Therefore, the separate processes of saccharification from fermentation, in which high concentration of fermentable sugars are obtained from efficient hydrolysis, may be more suitable for organic acid production. In this study, we selected *Aspergillus carbonarius*, which was previously reported for its capabilities of producing organic acids from various types of carbon sources including glucose, xylose, sucrose and galactose [18-21], to investigate its production of organic acids in a wheat straw hydrolysate. *A. carbonarius* is cultivated in static culture which has lower sensitivity to trace metals and better aeration than submerged culture in shake flasks (Darouneh et al. 2009). For investigation of inhibitor tolerance, fungal mycelia were recycled for organic acid production in the hydrolysate and compared with direct spore inoculation. In addition, an engineered strain devoid of glucose oxidase (Yang et al. 2014a) was used in a comparative study with the wild-type to examine the impacts of eliminating the extracellular conversion of glucose into gluconic acid on the production of other organic acids from the hydrolysate.

Materials and Methods

Strains and culture media

A. carbonarius wild-type strain ITEM 5010 (ATCC® MYA-4641™) and a previously constructed Δ*gox* mutant were employed in this study [20]. For preparation of spore suspensions, strains were grown in potato dextrose agar (PDA) medium at 30 ^{o}C for 5 days. For organic acid production, the defined medium contained: glucose, 70 g/L; xylose, 58 g/L, NH_3NO_4, 2.5 g/L; KH_2PO_4, 2.5 g/L; $MgSO_4$ $7H_2O$, 0.25 g/L; ZnSO4, 0.00062 g/L; $CuSO_4$, 0.00015 g/L; $FeSO_4$ $7H_2O$, 0.0065 g/L, and the initial pH in the defined medium was adjusted to 7. The wheat straw hydrolysate used in this study was provided by Biogasol®, Denmark. The pretreatment and hydrolysis of wheat straw was carried out by Biogasol®, Denmark under the conditions described previously by Baroi et al (2015). The composition of the liquid fraction of the wheat straw hydrolysate (Table 1) was determined in the same procedure as previously described [22], and the liquid fraction of wheat straw hydrolysate for fungal culture was prepared as follows: the wheat straw hydrolysate was centrifuged at 10,000 rpm for 20 min, and the liquid fraction of the wheat straw hydrolysate was collected and filtered through filter paper Whatman® no. 42 to remove the remaining particles in the liquid; the filtrate was supplemented with the same amounts of nutrients (except glucose and xylose) for organic acid production as mentioned in the defined medium. The original pH value in the prepared hydrolysate was approx. 5. For organic acid production, the initial pH values were adjusted to 3 and 7 by adding 10 M HCl and NaOH powder, respectively. After pH

adjustment, the hydrolysate was sterilized with 0.2 μM sterile filter (Nalgene®) and used immediately for fungal cultivation.

Table 1 Composition of wheat straw hydrolysate

Hydrolysate	Concentration g/L
Glucose	70.1 ±0.3
Xylose	56.8 ±0.2
Arabinose	6.2 ±0.2
Cellobiose	2.1 ± 0.1
Acetic acid	7.1 ±0.2
Furfural	0.24 ±0.02
5-hydroxymethylfurfural	N.D

(Data shown are mean values from triplicates with the standard deviation after "±"; "N.D" stands for "not detected")

Organic acid production

The spores were harvested from the PDA medium with 5 mL sterile water, and the spore suspension was collected and filtered through the Mira-cloth for removal of excess mycelia. For the direct spore inoculation, the spores were inoculated into 50 mL liquid media (the hydrolysate or the defined medium) in 250 mL Erlenmeyer flasks at the final concentration of 5 x 10^4 /mL, and the flasks were incubated stationary at 30 ^oC. For the cultivation with mycelia-recycling in the hydrolysate, the spores were inoculated into 50 mL hydrolysate (pH 7) in 250 mL Erlenmeyer flasks at the same concentration as described above. After 10 days stationary cultivation at 30 ^oC, cultivation broth under the mat-like mycelia was removed from the flasks. The fresh hydrolysate (50 mL) was added into the flask under the grown mycelia. The flasks were incubated stationary at 30 ^oC. All the cultivations in this study were carried out in triplicates.

Analysis

The samples were taken from the cultivation broth after gently shaking the flasks, and then filtered through 0.45 μM filters for the following analysis. HPLC analysis for sugars and organic acids were carried out in an Aminex 87H column (Biorad®)

at 60 ^{o}C by using HPLC mobile phase (5 mM H_2SO_4) at a flow rate of 0.6 mL/min. The concentration of gluconic acid was measured using a D-gluconate kit and following the protocol provided by the supplier (Megazyme®).

Fungal biomass measurement

The fungal cultures were filtered through filter paper followed by a thoroughly washing step with distilled water until pH reached 6.0. The washed fungal cells on the filter paper were dried at 100 ^{o}C for 48 h before weighing. The filter paper was dried at 100 ^{o}C for 48 h before use.

Results

Morphology of *A. carbonarius* in static culture

The wild-type was grown in the hydrolysate for organic acid production with initial pH adjusted to three different values (3, 5 and 7). The cultivation was carried out stationary in 250 mL Erlenmeyer flasks at 30 ^{o}C. The spores that were inoculated to the hydrolysate with initial pH values of 3 and 5 remained dormant throughout the cultivation. In the hydrolysate with initial pH adjusted to 7, the germination of spores was observed on day 2, and the mycelial growth became visible on the surface of the hydrolysate from day 3. On day 5, mat-like mycelia had formed on the surface of the hydrolysate. On day 7, intensive sporulation was observed on the top of the mycelia (Fig. 1a-c).

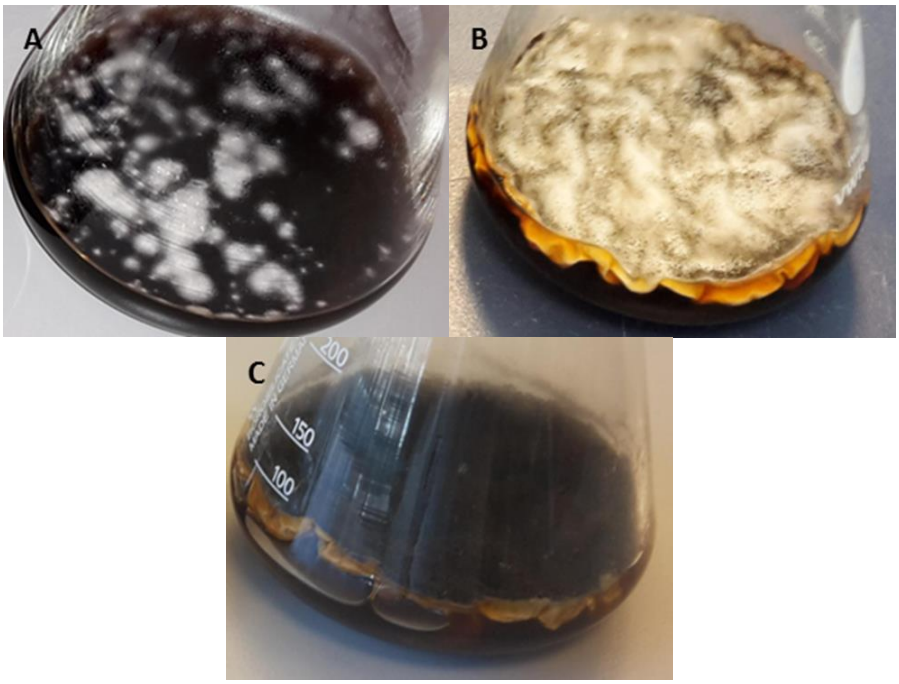

Fig. 1 Morphology of *A. carbonarius* wild-type in the wheat straw hydrolysate with initial pH adjusted to 7 (**a)** mycelia growth on day 3; (**b**) mycelia growth on day 5; (**c**) mycelia growth on day 7

Organic acid production by *A. carbonarius* wild-type from the hydrolysate

The initial concentrations of measured compounds in the hydrolysate are shown in Table 1. In the hydrolysate with initial pH 7, furfural was depleted by the wild-type after 2 days cultivation, and a significant decrease in the concentrations of acetic acid and glucose was observed from day 3 (Fig. 2a-b). Acetic acid was depleted very rapidly by day 5 (Fig. 2b). The glucose consumption increased dramatically from day 4 and approx. 90% of glucose was consumed from day 4 to day 7. Xylose was also consumed by the wild-type from day 5 while there was still plenty of glucose left in the hydrolysate. However, the consumption of xylose was slower than that of glucose and in total, 30 g/L xylose was consumed by the wild-type after 10 days cultivation (Fig. 2a). In the hydrolysate with initial pH values at 3 and 5, no sugar consumption was observed by the end of the cultivation, which was also in consistency with the morphological observations (Fig. 2c).

The production of organic acids was therefore observed only in the hydrolysate with initial pH 7. From day 3, gluconic acid was produced by *A. carbonarius* prior to other organic acids. The concentration of gluconic acid increased rapidly to 19.8

g/L until day 4 and remained at this value for the rest of the cultivation (Fig. 2a). Citric acid and oxalic acid were detected at concentrations of 1.9 g/L and 2.1 g/L respectively on day 4. From day 6, the production of citric acid and oxalic acid slowed down significantly and reached 12.6 g/L and 6.5 g/L after 10 days, respectively (Fig. 2a). The wild-type consumed xylose from day 5 to day 10. However, the utilization of xylose did not result in a further increase in the concentration of any organic acid after day 7 when the glucose was depleted. The pH value changed in full compliance with the organic acid production obtained during the cultivation. The cultivation pH decreased gradually from day 3 and reached approx. 3 on day 5(Fig. 2b). No further decrease in culture pH was observed after day 5.

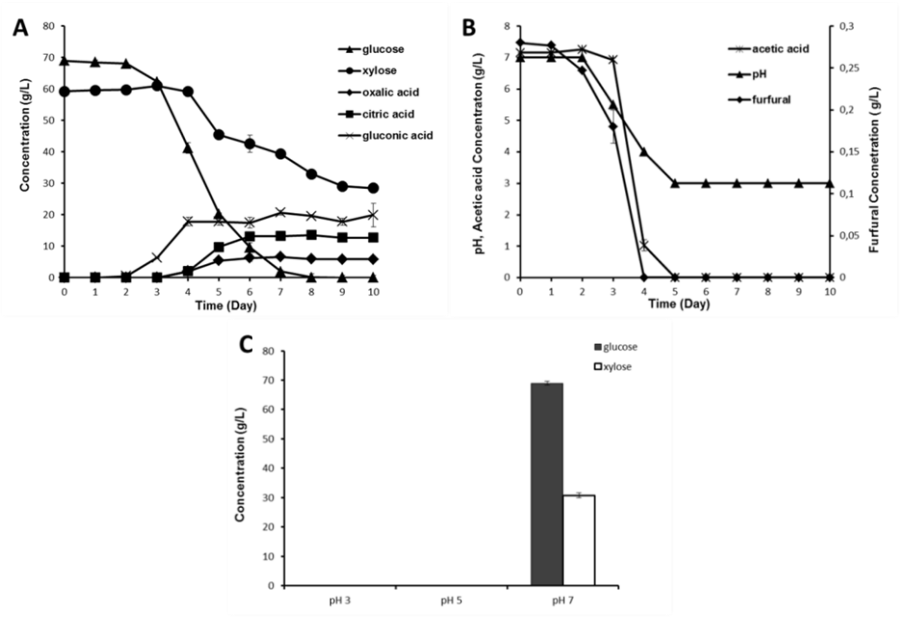

Fig. 2 Cultivation of *A. carbonarius* wild-type in the hydrolysate with initial pH 7 (**a**) sugar consumption and organic acid production; (**b**) conversion of detected inhibitors; (**c**) total sugar consumption at three pH values after 10 days (Data shown are mean values from triplicates with error bars indicating the standard deviation)

Recycling fungal mycelia for organic acid production from the hydrolysate

Mat-like mycelia obtained from the first batch cultivation for 10 days in the hydrolysate with initial pH 7 was used in the following cultivation. After fresh hydrolysate with three different pH values (3, 5 and 7) was added into the second batch cultivation, respectively, recycled mycelia floated on the surface of the

culture. In the hydrolysate with initial pH 3, neither fungal growth nor sugar consumption was observed by the end of the cultivation (Fig. 3c). Oppositely, both sugar consumption and organic acid production were obtained in the hydrolysate with initial pH 5 and 7, but the fungal biomass only increased significantly in the hydrolysate with initial pH 7 (Fig. 3d). Rapid glucose consumption was observed in the hydrolysate with initial pH 5 and 7 on day 1. However, there was no further glucose consumption in the hydrolysate with initial pH 5. In the hydrolysate with initial pH 7, glucose consumption slowed down from day 2 to day 4, and the recycled mycelia consumed only 4.2 g/L glucose during this period. After day 4, the recycled mycelia utilized glucose more rapidly, and 58.2 g/L glucose was consumed by day 10 (Fig. 3a). Acetic acid and furfural were depleted before day 4, and 25.8 g/L xylose was also consumed simultaneously with glucose by the recycled mycelia (Fig. 3a-c).

For organic acid production, gluconic acid production emerged after 1 day from the hydrolysate with initial pH 7. The recycled mycelia produced 10.5 g/L gluconic acid during the first day, but the concentration of gluconic acid only reached 13.5 g/L after 10 days cultivation (Fig. 3a). The production of oxalic acid and citric acid began from day 3, and reached 4.6 g/L and 3.9 g/L respectively on day 6 (Fig. 3a). After day 6, there was no further increase in either citric acid or oxalic acid production. Meanwhile, the cultivation pH decreased from 7 to 5.5 after 1 day and remained at this level until day 3. The final cultivation pH reached 3 from day 6 until the end of the cultivation (Fig. 3b). In the hydrolysate with initial pH 5, pH decreased from 5 to 4.5 after 1 day and remained at this level for the rest of the cultivation. In total, 8.4 g/L gluconic acid was produced by the recycled mycelia (Fig. 3c).

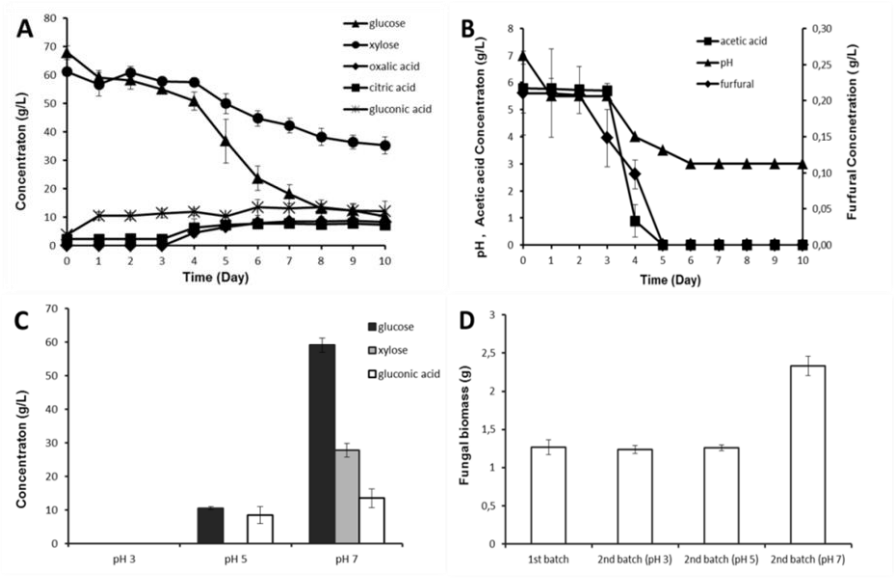

Fig. 3 Cultivation of recycled mycelia in the hydrolysate at three pH values (**a**) sugar consumption and organic acid production in the hydrolysate at pH 7; (**b**) the conversion of detected inhibitors and cultivation pH in the hydrolysate at pH 7; (**c**) total sugar consumption and gluconic acid production at three pH values after 10 days of cultivation; (**d**) Comparison of fungal biomass between two batch cultivations at three pH values (Data shown are mean values from triplicates with error bars indicating the standard deviation)

Comparison of organic acid production between the wild-type and Δ*gox* mutant

The organic acid production by the Δ*gox* mutant and the wild-type were compared during cultivation for 10 days in the hydrolysate with initial pH 7 and in the defined medium mimicking the concentrations of glucose and xylose in the hydrolysate. In the defined medium, only slight differences in the production of organic acids were observed between the Δ*gox* mutant and the wild-type (Table 2). Both the wild-type and the Δ*gox* mutant lowered the pH from 7 to 2.5 within 3 days after inoculation, and co-consumption of xylose and glucose was observed in both of strains. After 10 days, glucose and xylose were almost depleted (Fig. 4a-b).

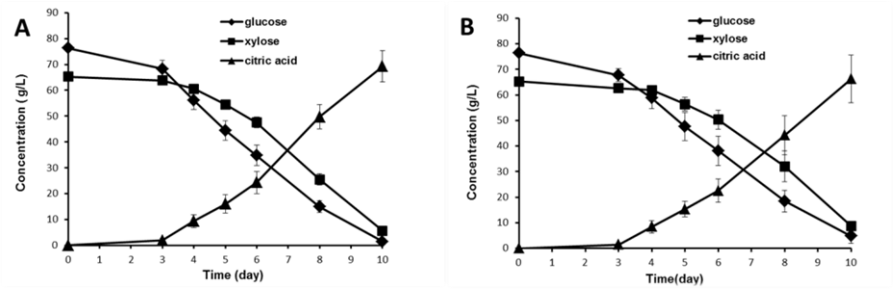

Fig. 4 Cultivation of the wild-type and the Δ*gox* mutant in the defined medium (**a**) the wild-type; (**b**) Δ*gox* mutant (Data shown are mean values from triplicates with error bars indicating the standard deviation)

Table 2 Comparison of organic acid production in the defined medium on Day10

Organic acid	**wild-type strain**	**Δ*gox* mutant**
Citric acid	69.3 ± 6.1 g/L	67.4 ± 7.3 g/L
Oxalic acid	0.12 ± 0.04 g/L	0.10 ± 0.02 g/L
Gluconic acid	0.25 ± 0.09 g/L	N.D

(Data shown are mean values from triplicates with the standard deviation after "±"; "N.D" stands for "not detected".)

In the hydrolysate adjusted to pH 7, both the wild-type and the Δ*gox* mutant consumed sugars and converted acetic acid and furfural in the same way (Fig. 5a-b). Furfural and acetic acid were depleted within the first three days before the utilization of sugars started. The organic acid production profile in the hydrolysate was different from the profile in the defined medium (Table 3). The maximum titers of organic acids were already reached after day 5 for the Δ*gox* mutant and after day 6 for the wild-type until day 10 where the experiment stopped (Fig. 5c-d). Probably due to the elimination of gluconic acid production in the Δ*gox* mutant, the pH decreased more slowly in the hydrolysate with the Δ*gox* mutant than the wild-type during the cultivation (Fig. 5e). Although the pH started decreasing in both of the cultures from day 3, the pH level was lowered to 3 in the culture with the wild-type on day 6 while it was still 3.5 in the culture with the Δ*gox* mutant. The wild-type consumed glucose more rapidly than the Δ*gox* mutant. However, the utilization of sugars (glucose and xylose) slowed down dramatically in both the wild-type and the Δ*gox* mutant after day 6. Then no further increase in organic acid production was observed in the two strains.

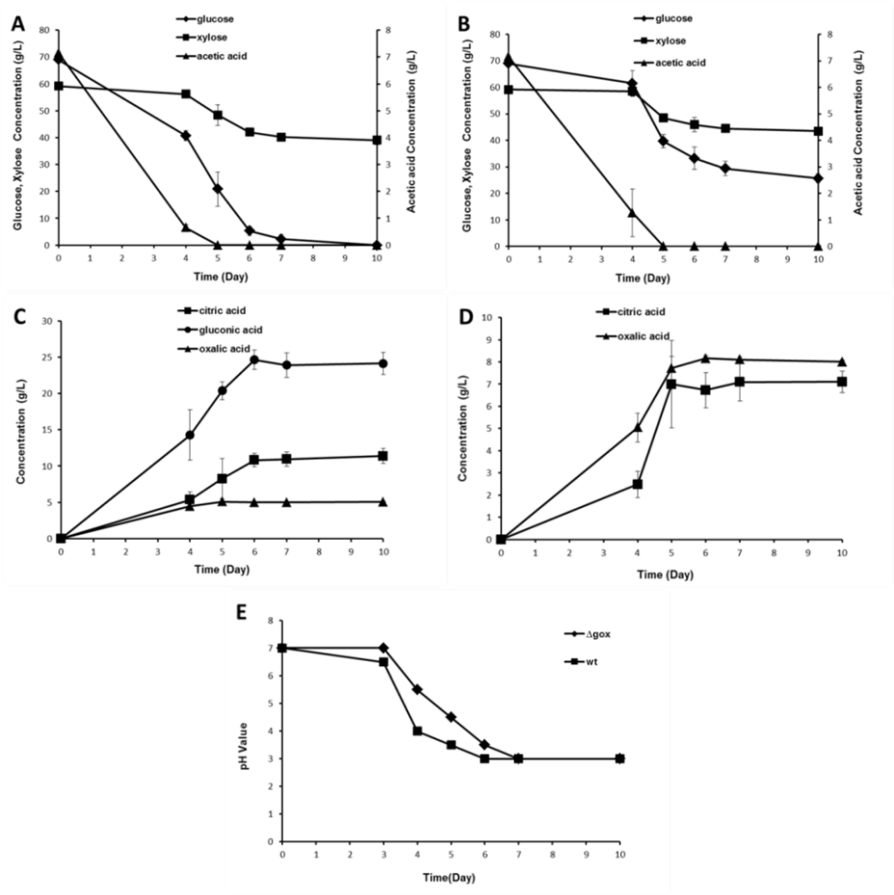

Fig. 5 Cultivation of the wild-type and the Δ*gox* mutant in the hydrolysate at pH 7 (**a**) sugar consumption and the conversion of acetic acid by the wild-type; (**b**) sugar consumption and the conversion of acetic acid by Δ*gox* mutant; (**c**) organic acid production by the wild-type; (**d**) organic acid production by the Δ*gox* mutant; (**e**) cultivation pH (Data shown are mean values from triplicates with error bars indicating the standard deviation)

Table 3 Comparison of organic acid production in the hydrolysate on day 10

Organic acid	**wild-type strain**	**Δ*gox* mutant**
Citric acid	11.5 ± 0.8 g/L	7.1 ± 0.5 g/L
Oxalic acid	5.1 ± 0.1 g/L	8.0 ± 0.1 g/L
Gluconic acid	25.9 ± 1.7 g/L	N.D

(Data shown are mean values from triplicates with the standard deviation after "±"; "N.D" stands for "not detected".)

Discussion

The abilities to utilize varieties of carbon sources for organic acid production (e.g. citric acid and fumaric acid) have been demonstrated with a number of filamentous fungi [17, 23-25]. However, the efficiency of sugar utilization varies among different species. In this study, utilization of sugars from lignocellulosic biomass was investigated in *A. carbonarius* using a wheat straw hydrolysate and a defined medium with the same concentrations of glucose and xylose as in the hydrolysate. The results showed that *A. carbonarius* consumed glucose and xylose simultaneously during the cultivation, especially when it was grown in the defined medium. An efficient co-consumption of glucose and xylose was obtained during the production phase of cultivation in the defined medium, and the consumption of xylose was only slightly lower than glucose. When the wild-type was grown in the hydrolysate, co-consumption of glucose and xylose was also obtained but the consumption of xylose was slower than that in the defined medium. In some microorganisms, the presence of glucose can repress the utilization of pentose sugars in the culture containing both glucose and pentose [26-28], for example, *A. niger* and *Saccharomycopsis lipolytica* have been studied for their citric acid production in a defined medium containing both glucose and xylose, and the consumption of xylose was very low compared with glucose [29]. The rapid consumption of glucose by *A. carbonarius* wild-type in the hydrolysate partially resulted from the conversion of glucose to gluconic acid. Therefore, the consumption of glucose by the wild-type was faster than the xylose consumption in the hydrolysate, which differed from the sugar consumption obtained in the defined medium. Consumptions of glucose and xylose by the Δ*gox* mutant in the hydrolysate were more similar, which might be a result of the deletion of the gluconic acid producing pathway. The efficient co-utilization of glucose and xylose in the hydrolysate indicates the potential for applying *A. carbonarius* to organic acid production from lignocellulosic biomass.

For microorganisms, one of the challenges in utilization of lignocellulosic hydrolysate is the microbial growth inhibition resulting from the different inhibitory compounds which are generated mainly from the biomass pretreatment. Although the microbial resistance to various types of inhibitors varies among species, there are some well-known inhibitors in lignocellulosic hydrolysates such as furfural, 5-hydroxymethylfurfural and acetic acid [6, 8]. To study the inhibitor tolerance by *A. carbonarius* in the hydrolysate, the initial cultivation pH was adjusted to three different values. The original pH in the hydrolysate was 5 which is the optimal pH for the enzymatic hydrolysis. A lower pH value of 3 was also chosen because it is the optimal initial pH for *A. carbonarius* to accumulate high amount of citric acid [21]. However, no fungal growth was observed during the cultivation when the initial pH was adjusted to those two values. The inhibitory effect seemed to occur in the early phase of the cultivation as the fungal spores remained dormant. Based on the composition analysis of the hydrolysate, acetic acid was present at relatively high concentration compared to other known inhibitors (e.g. furfural). The presence of acetic acid especially in the undissociated form can lead to toxic effects on microbial growth, especially on spore germination. In *A. niger*, acetic acid can strongly inhibit the spore germination at low concentration [30]. A similar inhibitory effect of acetic acid on *A. carbonarius* was found in the defined media containing the same concentration of acetic acid as that measured in the hydrolysate (Appendix B). An approach to eliminate this inhibitory effect is to convert acetic acid into the dissociated form by increasing cultivation pH. Addition of alkali is also considered an efficient detoxification method for lignocellulosic hydrolysates. It can decrease the furan aldehydes and phenols in the hydrolysate when the cultivation pH is increased above 9 [6, 31, 32]. In the hydrolysate with initial pH 7, the germination of spores started from day 2 followed by a rapid depletion of acetate, as well as furfural, indicating that the increased pH value can eliminate the inhibition on spores germination, and *A. carbonarius* is able to consume acetate and furfural. When the fungal mycelia from first batch cultivation were recycled and used directly in the following cultivation, an inhibition on fungal growth was observed at the same pH values as in the first batch cultivation. At pH 3, there was still no fungal growth or glucose consumption during the cultivation, whereas at pH 5, glucose consumption occurred on day 1 mainly due to the conversion of glucose into the gluconic acid production. Fungal growth was observed only in the hydrolysate at pH 7, and the consumption of glucose and the conversion of acetic acid emerged in the recycled mycelia on day 1, which was earlier than that observed in the first batch cultivation. However, the conversion of acetic acid slowed down after day 1 while the pH value decreased from 7 to 5.5 during the cultivation. One possible explanation for this phenomenon is that the decreased pH value in the hydrolysate will lead to an increased concentration of undissociated acetic acid which can also inhibit the growth of pre-grown fungal cells as previously reported in *A. niger* [33]. The comparison of growth patterns between the cultivation with the spore inoculation and the recycled mycelia reveals that the

inhibitory effects on the growth of *A. carbonarius* occur in both phases of spore germination and mycelial growth.

The organic acid production by *A. carbonarius* was strongly influenced by the pH in the hydrolysate. When the wild-type was grown in the hydrolysate adjusted to pH 7, gluconic acid was produced prior to other organic acids after day 2 in response to the high glucose concentration. Normally, *A. carbonarius* decreases cultivation pH rapidly in the defined medium omitting additional pH maintenance, and low cultivation pH can significantly inhibit the gluconic acid production. However, the buffering capacity of hydrolysate slowed down the pH decrease in the hydrolysate. A shift from production of gluconic acid to oxalic acid and citric acid could clearly be observed along with the pH decrease in the hydrolysate. After the cultivation pH reached 3, citric acid was produced as the main organic acid until the glucose was exhausted in the hydrolysate. In contrast, when the wild-type was grown in the defined medium, the cultivation pH decreased to 2.5 within three days, which significantly inhibited the production of gluconic acid and oxalic acid, and citric acid accumulated as the main organic acid. When the fungal mycelia were recycled for the organic acid production in the hydrolysate with initial pH 7, the pattern of organic acid production was similar to that observed in the first batch cultivation. *A. carbonarius* produced gluconic acid immediately in response to high glucose concentration after the recycled mycelia was inoculated to the fresh hydrolysate. The gluconic acid production slowed down dramatically after day 1 followed by a lag phase of organic acid production until day 3, implying that the gluconic acid production on day 1 might result from an incomplete inhibition on the grown fungal mycelia in the hydrolysate. On the other hand, recycling the fungal mycelia did not give any beneficial effects on the production of citric acid or oxalic acid. The production of citric acid and oxalic acid by the recycled mycelia was lower than that obtained with the spore inoculation.

A previously constructed Δ*gox* mutant was used in a comparative analysis of the impacts of deleting a glucose oxidase encoding gene (*gox*) on organic acid production in the wheat straw hydrolysate. In our previous study, it was shown that the disruption of the *gox* gene in *A. carbonarius* efficiently prevented the conversion of glucose to gluconic acid and led to increased production of oxalic acid and citric acid, when the Δ*gox* mutant was cultivated in a defined medium with pH maintained at 5.5 by adding $CaCO_3$ [20]. When the Δ*gox* mutant in the present study was cultivated in the hydrolysate adjusted to pH 7, the disruption of the *gox* gene had similar impacts on the production of oxalic acid and gluconic acid as previously reported but not on citric acid production. For comparison, the Δ*gox* mutant was also cultivated in the defined medium mimicking the concentrations of glucose and xylose in the hydrolysate. The results showed no significant difference in the production of citric acid by the Δ*gox* mutant and the wild-type. The production of gluconic acid and oxalic acid were highly inhibited by low pH while the citric acid production was enhanced, which is in consistence with the previous

findings in the well-known citric acid producer *A. niger* [34, 35]. Therefore, the wild-type produced more citric acid in the hydrolysate than the Δ*gox* mutant probably because the rapid pH decrease in the cultivation benefited citric acid production but inhibited the production of oxalic acid and gluconic acid. In contrast, when the Δ*gox* mutant decreased pH more slowly than the wild-type due to the elimination of gluconic acid production, it produced more oxalic acid instead of citric acid compared with the wild-type. The impacts of disrupting the *gox* gene on citric acid production differed in the hydrolysate and the defined media. This comparative study implies that it is necessary to take into consideration the complexity of the lignocellulosic hydrolysates, including pH buffering capacity and potential inhibitors, when the genetic engineered strain was applied in organic acid production using lignocellulosic hydrolysates as substrate.

Conclusion

In this study, organic acid production of *A. carbonarius* was investigated in a wheat straw hydrolysate and compared with production in a defined medium. The efficient co-utilization of glucose and xylose and the high tolerance to the inhibitory compounds in the hydrolysate indicate the potentials of *A. carbonarius* as a fungal cell factory for production of organic acids using lignocellulosic biomass. Moreover, the comparative study of the wild-type and the Δ*gox* mutant showed that impacts of a specific genetic modification on organic acid production were different in the defined media and the ligocellulosic hydrolysate.

Conflict of interest

The authors declare that they have no conflict of interest

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References

1. Nanda S, Azargohar R, Dalai AK, Kozinski JA. An assessment on the sustainability of lignocellulosic biomass for biorefining. Renewable and Sustainable Energy Reviews 2015;50:925-941.

2. Dionisi D, Anderson JA, Aulenta F, Mccue A, Paton G. The potential of microbial processes for lignocellulosic biomass conversion to ethanol: A review. Journal of Chemical Technology and Biotechnology 2015;90(3):366-383.

3. Elkins JG, Raman B, Keller M. Engineered microbial systems for enhanced conversion of lignocellulosic biomass. Curr Opin Biotechnol 2010;21(5):657-662.

4. Quevedo-Hidalgo B, Monsalve-Marín F, Narváez-Rincón PC, Pedroza-Rodríguez AM, Velásquez-Lozano ME. Ethanol production by *Saccharomyces cerevisiae* using lignocellulosic hydrolysate from *Chrysanthemum* waste degradation. World J Microbiol Biotechnol 2013;29(3):459-466.

5. Soo C-, Yap W-, Hon W-, Phang L. Mini review: hydrogen and ethanol co-production from waste materials via microbial fermentation. World J Microbiol Biotechnol 2015;31(10):1475-1488.

6. Jönsson LJ, Alriksson B, Nilvebrant N. Bioconversion of lignocellulose: Inhibitors and detoxification. Biotechnology for Biofuels 2013;6(1).

7. Sakai S, Tsuchida Y, Okino S, Ichihashi O, Kawaguchi H, Watanabe T, Inui M, Yukawa H. Effect of lignocellulose-derived inhibitors on growth of and ethanol production by growth-arrested Corynebacterium glutamicum R. Appl Environ Microbiol 2007;73(7):2349-2353.

8. Zha Y, Westerhuis JA, Muilwijk B, Overkamp KM, Nijmeijer BM, Coulier L, Smilde AK, Punt PJ. Identifying inhibitory compounds in lignocellulosic biomass hydrolysates using an exometabolomics approach. BMC Biotechnology 2014;14(5).

9. Liaud N, Liaud C, Giniés D, Navarro N, Fabre S, Herpoël Crapart I, Gimbert A, Levasseur S, Raouche J, Sigoillot. Exploring fungal biodiversity: organic acid production by 66 strains of filamentous fungi. Fungal Biology and Biotechnology 2014;1(1).

10. Magnuson JK, Lasure LL. Organic acid production by filamentous fungi. Advances in Fungal Biotechnology for Industry, Agriculture, and Medicine 2004;307-340.

11. Lu F, Ping K, Wen L, Zhao W, Wang Z, Chu J, Zhuang Y. Enhancing gluconic acid production by controlling the morphology of *Aspergillus niger* in submerged fermentation. Process Biochemistry 2015, 50(9)1342-1348.

12. Show PL, Oladele KO, Siew QY, Aziz Zakry FA, Lan JC-, Ling TC. Overview of citric acid production from *Aspergillus niger*. Frontiers in Life Science 2015;8(3):271-283.

13. Okabe M, Lies D, Kanamasa S, Park EY. Biotechnological production of itaconic acid and its biosynthesis in *Aspergillus terreus*. Appl Microbiol Biotechnol 2009;84(4):597-606.

14. Mohamad R, Mohamed M, Suhaili N, Ariff A. Kojic acid: Applications and development of fermentation process for production. Biotechnology and Molecular Biology Reviews 2010;5(2):24-37.

15. Pandey A, Soccol CR. Bioconversion of biomass: A case study of ligno-cellulosics bioconversions in solid state fermentation. Brazilian Archives of Biology and Technology 1998;41(4):379-390.

16. Saber WIA, El-Naggar NE-, Abdal-Aziz SA. Bioconversion of lignocellulosic wastes into organic acids by cellulolytic rock phosphate-solubilizing fungal isolates grown under solid-state fermentation conditions. Research Journal of Microbiology 2010;5(1):1-20.

17. Mondala AH. Direct fungal fermentation of lignocellulosic biomass into itaconic, fumaric, and malic acids: current and future prospects. Journal of Industrial Microbiology and Biotechnology 2015;42(4):487-506.

18. Weyda I, Lübeck M, Ahring BK, Lübeck PS. Point mutation of the xylose reductase (XR) gene reduces xylitol accumulation and increases citric acid production in *Aspergillus carbonarius.* Journal of Industrial Microbiology and Biotechnology 2014;41(4):733-739.

19. Ghareib M. Assimilation of galacturonate by *Aspergillus carbonarius*. Folia Microbiol (Praha) 1987;32(3):211-215.

20. Yang L, Lübeck M, Lübeck PS. Deletion of glucose oxidase changes the pattern of organic acid production in *Aspergillus carbonarius*. AMB Express 2014;4(1).

21. Linde T, Hansen NB, Lübeck M, Lübeck PS. Fermentation in 24-well plates is an efficient screening platform for filamentous fungi. Lett Appl Microbiol 2014;59(2):224-230.

22. Baroi GN, Baumann I, Westermann P, Gavala HN. Butyric acid fermentation from pretreated and hydrolysed wheat straw by an adapted *Clostridium tyrobutyricum* strain. Microb Biotechnol 2015;8(5):874-882.

23. Meng J, Zhou P-, Zhang J, Bao J. High titer citric acid fermentation from corn stover hydrolysate by *Aspergillus niger*. Huadong Ligong Daxue Xuebao/Journal of East China University of Science and Technology 2014;40(6):681-683.

24. Xu Q, Li S, Fu Y, Tai C, Huang H. Two-stage utilization of corn straw by Rhizopus oryzae for fumaric acid production. Bioresour Technol 2010;101(15):6262-6262-6264.

25. Yang Z, Bai Z, Sun H, Yu Z, Li X, Guo Y, Zhang H. Biomass pyrolysis liquid to citric acid via 2-step bioconversion. Microbial Cell Factories 2014;13(1).

26. Colabardini AC, Ries LNA, Brown NA, Dos Reis TF, Savoldi M, Goldman MHS, Menino JF, Rodrigues F, Goldman GH. Functional characterization of a xylose transporter in *Aspergillus nidulans*. Biotechnology for Biofuels 2014;7(1).

27. Ronne H.Glucose repression in fungi. Trends in Genetics 1995;11(1):12-17.

28. Subtil T, Boles E. Competition between pentoses and glucose during uptake and catabolism in recombinant *Saccharomyces cerevisiae*. Biotechnology for Biofuels 2012;5(11).

29. Maddox IS, Spencer K, Greenwood JM, Dawson MW, Brooks JD. Production of citric acid from sugars present in wood hemicellulose using *Aspergillus niger* and *Saccharomycopsis lipolytica*. Biotechnol Lett 1985;7(11):815-818.

30. Fencl Z, Leopold J. The inhibition of spore germination of the mould *Aspergillus niger* by acetic acid. Folia Microbiol (Praha) 1959;4(1):7-15.

31. Martinez A, Rodriguez ME, Wells ML, York SW, Preston JF, Ingram LO. Detoxification of dilute acid hydrolysates of lignocellulose with lime. Biotechnol Prog 2001;17(2):287-293.

32. Millati R, Niklasson C, Taherzadeh MJ. Effect of pH, time and temperature of overliming on detoxification of dilute-acid hydrolyzates for fermentation by *Saccharomyces cerevisiae*. Process Biochemistry 2002;38(4):515-522.

33. TANIGUCHI K, MATSUDA K, TERAMURA H, WAKE K. Effect of acetic acid on growth of *Aspergillus niger*. Journal of the Faculty of Science, Hokkaido University.Series 5, Botany 1977;10(4):189-198.

34. Papagianni M. Advances in citric acid fermentation by *Aspergillus niger:* Biochemical aspects, membrane transport and modeling. Biotechnol Adv 2007;25(3):244-263.

35. Yang L, Lübeck M, Lübeck PS. Effects of heterologous expression of phosphoenolpyruvate carboxykinase and phosphoenolpyruvate carboxylase on organic acid production in *Aspergillus carbonarius*. J Ind Microbiol Biotechnol 2015;42(11):1533-1545.

CHAPTER 5. ENHANCED SUCCINIC ACID PRODUCTION IN ASPERGILLUS SACCHAROLYTICUS BY HETEROLOGOUS EXPRESSION OF FUMARATE REDUCTASE FROM TRYPANOSOMA BRUCEI

Enhanced succinic acid production in *Aspergillus saccharolyticus* by heterologous expression of fumarate reductase from *Trypanosoma brucei*

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Abstract

Aspergillus saccharolyticus exhibits great potential as a cell factory for industrial production of dicarboxylic acids. In the analysis of the organic acid profile, *A. saccharolyticus* was cultivated in an acid production medium using two different pH conditions. The specific activities of the enzymes, pyruvate carboxylase (PYC), malate dehydrogenase (MDH) and fumarase (FUM), involved in the reductive tricarboxylic acid (rTCA) branch, were examined and compared in cells harvested from the acid production medium and a complete medium. The results showed that ambient pH had a significant impact on the pattern and the amount of organic acids produced by *A. saccharolyticus*. The wild-type strain produced higher amount of malic acid and succinic acid in the pH buffered condition (pH 6.5) compared with the pH non-buffered condition. The enzyme assays showed that the rTCA branch was active in the acid production medium as well as the complete medium but the measured enzyme activities were different depending on the media. Furthermore, a soluble NADH dependent fumarate reductase gene (*frd*) from *Trypanosoma brucei* was inserted and expressed in *A. saccharolyticus*. The expression of the *frd* gene led to an enhanced production of succinic acid in *frd* transformants compared with the wild-type in both pH buffered and pH non-buffered conditions with highest amount produced in the pH buffered condition (16.2 ± 0.5 g/L). This study demonstrates the feasibility of increasing succinic acid production through the cytosolic reductive pathway by genetic engineering in *A. saccharolyticus*.

Keywords

Aspergillus saccharolyticus; succinic acid; genetic engineering; malic acid; fumarate reductase

Introduction

Succinic acid, also known as butanedioic acid or amber acid, is a subject of significant research interest in recent years due to its use in various industrial applications. Succinic acid can be applied directly as flavor additives, pharmaceutical intermediates, detergents and surfactants, and can also be used as intermediates in the chemical industry for producing other commodity or specialty chemicals like 1,4-butanediol, gamma-butyrolactone, tetrahydrofuran and poly(1,3-propylene succinate)[1, 2]. For the last decade, there has been an increasing global demand of succinic acid, and the production of succinic acid will reach 100,000 ton per year by 2015 with a total market value of 2400 – 3000 US dollars/ton [3]. Presently, succinic acid is mainly produced via a chemical process by hydrogenation of maleic anhydride which is obtained from the C4 fraction of crude oil refineries [4, 5]. With increased interest in lowering carbon emission during production of chemicals, the focus has shifted toward producing chemicals from biomass materials instead of crude oil products. [6-8]. In the biotechnological processes, the efficiency in production of succinic acid has a significant impact on the cost of production. It is commonly agreed that the key factor influencing the yield and the productivity of a biotechnological process is the biocatalysts. So far, several species have been reported to naturally produce promising amounts of succinic acid during fermentation*: Actinobacillus succinogenes, Anaerobiospirillum succiniciproducens, Escherichia coli, Mannheimia succiniciproducens, Penicillium simplicissimum,* and *Aspergillus oryzae* [9-14]. Most of the identified strains with high yields of succinic acid production are bacteria and the fermentation processes developed for them are mainly anaerobic [3, 11]. In recent years, many efforts have also been made on genetic engineering bacterial strains (e.g. *E. coli*) and yeast strains (e.g. *Saccharomyces cerevisiae*) for industrial bio-based production of succinic acid [15, 16]. In bacteria, *E. coli* is one of the most intensively studied and engineered species for succinic acid production. The enhanced succinic acid production has been achieved in genetically engineered *E. coli* strains by increasing the carbon flux towards succinic acid producing pathways and eliminating the byproducts [11, 17, 18]. In yeast, the production of succinic acid was increased in *S. cerevisiae* after a series of genetic modifications were introduced in the primary metabolic pathways including the tricarboxylic acid (TCA) cycle and the glyoxylate pathway [16, 19-21]. Oppositely, very few studies have been carried out on filamentous fungi for succinic acid production since the reported fungi seem to be less efficient in their natural capacity to produce succinic acid compared with bacteria. In *Aspergillus niger*, overexpression of the genes involved in the cytosolic reductive tricarboxylic acid (rTCA) branch and the glyoxylate pathway had no significant impact on succinic acid production [22, 23]. However, several features of filamentous fungi, including excellent capabilities of using different types of carbon sources, high tolerance to low pH and various inhibitors, warrant their potential as competitive candidates for producing succinic acid via biotechnological processes [24, 25].

In this study, a black *Aspergillus*, *Aspergillus saccharolyticus*, was studied and genetic engineered for succinic acid production. *A. saccharolyticus* was first identified by Sørensen et al. and investigated for its enzyme production, in particular beta-glucosidase [26]. It is able to utilize different types of carbon sources including monosaccharides and polysaccharides for enzyme production [27]. In addition, *A. saccharolyticus* was found in preliminary studies to naturally produce relatively high amounts of malic acid and succinic acid, compared with other black Aspergilli, several of which are known as natural citric acid producers [28, 29]. In microorganisms, both malic acid and succinic acid can be produced via three main routes: the TCA cycle, the rTCA branch and the glyoxylate pathway. In filamentous fungi, the rTCA branch has been studied for the organic acid production by *Aspergillus flavus*, *A. oryzae* and *Rhizopus oryzae* [30-34]. All these fungi can produce high amounts of malic acid and it has been shown that the rTCA branch is a very active pathway during the production phase [30, 32, 33]. This leads us to the assumption that the natural production of malic acid by *A. saccharolyticus* may facilitate pathway engineering for increasing the succinic acid production via the cytosolic reductive pathway because an active rTCA branch with high malic acid production can theoretically provide the fumarate as the essential precursor for the reductive reaction to produce succinic acid. Succinic acid can be produced as the end product of the rTCA branch in bacteria, e. g. *E. coli*, through the reduction of fumarate which is normally carried out by the fumarate reductase (FRD) [35]. Although a positive correlation between the production of malic acid and succinic acid has been shown in filamentous fungi, a fumarate reductase has never been identified in any fungal species [34]. Generally, fumarate reductases are divided into two classes depending on their locations in the cell: membrane bounded enzymes and soluble enzymes. Most of fumarate reductases use $FADH_2$/$FMNH_2$ as electron donor during the reaction. There are two known soluble fumarate reductases in the protist *Trypanosoma brucei* and the thermophilic bacterium *Hydrogenobacter thermophilus*, respectively, both using NADH as electron donor [36-38]. In this study, a synthetic and codon optimized truncated gene (*frd*) encoding fumarate reductase naturally located in glycosomes of *T. brucei* was inserted and expressed in *A. saccharolyticus*. Our hypothesis was that the carbon flux could be continued to succinic acid via reduction of fumarate by expression of the *frd* gene and it would eventually result in an enhanced production of succinic acid.

Materials and Methods

Strains and culture media

The *A. saccharolyticus* strain IBT 28231 was cultivated in potato dextrose agar (PDA) medium at 30 ^{o}C for spore production. For genomic DNA extraction and biomass growth, the wild-type and transformants were cultivated in yeast extract peptone dextrose (YPD) medium containing: yeast extract, 10 g/L ; peptone, 20 g/L

and glucose, 20 g/L, at 30 ^{o}C for 2 days, after which mycelia were collected for DNA isolation. For fungal transformation, the transformants were cultivated at 30 ^{o}C in minimal medium with agar (MMA) containing: glucose, 10 g/L; sorbitol, 182 g/L; $NaNO_3$, 6 g/L; KCl, 0.5 g/L; $MgSO_4$ $7H_2O$, 0.5 g/L; KH_2PO_4, 1.5 g/L; $ZnSO_4$, 0.005 g/L; $FeSO_4$ $7H_2O$, 0.003 g/L; $CuSO_4$, 0.001 g/L; $MnCl_2$, 0.002 g/L; biotin, 0.001 g/L; thiamine, 0.001 g/L; riboflavin, 0.001 g/L; para-aminobenzoic acid, 0.001 g/L; agar, 18 g/L. The pre-cultivation was carried out in a medium containing: yeast extract, 1.8 g/L and peptone, 5 g/L. Cultivation was carried out in two different production media for organic acid production. For pH buffered conditions, the organic acid production medium, which was modified from the malic acid production medium described by Peleg et al, consisted of: glucose, 80 g/L; NH_4NO_3, 1.5 g/L; KH_2PO_4, 0.15 g/L; $MgSO_4$ $7H_2O$, 0.8 g/L; $CaCl_2$ $2H_2O$, 0.2 g/L; NaCl, 0.15 g/L; $ZnSO_4$, 0.0015 g/L; $FeSO_4$ $7H_2O$, 0.03 g/L; biotin, 1×10^{-5} g/L and $CaCO_3$, 40 g/L [39]. For pH non-buffered conditions, the organic acid production medium was made using the same recipe as described above but omitting calcium carbonate.

Construction of plasmid expressing *frd* gene

A truncated version of the *frd* gene (accession no. KT026107), which was derived from the *frd* gene (accession no. AF457132) in *T. brucei*, was synthesized and codon optimized for expression in *A. saccharolyticus* by GenScript (USA) using the codon usage information from *A. niger*. The putative glycosomal targeting sequence serine-lysine-isoleucine (SKI) of the *frd* gene was omitted in the synthesized gene. The truncated gene was cloned into the vector pSBe1 between the *gpdA* promoter and the *TrpC* terminator using the primers Frd uFw1 and Frd uRv1 (Table 1) via USER cloning as described previously [40]. The plasmid pSBe1frd was verified by PCR using the primers Frd Fw1 and Frd Rv1 (Table 1) and used for fungal transformation (Fig. 1). The primers and plasmid maps were designed using the CLC workbench (CLC Bio).

Table 1 Primers used in this research

Name	Sequence (5'→3')	Annotation
Frd Fw1	ATGGTTGATGGTCGGTCGT	PCR amplification of *frd* gene
Frd Rv1	TTAGCTACCCGACGGTTCAGTT	PCR amplification of *frd* gene
Frd uFw1	AGAGCGAUATGGTTGATGGTCGGTCGT	USER cloning of *frd* gene
Frd uRv1	TCTGCGAUTTAGCTACCCGACGGTTCAGT	USER cloning of *frd* gene

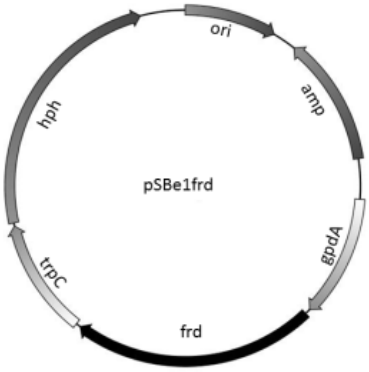

Fig. 1 Plasmid map for pSBe1frd. *gpdA*, constitutive promoter; *frd*, fumarate reductase encoding gene; *trpC*, terminator; *hph*, hygromycin resistance gene; *amp*, ampicillin resistance gene; *ori*, origin of replication in the plasmid

Protoplast transformation

Protoplasts of *A. saccharolyticus* were made from freshly harvested mycelia as previously described [41]. The final concentration of protoplasts for aliquots was adjusted to 2 x 10^7 /mL and the protoplasts were preserved at -80 ^oC with the addition of 40% PEG4000 and 7% DMSO. The protoplast transformation was made as previously described [41]. Plasmid DNA (8 µg) in a total volume of 10 µL TE buffer was added into 100 µL protoplast suspension. Transformants were selected after sporulation on MMA plates using 100 µg/mL hygromycin as a selective agent. Spores from each transformant were transferred to a new plate by streaking out on PDA medium containing 100 µg/mL hygromycin and incubated at 30 ^oC overnight. Next day, single colonies were identified and picked up to verify insertion of the target gene in the genome by extracting genomic DNA from the transformants and amplifying the fragments with gene specific primers Frd Fw1 and Frd Rv1 in PCR (Table 1).

Enzyme assays

For measurement of enzyme activities in cell free extracts, *A. saccharolyticus* wild-type (WT) strain and the *frd* transformants were cultivated in shaking flasks containing YPD medium at 30 ^oC for 2 days. For comparison of enzyme activities

in the wild-type strain between different media, the wild-type strain was also cultivated in the acid production medium using the same conditions. Fungal mycelia were filtered through Miracloth and washed thoroughly with cold 0.01 M Tris-HCl buffer (pH 8.0), before being suspended in 0.01 M Tris -HCl buffer (pH 8.0) supplemented with 0.2 M KCl. Cell free extracts were prepared by homogenizing mycelia suspension briefly with glass beads for 30 s followed by centrifugation at 24,000 x g for 30 min at 4 ^{o}C. The supernatants were obtained and used in enzyme activity assays as cell cytosol fractions [31]. The concentration of protein in cell extract was measured by using the Pierce BCA protein assay reagent kit (Thermo Scientific).

All the enzymes were assayed spectrophotometrically at room temperature in 1.5 mL cuvettes (1.0 cm light path) using the Spectrophotometer DR3800 (Hach company). The enzyme activities of pyruvate carboxylase (PYC) and malate dehydrogenase (MDH) were measured as previously described by Knuf et al (2013): PYC activity was measured in an assay mixture containing 100 mM Tris, pH 8.0, 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 1 mM pyruvate, 0.2 mM NADH, 1 mM ATP and 5 unit malate dehydrogenase; MDH activity was determined in an assay mixture containing 100 mM Tris, pH 8.0, 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.667 mM oxaloacetic acid and 0.2 mM NADH. The reaction was initiated by adding freshly prepared cell extract, and the rate of decrease in absorbance of NADH was monitored at 340 nm. One unit of enzyme activity was defined as the oxidation of 1 μmol NADH per milligram of total protein per minute at 30 ^{o}C and pH 8.0 [34]. Fumarase (FUM) activity was measured as previously described: the assay mixture contained 100 mM Tris, pH 8.0, 0.04 mM NAD, 4 mM $MgCl_2$, 5 mM K_2HPO_4, and 10 units malate dehydrogenase. The FUM activity was measured by monitoring the increase of absorbance due to reduction of NAD at 340 nm after adding the cell extract. One unit FUM activity was defined as the formation of 1 μmol NADH per milligram of total protein per minute at 30 ^{o}C and pH 8.0 [42]. The fumarate reductase (FRD) activity was measured as previously described: the assay mixture contained 50 mM phosphate buffer (pH 6.5), 20 mM fumarate and 0.2 mM NADH. The reaction was carried out at 30 ^{o}C after adding the cell extract. The FRD activity was determined by monitoring the decrease of absorbance due to the oxidation of NADH at 340 nm. One unit of FRD activity was defined as the oxidation of 1 μmol NADH per milligram of total protein per minute at 30 ^{o}C and at pH 6.5 [43].

Organic acid production and analysis of extracellular organic acids

Spores of the wild-type strain and transformants were harvested from the PDA medium with sterile water after 4-6 days incubation at 30 ^{o}C. The spore suspensions were prepared by filtering through sterilized Miracloth (Fisher Scientific) for removal of the mycelia. The pre-cultivation was carried out as previously described [41], and the pellets formed in the 20 mL pre-culture were filtered through the sterile Miracloth, added to 50 mL organic acid production medium in 250 mL flasks

and incubated at 30 ^{o}C with agitation speed of 180 rpm for 6 days. The initial pH of the production media were adjusted to 6.5 before inoculation. All the cultivations were carried out in triplicates.

The samples taken from the pH buffered culture were acidified with 50% sulfuric acid to precipitate the calcium ion in form of calcium sulfate and exchange the organic acid back to the liquid phase. The acidified samples were incubated at 80 ^{o}C for at least 15 min to complete the reaction followed by centrifugation at 14000 rpm for 1 min. The samples taken from the pH non-buffered culture were filtered directly without acidification. HPLC analysis of the supernatants for sugar and organic acids was carried out in an Aminex 87H column (Biorad) at 60 ^{o}C by using a HPLC mobile phase (5 mM H_2SO_4) at a flow rate of 0.6 mL/min. The total yield of the two C4 – dicarboxylic acids, malic acid and succinic acid, was calculated in mole concentration instead of g acid/g glucose to eliminate the deviations caused by the different molecular weights of succinic acid and malic acid.

Fungal biomass determination

For determination of the dry weight of fungal biomass from the pH buffered culture, the fermentation broth was acidified with 1N HCl solution to dissolve the calcium carbonate and filtered through the filter paper (Whatman^{TM} no.10404026) which was dried overnight at 100 ^{o}C before use. The mycelia on the filter paper were then washed with distilled water until pH in the filtrate reached 6.0. The filter paper with washed mycelia was dried at 100 ^{o}C for 2 days before weighing. The determination of fungal biomass from the pH non-buffered culture was carried out as described for the pH buffered culture but without the HCl acidification step.

Statistical analysis

Unless annotated in the text, all the experiments in this study were carried out in triplicates and the average values were presented with standard errors. The significant differences indicated in the results were confirmed by performing the *t*-test individually between two sets of experimental data (e.g. between the wild-type strain and an individual transformant). The threshold of significance in the *t*-test was set as p=0.05.

Results

Organic acid profiling in pH buffered and pH non-buffered conditions

The wild-type strain of *A. saccharolyticus* was cultivated in shaking flasks for the production of succinic acid and other TCA organic acids in two different pH conditions. *A. saccharolyticus* formed similar pellet morphology in both pH conditions as shown in Fig. 2. In the production medium buffered with addition of

$CaCO_3$, malic acid was produced as the main organic acid. On day 1, 0.4 ± 0.02 g/L malic acid was measured, and it reached 17.6 ± 1.2 g/L on day 6 after glucose was consumed (Fig. 3a and 3c). In addition to malic acid, succinic acid was detected at the highest amount of 3.8 ± 0.1 g/L on day 4, which held at the same level until day 6 (Fig. 3b). Moreover, *A. saccharolyticus* produced up to 1.9 ± 0.1 g/L citric acid (Fig. 3d). An elevation in the production of malic acid and succinic acid was observed after 1 day (Fig. 3b and 3c). In the production medium without $CaCO_3$, pH in the culture decreased from the initial pH 6.5 to approx.2.5 after 1 day. *A. saccharolyticus* still produced malic acid but at a relatively low concentration (2.8 ± 0.3 g/L) after 6 days compared with the pH buffered condition. In addition, the production of citric acid (0.16 ± 0.02 g/L) also decreased and succinic acid was not produced at a detectable quantity (Fig. 4b-d). The consumption of glucose was slower during the cultivation in the pH non-buffered condition and there was 13.6 ± 1.4 g/L glucose left in the culture after 6 days (Fig. 4a), whereas, the glucose was almost exhausted on day 5 in the pH buffered condition.

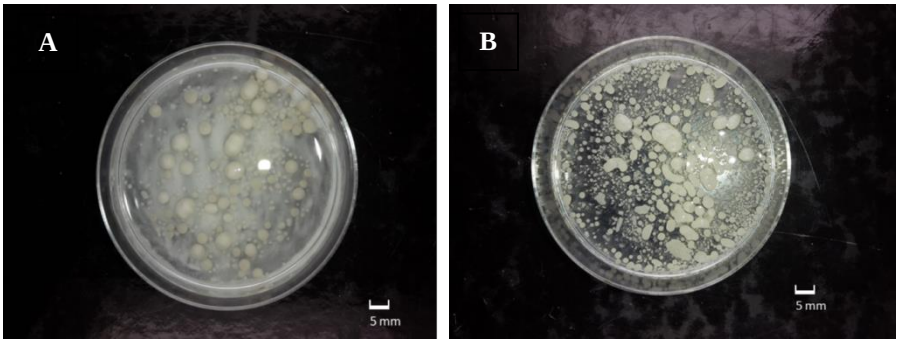

Fig. 2 Morphology of *A. saccharolyticus* wild-type in acid production media. **a** pH buffered condition. **b** pH non-buffered condition

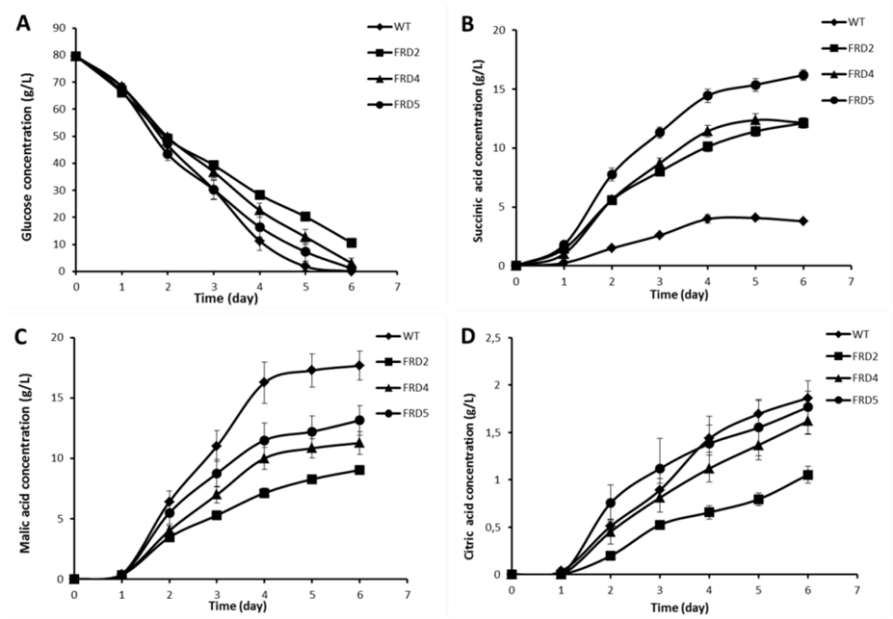

Fig. 3 The production of organic acids by *A. saccharolyticus* wild-type strain and *frd* transformants in pH buffered condition. **a** Glucose concentration. **b** Succinic acid production. **c** Malic acid production. **d** Citric acid production (Data shown are mean values from triplicates with error bars indicating the standard error)

Protoplast transformation of *A. saccharolyticus* with *frd* gene

For expression of the *frd* gene in *A. saccharolyticus*, the *frd* gene was inserted into the plasmid via the *AsisI* cassette located between the constitutive promoter *gpdA* and the *TrpC* terminator. The resulting plasmid was used for protoplast transformation and colonies appeared on MMA plates after 3-4 days incubation. In total, six colonies were obtained after protoplast transformation, and among them, three colonies carried the inserted *frd* gene based on PCR verification.

Comparison of the activities of enzymes involved in the rTCA branch

The activities of enzymes involved in the rTCA branch in the *A. saccharolyticus* wild-type strain were assayed in the cytosol fraction of the cell extract prepared from the cells cultivated in the YPD medium and in the pH buffered acid production medium. Compared with the acid production medium, the wild-type strain produced more fungal biomass but no malic acid or succinic acid in the YPD medium (Appendix C). As showed in Table 2, the activities of all three enzymes (PYC, MDH and FUM) in the rTCA branch were detected in both the YPD and the

acid production media. The PYC activity in the cell cultivated in the YPD medium was significantly higher than the activity measured from the cells cultivated in the acid production medium (0.019 ± 0.001 U/mg vs 0.007 ± 0.001 U/mg, $p < 0.05$). On the contrary, the specific activities of MDH and FUM increased 2 times and 1.5 times, respectively, in the cells cultivated in the acid production medium compared with the YPD medium (16.5 ± 0.1 U/mg MDH activity and 0.015 ± 0.001 U/mg FUM activity in the acid production medium vs 7.9 ± 0.2 U/mg MDH activity and 0.01 ± 0.001U/mg FUM activity in the YPD medium, $p<0.05$).

Table 2 Enzyme activities measured in *A. saccharolyticus* wild-type strain

	PYC	MDH	FUM
YPD medium	0.019 ± 0.001	7.9 ± 0.31	0.010 ± 0.001
AP medium	0.007 ± 0.001	16.5 ± 0.13	0.015 ± 0.002

The enzyme activity (U/mg protein) was measured in cells harvested after 48 h of incubation in YPD medium or in the acid production (AP) medium under pH buffered conditions. PYC – pyruvate carboxylase; MDH – malate dehydrogenase; FUM – fumarase (Data shown are mean values from triplicates with the standard deviation after "±")

Enzyme activity assays of fumarate reductase

The expression of the *frd* gene was verified in *A. saccharolyticus* by measuring the FRD activity in the wild-type strain and in the selected *frd* transformants cultivated in the YPD medium since the transcription of the *frd* gene was initiated by the constitutive promoter *gpdA*. As shown in Table 3, all three *frd* transformants exhibited FRD activity compared with the wild-type strain, which verified the heterologous expression of *frd* gene in *A. saccharolyticus*. The *frd* transformant no. 5 showed much higher enzyme activity (0.027 ± 0.001 U/mg) than the other two transformants (0.014 ± 0.001 U/mg and 0.019 ± 0.002 U/mg).

Table 3 The activity of fumarate reductase in the wild-type and *frd* transformants

	FRD
WT	ND
FRD2	0.014 ± 0.002
FRD4	0.019 ± 0.003
FRD5	0.027 ± 0.001

The enzyme activity (U/mg protein) was measured in the cells after 48 h of incubation in YPD medium. WT – wild-type strain; FRD 2, 4 and 5 – transformants with expression of fumrate reductase; FRD – fumarate reductase (Data shown are mean values from triplicates with the standard deviation after "±")

Impact of expressing the *frd* gene on succinic acid production in *A. saccharolyticus*

In the pH buffered acid production medium, the production of succinic acid increased dramatically in the *frd* transformants compared with the wild-type strain. As shown in Fig. 3b, the three selected *frd* transformants produced 12.1 ± 0.4 g/L, 12.1 ± 0.5 g/L and 16.2 ± 0.5 g/L succinic acid, respectively, after 6 days cultivation, while the wild-type strain produced only 3.8 ± 0.1 g/L ($p< 0.05$). The production of succinic acid slowed down after 5 days in the tested strains when the glucose was almost exhausted. On the other hand, the production of malic acid decreased significantly in the *frd* transformants compared with the wild-type. The wild-type produced 17.6 ± 1.2g/L malic acid after 6 days and the selected transformants produced at most 13.1 ± 1.2 g/L, $p < 0,05$ (Fig. 3c). The production of citric acid was lower in the *frd* transformants than the wild-type (Fig. 3d).

In the acid production medium under the pH non-buffered condition, as seen in Fig. 4b-d, succinic acid was produced by the *frd* transformants from day 1 and was accumulating until day 3. There was no succinic acid detected from the wild-type strain during cultivation. The highest amount of succinic acid (1.8 ± 0.2 g/L) was obtained from transformant no. 5. However, all transformants started consuming succinic acid after day 3 or 4 when there were still plenty of glucose left in the medium (Fig. 4b). In addition to succinic acid, the production of malic acid was observed in all the *frd* transformants and particularly in the wild-type during cultivation. The wild-type strain produced more malic acid than the *frd* transformants in the pH non-buffered condition, even though that the yield of malic acid was much lower than that measured in the pH buffered condition. However, as

another intermediate in the rTCA branch, fumaric acid was not detected in the culture under any of the tested pH conditions.

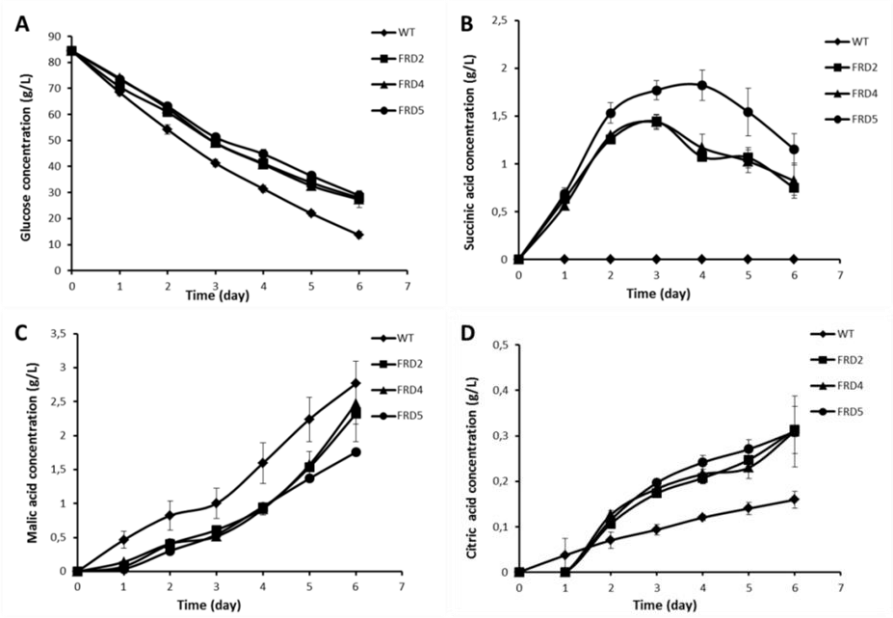

Fig. 4 The production of organic acids by *A. saccharolyticus* wild-type and *frd* transformants in pH non-buffered condition **a** Glucose concentration; **b** Succinic acid production; **c** Malic acid production; **d** Citric acid production (Data shown are mean values from triplicates with error bars indicating the standard error)

For an overview of the impact of expressing the *frd* gene on carbon flux towards C4-dicarboxylic acid, the total C4-dicarboxylic acid production was calculated based on the measured concentrations of malic acid and succinic acid after 6 days. As shown in Fig. 5a, the molar yield of C4-dicarboxylic acids increased in the transformants compared to the wild-type. In the pH buffered condition, the highest yield of C4-dicarboxylic acids (0.54 ± 0.01 mole/mole glucose) was reached by transformant no.5 while the wild-type produced 0.37 ± 0.02 mole/mole glucose. Similar results were also obtained in the pH non-buffered condition but the increase in the molar yield of C4-dicarboxylic acids was not significant in the transformants, probably due to the low C4-dicarboxylic acid production (Fig.5a). The fungal biomass from the wild-type and the transformants was also measured and compared after 6 days. In general, the selected transformants and the wild-type reached a similar yield of fungal biomass in pH buffered and non-buffered conditions, and no significant differences in the fungal biomass were observed between the individual transformants and the wild-type (Fig. 5b).

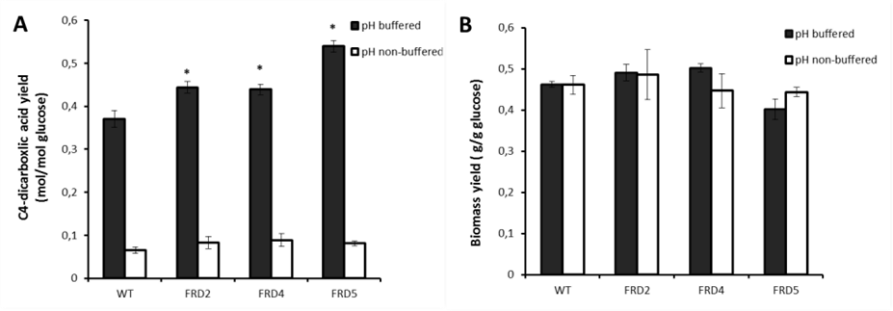

Fig. 5 The molar yield of total C4-dicarboxylic acid and the fungal biomass growth in pH buffered and non-buffered conditions. **a** The molar yield of total C4-dicarboxylic acids by *A. saccharolyticus* wild-type strain and *frd* transformants after 6 days measured as mol acid/ mol consumed glucose (the values from transformants which differed significantly ($p<0.05$) from the wild-type are indicated with an asterisk). **b** The yield of fungal biomass of the wild-type strain and *frd* transformants after 6 days (Data shown are mean values from triplicates with error bars indicating the standard error)

Discussion

Filamentous fungi, especially Aspergilli, are well known as industrial workhorses for organic acid production [25, 44, 45]. However, there are only few fungal species reported for their abilities to naturally secrete high amounts of succinic acid or other C4-dicarboxylic acids [14, 46, 47]. A black *Aspergillus* species, *A. saccharolyticus*, was chosen in this study for its ability to produce relatively high amounts of organic acids including malic acid, succinic acid and citric acid. For investigation of the profile of the main organic acids produced by *A. saccharolyticus*, a pH value at approximately 6.5 was adopted since it is widely used for the production of malic acid and fumaric acid by different fungal strains [32, 39, 48, 49]. As shown in Fig. 3, the obtained organic acid profile of *A. saccharolyticus* is very similar to the organic acid pattern formerly reported in other malic acid producers such as *A. flavus* and *A. oryzae* [14, 46]. In both of these species, malic acid is produced as the main organic acid under similar conditions, and the rTCA branch is shown to be involved in malic acid production. Succinic acid, which is normally regarded as a byproduct in malic acid fermentation, can theoretically be produced via the same pathways as malic acid. However, the lack of a fumarate reductase in the cytosol implies that succinic acid in fungi cannot be produced via the rTCA branch without genetic engineering.

The negative impacts of low ambient pH on production of malic acid and other measured organic acids by *A. saccharolyticus* were observed after the cultivation pH decreased to 2.5 in the pH non-buffered condition. The glucose consumption in

the pH non-buffered condition did not decline dramatically compared with the pH buffered condition, which indicates that the low yield of measured organic acids is not caused by the intolerance of *A. saccharolyticus* to low pH. The impacts of ambient pH on organic acid production have been investigated in a number of fungi, for example, in *A. niger*, the production of oxalic acid and gluconic acid are strongly inhibited when the cultivation pH is lower than 4, whereas the production of citric acid increased at low pH [44, 45, 50-52]. For *A. saccharolyticus*, further studies are required to reveal the impacts of ambient pH on organic acid production.

For examination of the role of the rTCA branch in organic acid production by the *A. saccharolyticus* wild-type strain, a comparative study of the activities of enzymes involved in the rTCA branch was made on the cells harvested from the acid production medium and the YPD medium, respectively. The rTCA branch in filamentous fungi normally involves three cytosolic enzymes: PYC, MDH and FUM. The pathway plays a very important anaplerotic role in fungal metabolism and it is an efficient pathway for producing malic acid and fumaric acid in several fungal species [31, 33, 34]. The specific enzyme activities of MDH and FUM in *A. saccharolyticus* increased in cells cultivated in the acid production medium compared with the YPD medium, which is consistent with the accumulation of malic acid in the acid production medium. However, the PYC activity was lower in the cells from the acid production medium than in the YPD medium. The increase in PYC activity has been reported in fungi during the transition from early phase to stationery phase of malic acid production [14]. However, as shown in this study, when *A. saccharolyticus* started producing malic acid in the acid production medium, the activity of PYC in *A. saccharolyticus* was not induced to a higher level than in the YPD medium. The decrease in the activity of PYC and the increase in the enzyme activities of MDH and FUM in the rTCA branch may form a bottleneck in the carbon flux flowing towards malic acid in the acid production medium. It was shown in *A. oryzae* that the overexpression of PYC led to increased malic acid production by increasing the PYC activity in the production phase [14, 32]. By examining the enzyme activities of PYC, MDH and FUM, the integrity of the rTCA branch in *A. saccharolyticus* was verified, which also provided the possibility to continue the carbon flux further to succinic acid in the cytosol by expressing a *frd* gene encoding a fumarate reductase (FRD) that catalyzes the conversion of fumarate to succinate.

In the *frd* transformants, the expression of *frd* gene encoding FRD located in the glycosomes of *T. brucei* increased the succinic acid production via the reduction of cytosolic fumarate. Unlike most of the identified fumarate reductases from different microorganisms which are membrane-bounded or using $FADH_2$/$FMNH_2$ as electron donors, the FRD in *T. brucei* is soluble and NADH-dependent [36, 38]. This characteristic makes the *T. brucei* FRD more suitable for converting fumarate to succinate in *A. saccharolyticus* than other fumarate reductases since $FADH_2$/$FMNH_2$ cannot be directly generated from glycolysis and the expression of

membrane bounded fumarate reductases may be influenced by uncertain factors, like malfunction of the electron transferring chain in the cell membrane or the recognition of the membrane anchor of the expressed FRD in *A. saccharolyticus*. Compared with the wild-type, the expression of FRD in the transformants increased the production of succinic acid. It indicates that the carbon flux is successfully increased to succinic acid in cytosol via the reduction of fumarate carried out by heterologous expressed FRD in *A. saccharolyticus*. A positive correlation between the yield of succinic acid and the specific enzyme activities of FRD in the three selected transformants was found as the highest succinic acid yield was observed in the transformant no.5 which exhibited the highest FRD activity.

In addition to enhanced production of succinic acid, the production of malic acid decreased in the transformants compared with the wild-type strain. One possible explanation might be that the carbon flux was continued to succinic acid production in the rTCA branch so malic acid was partially converted to fumarate which was further used as the substrate for FRD. However, due to the complexity of fungal metabolism, cytosolic fumarate might also be supplied from other metabolic pathways, e.g. the mitochondrial TCA cycle. Further analysis of intracellular carbon flux is required to reveal the carbon flow between succinic acid and malic acid, and to determine the actual contribution of rTCA branch to enhanced production of succinic acid in the *frd* transformants. At last, the total molar yield of C4-dicarboxylic acids (malic acid plus succinic acid) also increased in the *frd* transformants compared with the wild-type strain but at a limited level. It indicates the total carbon flow towards organic acid production is, to some extent, strictly regulated in *A. saccharolyticus*. Further study on regulation of different organic acid producing pathways is necessary for rerouting more carbon flux into succinic acid production.

Succinic acid was produced by the *frd* transformants in both pH buffered and non-buffered conditions. It indicates that the expressed FRD is able to function in *A. saccharolyticus* at low pH (2.5) and neutral pH (6.5). Low cultivation pH is normally considered to be more economically feasible for organic acid production processes since it can reduce the cost of processes including pH maintenance and product purification [53]. For most of the reported succinic acid producers, the efficient production of succinic acid is highly dependent on neutral cultivation pH, except the low pH process applied in the engineered yeast strain [3, 9, 10, 16]. The ability of *A. saccharolyticus* to secrete succinic acid in low pH culture provides a possibility to apply it as a cell factory for succinic acid production in low pH processes. On the other hand, the concentration of measured organic acids decreased sharply in the low pH culture. Although no direct information regarding the biochemical reactions or enzymes affected by the change in ambient pH in *A. saccharolyticus* was revealed in this study, the potential impacts of ambient pH change on relevant pathways might be expected. Further investigations and genetic

engineering on relevant pathways are necessary for improving the production of succinic acid in low pH conditions.

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Conflict of interest

The authors declare that they have no conflict of interest

Ethical approval

This study does not contain any experiment with human participants or animals performed by any of the authors

References

1. Werpy T, Frye J, Holladay J. Succinic Acid - A Model Building Block for Chemical Production from Renewable Resources. Biorefineries-Industrial Processes and Products: Status Quo and Future Directions 2008;2:367-379.

2. Zeikus JG, Jain MK, Elankovan P. Biotechnology of succinic acid production and markets for derived industrial products. Appl Microbiol Biotechnol 1999;51(5):545-552.

3. Cheng K-, Zhao X-, Zeng J, Zhang J. Biotechnological production of succinic acid: Current state and perspectives. Biofuels, Bioproducts and Biorefining 2012;6(3):302-318.

4. Muzumdar AV, Sawant SB, Pangarkar VG. Reduction of maleic acid to succinic acid on titanium cathode. Organic Process Research and Development 2004;8(4):685-688.

5. Zhao F, Yan F, Qian Y, Chu Y, Ma C. Electrochemical synthesis of succinic acid at a TiO2 film electrode prepared by in-situ anodic oxidation. International Journal of Electrochemical Science 2012;7(12):12931-12940.

6. Hatti-Kaul R, Törnvall U, Gustafsson L, Börjesson P. Industrial biotechnology for the production of bio-based chemicals - a cradle-to-grave perspective. Trends Biotechnol 2007;25(3):119-124.

7. Ma R, Guo M, Zhang X. Selective conversion of biorefinery lignin into dicarboxylic acids. ChemSusChem 2014;7(2):412-415.

8. Cao Y, Zhang R, Sun C, Cheng T, Liu Y, Xian M: Fermentative succinate production. An emerging technology to replace the traditional petrochemical processes. BioMed Research International 2013;10(2).

9. van Heerden CD, Nicol W. Continuous and batch cultures of *Escherichia coli* KJ134 for succinic acid fermentation: Metabolic flux distributions and production characteristics. Microbial Cell Factories 2013;12(1).

10. Corona-González RI, Bories A, González-Álvarez V, Pelayo-Ortiz C. Kinetic study of succinic acid production by *Actinobacillus succinogenes* ZT-130. Process Biochemistry 2008;43(10):1047-1053.

11. Beauprez JJ, De Mey M, Soetaert WK. Microbial succinic acid production: Natural versus metabolic engineered producers. Process Biochemistry 2010;45(7):1103-1114.

12. Song H, Jang SH, Park JM, Lee SY. Modeling of batch fermentation kinetics for succinic acid production by *Mannheimia succiniciproducens.* Biochem Eng J 2008;40(1):107-115.

13. Gallmetzer M, Meraner J, Burgstaller W. Succinate synthesis and excretion by *Penicillium simplicissimum* under aerobic and anaerobic conditions. FEMS Microbiol Lett 2002;210(2):221-225.

14. Knuf C, Nookaew I, Brown SH, McCulloch M, Berry A, Nielsen J. Investigation of malic acid production in *aspergillus oryzae* under nitrogen starvation conditions. Appl Environ Microbiol 2013;79(19):6050-6058.

15. Dole S, Yocum RR. Organic acid production in microorganisms by combined reductive and oxidative tricaboxylic acid cylce pathways. U.S. Patent and Trademark Office 2014;NO.8:778-656.

16. Jansen MLA, Segueilha L, Verwaal R, Louchart M. Dicarboxylic acid production process. U.S. Patent Application 2011;13:821-125.

17. Kim P, Laivenieks M, Vieille C, Zeikus JG. Effect of Overexpression of *Actinobacillus succinogenes* Phosphoenolpyruvate Carboxykinase on Succinate Production in *Escherichia coli*. Appl Environ Microbiol 2004;70(2):1238-1241.

18. Jantama K, Zhang X, Moore JC, Shanmugam KT, Svoronos SA, Ingram LO. Eliminating side products and increasing succinate yields in engineered strains of *Escherichia coli* C. Biotechnol Bioeng 2008;101(5):881-893.

19. Raab AM, Gebhardt G, Bolotina N, Weuster-Botz D, Lang C. Metabolic engineering of *Saccharomyces cerevisiae* for the biotechnological production of succinic acid. Metab Eng 2010;12(6):518-525.

20. Raab AM, Lang C.Oxidative versus reductive succinic acid production in the yeast *Saccharomyces cerevisiae.* Bioengineered Bugs 2011;2(2).

21. Arikawa Y, Kuroyanagi T, Shimosaka M, Muratsubaki H, Enomoto K, Kodaira R, Okazaki M. Effect of gene disruptions of the TCA cycle on production of succinic acid in *Saccharomyces cerevisiae*. Journal of Bioscience and Bioengineering 1999;87(1):28-36.

22. Meijer S, Otero J, Olivares R, Andersen MR, Olsson L, Nielsen J. Overexpression of isocitrate lyase—glyoxylate bypass influence on metabolism in *Aspergillus niger*. Metab Eng 2009;11(2):107-116.

23. de Jongh WA, Nielsen J. Enhanced citrate production through gene insertion in *Aspergillus niger.* Metab Eng 2008;10(2):87-96.

24. May G. Fungal technology. Applied Molecular Genetics of Filamentous Fungi 1992;6:1-27.

25. Magnuson JK, Lasure LL. Organic acid production by filamentous fungi. Advances in Fungal Biotechnology for Industry, Agriculture, and Medicine 2004;307-340.

26. Sørensen A, Lübeck PS, Lübeck M, Teller PJ, Ahring BK. ß-Glucosidases from a new *Aspergillus* species can substitute commercial ß-glucosidases for saccharification of lignocellulosic biomass. Can J Microbiol 2011;57(8):638-650.

27. Sørensen A, Andersen JJ, Ahring BK, Teller PJ, Lübeck M. Screening of carbon sources for beta-glucosidase production by *Aspergillus saccharolyticus*. International Biodeterioration and Biodegradation 2014;93:78-83.

28. Bercovitz A, Peleg Y, Battat E, Rokem JS, Goldberg I. Localization of pyruvate carboxylase in organic acid-producing *Aspergillus* strains. Appl Environ Microbiol 1990;56(6):1594-1597.

29. Liaud N, Liaud C, Giniés D, Navarro N, Fabre S, Herpoël Crapart I, Gimbert A, Levasseur S, Raouche J, Sigoillot: Exploring fungal biodiversity. organic acid

production by 66 strains of filamentous fungi. Fungal Biology and Biotechnology 2014;1(1).

30. Peleg Y, Stieglitz B, Goldberg I. Malic acid accumulation by *Aspergillus flavus* - I. Biochemical aspects of acid biosynthesis. Appl Microbiol Biotechnol 1988;28(1):69-75.

31. Peleg Y, Barak A, Scrutton MC, Goldberg I. Malic acid accumulation by *Aspergillus flavus* - III. 13C NMR and isoenzyme analyses. Appl Microbiol Biotechnol 1989;30(2):176-183.

32. Brown SH, Bashkirova L, Berka R, Chandler T, Doty T, McCall K, McCulloch M, McFarland S, Thompson S, Yaver D, Berry A. Metabolic engineering of *Aspergillus oryzae* NRRL 3488 for increased production of l-malic acid. Appl Microbiol Biotechnol 2013;97(20):8903-8912.

33. Zhang B, Skory CD, Yang S. Metabolic engineering of *Rhizopus oryzae*: Effects of overexpressing pyc and pepc genes on fumaric acid biosynthesis from glucose. Metab Eng 2012;14(5):512-520.

34. Knuf C, Nookaew I, Remmers I, Khoomrung S, Brown S, Berry A, Nielsen J. Physiological characterization of the high malic acid-producing *Aspergillus oryzae* strain 2103a-68. Appl Microbiol Biotechnol 2014;98(8):3517-3527.

35. Wang X, Gong CS, Tsao GT. Bioconversion of fumaric acid to succinic acid by recombinant *E. coli*. Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology 1998;70(2):919-928.

36. Besteiro S, Biran M, Biteau N, Coustou V, Baltz T, Canioni P, Bringaud F. Succinate secreted by *Trypanosoma brucei* is produced by a novel and unique glycosomal enzyme, NADH-dependent fumarate reductase. J Biol Chem 2002;277(41):38001-38012.

37. Cecchini G, Schröder I, Gunsalus RP, Maklashina E. Succinate dehydrogenase and fumarate reductase from *Escherichia coli*. Biochimica et Biophysica Acta - Bioenergetics 2002;1553(1-2):140-157.

38. Coustou V, Besteiro S, Rivière L, Biran M, Biteau N, Franconi J-, Boshart M, Baltz T, Bringaud F. A mitochondrial NADH-dependent fumarate reductase involved in the production of succinate excreted by procyclic *Trypanosoma brucei*. J Biol Chem 2005;280(17):16559-16570.

39. Peleg Y, Rahamim E, Kessel M, Goldberg I. Malic acid accumulation by *Aspergillus flavus* - II. Crystals and hair-like processes formed by *A. flavus* in a l-malic acid production medium. Appl Microbiol Biotechnol 1988;28(1):76-79.

40. Hansen NB, Lübeck M, Lübeck PS. Advancing USER cloning into simpleUSER and nicking cloning. J Microbiol Methods 2014;96(1):42-49.

41. Yang L, Lübeck M, Lübeck PS. Deletion of glucose oxidase changes the pattern of organic acid production in *Aspergillus carbonarius*. AMB Express 2014;4(1).

42. Hatch MD. A simple spectrophotometric assay for fumarate hydratase in crude tissue extracts. Anal Biochem 1978;85(1):271-275.

43. Miura A, Kameya M, Arai H, Ishii M, Igarashi Y. A soluble NADH-dependent fumarate reductase in the reductive tricarboxylic acid cycle of *Hydrogenobacter thermophilus* TK-6. J Bacteriol 2008;190(21):7170-7177.

44. Papagianni M. Advances in citric acid fermentation by *Aspergillus niger:* Biochemical aspects, membrane transport and modeling. Biotechnol Adv 2007;25(3):244-263.

45. Ramachandran S, Fontanille P, Pandey A, Larroche C. Gluconic acid: Properties, applications and microbial production. Food Technology and Biotechnology 2006;44(2):185-195.

46. Battat E, Peleg Y, Bercovitz A, Rokem JS, Goldberg I. Optimization of L-malic acid production by *Aspergillus flavus* in a stirred fermentor. Biotechnol Bioeng 1991;37(11):1108-1116.

47. Kenealy W, Zaady E, Du Preez JC. Biochemical aspects of fumaric acid accumulation by *Rhizopus arrhizus.* Appl Environ Microbiol 1986;52(1):128-133.

48. RHODES RA, MOYER AJ, SMITH ML, KELLEY SE. Production of fumaric acid by *Rhizopus arrhizus.* Appl Microbiol 1959;7(2):74-80.

49. Ding Y, Li S, Dou C, Yu Y, He H. Production of fumaric acid by *Rhizopus oryzae*: Role of carbon-nitrogen ratio. Appl Biochem Biotechnol 2011;164(8):1461-1467.

50. CLELAND WW, JOHNSON MJ. Studies on the formation of oxalic acid by *Aspergillus niger*. The Journal of biological chemistry 1956;220(2):595-606.

51. Mandal SK, Banerjee PC. Submerged production of oxalic acid from glucose by immobilized *Aspergillus niger*. Process Biochemistry 2005;40(5):1605-1610.

52. Singh OV, Jain RK, Singh RP. Gluconic acid production under varying fermentation conditions by *Aspergillus niger*. Journal of Chemical Technology and Biotechnology 2003;78(2-3):208-212.

53. Sauer M, Porro D, Mattanovich D, Branduardi P. Microbial production of organic acids: expanding the markets. Trends Biotechnol 2008;26(2):100-108.

CHAPTER 6. CONCLUDING REMARKS AND FUTURE PERSPECTIVE

This project aims to develop fungal cell factories for biotechnological production of organic acid from lignocellulosic biomass. Two *Aspergillus* strains, *Aspergillus carbonarius* ITEM5010 and *Aspergillus saccharolyticus* IBT28231 were selected and genetically engineered for enhanced succinic acid production based on their metabolic traits. The main focus in the thesis was on *A. carbonarius* strain due to the high secretion capacity of organic acids and the availability of the fully sequenced genome. In contrast among the drawbacks of using *A. saccharolyticus* for metabolic engineering compared with *A. carbonarius* was especially the lack of a fully sequenced genome. However, at present the genome is underway and will soon be released, facilitating future design of this species into cell factories.

For *A. carbonarius*, a cytosolic bypass was constructed by heterologous expression of phosphoenolpyruvate carboxylase from *Escherichia coli* and phosphoenolpyruvate carboxykinase from *Actinobacillus succinogenes* in the wild-type. Impacts of the corresponding genetic modifications on organic acid production were examined in the derived mutants. Enhanced production of citric acid was obtained in all the selected mutants, but there were only limited impacts on production of malic acid and succinic acid. On the other hand, deletion of the *gox* gene, responsible for conversion of glucose to gluconic acid by *A. carbonarius*, changed the pattern of organic acid production in buffered pH condition. The production of gluconic acid by the Δ*gox* mutant was eliminated and production of citric acid, oxalic acid and malic acid were increased in the buffered pH condition.

For *A. saccharolyticus*, the organic acid profile in the buffered pH condition showed that *A. saccharolyticus* wild-type could naturally secrete high amounts of malic acid and succinic acid. Expression of a NADH dependent fumarate reductase increased succinic acid production significantly in *frd* mutant, implying the carbon flux could be continued to succinic acid via the cytosolic reductive pathway. On the other hand, succinic acid production by *frd* mutants was also detected in low pH conditions, indicating the possibility to apply *A. saccharolyticus* in low pH processes of succinic acid production.

Through the research attempts in genetic engineering of *A. carbonarius* and *A. saccharolyticus* for succinic acid production, the difficulties in re-routing the carbon flux towards the desired organic acids have been demonstrated by comparing the impacts of genetic modifications on organic acid production in these two strains. It is evident in the current phase of this project that it is more difficult

to increase the production of succnic acid by *A. carbonarius* compared with *A. saccharolylticus*.

In addition, a wheat straw hydrolysate was used as a substrate for organic acid production by *A. carbonarius*. Several advantages of *A. carbonarius* were verified for its future use in the bio-based organic acid production from lignocellulosic biomass including efficient co-utilization of glucose and xylose, promising tolerance to inhibitors and varieties of organic acid products. Furthermore, the Δ*gox* mutant was also cultivated in the wheat straw hydrolysate, and different impacts of the same genetic modification on organic acid production were found in the defined media and the wheat straw hydrolysate.

For future perspectives, the strain development of *A. carbonarius* and *A. saccharolyticus* for succinic acid production may be carried out in the following directions:

First, the organic acid production by those two strains was significantly influenced by cultivation pH and aeration which cannot be monitored in shake flasks. Therefore, the cultivation conditions for organic acid production may be optimized in bioreactors from which those key parameters can be precisely controlled.

Second, for *A. carbonarius*, the fact that enhanced carbon flux via the genetic modifications introduced in *A. carbonarius* eventually flowed towards citric acid production implies the importance of elucidating the mechanism of citric acid accumulation by *A. carbonarius* for future strain improvement. An effective means of controlling carbon flux towards citric acid will provide great flexibility for applying *A. carbonarius* as versatile cell factories for production of other organic acids. In addition, a number of genetic modifications targeting to succinic acid producing pathways might be combined in order to further increase the carbon flux towards succinic acid production. Application of pathway modeling tools in metabolic engineering of *A. carbonarius* may benefit in designing strategies for strain improvement.

Third, for *A. saccharolyticus*, since enhanced succinic acid production was obtained via reduction of fumarate in cytosol, future genetic manipulation might be carried out in the *frd* mutants to increase the carbon flux towards succinic acid via the rTCA branch. On the other hand, high amount of malic acid was still produced as byproduct by the *frd* mutant, so new genetic engineering strategies also need to focus on reducing formation of malic acid as byproduct in the future strain development.

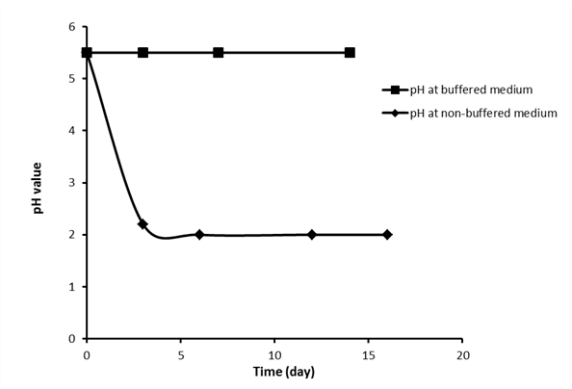
Fourth, this project has verified the feasibility of using hydrolysate of lignocellulosic biomass for organic acid production by *A. carbonarius*. Compared with defined media, sugar consumption and organic acid productivities by *A.*

carbonarius decreased significantly in the hydrolysate. In the next phase, more efforts might be made to investigate the inhibitory effects resulted from other potential inhibitors in addition to acetic acid and furfural, and to also improve the resistance of *A. carbonarius* to the inhibitory compounds in the hydrolysate via strain improvement or detoxification methods.

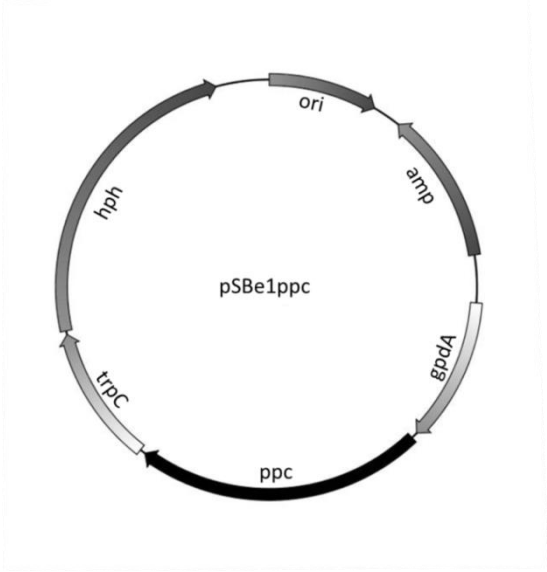
APPENDICES

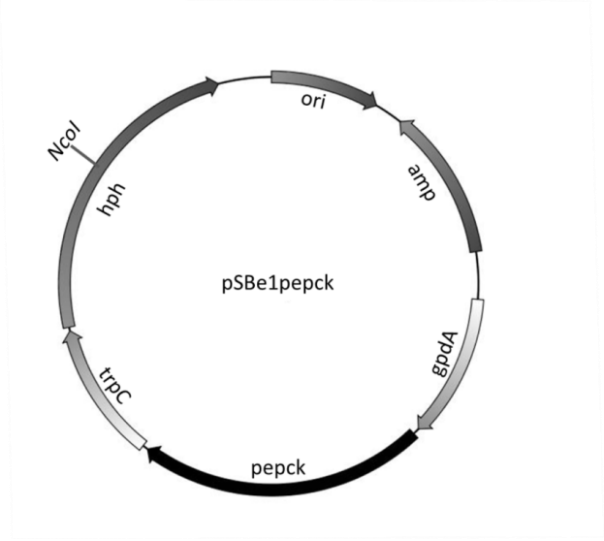
Appendix A. Supplementary materials for Chapter 2

1. pH value in the glucose media under pH buffered and non-buffered conditions



2. Plasmid maps





3. pH values in the non-buffered pH cultures containing glucose and xylose

	Glucose based medium				Xylose based medium			
	pepck	ppc	pepck+ppc	WT	pepck	ppc	pepck+ppc	WT
Day 3	2-2.5	2-2.5	2-2.5	2-2.5	3.5	3.5	3.5	3.5
Day 7	2	2	2	2	3-3.5	3-3.5	3-3.5	3-3.5

Appendix B. Supplementary materials for Chapter 4

Growth of *A. carbonarius* in the defined media containing acetic acid and furfural

pH values	Acetic acid (7 g/L)	Furfural (0.25 g/L)	Acetic acid (7 g/L) + Furfural (0.25 g/L)
3	-	+	-
5	-	+	-
7	+	+	+

The growth test of *A. carbonarius* was carried out in duplicates and checked on day 10 after the spores were inoculated into the media. "+" indicates that growth was observed; "-"indicates that growth or spore germination was not detected.

Appendix C. Supplementary materials for Chapter 5

Organic acid production by *A. saccharolyticus* in the YPD medium and in the acid production medium

	Malic acid (g/L)	Succinic acid (g/L)	Citric acid (g/L)	Fungal biomass (g)
YPD medium	N.D	N.D	N.D	0.61 ± 0.03
AP medium	4.4 ± 0.6	0.95 ± 0.03	0.29 ± 0.02	0.44 ± 0.01

The samples were taken after 2 days cultivation in the YPD medium or in the acid production (AP) medium in pH buffered condition. ("N.D" indicates that no production was detected; Data shown are mean values from triplicates with the standard error after "±")

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