Itaconic acid production in Aspergillus

Li, A.

Summary

Itaconic acid (methylene succinic acid) is a white crystalline unsaturated C5 dicarboxylic acid. Because of its specific favorable properties and the unique structure, itaconic acid is used as monomer or co-monomer in the manufacture of plastics, resins, synthetic fibers, paints, surfactant and elastomers etc. Besides, in 2004 it was selected by the Department of Energy in the US as one of the 12 building block chemicals that are the most interesting to be produced by industrial biotechnology. Currently itaconic acid is produced commercially by *Aspergillus terreus* via fungal fermentation. Due to its limited availability, the use of itaconic acid is restricted. To increase itaconic acid production levels and make it economically available, *Aspergillus niger* is chosen as the production host strain due to its flexibility as a production platform. *A. niger* (one of the black aspergilli) has a long tradition of safe use (Schuster et al. 2002) in the production of enzymes and organic acids, and is widely applied in biotechnology as host for the production of food ingredients, pharmaceuticals and industrial enzymes. Besides, *A. niger* accumulates organic acids on a wide range of substrates under various environmental conditions. Most important, *A. niger* produces large amounts of citric acid (360 g/L) which in *A. terreus* actually is a precursor in the biosynthetic pathway towards itaconic acid. This thesis describes the research carried out for producing itaconic acid in *A. niger*, and illustrates research approaches of organic acids production in fungi in general.

Studies on the metabolic pathways for organic acid production by fungi show that the major commercial products are either the result of the simple oxidation of a monosaccharide, or are intermediates or derivatives of the tricarboxylic acid (TCA) cycle. **Chapter 1** reviews the industrial production of organic acids especially di, tri- carboxylic acids in fungal species. Among them, some organic acids such as citric- and gluconic acid have been already commercialized for years. Other ones may have possibilities to be produced via industrial bio-based processes as well. Therefore, existing or potential metabolic pathways for each organic acid are described including the key enzymes and their encoding genes. Furthermore, state of the art knowledge on the relevant transporters in fungi, production medium and conditions involved in the production process are summarized.

A. niger which is applied for itaconic acid production in this study, is not a natural production host. The genes encoding enzymes involved in

itaconic acid production are derived from a natural itaconic acid producer, *A. terreus*. As a starting step to identify the crucial genes in the biosynthesis of itaconic acid, **Chapter 2** describes a clone-based transcriptomics approach. From a number of differently controlled *A. terreus* batch fermentations, those cultures with the largest difference in itaconic acid titer and productivity were selected for mRNA isolation. From a clone based microarray experiment, the clones resulting in the strongest (> 10 fold) differential hybridization signals were identified. One of these clones contained (part of) a gene of the MmgE-PrpD family. The full length coding region of this gene was shown to encode the *cis*-aconitate decarboxylase by heterologous expression in *E. coli*. Expression of this gene in *A. niger*, resulted in itaconic acid production. Genome analysis suggests that in *A. terreus* the *cis*-aconitate decarboxylase gene is part of an itaconic acid related gene cluster including genes encoding two pathway specific transporters and a regulatory Zinc finger protein.
In order to increase the production level of itaconic acid in *A. niger*, we continued by strain engineering and optimizing cultivation media. In **Chapter 3**, based on research described in Chapter 2 and research from other groups, two genes – *gpdA* encoding glyceraldehyde-3-phosphate dehydrogenase (GPD) and *hbd1* encoding a flavohemoglobin domain (HBD) were selected to be overexpressed in *A. niger*. Beyond that, new media were designed based on a reference medium for *A. terreus*. To be able to analyze large numbers of cultures, we developed an approach for screening fungal transformants and various media in 96-well micro-titer plates. Although the performance of GPD and HBD transformants were not found to be better than their parental strain under the tested cultivation conditions, copper (Cu^{2+}) was shown to be positively correlated with improving itaconic acid production. Surprisingly, the conditions for optimizing itaconic acid production clearly differ from conditions optimal for citric- and oxalic acid production.
As mentioned from the transcriptomics study described in Chapter 2, *cadA* (gene encoding *cis*-aconitate decarboxylase) and *mttA* (gene encoding a mitochondrial carrier protein) are highly up-regulated in itaconic acid production conditions. By exploring the genome of *A. terreus*, another interesting gene *mfsA* (a putative dicarboxylate carrier) which was found to be clustered with *cadA* and *mttA* might also be related to itaconic acid production. Gene *mttA* encodes a protein potentially functioning in transporting tri-carboxylic acids out of mitochondria and gene *mfsA* encodes a protein potentially involved in exporting di-carboxylic acids out of the cells. In **Chapter 4**, individual expression of these two transporters in *A. niger* was shown to improve itaconic acid production. However, the production was not further

improved when *mttA* and *mfsA* were both expressed in strain CAD 10.1, an *A. niger cadA* transformant. Besides, oxalic acid accumulated as a by-product in the culture. In order to increase itaconic acid production and eliminate by-product formation, two new *A. niger* production hosts, D15 #26 (a non-acidifying strain) and the *oahA* deletion strain AB 1.13 Δ*oahA* #76 (no oxalic acid production) have been tested for improving itaconic acid production. Whereas expressing the *cadA* gene in D15#26 did not result in increased itaconic acid production, the one step expression of *cadA* in AB 1.13 Δ*oahA* #76 did accumulate similar amounts of itaconate as the *mttA* and *mfsA* transformants. Furthermore, the transformant Δ*oahA* #76 CAD5 accumulated higher amount of citric acid, which could be considered as a benefit in using this strain for further strain development towards improved itaconic acid production.

The study was expanded by analysis of improved host strains and fermentation conditions. An improved citric acid host strain *A. niger* strain ATCC 1015 N201 was introduced as a new production host. The selected *cadA* transformant from N201 and the previous developed strain HBD 2.5 with an overexpressed fungal hemoglobin domain (*hbd1*) were tested in various dissolved oxygen (D. O.) conditions, showing that reduced levels of dissolved oxygen have a positive influence on itaconic acid production in *A. niger* strains. This coincided with the observation that overexpressing of a fungal hemoglobin domain in *A. niger* improved itaconic acid production under controlled cultivation conditions at low D. O. levels. The most favorable D.O. for producing itaconic acid was around 10% -20% . Replacing the lab strain AB 1. 13 CAD with the industrial organic acid producing strain N201 CAD increased the amount of itaconic acid production. The above results provided us with new insights of improving itaconic acid production in *A. niger*.

In **Chapter 5** , as a demonstration for the final step of bio-based itaconic acid production, a novel downstream recovery procedure using *A terreus* strain as a study model was explored. This chapter presents preliminary work to integrate biotechnological production of itaconic acid with in-stream product recovery (ISPR). Solvent extraction was selected among the current major techniques for recovering itaconic acid using 2-methyl tetrahydrofuron (2m-THF) as extractant.