General discussion

The study described in this thesis is aimed at improving the production level of the commercially interest C5 di-carboxylic acid itaconic acid using the filamentous fungus *Aspergillus niger*. The initial idea of using *A. niger* as a production host was based on its versatile and tolerant character in various growth environments, and its extremely high capacity of accumulating the precursor of itaconic acid: citric acid (up to 360 g/L). As *A. niger* is not a native itaconic acid production host, genetic modifications of the production host would be required. Two research directions were followed to produce and further improve the levels of itaconic acid in *A. niger*. One direction focused on host selection and genetic modification of the itaconic acid production pathway and related genes, the other direction dealt with optimizing the production process of itaconic acid including development of a suitable production medium, defining pH and dissolved oxygen conditions etc.

A clone based transcriptomics approach identified genes crucial in the biosynthesis pathway of itaconic acid in *Aspergillus terreus*. Expressing the *cis*-aconitate decarboxylase (*cadA*) gene from *A. terreus* opened the metabolic pathway towards itaconic acid in *A. niger*. In the subsequent route of improving itaconic acid production by *A. niger* via genetic modification, studies on expression of two transporter genes (*mttA*, *mfsA*) from the itaconic acid gene cluster to improve intracellular transport of the precursor and export of itaconic acid to the culture medium, over-expression of a hemoglobin domain gene (*hbd1*) to improve intracellular oxygen availability, deletion of oxaloacetate acetyl hydrolase gene (*oahA*) to get rid of oxalic acid as a by-product, and replacing the *A. niger* lab strain with an industrial citric acid producing strain *A. niger* N201, all resulted in increased itaconic acid production levels. Meanwhile, the other route on optimizing the itaconic acid production by modifying medium and process parameters showed that increased levels of copper positively correlated to itaconic acid production, and reduced dissolved oxygen (D. O.) levels in fermentation process were beneficial for the production of itaconic acid.

Although in the route towards obtaining an improved *A. niger* production host, we identified 3 genes constituting the itaconic acid production pathway, the levels and yield of itaconic acid produced in *A. niger* were still low in comparison to the levels observed in the native host. To further improve itaconic acid production in *A. niger*, we need to understand the major difference in production profile between *A. terreus*

and *A. niger*. Where citric acid is completely absent in cultivation medium of *A. terreus* producing itaconic acid, in most cases it is still coproduced in large amounts in *A. niger* strains expressing the itaconic acid pathway genes. Replacement of the initial lab strain used for the transformation of *cadA* with the industrial citric acid producing strain N201 did improve itaconic acid levels, supporting our initial hypothesis that improved levels of citric acid could result in improved itaconic acid production, but also still showed citric acid production. To completely direct the flux towards itaconic acid, further genetic modification of genes involved in itaconic acid production is required. Towards this goal, more genes from the *A. terreus* transcriptome analysis (Li et al., 2011) are being identified (Li et al., manuscript in preparation). Interestingly, among the genes highly up-regulated in conditions of itaconic acid production, several genes encoding enzymes involved in the pentose phosphate pathway were present. Up-regulation of the pentose phosphate pathway in *A. terreus* may result in improved flux towards intermediate metabolites in the glycolysis pathway relevant for itaconic acid production conditions.

Another aspect for further improvement could be the fact that in the current *A. niger* strains different sub-cellular compartments are involved in the biosynthesis of itaconic acid. A recent paper by Blumhoff et al (2013) showed that, expression of the final part of the itaconic acid pathway (*cadA*) in the mitochondrial compartment from *A. niger* could be beneficial. Still as also in *A. terreus* localization of the proteins encoded by TCA cycle, producing the citric acid precursor, and the itaconic acid pathway gene is similar as in *A. niger* this does not explain the abovementioned discrepancy of citrate coproduction. Current research in our laboratory focusses on further understanding of this aspect in more detail.

The other research route focused on optimizing the production process to increase itaconic acid levels. Copper as a trace-element was shown to be positively related to itaconic acid production (Chapter 3). Although the role of copper in itaconic acid production in *A. niger* not understood, in the extended transcriptome study described above (Li et al., manuscript in preparation), also coregulation of a copper-transport protein with itaconic acid production was observed.

Our observations that reduced D. O. supply improved itaconic acid production (Chapter 4, Table 3), seems counter-intuitive given the fact that expression of a hemoglobin domain resulted in increased levels of itaconic acid. Although also here our understanding is far from complete, these results may suggest that reduced DO levels during fermentation result in a better flux towards itaconic acid and that expression of the

hemoglobin domain protein may protect the host strain from intracellular oxygen depletion which might be an effect of the reduced DO levels in the medium.

An aspect that was not covered in large detail in our study, is the fact that high levels of itaconic acid show toxicity towards *A. niger* (and *A. terreus*) (Li et al. unpublished results). Obviously this would possibly limit the production of itaconic acid in this host. Therefore, besides the aspects described above we also addressed issues of recovering itaconic acid during fermentation (Chapter 5). Itaconic acid was successfully recovered via a solvent extract method in a petraction module coupled in-stream using *A. terreus* as a study model. Based on these results, an obvious next step will be to optimize the in-situ recovery process. Currently, the major bottle neck is the inhibitory effect of the organic solvent used for recovery on fungal growth. Possible solutions would be using less toxic organic solvents or integrate a solvent recovery module in the process at a stage prior to looping back the broth to the bio-reactor.

As presented in Chapter 1, producing organic acids as industrial building blocks using fungi has a great potential for the future biobased chemical industry. Our work illustrated a promising research route initiated with identifying crucial genes using a clone-based transcriptomics approach, further developed by combining the genetic modification of a novel fungal host, optimization of the production conditions together with an in-stream product recovery process.

References

Li, A., Caspers, M., Punt, P. (Unpublished) The systems biology of itaconic acid production host *Aspergillus terreus* and the new production host *Aspergillus niger*

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