Expression of the *Aspergillus terreus* itaconic acid biosynthesis cluster in *Aspergillus niger*

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**Abstract**

**Background:** *Aspergillus terreus* is a natural producer of itaconic acid and is currently used to produce itaconic acid on an industrial scale. The metabolic process for itaconic acid biosynthesis is very similar to the production of citric acid in *Aspergillus niger*. However, a key enzyme in *A. niger*, cis-aconitate decarboxylase, is missing. The introduction of the *A. terreus cadA* gene in *A. niger* exploits the high level of citric acid production (over 200 g per liter) and theoretically can lead to production levels of over 135 g per liter of itaconic acid in *A. niger*. Given the potential for higher production levels in *A. niger*, production of itaconic acid in this host was investigated.

**Results:** Expression of *Aspergillus terreus* cis-aconitate decarboxylase in *Aspergillus niger* resulted in the production of a low concentration (0.05 g/L) of itaconic acid. Overexpression of codon-optimized genes for cis-aconitate decarboxylase, a mitochondrial transporter and a plasma membrane transporter in an oxaloacetate hydrolase and glucose oxidase deficient *A. niger* strain led to highly increased yields and itaconic acid production titers. At these higher production titers, the effect of the mitochondrial and plasma membrane transporters was much more pronounced, with levels being 5–8 times higher than previously described.

**Conclusions:** Itaconic acid can be produced in *A. niger* by the introduction of the *A. terreus* cis-aconitate decarboxylase encoding *cadA* gene. This results in a low itaconic acid production level, which can be increased by codon-optimization of the *cadA* gene for *A. niger*. A second crucial requirement for efficient production of itaconic acid is the expression of the *A. terreus mttA* gene, encoding a putative mitochondrial transporter. Expression of this transporter results in a twenty-fold increase in the secretion of itaconic acid. Expression of the *A. terreus* itaconic acid cluster consisting of the *cadA* gene, the *mttA* gene and the *mfsA* gene results in *A. niger* strains that produce over twenty-five-fold higher levels of itaconic acid and show a twenty-fold increase in yield compared to a strain expressing only CadA.

**Keywords:** *Aspergillus niger*, *Aspergillus terreus*, cis-aconitate decarboxylase *cadA*, Mitochondrial transporter *mttA*, Plasma membrane transporter *mfsA*, Itaconic acid

**Background**

Increased awareness of the environmental pressure caused by petroleum-based production processes and products has stimulated and intensified research on bio-based production methods and products. Efficient bio-based production is economically problematic due to the relative low-cost of petroleum-based chemicals and is also technically complex. The design and construction of efficient cell factories requires a modification of the host cell or chassis at a systems level rather than at a single gene level. Itaconic acid or methylsuccinic acid is a C⁵ dicarboxylic acid. The methylene group of itaconic acid can participate in polymerization reactions. On the basis of this characteristic, itaconic acid can be used for the production of synthetic polymers [1]. Furthermore, it can be used as a bioactive component in agriculture and pharmacy, as a medicine [2] and as a starting compound in enzymatic conversions to form useful poly-functional building blocks [3]. For all of these reasons, itaconic acid has been designated by the U.S. Department of Energy as one of the top twelve building-block chemicals that can...
be produced from plant biomass sugars via a fermentative process [4].

Currently, A. terreus is used for the commercial production of itaconic acid by submerged fermentation [2,5]. The pathway for the production of itaconic acid is a metabolic variant of the pathway for citric acid production in A. niger (Figure 1). Citric acid is produced commercially using A. niger, reaching production levels over 200 g/L [6], which, in a molar ratio, corresponds to over 135 g/L itaconic acid. A. terreus reaching itaconic acid titers of 80 g/L shows the potential of A. niger to reach far higher production titers. A second advantage is that the existing citric acid fermentation infrastructure can be used for this A. niger-based fungal itaconic acid cell factory.

A. niger does not naturally produce itaconic acid because it lacks the essential enzyme cis-aconitate decarboxylase. The cadA gene encoding this enzyme in A. terreus has been identified using different approaches, including an enzyme purification approach [7] and a clone-based transcriptomics approach [8]. The expression of the cadA gene in A. niger leads to extremely low levels of itaconic acid production (0.05 g/L), indicating that the sole expression of the enzyme is insufficient for efficient production of itaconic acid. In the A. terreus genome, the cadA gene is located close to the lovastatin cluster [9] and flanked by a putative mitochondrial transporter (mttA) and a putative plasma membrane transporter (mfsA). The co-regulation of these transporters with cadA, as reported by Li et al. [8], suggested that the putative mitochondrial transporter might be involved in itaconic acid production in A. terreus. Recently, Li et al. [10] showed that the effect of these putative transporters on itaconic acid production in A. niger resulted in a slight increase in itaconic acid production levels. However, the maximum titer of 1.5 g/L itaconic acid that was reached is far from the theoretical titer of over 135 g/L under conditions of high citric acid production.

For our studies, we have used a specific mutant of A. niger to serve as a chassis for the production of itaconic acid. This strain carries two specific mutations, one in the oahA gene encoding oxaloacetate hydrolase and one in the goxC gene encoding glucose oxidase. This strain has certain advantages; the production of by-products is reduced because it is not able to produce oxalic acid or gluconic acid. As a result, this leaves more carbon available for citrate and itaconate production. Finally, as reported by Ruijter et al., strains carrying both the oahA mutation and the goxC mutation are insensitive to Mn2+ ions in the medium, which results in constitutive citrate production irrespective of the fermentation regime [11]. In this study, we use a robust fermentation regime developed by van der Veen et al. [12] that is optimized to reduce variance in the experiments. In the fermentation medium, sorbitol is used as the main carbon source as it is essentially non-inducing and non-repressing for the D-xylose inducible xlnD promoter [13]. Sorbitol is metabolized to form fructose [14], which is phosphorylated by hexokinase to fructose-6-phosphate and further metabolized via the glycolysis pathway and the TCA cycle. In the TCA cycle, citrate is converted into isocitrate in a reaction that yields cis-aconitate as an intermediate. Iaconic acid can be formed from cis-aconitate by a cis-aconitate decarboxylase-catalyzed reaction.

In our study, we show that the overexpression of the codon-optimized cadA, mttA and mfsA genes in the oxaloacetate hydrolase- and glucose oxidase-deficient strain leads to increased yields and itaconic acid production titers. At these higher production titers, the effect of the mitochondrial and plasma membrane transporters is much more pronounced than previously described [10].

**Results and discussion**

Our strategy for the design of a fungal cell factory was based on the use of a specific chassis for the production of itaconic acid in A. niger. The A. niger strain that we chose is a mutant strain that is not able to produce oxalic acid or gluconic acid due to mutations in the oahA and goxC genes, respectively. This is an important advantage because this strain does not produce these unwanted side products. Due to the reduced formation of by-products, more carbon can be converted into the final product - itaconic acid. The oahA mutation also leads to constitutive citric acid production that is insensitive to the presence of...
metal ions, as discovered by Ruijter et al. [11]. The constitutive production of citric acid is a great benefit because itaconic acid production is directly derived from citric acid production.

Expression of the A. terreus cadA gene in A. niger
The gene encoding cis-aconitate decarboxylase was identified in the A. terreus genome using a proteomics approach in which the enzyme was partially purified. Both a cDNA fragment from A. terreus and a codon-optimized cadA synthetic gene were used for the expression of cis-aconitate decarboxylase in A. niger NW186. The A. terreus coding sequences have a slightly higher GC content in comparison to the A. niger coding sequences (56.2% vs 53.8%, respectively) [15]. This higher GC content is mostly found at the third position; in A. terreus, 65.3% GC and in A. niger, 59.3%. A total of 305 out of 490 codons were changed in the cadA sequence, including the codons to remove restriction enzyme sites.

The transformants from both plasmids yielded varying low amounts of itaconic acid. This variation in itaconic acid production could result from differences in copy numbers amongst the strains and variation in the site of integration of the construct in the different transformants. Surprisingly, codon-optimization of the cadA gene for A. niger resulted in a more than three-fold increase in itaconic acid production. The transformants containing the codon-optimized gene (sCAD) (Figure 2B) produced higher amounts of itaconic acid compared to the ones expressing the cDNA fragment (cCAD) (Figure 2A). Based on these results, the two putative transporters mttA and mfsA from A. terreus were also synthetically made and codon-optimized for expression in A. niger.

No itaconic acid production was detected in the A. niger strains that did not contain the cis-aconitate decarboxylase encoding gene. The sCAD4 strain was selected for our further studies.

Copy number of the cadA gene in A. niger transformants
The copy number was determined by qPCR using genomic DNA as template. The Pfaffl method was applied to calculate the copy number [16]. The qPCR results for cadA were compared with those of the single copy gene pkiA in order to determine the copy number of cadA. For the transformants cCAD4, cCAD6 and cCAD11, expressing the A. terreus cadA cDNA, a copy number of 1 was determined. These strains also produced the same levels of itaconic acid for 10 days. After 14 days, the cCAD6 strain produced a higher level of itaconic acid. Of the strains expressing the cadA cDNA, cCAD3 and cCAD5 had the highest copy number at 4. These strains also produced the highest levels of itaconic acid of the strains that expressed the non-optimized cadA cDNA. The only atypical transformant was cCAD7, which had a copy number of 21 but produced hardly any itaconic acid. Although this was a striking result, it has previously been observed in A. niger [17].

The highest itaconic acid producing transformants, sCAD1 and sCAD4, expressed the synthetic codon-optimized cadA gene and had the highest copy numbers at 11 and 6, respectively. The transformants sCAD7, sCAD8 and sCAD10, which produced between 4 and 8 mM itaconic acid after 14 days, had copy numbers of 2, 2 and 4, respectively. Although the sCAD10 strain had 4 copies of cadA, it did not produce more than the strains with only 2 copies of cadA. In this particular case, the place of integration could negatively influence the level of expression compared to sCAD7 and sCAD8. The sCAD6 strain, which did not produce any itaconic acid, also did not have a copy of the cadA gene.

These results also suggest a positive effect of codon-optimization because the strains that produced the highest levels of itaconic acid, namely sCAD7, sCAD8 and sCAD10, were the strains expressing the codon-optimized cadA gene. The copy number of these strains was determined to be 2, 2 and 4, respectively. This is in contrast to the cCAD5 and cCAD3 strains, which carried 4 copies of
the non-optimized cadA gene but produced less itaconic acid.

Expression of the A. terreus itaconic acid biosynthesis cluster in A. niger

Based on our findings on the expression of the cadA in A. niger, we extended our studies by co-expressing the two putative transporter encoding genes flanking the cadA gene in the A. terreus genome. In these studies, we also used synthetic codon-optimized fragments of the mttA and mfsA genes for expression in the A. niger strain that contains the codon-optimized cadA gene, sCAD4. For our first analysis, these strains were grown in Erlenmeyer culture flasks to analyze the effects of the transporters that were introduced. In these experiments, strains that contained the cadA and mfsA genes produced slightly higher levels of itaconic acid compared to the sCAD4 strain carrying only the cadA gene (Table 1).

The putative mitochondrial transporter mttA had a much more pronounced effect on itaconic acid production, as expression of this gene led to increased itaconic acid production in all transformants analyzed in comparison to the strain that had only the cadA gene. The increase found ranged between a factor of 6 and 25 (Table 1), which is far more than the previously described increase for an A. niger strain carrying only the oahA mutation and in which the genes were not codon-optimized [10].

We performed batch fermentations to study the improved itaconic acid production in a more controlled way. To investigate the effect of the mttA and mfsA transporters in the production of itaconic acid in A. niger, the best performing transformant of the cadA + mttA and cadA + mfsA strains were selected along with four transformants of newly constructed strains carrying the combination of cadA, mttA and mfsA. The parent strain sCAD4 was chosen as the control.

When grown in batch fermenters, we did not find the slight increase in itaconic acid production in the strains that co-expressed mfsA with cadA (Table 1, Figures 3 and 4) that we found in the Erlenmeyer cultures. This was in contrast to previously published data in which nearly five-fold higher itaconic acid levels were found when cadA was co-expressed with mfsA [10]. However, we did find increased citric acid concentrations (Figure 4). This implies that this transporter is also able to export citric acid. Although we did not find a positive effect on the levels of itaconic acid production from strains expressing mfsA and cadA, we did find a positive effect on itaconic acid production levels in strains expressing cadA, mttA and mfsA in NW186 (Table 1, Figures 3 and 4). Apparently, the plasma membrane transporter MfsA is able to secrete both itaconic acid and citric acid. Obviously, A. niger is able to secrete itaconic acid without a heterologous plasma membrane transporter as is shown by the expression of cadA in A. niger. The transformant cadA + mfsA 2.5 did not show increased itaconic acid production in the fermenter studies, but because the itaconic acid levels are relatively low, it is possible that there was no bottleneck in itaconic acid export in this strain under these conditions.

In the case of the cadA + mttA + mfsA transformants, levels of itaconic acid production were strongly increased in comparison to the strain that only carried the cadA gene, with the highest increase being over 25-fold (Figure 3). These increased production levels were parallelled with an increased yield, defined as the product yield on the substrate in % (C-mol/C-mol), in the transformants that contained both putative transporters. The strain expressing only cadA gave a yield of approximately 1%, which is of the same order as was found by

![Figure 3 Itaconic acid production in fermenter cultures.](http://www.microbialcellfactories.com/content/13/1/11)

The itaconic acid production is given in gram per gram dry weight at T = 78 hours after induction of the transformants carrying the complete itaconic acid biosynthesis cluster from A. terreus compared to the best performing CadA, CadA + MfsA and CadA + MttA transformants.

Table 1 Production of itaconic acid in Erlenmeyer cultures

|                     | Itaconic acid produced (mM) | Factor difference |
|---------------------|-----------------------------|-------------------|
| Control             | 0.13 ± 0.02                 |                  |
| cadA + mfsA 2.4     | 0.14 ± 0.01                 | 1                |
| cadA + mfsA 2.5     | 0.25 ± 0.06                 | 2                |
| cadA + mttA 1.1     | 0.98 ± 0.09                 | 8                |
| cadA + mttA 1.2     | 3.23 ± 0.94                 | 25               |
| cadA + mttA 1.4     | 0.74 ± 0.04                 | 6                |
| cadA + mttA 1.5     | 0.74 ± 0.00                 | 6                |
| cadA + mttA 1.6     | 1.21 ± 0.24                 | 10               |

The itaconic acid production is given in mM in the CadA + MfsA and CadA + MttA transformants at 30 hours after induction. Measurements were carried out in duplicate; the ± represents the standard error of the mean.
Li et al. [10], although they used different carbon sources and concentrations of carbon sources. The strongest increase in yield was caused by the expression of the mitochondrial transporter \textit{mttA} in the \textit{sCAD4} strain, which resulted in a yield of 24%. But, when the complete itaconic acid cluster from \textit{A. terreus} was expressed, the yield further increased to 32% in the best performing strain (Table 2).

Although several other acids including \textit{cis}-aconitic acid, succinic acid, malic acid, pyruvic acid and \textit{α}-ketoglutaric acid are secreted, itaconic acid was the predominantly produced acid in the highest producing strains. One exception was the strain expressing \textit{cadA} in combination with \textit{mfsA} where, instead of itaconic acid, citric acid was the predominantly produced acid (Table 3).

The production of itaconic acid levels obtained were relatively low compared to those in industrial production processes. It is not surprising that our strain was less efficient in itaconic acid production than the commercial \textit{A. terreus} strain since the \textit{A. niger} strain used is not producing high levels of citrate. The citrate concentrations produced by our strain are far lower than those obtained in an industrial environment using an industrial \textit{A. niger} strain. Although itaconic acid production in \textit{A. niger} was

### Table 2 Comparison of itaconic acid producing strains

|                         | Itaconic acid production (g/L) | Sorbitol and xylose consumed (C-mmol) | Itaconic acid yield on consumed sorbitol and xylose (%) | Biomass (g dry weight per L culture broth) | Data from |
|-------------------------|--------------------------------|--------------------------------------|-------------------------------------------------------|------------------------------------------|-----------|
| \textit{sCAD4}          | 0.3                            | 619                                  | 1.6                                                   | 2.4                                      | This research |
| \textit{cadA} + \textit{mfsA 2.5} | 0.1                            | 632                                  | 0.8                                                   | 1.2                                      | This research |
| \textit{cadA} + \textit{mttA 1.2} | 5.4                            | 843                                  | 24.4                                                  | 3.2                                      | This research |
| \textit{cadA} + \textit{mttA} + \textit{mfsA 1} | 5.6                            | 806                                  | 26.8                                                  | 1.6                                      | This research |
| \textit{cadA} + \textit{mttA} + \textit{mfsA 2} | 6.0                            | 848                                  | 27.4                                                  | 3.1                                      | This research |
| \textit{cadA} + \textit{mttA} + \textit{mfsA 3} | 5.5                            | 702                                  | 30.0                                                  | 2.1                                      | This research |
| \textit{cadA} + \textit{mttA} + \textit{mfsA 4} | 7.1                            | 844                                  | 32.1                                                  | 2.6                                      | This research |
| \textit{AB 1.13 CAD}    | 0.9                            | 1.0                                  | 1.0                                                   | Li et al. [13]                           |
| \textit{MTT 1.4}       | 1.4                            | 1.6                                  | Li et al. [13]                                        |
| \textit{MFS 3.9}       | 1.4                            | 1.6                                  | Li et al. [13]                                        |
| \textit{CAD + MTT + MFS 3} | 0.9                            | 1.0                                  | Li et al. [13]                                        |

The production of itaconic acid is given in g/L at 78 hours after induction. The yield in percentage (C-mol/C-mol) is calculated based on consumed sorbitol and D-xylose. All strains from Li et al. [13] are uridine prototrophs of \textit{AB 1.13}. 
still far less efficient than in *A. terreus*, major improvements were made. Under lab conditions, we were able to improve the titer of itaconic acid produced in *A. niger* by a factor of over twenty-five and the yield by approximately twenty-fold. Compared to the values for itaconic acid production in *A. niger* that have been published [10], the strains we constructed showed a five-fold higher production level and a twenty-fold increase in yield. These improvements mainly resulted from the use of codon-optimized genes and from the increased efficiency of substrate-use by the elimination of oxalate and gluconate production.

**Conclusions**

Itaconic acid can be produced in *A. niger* by the introduction of the *A. terreus* cis-aconitate decarboxylase encoding *cadA* gene. However, this results in very low production levels. The production levels can be increased if the *A. terreus* *cadA* gene is codon-optimized for *A. niger*. When the expression of *cadA* in *A. niger* was combined with the expression of the *A. terreus* *mfsA* gene encoding a putative plasma membrane transporter, no effect on the production levels of itaconic acid was found. This suggests that the itaconic acid produced in *A. niger* is efficiently secreted by an endogenous *A. niger* transporter. The expression of *mfsA* in combination with *cadA* led to increased citrate production suggesting that MfsA is a transporter that is able to secrete citric acid as well as itaconic acid.

Our results show that in addition to the *cadA* gene, the *mttA* gene from *A. terreus* is also crucial for efficient itaconic acid production in *A. niger*. Expression of the *mttA* gene, encoding a putative mitochondrial transporter, in the strain that expresses *CadA* resulted in an over twenty-fold increased secretion of itaconic acid. Expression of the *A. terreus* itaconic acid cluster, consisting of the *cadA*, *mttA* and *mfsA* genes, led to *A. niger* strains with over twenty-five-fold higher levels of itaconic acid and a 20-fold increase in yield when compared to a strain that expressed only *CadA*.

**Methods**

**Strains and spore preparations**

The fungal strains used in this study were *Aspergillus terreus* NRRL 1960 (Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands, CBS 116.46) and *Aspergillus niger* NW186 (*cspA1, goxC17, prtF28 ΔargB, pyrA6*), which is a *pyrA* mutant of *Aspergillus niger* NW185 [11]. To obtain spores, 20 spores per mm² were plated onto complete medium plates [18], incubated for 5 days at 30°C and allowed to mature at 4°C for 24 h. The spores were harvested in 0.9% (w/v) NaCl and 0.005% (v/v) Tween-80, washed with 0.9% (w/v) NaCl and stored at 4°C until use.

**Fermentation and induction of itaconic acid production in *A. terreus* NRRL 1960**

*A. terreus* was grown at 30°C and 200 rpm by inoculating spores (10⁸ spores per mL) in 100 mL pre-cultures in 1 L flasks containing 25 g/L glucose, 4.5 g/L MgSO₄.7H₂O, 0.4 g/L NaCl, 4 mg/L ZnSO₄.7H₂O, 100 mg/L KH₂PO₄, 2 g/L NH₄NO₃ and 0.5 g/L Corn Steep Liquor (CSL). After two days, a 10% (w/v) inoculum was transferred to

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### Table 3 Overview of extracellular acid concentrations (mM) measured in time during fermentation

|          | Citric acid | Itaconic acid | cis-aconitic acid | Succinic acid | Malic acid | Pyruvic acid | α-ketoglutaric acid |
|----------|-------------|---------------|-------------------|--------------|------------|--------------|---------------------|
| 6 h      |             |               |                   |              |            |              |                     |
| sCAD4    | 3.9         | ND            | ND                | ND           | ND         | ND           | ND                  |
| cadA + mfsA 2.5 | 4.0        | ND            | ND                | ND           | ND         | ND           | ND                  |
| cadA + mttA 1.2 | 2.7        | 4.0           | ND                | 0.2          | ND         | ND           | ND                  |
| cadA + mttA + mfsA 4 | 3.3        | 2.7           | ND                | ND           | ND         | ND           | ND                  |
| 30 h     |             |               |                   |              |            |              |                     |
| sCAD4    | 1.8         | 1.45          | 0.3               | ND           | 2.4        | ND           | ND                  |
| cadA + mfsA 2.5 | 8.5        | 0.6           | ND                | ND           | ND         | ND           | ND                  |
| cadA + mttA 1.2 | 2.5        | 28.1          | ND                | 0.7          | ND         | ND           | 1.5                 |
| cadA + mttA + mfsA 4 | 1.9        | 21.1          | 1.2               | ND           | 0.8        | ND           | ND                  |
| 54 h     |             |               |                   |              |            |              |                     |
| sCAD4    | 1.9         | 1.7           | 0.3               | ND           | 2.1        | ND           | ND                  |
| cadA + mfsA 2.5 | 11.1       | 0.8           | ND                | 0.8          | 0.7        | ND           | ND                  |
| cadA + mttA 1.2 | 1.8        | 46.0          | ND                | 1.4          | ND         | ND           | 0.4                 |
| cadA + mttA + mfsA 4 | 1.8        | 43.6          | 1.8               | ND           | 0.6        | ND           | ND                  |
| 78 h     |             |               |                   |              |            |              |                     |
| sCAD4    | 2.3         | 2.0           | 0.3               | ND           | 2.2        | ND           | ND                  |
| cadA + mfsA 2.5 | 12.7       | 1.0           | ND                | 0.9          | 0.7        | ND           | ND                  |
| cadA + mttA 1.2 | 1.5        | 41.1          | ND                | 0.9          | ND         | ND           | ND                  |
| cadA + mttA + mfsA 4 | 1.8        | 54.3          | 2.2               | 0.9          | ND         | ND           | ND                  |

ND: the compound was not detected in the sample.
the CAD production medium, as described by Cros and Schneider [19] with the following changes: 3 g/L NH₄NO₃ instead of urea and 1.5 g/L MgSO₄·7H₂O with a final pH of 2.0.

**Gene cloning and plasmid design**

Standard methods were used to carry out DNA manipulations and *E. coli* transformations [20]. The gene encoding CadA was obtained by PCR from the *A. terreus* genome and cloned in a pUC19 derived vector under the control of the *A. niger* _pkIA_ promoter [17] and the terminator of the _trpC_ gene of *A. niger*. The codon-optimized _cadA_ gene was synthesized by Geneart (Invitrogen, Carlsbad, CA, US) and cloned in pUC19. Codon-optimized genes _mttA_ and _mfsA_ were synthesized by DNA 2.0 (Menlo Park, USA) and cloned in a pUC19 derived vector under the control of a modified _xlnD_ promoter and the terminator of the _xlnD_ gene of *A. niger* [13]. The promoter modification inactivated the _CreA_ sites leaving the promoter inducible by D-xylose. For the construction of plasmid pLS001 the [p_p_xlnD* – MTT – _xlnD] fragment was obtained by PCR using pMTT as a template and the following primers: LS_p_xlnD_HindIII_for (5'-GAG-AAA-GCT-TCG-AAT-GAT-AAG-ACC-GGC-GAT-AGT-GG 3') and LS_t_xlnD_XbaI_rev (5'-GAG-ATC-TAG-GCA-GTC-GCA-CTC-CCG-ACC 3'). This fragment was cloned into pMFS and digested with HindIII and XbaI.

The plasmids were propagated in DH5α *E. coli*, in LB medium (10 g/L Bacto tryptone, 5 g/L Yeast extract, 10 g/L NaCl) supplemented with the appropriate antibiotics (100 mg/L ampicillin, 50 mg/L kanamycin).

**Fungal transformation**

For transformation of *A. niger*, protoplasts were generated using Novozyme 234. The _cadA, mttA_ and _mfsA_ genes were introduced in *A. niger* NW186 by co-transformation as previously described [21] using the pGW635 plasmid, which contains the _pyrA_ gene [22] as a primary selection marker. The pLS001 plasmid was introduced in the *A. niger* strain containing the _cadA_ gene by co-transformation using the pAL69 plasmid, which contains the _pyrA_ gene of *A. terreus* [18]. The identification of the transformants was carried out on the extracted DNA using PCR with Taq polymerase and the _LS_mttA_for (5'- ATT-AAG-ACC-GCG-ATG-GAG-GAG-ATG-TGG-CAT-G 3') and _LS_mttA_rev (5'- CTT-CTC-GTA-GAC-GGG-GAA-CA 3') primers to check for the presence of the _mttA_ gene. The _LS_mfsA_for (5'- ACC- TTC-AGC- TGG-CGT-GGT-GCT-CCT-TTA- ACT-GC 3') and _LS_mfsA_rev (5’- GAT-AAG-ACC-GGC-GAT-AGT-GG 3’). primers were used to check for the presence of the _mfsA_ gene.

**DNA extraction and PCR of _cadA + mttA_ and _cadA + mfsA_ from *A. niger* transformants**

DNA extraction and PCR was carried out to identify the colonies that randomly integrated the genes of interest. Fresh mycelium was disrupted using Fastprep and 400 μL extraction buffer (100 mM TrisHCl pH 8.0, 5 mM EDTA, 1.2 M NaCl). DNA was extracted using phenol-chloroform, and the pellet was washed with 70% cold ethanol, air-dried and resuspended in 50 μL MQ water. The identification of the transformants was carried out on the extracted DNA using PCR with Taq polymerase and the _LS_mttA_for (5'- ATT-AAG-ACC-GCG-ATG-GAG-GAG-ATG-TGG-CAT-G 3') and _LS_mttA_rev (5'- CTT-CTC-GTA-GAC-GGG-GAA-CA 3') primers to check for the presence of the _mttA_ gene. The _LS_mfsA_for (5'- ACC- TTC-AGC- TGG-CGT-GGT-GCT-CCT-TTA- ACT-GC 3') and _LS_mfsA_rev (5’- GAT-AAG-ACC-GGC-GAT-AGT-GG 3’). primers were used to check for the presence of the _mfsA_ gene.

**Growth experiments of transformants in shake flasks**

All positively identified _cadA + mttA_ and _cadA + mfsA_ transformants were grown at 30°C and 200 rpm in 250 mL shake flasks containing 25 mL PM medium (1.2 g NaNO₃, 0.5 g KH₂PO₄, 0.2 g MgSO₄·7 H₂O, 0.5 g yeast extract and 40 μL Vishniac per liter with 100 mM sorbitol as a carbon source [11]). Induction at _t_ = 0 with 10 mM D-xylose was carried out 18 hours after inoculation. HPLC analysis was carried out on the samples after 30 hours.
Transformants containing the cadA, mttA and mfsA genes were grown in 1 L shake flasks containing 200 mL PM medium as described above. Samples were taken at 6, 30, 54 and 78 h after induction.

Fermentation studies
The transformants containing the cadA, mttA and mfsA genes and the control strains, sCAD4, cadA + mttA 1.2 and cadA + mfsA 2.5, were inoculated (10^7 spores/mL) in 1 L fermenters (Sartorius) containing 0.75 L of PM medium with 100 mM sorbitol. After 18 hours of growth at 30°C, the strains were induced by the addition of 50 mM xylose. The strains were further grown for 5 days at 30°C at a stirrer speed of 1000 rpm. The pH in the culture broth was not controlled. Samples were taken at 6, 30, 54 and 78 hours after induction.

Dry weight measurement
To determine dry weight, 10 mL fermentation broth was sampled and filtered using a 5 micron nylon gauze. The biomass was washed with demineralized water and completely dried on pre-weighed aluminum trays in an oven at 120°C for 24 hours.

HPLC analysis
High-pressure liquid chromatography (HPLC) was used to determine the extracellular concentrations of sorbitol, xylose, itaconic acid, citric acid, cis-aconitic acid, pyruvic acid, α-ketoglutaric acid, lactic acid, succinic acid, fumaric acid and oxalic acid in the samples. For organic acid measurements, a Shodex KC811 column was used and eluted with 0.01 N H_2SO_4 at a flow rate of 0.8 mL min^{-1} and sampling was carried out at 30°C for 25 min. Detection was carried out using both a refractive index detector (Spectra system RI-150, sample frequency 5.00032 Hz) and a UV–VIS detector (Spectrasystem UV1000, λ = 210 nm). 6 mM crotonate was used as an internal standard. The sugars were measured using a Dionex Carbotrap MA-1 column.

Determination of copy number
DNA was extracted using the method described in “DNA extraction and PCR of cadA + mttA + mfsA transformants”. The copy number of the cadA genes in the transformants expressing cadA from A. terreus or the synthetic codon-optimized cadA gene was determined in triplicate using a Rotor-Gene Q Cycler. The reaction mixture contained 8 μL 2× Absolute QPCR SYBR Green mix (Thermo Scientific), 100 nM forward and reverse primers and 2 μL 100 times diluted gDNA. Primers LS_qcadAs_F (5′-GAGATCCTATGGCGGTTCCTTCC - 3′) and LS_qcadA_R (5′-CAAGAGCTGGGATATCTCC - 3′) were used to determine the copy number of the A. terreus cadA gene and the primers LS_qcadAs_F (5′-ACTCCGAAGAGTTCG ACCAG - 3′) and LS_qcadAs_R (5′-ACCAGGTCCTC GATTTCCTT - 3′) were used to determine the copy number of the synthetic cadA gene. The pkiA gene, of which only one copy is present, was used as a reference gene using the primers LS_qpkiA_F (5′-GGTAAACGACAGC GATTGGATA - 3′) and LS_qpkiA_R (5′-GGGCTCACA GTGAATGTTGT - 3′). Water and SDS samples were used as controls. The qPCR cycling program was as follows: 15 min initial polymerase activation at 95°C followed by 40 cycles of 95°C for 15 sec, 59°C for 15 sec and 72°C for 30 sec. The calculations were carried out using the Pfaffl method [16].

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
LS, MV, ML, WB, TS, JC designed and performed the experimental work and participated in writing the manuscript. IM and AK collaborated in the coordination of the research and helped to draft the manuscript. LG designed the study and participated in writing of the manuscript. All authors read and approved the submission of the manuscript.

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