Itaconic acid is an important building block for the chemical industry. Currently, Aspergillus terreus is the main organism used for itaconic acid production. Due to the enormous citric acid production capacity of Aspergillus niger, this host is investigated as a potential itaconic acid production host. Several strategies have been tried so far: fermentation optimization, expression of cis-aconitate decarboxylase (cadA) alone and in combination with aconitase targeted to the same compartment, chassis optimization, and the heterologous expression of two transporters flanking the cadA gene. We showed that the heterologous expression of these two transporters were key to improving itaconic acid production in an A. niger strain that was unable to produce oxalic acid and gluconic acid. The expression of transporters has increased the production levels of other industrially relevant processes as well, such as β-lactam antibiotics and bioethanol. Thus far, the role of transporters in production process optimization is a bit overlooked.

Introduction

Over the past few years there is an increasing interest in itaconic acid as a bio-based building block to produce, e.g., plastics, adhesives, elastomers, and coatings. Besides these products, itaconic acid is also used as a building block for materials used for biomedical purposes like drug delivery systems and as a material in restorative dentistry. In search of optimized production processes for the production of itaconic acid from plant waste streams Aspergillus niger is investigated as a cell factory by several research groups.

A. niger is one of the organisms of choice to exploit plant derived waste streams due to its extensive capacity to degrade polysaccharides. In addition, A. niger has a long history in industrial fermentations and can be easily genetically modified making the organism an attractive host for the production of enzymes and metabolites.

Itaconic acid is nowadays produced on an industrial scale using the filamentous fungus Aspergillus terreus. Titers up to 80 g/L are achieved and several strategies have been investigated to further improve the production in A. terreus.

Itaconic acid biosynthesis is very similar to citric acid biosynthesis because it is basically an extension of the citric acid biosynthesis pathway (Fig. 1). Aconitase catalyzes the isomerization of citrate to isocitrate via cis-aconitate. The decarboxylation of the intermediate cis-aconitate by cis-aconitate decarboxylase leads to the formation of itaconate. A. niger has been exploited for citric acid production for many years and extreme high citrate production levels, over 200 g/L, are achieved nowadays. If these citrate production strains can be modified for itaconic acid production, titers over 135 g/L could be reached.

Itaconic Acid Production in Aspergillus niger

The pathway transfer of itaconic acid production to A. niger started with the
overexpression of the *A. terreus cadA* gene encoding *cis*-aconitate decarboxylase in *A. niger*. This led to the production of low amounts of itaconic acid. Oxalic acid is the major organic acid being produced by *A. niger* laboratory strains. Besides oxalic acid also citric acid and gluconic acid are produced in large amounts.

These initial results showed that the production of itaconic acid in *A. niger* is possible, but the production level obtained is far from the theoretical levels if only *cadA* is expressed. To improve the itaconic acid production several strategies have been applied. These strategies target different levels of the production process.

**Fermentation optimization**

One of the strategies applied is to optimize the fermentation conditions and in particular the medium composition. Though positive and negative effects of several components were found, the most pronounced positive effect was the addition of copper. By optimizing the copper concentration the oxalic acid production was reduced, while the citric acid production increased. Nevertheless, even after medium optimization, the itaconic acid concentration obtained was only 2 g/L.

**Chassis optimization**

An early example of host or chassis optimization for citric acid production is the work of Ruijter et al. (1999). With the aim to reduce side product formation in citric acid production and to increase the carbon flow toward citric acid production, these authors constructed strains deficient in oxalic acid and gluconic acid biosynthesis. Loss-of-function mutations in oxaloacetatehydrolase (*OahA*) and glucose oxidase (*GoxC*) result in the inability to produce any oxalic acid and gluconic acid. Surprisingly, combining these mutations in *A. niger* results in constitutive citric acid production, which is then no longer influenced by the carbon source used or the medium composition used. Li et al. showed the importance of chassis optimization in side product formation in itaconic acid production by *A. niger* by expressing *cadA* in an *oahA* *A. niger* strain. This resulted in 0.4 g/L itaconic acid, which was also an improvement compared the 0.24 g/L that was obtained by the expression of *cadA* in a wild type background. Our studies on the production of itaconic acid in *A. niger* have been done in the genetic background lacking both *OahA* and *GoxC* activity resulting in itaconic acid production levels that are far above the levels thus far described.

**Pathway optimization**

An elegant way to improve itaconic acid production was shown by Blumhoff et al. They targeted the two important enzymes, *aconitate* and *cis*-aconitate decarboxylase, to the same compartment. It is believed that *CadA* is localized in the cytosol while *aconitate* activity is mainly found in the mitochondria as part of the TCA cycle (Fig. 1). The itaconic acid production level increased significantly when both enzymes were targeted to the mitochondria. This indicates that the substrate limitation might actually be a transport limitation between the different compartments.

Another approach was the combined overexpression of a modified phosphofructokinase in combination with the itaconic acid biosynthesis cluster. Using phosphofructokinase that was relieved of citrate inhibition led to increased productivity levels but not to improved production levels.

**Optimization of cellular transport**

In our recently published paper we showed that besides the overexpression of the catalyzing enzyme *cis*-aconitate decarboxylase and the chassis optimization, two transporters play a crucial role in establishing an efficient production process. Especially the heterologous expression of the mitochondrial transporter improved the production significantly in this particular *A. niger* strain.

An overview of the most interesting modifications to establish and optimize itaconic acid production in *A. niger* is given in Table 1.

**Cellular Transport**

Heterologous expression of transporters is rarely done as part of strain improvement, while it does have a great potential to improve production levels of industrially relevant compounds. We showed that the overexpression of the mitochondrial transporter *MttA* improved...
Due to increasing penicillin resistance, the production of penicillin by *Penicillium chrysogenum* is highly optimized over the years. The majority of these strategies appeared on the functioning of a few important transporters in β-lactam antibiotic production. One very interesting transporter is the *cefT* gene from *A. chrysogenum*. The heterologous expression of this transporter in different *P. chrysogenum* strains resulted in an increased secretion of the desired β-lactam antibiotic. Part of the β-lactam antibiotics biosynthesis takes place in the peroxisomes while the major part of the biosynthesis takes place in the cytosol. Thus besides an efficient import of the substrate and export of the product also peroxisomal transport is an important process in β-lactam antibiotics production. In *A. chrysogenum* the transporter CefM was found that is essential for the transport of penN from the peroxisomes to the cytosol.17

### L-galactonic acid production by filamentous fungi

Pectin is a major plant cell wall component in certain agricultural waste streams like sugar beet pulp. Since sugar beet pulp is mostly dumped, developing a process for the production of metabolites using sugar beet pulp is economically attractive. Pectin is easily hydrolysed by filamentous fungi to D-galacturonic acid, which is subsequently metabolised. Kuivanen et al. describe the production of L-galactonic acid from D-galacturonic acid by *Trichoderma reesei* and *A. niger*.18 In this study two effects on cellular transport were found; increased production of a putative D-galacturonic acid transporter and intracellular accumulation of L-galactonic acid. The latter suggests a bottleneck in L-galactonic export that may be relieved by the introduction on

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**Table 1. Overview of the constructed itaconic acid producing Aspergillus niger strains**

| Strain | A. niger parent strain | Expressed genes | Itaconic acid production in g/L | Description growth conditions |
|--------|------------------------|-----------------|---------------------------------|------------------------------|
| sCAD4⁹ | NW186                  | cadA            | 0.25                            | 100 mM sorbitol + 50 mM xylose, 78 h of fermentation |
| cadA + mfsA 2.5⁹ | sCAD4 | cadA + mfsA | 0.14                            | 100 mM sorbitol + 50 mM xylose, 78 h of fermentation |
| cadA + mttA 1.2⁹ | sCAD4 | cadA + mttA | 5.4                             | 100 mM sorbitol + 50 mM xylose, 78 h of fermentation |
| cadA + mttA + mfsA 4⁹ | sCAD4 | cadA + mttA + mfsA | 7.1                            | 100 mM sorbitol + 50 mM xylose, 78 h of fermentation |
| CAD10.1⁴ | AB 1.13               | cadA            | 0.7                             | 100 g/L glucose after 90 h of fermentation |
| CAD10.1⁸ | AB 1.13               | cadA            | 0.24                            | Micro-titer cultures, 60 h |
| ΔoahA#76 CAD 5 | AB 1.13 ΔoahA#76 | cadA | 0.4                             | Micro-titer cultures, 60 h |
| MTT 1.4⁶ | CAD10.1               | cadA + mttA | 1.4                             | Micro-titer cultures, 60 h |
| MFS 3.9⁸ | CAD10.1               | cadA + mfsA | 1.4                             | Micro-titer cultures, 60 h |
| CAD + MTT + MFS ₃⁸ | MTT 1.4 | cadA + mttA + mfsA | 0.9                            | Micro-titer cultures, 60 h |
| cCADa¹¹ | ATCC 1015             | cytosolic cadA | 0.05                           | Shakeflask culture with 20% glucose for 240–312 h |
| mCADa¹¹ | ATCC 1015             | mitochondrial cadA | 0.17                          | Shakeflask culture with 20% glucose for 240–312 h |
| mCADa + mAcNA¹¹ | ATCC 1015 | mitochondrial cadA + mitochondrial acoA from A. niger | 1.2                          | Shakeflask culture with 20% glucose for 240–312 h |
| cCADa + cAcNA + mCADa + mAcNA¹¹ | ATCC 1015 | cytosolic cadA + cytosolic acoA from E. coli + mitochondrial cadA + mitochondrial acoA from A. niger | 1.4                          | Shakeflask culture with 20% glucose for 240–312 h |
an L-galactonic acid transporter. As the uptake of D-galacturonic acid might also be a bottleneck, increased expression of the D-galacturonic acid transporter may increase the efficiency of the process as well. Overexpression of a D-galacturonic acid transporter in A. niger leads to preferred use of D-galacturonic acid paralleled by increased citric acid formation (Sloothaak and de Graaff, personal communication). This might open ways to exploit the pectin fraction of plant biomass for biobased production.

Expression of a xylose transporter in Saccharomyces cerevisiae

Saccharomyces cerevisiae is one of the most-studied organisms in biotechnology and serves as a model organism for eukaryotes. S. cerevisiae is used in industry for the production of ethanol. Currently, the production of ethanol from plant waste materials is a trending topic in biotechnology. One of the major drawbacks of wildtype S. cerevisiae is its inability to grow on xylose. After glucose, xylose is the most abundant sugar present in plant cell wall polysaccharides. Therefore, a lot of research is done on the heterologous expression of xylose metabolizing pathways in S. cerevisiae.\(^2\,3\)

Besides the enzymatic conversion of xylose also the import of xylose into the cell has been investigated. The heterologous expression of xylose transporters in an S. cerevisiae strain that is able to use xylose for the production of ethanol showed that the production rate of ethanol increased up to 70%.\(^4\) This example also shows that in this efficient ethanol production process import of xylose was a limiting step and major improvements can still be made by the expression of transporters. Recently, Young et al. showed that they were able to improve xylose transporters even further using directed evolution. Only a few point mutations already led to an improved growth rate by 70%. Other mutations caused differences in efficiency and changes in affinity of the transporter.\(^2\)

Outlook

The transfer of biosynthetic pathways that lead to the production of metabolites of industrial importance from one organism to a biotechnologically more preferred organism has become more feasible than ever. Pathway transfer became a realistic possibility after the development of modern cloning techniques. Nowadays, synthetic biology increases the possibilities for effective pathway transfer enormously. The use of standardized modules combined with metabolic modeling allows the rational design of a desired pathway suitable for the new host or chassis. Routinely, codon usage is optimized to achieve efficient expression of the coding sequence by the production host. Combining codon optimization usage with the use of well-defined and characterized parts like promoters, terminators, and signal sequences will significantly decrease the time needed for the development of future processes.

However, transporters are often overlooked when transferring pathways from one organism to the other. This is not surprising since it is more difficult to identify and characterize transporters compared with enzymes. Despite the difficulties, new strategies became available to characterize transporters.

A nice example is the expression of eukaryotic transporters in Lactococcus lactis as an alternative host for E. coli.\(^23\)\(^\text{a}\) A success story using L. lactis as an expression host is the characterization of the mitochondrial pyruvate transporter that is encoded by two genes.\(^2\)

As the evolutionary distance between different organisms increases, differences in membrane composition and membrane structure become apparent. Even though the function of a given transporter might be known in one organism it might be a challenge to functionally express the transporter in the host of choice due to these differences.

Transporters play a crucial role in optimizing pathways for the production of industrially relevant compounds. The here-described studies on the production of itaconic acid in A. niger, β-lactam antibiotics production in P. chrysogenum, and the bioethanol production from xylose in S. cerevisiae are a showcase of the knowledge that needs to be developed. Many more success stories will follow in the future when more knowledge is acquired about the function of transport proteins. Transporters might be the key to success when transferring pathways to fungi.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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