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Chapter 4

Reduced by-product formation and modified oxygen availability improve itaconic acid production in *Aspergillus niger*

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Abstract

Aspergillus niger has an extraordinary potential to produce organic acids, as proven by its application in industrial citric acid production. Previously, it was shown that expression of the *cis*-aconitate decarboxylase gene (*cadA*) from *Aspergillus terreus* converted *A. niger* into an itaconic acid producer (Li et al. 2011). After some initial steps in production optimization in the previous research (Li et al. 2012), this research aims at modifying host strains and fermentation conditions to further improve itaconic acid production.

Expression of two previously identified *A. terreus* genes encoding putative organic acid transporters (*mttA*, *mfsA*) increased itaconic acid production in an *A. niger cis*-aconitate decarboxylase expressing strain. Surprisingly, the production did not increase further when both transporters were expressed together. Meanwhile, oxalic acid was accumulated as a by-product in the culture of *mfsA* transformants. In order to further increase itaconic acid production and eliminate by-product formation, a non-acidifying strain D15 #26 and the oxaloacetate acetylhydrolase (*oahA*) deletion strain AB 1.13 $\Delta oahA$ #76 have been analysed for itaconic acid production. Whereas *cadA* expression in AB 1.13 $\Delta oahA$ #76 resulted in higher itaconic acid production than strain CAD 10.1, this was not the case in strain D15#26. As expected, oxalic acid production was eliminated in both strains. In a further attempt to increase itaconic acid levels, an improved basal citric acid producing strain, N201, was used for *cadA* expression. A selected transformant (N201 CAD) produced more itaconic acid than strain CAD 10.1, derived from *A. niger* strain AB1.13. Subsequently, we have focused on the influence of dissolved oxygen (D. O.) on itaconic acid production. Interestingly, reduced D. O. levels (10-25%) increased itaconic acid production using strain N201 CAD. Similar results were obtained in strain AB 1.13 CAD+HBD 2.5 (HBD 2.5) which overexpressed a fungal hemoglobin domain. Our results showed that overexpression of the hemoglobin domain increased itaconic acid production in *A. niger* at lower D.O. levels. Evidently, the lower levels of D.O. have a positive influence on itaconic acid production in *A. niger* strains.

Introduction

Itaconic acid, is a C5-dicarboxylic acid which is produced by *Aspergillus terreus* (*A. terreus*) for industrial use (Okabe et al. 2009; Willke and Vorlop 2001). Itaconic acid possesses two carboxyl moieties which give it the ability to act as a co-monomer in the manufacture of polymers (Okabe et al. 2009; Willke and Vorlop 2001). Its application areas are mainly in plastics, paper industry and adhesives (Okabe et al. 2009; Willke and Vorlop 2001). The biological pathway of itaconic acid production in *A. terreus*, occurs in two sub-cellular compartments (Bonnarme et al. 1995; Jaklitsch et al. 1991). In this pathway, pyruvate is converted to acetyl-CoA in the mitochondria, and together with oxaloacetate, is converted by citrate synthase to citric acid. Further in the TCA cycle, citrate is dehydrated to *cis*-aconitate and then, as *cis*-aconitate decarboxylase (CAD) is assumed to convert *cis*-aconitate into itaconate inside the cytosol, mitochondrial transporter is involved in transporting *cis*-aconitic acid from the mitochondrion to the cytosol. Alternatively, citric acid is first transported to the cytosol by a tri-carboxylic acid transporter and further converted to itaconic acid in two steps. Finally, itaconic acid is exported out of the cell most likely via a di-carboxylic acid transporter. Although the details of tri-/di- carboxylic acid transport in relation to itaconic acid are unknown, an important role of the two transporters in shuttling metabolites required for the itaconic acid metabolic pathway is suggested.

The role of this type of transporters is also suggested from our previous study on the identification of the *cis*-aconitate decarboxylase, as in this study also a mitochondrial carrier protein (*mttA*) and a putative dicarboxylate carrier (*mfsA*) were found to be expressed in concordance with *cadA* in a hypothetical itaconic acid gene cluster in *A. terreus* (Li et al. 2011). These genes lack true orthologous in *A. niger*, which suggest a unique transport system specific to itaconic acid. Therefore, expression of these two transporters in *A. niger* CAD strains seems to be a promising approach to increase the itaconic acid production.

To further increase the production capacity and reduce by-product formation, other parental *A. niger* strains were evaluated. *A. niger* was chosen as a production host for itaconic acid due to the high capacity of accumulating citric acid which as described above is a precursor of itaconic acid production. We previously showed that one step *cadA* expression in *A. niger* converted the parental strain AB 1.13 into an itaconic acid producer (Li et al. 2011). In order to improve the production level of itaconic acid, a combination of genetic modification on the production host and production medium optimization has already been described (Li et al. 2012). Medium optimization led to an improved medium for itaconic acid production in *A. niger*. However, the batch fermentation of

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an *A. niger* transformant showed that oxalic acid as a by-product, was still produced in the selected medium condition (Li et al 2012). Although also citric acid was still co-produced with itaconic acid, we have observed that more citric acid production led to higher itaconic acid accumulation (Li et al 2012). Previous studies in *A. terreus* showed that reduced dissolved oxygen (D.O.) levels improved itaconic acid production (Li et al. 2011).

From these results, three aspects for further itaconic acid production improvement can be identified, i.e. reduction of oxalic acid by-product formation in the parental strain; use of a host strain with increased citric acid production; modification of oxygen availability.

Although oxalic acid production can be reduced by using alternative process conditions (Li et al. 2012), here we designed a molecular/genetic strategy to eliminate oxalic acid. One approach we followed was to delete the gene *oahA* encoding oxaloacetate hydrolase responsible for cleaving oxaloacetate into oxalate and acetate. An alternative approach to reduce by product formation was followed by using a previously isolated low acid producing mutant from AB 1.13 (Gordon et al. 2000).

Assuming that higher levels of citric acid production in a parental *A. niger* strain would lead to higher itaconic acid accumulation after introduction of the *A. terreus cadA* gene, *A. niger* strain N201 (ATCC 1015) was selected as parental strain for *cadA* transformation based on its high citric acid production level. This strain is a fully sequenced wild type strain used in the first patented citric acid production process (Currie 1917).

As previously shown, itaconic acid production conditions are not identical to citric acid production conditions. In *A. terreus*, itaconic acid production is influenced by multi-environmental factors such as media components, pH, oxygen availability, temperature etc. (Gyamerah 1995; Matthey 1992; Milsom and Meers 1985; MuralidharaRao et al. 2007; Okabe et al. 2009; Pfeifer et al. 1952; Riscaldati et al. 2000; Rychtera and Wase 1981; Vassilev et al. 1992; Willke and Vorlop 2001). Here we considered oxygen availability determined as dissolved oxygen (D. O.) as an potentially important parameter for successful itaconic acid production by *A. niger* in submerged cultures. Besides altering fermentation conditions, also a molecular genetic approach was followed to modify oxygen availability. Based on the fact that increased oxygen uptake through overexpression of a bacterial hemoglobin (*vgb*) was shown to have a strong effect on itaconic acid production in *A. terreus* (Lin et al. 2004), and overexpression of a fungal hemoglobin domain improved oxygen uptake in *Aspergillus oryzae* (te Biesebeke et al. 2006), we have overexpressed a fungal hemoglobin domain (*hbd1*) in *A. niger* strain previously (Li et al. 2012). Although the resulting transformant (HBD 2.5) did not show improved itaconic acid production (Li et al. 2012), we assumed that modification of D.O. levels could have an effect on itaconic acid production in this strain.

Materials and methods

Strains/Plasmids/Fungal transformation

Aspergillus niger strains AB 1.13 pyrG⁻, AB 1.13, CAD 10.1 (Li et al. 2011), AB 1.13 CAD, HBD 2.5 (Li et al. 2012), D15#26, and N201 (ATCC 1015) (Currie 1917) were used in this study (Table 1). They were all maintained on potato-dextrose agar (PDA) plates and in 20% glycerol spore suspensions frozen at -80°C.

Table 1. List of *A. niger* strains used in this study.

Strains and transformants	Abbreviation	Strain description	Reference
AB 1.13 pyrG ⁻			Mattern et al. 1992
AB 1.13 pyrG ⁺	AB 1.13	Uridine prototroph of AB 1.13 pyrG ⁻	
AB 1.13 CAD pyrG ⁺ 4.1	AB 1.13 CAD	Highest itaconic acid production among all AB 1.13 CAD transformants	Li et al. 2012
AB 1.13 CAD pyrG ⁻ 10.1	CAD10.1	Uridine deficient	Li et al. 2011
AB 1.13 CAD+MTT 1.4	MTT 1.4	Selected MTT transformant of CAD 10.1	
AB 1.13 CAD+MFS 3.9	MFS 3.9	Selected MFS transformant of CAD 10.1	
AB 1.13 CAD+MTT+MFS_3	CAD+MTT+MFS_3	Selected MFS transformant of MTT 1.4	
AB 1.13 CAD+HBD 2.5	HBD 2.5	Selected HBD transformant of CAD 10.1	Li et al. 2012
AB 1.13 $\Delta oahA$ # 76	$\Delta oahA$ # 76	AB 1.13 pyrG ⁻ deleted for oxaloacetate acetylhydrolase	
AB 1.13 $\Delta oahA$ # 76 CAD5	$\Delta oahA$ # 76 CAD 5	Selected CAD transformant of $\Delta oahA$ # 76	
D15# 26			Mattern et al. 1992
D15# 26 CAD7		Selected CAD transformant of D15# 26	
N201		High accumulation in citric acid	Currie 1917
N201 CAD		Selected CAD transformant of N201	

To constitutively express the two transporters *mttA* (ATEG_09970) and *mfsA* (ATEG_09972) in strain CAD 10.1, both genes were in vitro synthesized (Geneart) as compatible gene fragments to be cloned into vector pAN52-5doubleNotI using NcoI and BamHI restriction sites. The vector pAN52-5doubleNotI was derived from pAN52-4 (EMBL accession #Z32750) by introducing downstream of the *trpC* termination an extra

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NotI site. The plasmid pAB 4-1 (Van Hartingsveldt et al. 1987) containing the *pyrG*⁺ gene as a selection marker was used for co-transformation. Then, the *mttA* (pAN52-5doubleNotI+MTT) and *mfsA* (pAN52-5doubleNotI+MFS) expression vectors were co-transformed with pAB4-1 into *A. niger* strain CAD 10.1. All transformants were selected once for their ability to growth on Minimal Medium plates without uridine.

A selected itaconic acid producing cotransformants carrying *mttA* (AB 1.13 CAD MTT1.4) was further transformed with *mfsA* to obtain strain AB 1.13 CAD+MTT+MFS. The *Aspergillus* vector pAN7-1 containing the *E. coli* HmB-resistant gene (*hph*) (Punt and Van Den Hondel 1992) as a selection marker was used to co-transform with *mfsA*. These transformants were selected on hygromycin containing Minimal Medium agar plates.

To express the *cadA* gene in strains D15#26, AB 1.13 $\Delta oahA$ #76 and N201, the plasmid pAN52-amdS+CAD was used (Li et al. 2011). These transformants were selected on acrylamide containing Minimal Medium agar plates.

Construction of a non-oxalic acid producing strain

To delete the oxaloacetate hydrolase gene *oahA* from strain AB 1.13, the deletion plasmid was made as follows: the primers *oahA*-1535for: 5'-CCATCGGCTTCTCCGTCGG-3' and *oahA*+ 506rev: 5'-GGCAATAAGGTTTGTGGGTG-3' were used for amplifying gene *oahA* (An10g00820) from chromosomal DNA of *A. niger*. The resulting

3.2 kb fragment was cloned in pGEM[®]-T Easy vector using the kit from Promega. From the *oahA* vector, a 526 bps Ehel fragment within *oahA* was replaced with the *pyrG*⁺ selection marker isolated as a Ehel fragment. For further fungal transformation, the $\Delta oahA$ deletion fragment was cut with NotI from the resulting *oahA* deletion vector.

To generate a non-oxalic acid producing strain, the $\Delta oahA$ deletion fragment was transformed into *A. niger* AB 1.13 *pyrG*⁻. 96 selected transformants were checked via PCR using two *oahA* specific primers *oahA*4486for: 5'-CCTGATGGTCGCCCCGTTCC-3' and *oahA*1099rev: 5'-CACCATTAGCAAACCATCTCC-3'. In total, 33 PCR positive strains were selected for Southern blot analysis as described (Punt et al. 2008). From these the transformant AB 1.13 $\Delta oahA$ # 76 was selected for fungal transformation with *cadA*.

Northern blot analysis

Northern analysis was carried out essentially as described (Sambrook et al., 1989). With the 'PCR DIG Probe Synthesis Kit' (Roche, 2006), the DIG (digoxigenin) labelled fragments derived from *cadA* and *gpdA* genes were used as probes for Northern analysis.

PCR was used to amplify *cadA* probe fragments from plasmid pAN52-4amdS-CAD using primers 5'-GGTCTTAGCCGAGCAAGGC -3', and 5'-

GCGACACTCATCTGCCCTG -3'; and to amplify *gpdA* probe from plasmid pAB 5-1 (Verdoes et al. 1994) using primers 5'-ATCGAGACCTACGAGGAGGG-3', and 5'-CCGGGAGTTCCTGCGAAGG-3'.

Cultivation conditions

For the screening and selection of *A. niger* CAD or transporter transformants, our previously developed screening assay was used (Li et al. 2012). All plates were placed in a plastic air bag and cultivated in a 33°C, 850 rpm incubator (Microtron, Infors-ht) for 60 hours, except for N 201CAD transformants which were incubated for 48 hours. In the end of the cultivation, culture medium was harvested and used for HPLC analysis.

For controlled batch fermentations, the production medium (M12) described in our previous study (Li et al. 2012) with the following composition was used (per litre): 100 g glucose, 2.36 g (NH₄)₂SO₄, 0.11 g KH₂PO₄, 0.5 g MgSO₄ • 7H₂O, 0.6 mg FeSO₄ • 7H₂O, 2.5 mg CuSO₄ • 5H₂O, 0.6 mg ZnSO₄ • 7H₂O, 0.074 g NaCl and 0.13 g CaCl₂ • 2H₂O. This medium was prepared in demineralized water. The production medium M12+ Cu has an extra addition of 2.5 mg CuSO₄ • 5H₂O (0.01 mM).

Pre-cultures were prepared by inoculation of 10⁶ spores/ml in 2 × 100 ml production medium in two 500 ml baffled Erlenmeyer flasks. After 64 hours at 33°C and shaking at 125 rpm, the pre-cultures were used for inoculation of the fermenters.

Fermentations were performed in 5 liter Benchtop Fermentors (BioFlo 3000, New Brunswick Scientific Co., Inc.) at 33°C. The basic pH regime was initiated at 3.5 and subsequently regulated at 2.3, by addition of 4M KOH (Base). Struktol was applied as antifoam agent (Schill & Seilacher) in all cultures throughout the fermentation.

Air was used for sparging the bioreactor at a constant flow of 0.25 vvm [(vol.liquid)⁻¹ min⁻¹]. The solubility of oxygen in the medium is around 225 μMol at 33°C. Pure air sparging was calibrated as 100% D.O. whereas pure nitrogen sparging was calibrated as 0% D.O. In the basic D.O. regime, D.O. was set at 100% from the start of the fermentation. As soon as due to mycelial growth D.O. levels dropped below 25%, stirrer agitation was increased automatically to maintain D.O. at 25%.

For studying the influence of oxygen availability on itaconic acid production, D.O was fixed throughout the whole fermentation at 10%, 15%, 20%, 25% for strain N201 CAD, and at 5%, 10%, 20% for strain HBD 2.5. The different percentage of D.O. was obtained by varying the mixture of air/nitrogen in the inlet gas.

Analytical Methods

Metabolite analysis

To quantify metabolites, samples collected from fermentation cultures were analyzed by high performance liquid chromatography (HPLC). Dionex ICS 3000 from Thermo Fisher was used for analyzing organic acids through an organic acids column IonPac[®] ICE AS6, with 1.6 mM Heptafluorobutyric acid as eluent and a detector of suppressed conductivity CD25. Standard compounds (oxalic acid, citric acid, *cis*-aconitic acid and itaconic acid) with concentrations of 100 mg/L, 200 mg/L, 500 g/L, 750 mg/L, 1000 mg/L and 2000 mg/L were used for calibration.

Biomass dry weight

Biomass of the batch cultivation was harvested by filtration of a fixed volume (10 ml) of the culture through the pre-weight dry filters and washed with demineralised water. Subsequently, the harvested biomass with the filter was dried in an oven at 110°C for 24 hours and weighed. The biomass dry weight was established by subtracting the weight of the dry filter.

Results

To further improve the production level of itaconic acid based on our previous research in *A. niger*, we explored four aspects. The two transporters of the itaconic acid gene cluster from *A. terreus* (Li et al. 2011) were expressed in *A. niger* to obtain a better itaconic acid transport system in *A. niger*. To eliminate accumulation of the by-product oxalic acid in an *A. niger* itaconic acid producing host, a low acid secretion strain D15#26 and an oxaloacetate hydrolase deficient strain AB 1.13 Δ *oahA*#76 were used. As higher citric acid production was previously shown to lead to higher itaconic acid formation (Li et al. 2012), a basal citric acid producing strain, *A. niger* N 201, was modified into a new itaconic acid production host by introducing the *A. terreus cadA* gene as previously described (Li et al., 2011) resulting in strain (N201 CAD). Furthermore, oxygen availability was considered as an important parameter in itaconic acid fermentation process. The new host N201CAD together with a hemoglobin domain overexpressing strain (HBD 2.5: Li et al. 2012) were studied in fermentations carried out with various D.O. regimes.

Selection of *A. niger* itaconic acid transformants

To select the best itaconic acid producing strains from *mttA* and *mfsA* transformants, 8 *mttA* and 24 *mfsA* transformants taken from minimal medium selection plates were cultivated in micro-titer plate. As shown in

Figure 1A, nearly every *mfsA* transformant produced more itaconic acid than strain AB 1.13 CAD. The best one among them was strain AB 1.13 CAD +MFS (MFS 3.9) which had nearly 5 fold higher itaconic acid production (0.292 g/L). Surprisingly, among the *mttA* transformants, strain AB 1.13 CAD+ MTT (MTT 1.4) was the only one producing significantly more itaconic acid (0.492 g/L) than strain AB 1.13 CAD (0.054 g/L) (Figure 1B), whereas Southern analysis confirmed the presence of the *mttA* gene in MTT 1.4 /MTT 1.1 and MTT 1.5. However, as strain MTT 1.4 also produced 2 fold higher itaconic acid than MFS 3.9, we decided to also introduce *mfsA* into strain MTT 1.4 to obtain a itaconic acid strain expressing both *mttA* and *mfsA*. Unexpectedly, the screening results show no improved itaconic acid production compared to MTT 1.4 (Figure 2). Among 12 selected transformants, only transformant 3 (CAD+MTT+MFS_3) had a similar production level as the reference, the rest even had somewhat decreased production.

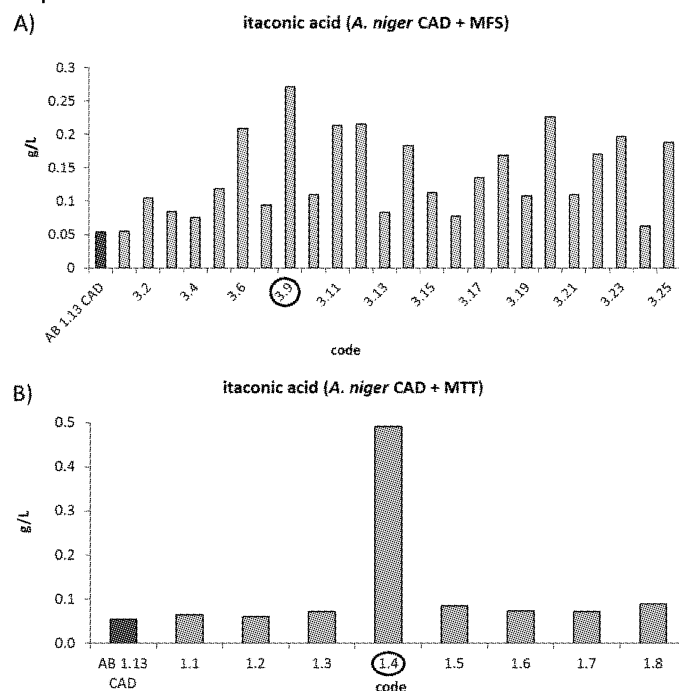


Figure 1. Itaconic acid production levels of MFS (A) and MTT (B) transformants. Strain AB 1.13 CAD was used as a control. In the screening experiment only single cultures were analyzed. For the selected strains repeated cultures were carried out showing a standard deviation of less than 6% .

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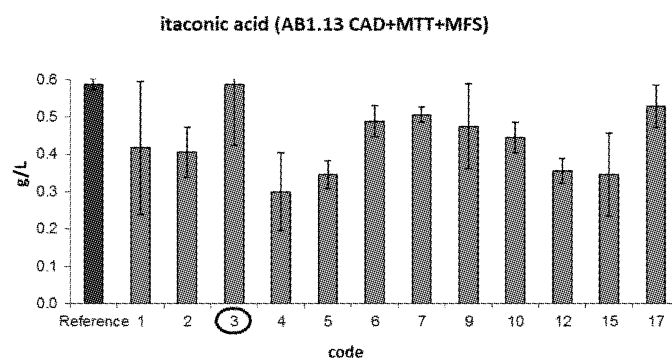


Figure 2. Itaconic acid production levels of AB 1.13 CAD+MTT+MFS transformants. Strain MTT 1.4 was used as reference.

Batch fermentations with transporter strains

To analyze improved itaconic acid levels under more relevant condition, batch fermentation experiments were performed with five *A. niger* strains (AB 1.13, AB 1.13 CAD, MTT 1.4, MFS 3.9 and CAD+MTT+MFS_3). During fermentation, mycelium was harvested to determine the biomass. Cultivation medium was analyzed for organic acids (Figure 3).

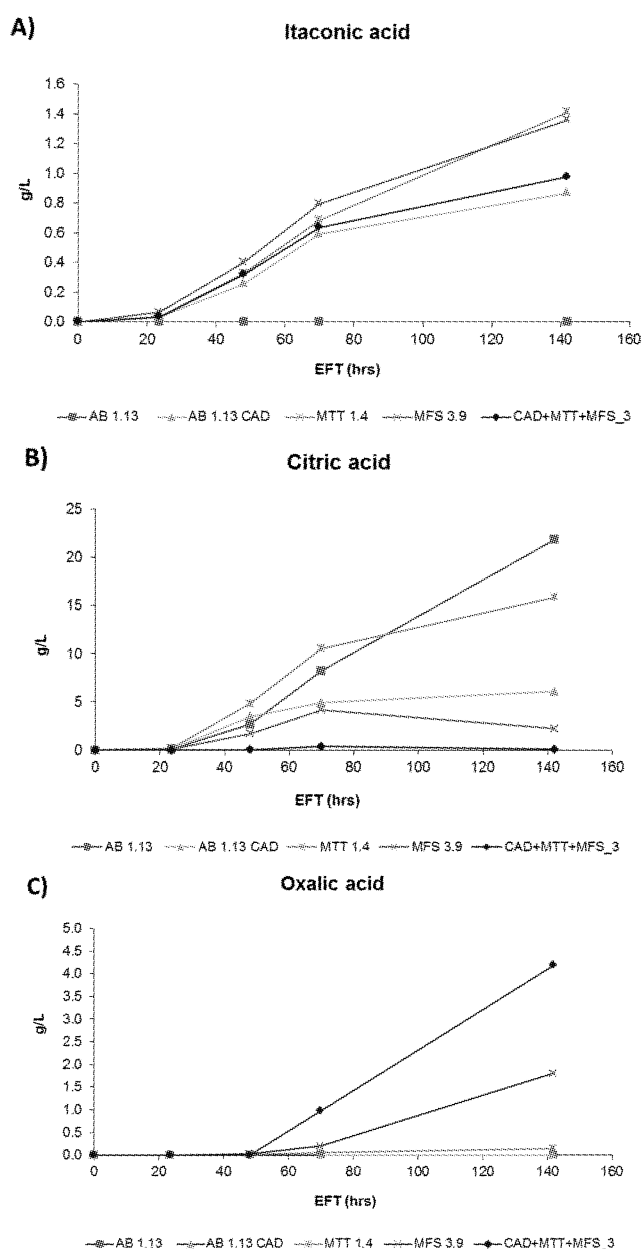


Figure 3. Organic acids production from batch bio-reactor cultivations of five *A. niger* strains: AB 1.13, AB 1.13 CAD, MTT 1.4, MFS 3.9 and CAD+MTT+MFS_3 were shown. A) itaconic acid; B) citric acid; C) oxalic acid. (■: AB 1.13; ▲: AB 1.13 CAD; *: MTT 1.4; X: MFS 3.9; ◆: CAD+MTT+MFS_3)

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As shown, the best itaconic acid producing strains were MTT 1.4 and MFS 3.9, both producing a similar amount of itaconic acid about 1.5 g/L. Both of these two transporter strains produced more itaconic acid than AB 1.13 CAD. The itaconic acid production of the double transporter strain CAD+MTT+MFS-3 produced less than MTT 1.4 and MFS 3.9, similar to AB 1.13 CAD (0.9 g/L).

AB 1.13 produced the highest amount of citric acid (22 g/L) and no oxalic acid. Strains MTT 1.4 and AB 1.13 CAD produced less citric acid (16 g/L and 6 g/L) and no oxalic acid. In comparison, strain MFS 3.9 produced far less citric acid (2.2 g/L), but accumulated oxalic acid (1.8 g/L). In case of the double transporter strain CAD+MTT+MFS_3, hardly any citric acid was produced. However, the oxalic acid (4.3 g/L) accumulation in this strain was increased. The mycelium growth of all strains was similar as presented in Figure 4, except AB 1.13 CAD having somewhat less biomass than the others.

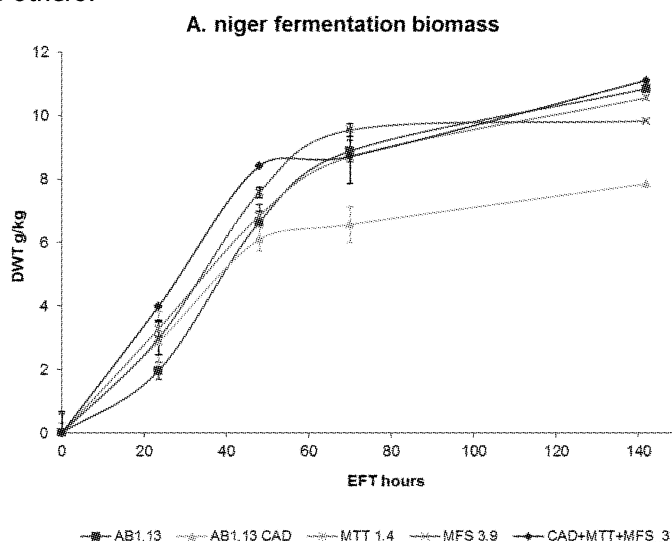


Figure 4. Biomass determination of mycelium in batch fermentation. (■: AB 1.13; ▲: AB 1.13 CAD; *: MTT 1.4; X: MFS 3.9; ◆: CAD+MTT+MFS_3)

Comparison of *A. niger* host strains for itaconic acid production

As mentioned, to improve producing itaconic acid, one aspect was to seek for a better host strain with higher citric acid production, the other aspect was to eliminate producing oxalic acid by-product. Therefore, a basal citric acid producing strain N201 and a oxaloacetate hydrolase deficient strain AB 1.13 *oahA#76* (*oahA#76*) were selected.

In order to compare citric acid production levels, strain AB 1.13, *oahA#76* and N201 were cultivated in controlled batch fermentation with a

previously selected itaconic acid production medium (medium 12; Li et al., 2012). As shown in Figure 5A in 96 hours strain N201 produced about 0.80 g (cit)/g (biomass) citric acid, which is over three times more than strain AB 1.13 (0.23 g (cit)/g (biomass)). Furthermore, strain N201 did not produce oxalic acid under this condition (Figure 5B), while glucose consumption was the same and the biomass growth was similar (data not shown). The same production levels of citric acid and oxalic acid were shown with strain *oahA*#76. Based on this result, we decided to use both of them as production host for itaconic acid.

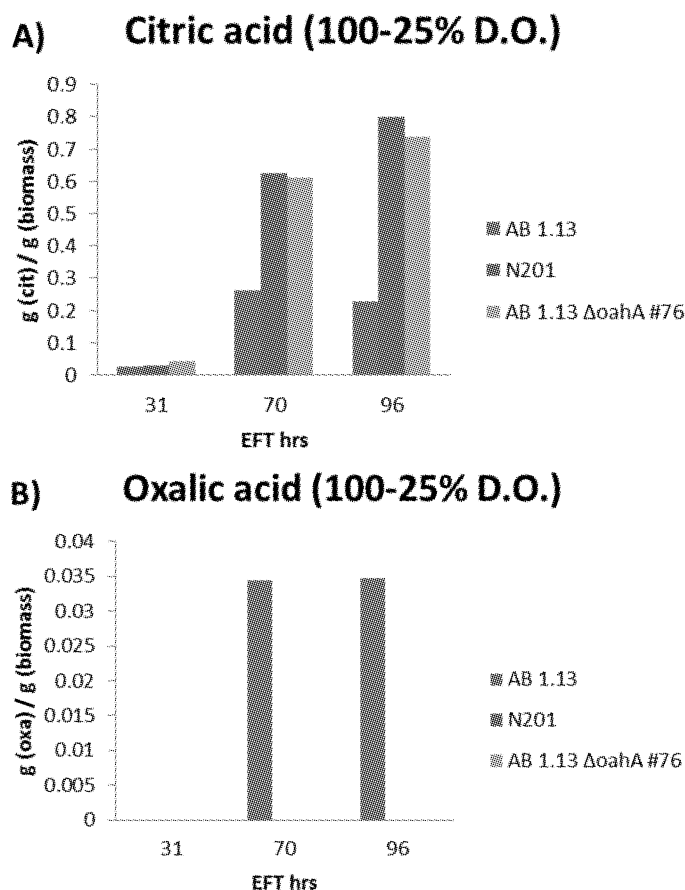


Figure 5. Organic acid content (citric acid (A) and oxalic acid (B)) of two *A. niger* strains under Medium 12 in controlled batch fermentation.

Similar to the transporter transformants, among 14 *cadA* transformants from D15# 26 and 7 from AB 1.13 Δ oahA#76, the best itaconic acid producing strains (D15#26 CAD7 and AB 1.13 *oahA*#76 CAD5 (*oahA*#76

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CAD5) were selected using the microplate screening assay. All selected itaconic acid strains were compared for itaconic acid production in the micro-titer assay. The results are presented in Table 2. Strains D15#26 CAD7 did not produce higher itaconic acid than CAD 10.1. However, strain *oahA#76* CAD5 produced two-fold more itaconic acid compared to CAD 10.1, and the production level (0.4 g/L) was similar to MTT 1.4 (0.47 g/L) and MFS 3.9 (0.44 g/L). Moreover, *oahA#76* CAD5 also yielded the highest citric acid levels (2.12 g/L). No detectable levels of oxalic acid were produced in the micro-titer cultivations.

Table 2. Organic acids contents of the *A. niger* micro-titer cultures in 60 hours.

strains	itaconic acid		citric acid	
	g/L	STDEV	g/L	STDEV
D15#26	0.00	0.00	0.07	0.00
D15#26 CAD 7	0.15	0.11	0.00	0.00
AB 1.13	0.00	0.00	0.28	0.11
CAD 10.1	0.24	0.02	0.68	0.01
$\Delta oahA\#76$	0.00	0.00	0.56	0.11
$\Delta oahA\#76$ CAD 5	0.40	0.08	2.12	0.32
MTT 1.4	0.47	0.04	0.94	0.08
MFS 3.9	0.44	0.07	0.44	0.05
CAD+MTT+MFS_3	0.39	0.03	0.79	0.13

Also strain N201 was transformed with *cadA* and the transformants were screened for itaconic acid production. In this case, also *cadA* expression of several transformants was analysed by Northern blot analysis (Figure 6). Strain N201 CAD 02 was selected for its high itaconic acid production and *cadA* expression. Its itaconic acid production level (135 mg/L) was almost two times higher than strain AB 1.13 CAD (72 mg/L). This strain was used to study the effect of cultivation conditions.

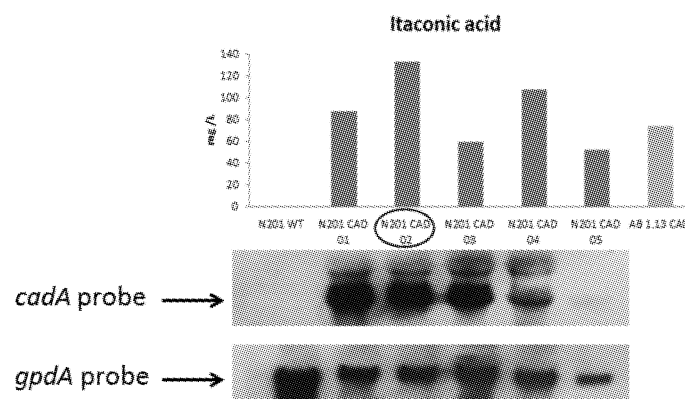


Figure 6. Northern blot analysis (graph above) and micro-titer plate cultivation (60 hours) (below) results of the N201 CAD transformants. The wild type strain N201 was used as a reference. The previous strain AB 1.13 CAD was used as a comparison.

Influence of dissolved oxygen level on organic acids production in *A. niger*

Based on previous research (Li et al. 2011), it was suggested that D.O. could be an important factor for itaconic acid production. Moreover, as previous results in *A. terreus* (Lin et al. 2004) have shown that undisturbed aeration is required for optimal itaconic acid production, we have studied the effect of oxygen availability in more detail. Reduced D.O. levels were shown to increase itaconic acid levels in *A. terreus* batch cultivation (Li et al. 2011). Therefore we studied the effect of reduced D.O. on itaconic acid production in *A. niger*. We used strain N201 CAD in controlled batch fermentation with four D.O. levels. The amount of itaconic acid produced by strain N201 CAD is shown in Table 3. The basic cultivation conditions based on a 100% -25% D.O. gradient produced less itaconic acid (0.05 g(ita)/g(biomass)) then the other D.O. cultures (10% , 15% , 20% and 25%). The highest production of both itaconic acid (0.20 g(ita)/g(biomass)) and citric acid (0.40 g(cit)/g(biomass)) were observed with 20% D.O. (Table 3). The production level of strain N201 CAD on oxalic acid was presented in Table 3. The highest oxalic acid was accumulated at 100-25% D. O.. There was no oxalic acid being accumulated in other itaconic acid producing D. O. conditions. As expected (Table 3), strain $\Delta oahA\#76$ CAD5, did not produce any oxalic acid under controlled fermentation conditions. Moreover, this strain produced two-fold more itaconic- and citric acid in comparison to N201 CAD under standard DO conditions (100-25% D.O.).

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Table 3. Organic acid production of *A. niger* strains cultured in controlled batch cultivations (200 hours). Strain N201 CAD and HBD 2.5 were cultivated with various levels of D.O.. (For HBD 2.5, the parental strain AB 1.13 CAD was used as a reference.)

	D.O.	Itaconic acid	citric acid	oxalic acid
		<i>g/g biomass</i>	<i>g/g biomass</i>	<i>g/g biomass</i>
$\Delta oahA\#76$ CAD	100-25%	0.11	0.38	0.00
N201 CAD	100-25%	0.05	0.19	0.30
	25%	0.10	0.15	0.00
	20%	0.20	0.38	0.00
	15%	0.10	0.12	0.00
	10%	0.11	0.20	0.01
HBD 2.5	20%	0.16	0.68	0.00
	10%	0.17	0.52	0.00
	5%	0.16	0.13	0.00
AB 1.13 CAD	10%	0.07	0.55	0.00

Based on these results, we have analyzed the effect of reducing D.O. in an *A. niger* itaconic acid producing strain overexpressing a fungal hemoglobin domain in batch fermentations (Table 3). In our previous studies, using *A. niger* strain HBD 2.5 with overexpressed fungal hemoglobin domain (*hbd1*) did not improve itaconic acid production in 100-25% D.O.. Here we analyzed strain HBD 2.5 at reduced D.O. levels. As shown, strain HBD 2.5 produced 0.15 g(ita)/g(biomass) itaconic acid, which is two times higher than the parental strain AB 1.13 CAD at 10% D. O. (0.07 g(ita)/g(biomass)) (Table 3).

Moreover, in even lower oxygen supply conditions, itaconic acid production level of strain HBD 2.5 was maintained (0.157 g(ita)/g(biomass)). The citric acid production at 5% D.O. was much lower in comparison to the production of itaconic acid at 5% D.O. Similar to strain N201 CAD, the highest production of both itaconic acid (0.17 g(ita)/g(biomass)) and citric acid (0.68 g(cit)/g(biomass)) were at 20% D.O.. There was no accumulation of oxalic acid under these conditions.

Discussion

Effect of putative transporters from the *A. terreus* itaconic acid gene cluster on Itaconic acid production in batch fermentation experiments with *A. niger*

In order to increase secreted itaconic acid yields in *A. niger* production, we expressed two transporters genes from the *A. terreus* itaconic acid gene cluster gene cluster. The two transporters, *mfsA* and *mttA*, lacking orthologs in *A. niger* were expressed in *A. niger* strain CAD 10.1. As

observed, expression of *mfsA* increased itaconic acid production secretion in *A. niger*, indicating their role in the transport of itaconic acid (Figure 1). The *mfsA* transporter as a putative dicarboxylate carrier was assumed to facilitate the export of itaconic acid into the medium. Similar as presented in the research of Zelle to improve malic acid production in *Saccharomyces cerevisiae* (Zelle et al. 2008), we present positive effect on itaconic acid production of the putative pathway specific dicarboxylate carriers *mfsA*. Only in one of the MTT transformants a positive effect was observed. Moreover, expressing *mfsA* in this strain did not further improve the production level of itaconic acid (Figure 3A). Further research is underway to analyse this unexpected phenomenon.

Although metabolite transport across the mitochondrial membrane has been widely investigated for, the knowledge about the mechanism of mitochondrion carrier proteins in relation to organic acid transport in fungi is still very limited (Palmieri et al. 2004). From the results we have obtained it is clear that modifications in the mitochondrial and cytoplasmic membrane transport have profound effects on organic acid transport. As shown, in controlled batch fermentations the production profiles of citric acid and oxalic acid are not the same as itaconic acid for the different transporter strains. The *mttA* transformant (MTT 1.4) showed increased accumulation of citric acid while the *mfsA* transformant (MFS 3.9) showed decreased accumulation of citric acid (Figure 3B). Apparently, the *mfsA* transporter contributes to a better conversion of citric acid towards itaconic acid, possibly by reducing intracellular itaconic acids levels. However, introduction of the *mfsA* transporter resulted in an accumulation of the by-product oxalic acid, which is not observed with *mttA* transformant (Figure 3C). Therefore, both strain MTT 1.4 and MFS 3.9 have advantages and drawbacks in itaconic acid production. The effect of expressing both transporters in one strain (CAD+MTT+MFS_3) seem to be dominated by *mfsA* as citric acid secretion is completely absent and the accumulation of oxalic acid is highly increased.

As shown in the fermentation with the production strain AB 1.13 CAD, also without any of the two transporters from *A. terreus*, itaconic acid could also be exported out of the cell. Therefore, we assumed that in *A. niger*, other transporters e.g. for citric acid might function as an itaconic acid transporter. As citric acid is secreted in almost every of the strains, analyzed the citric acid transport system in *A. niger* is suggested to be very efficient and several putative homologs for mitochondrial and plasma membrane transport of di- and tri-carboxylic acids can be identified (Li and Punt, 2012 submitted for publication). However, until

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now detailed knowledge about this transport is lacking and further research will be required to improve secretion of alternative organic acids in *A. niger*.

Itaconic acid production test of two non-oxalic acid producing strains

In our previous studies, we observed that oxalic acid as a by-product was co-produced under preferred itaconic acid production conditions (Li et al. 2011/2012). To eliminate this by-product, two non-oxalic acid producing strains from *A. niger* (D15#26 and AB 1.13 $\Delta oahA$ #76) were evaluated in this study. Although D15#26 CAD7 could produce itaconic acid, the levels were clearly lower than in a wild type strain AB1.13 (Table 2). Strain $\Delta oahA$ #76 CAD5 deficient in oxaloacetate hydrolase activity, produced even higher itaconic acid especially in fermentation than its reference strain (Table 3). As previous research has shown that the absence of oxaloacetate hydrolase also positively effect citric acid production (Ruijter et al. 1999) and more recently also production of other metabolites was positively influenced by the absence of oxalic acid byproduct formation (Pedersen et al. 2000; Gombert et al. 2011), *oahA* knockout should be considered for further strain improvement in relation to alternative organic acids / building block chemicals production.

Influence of D.O. on Itaconic acid production under controlled batch fermentation.

Another aspect to increase itaconic acid production was to optimize the conditions of *A. niger* batch fermentations. Here we focused on the influence of different levels of dissolved oxygen (D.O.), which was based on a previous study that a lower oxygen supply (25% D.O.) was optimal for itaconic acid production in controlled batch fermentation using *A. terreus* (Li et al. 2011). Two itaconic acid producing strains from *A. niger* were included in this study. One was N201 CAD, expressing *cadA* in a basal organic acid producing strain. The other was HBD 2.5 which carried expressed a fungal hemoglobin domain (*hbd1*) (Li et al. 2012). We observed that the highest itaconic acid production was at 20% D.O. for strain N201 CAD (Table 3). Moreover, lower D.O. conditions (10-25%) eliminated oxalic acid accumulation in this strain (Table 3). As observed for strain HBD 2.5, overexpressing *hbd1* in strain AB 1.13 CAD under low D.O. levels (5% -20%) improved itaconic acid production (Table 3), and also diminished production of oxalic acid. Interestingly, at the lowest D. O. level (5%) this strain produced considerably reduced levels of citric acid, while itaconic acid levels were retained. Altogether these results proved our assumption that overexpression of hemoglobin domain could increase itaconic acid production, possibly by improving oxygen sequestration and transport in the cell (te Biesebeke et al. 2006)

In this study, further optimization of *A. niger* as an itaconic acid production host has been demonstrated. We increased the production level from 0.8 g/L to 2.5 g/L. Expression of the two transporters (*mttA* or *mfsA*), overexpression of the hemoglobin domain (*hbd1*), elimination of oxalic acid by-product ($\Delta oahA$), replacement with a basal organic acid production host N201 and lower D. O. (10-20%) supply in the fermentation culture have all shown to enhance itaconic acid production level in *A. niger*. To increase itaconic acid production capacity even further, combining these features into one platform will be the target of our ongoing research.

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