Integrated strain- and process design enable production of 220 g L$^{-1}$ itaconic acid with *Ustilago maydis*

Hamed Hosseinpour Tehrani¹, Johanna Becker¹, Isabel Bator¹, Katharina Saur¹, Svenja Meyer¹, Ana Catarina Rodrigues Lóia¹, Lars M. Blank¹ and Nick Wierckx¹,²*

**Abstract**

**Background:** Itaconic acid is an unsaturated, dicarboxylic acid which finds a wide range of applications in the polymer industry and as a building block for fuels, solvents and pharmaceuticals. Currently, *Aspergillus terreus* is used for industrial production, with titers above 100 g L$^{-1}$ depending on the conditions. Besides *A. terreus*, *Ustilago maydis* is also a promising itaconic acid production host due to its yeast-like morphology. Recent strain engineering efforts significantly increased the yield, titer and rate of production.

**Results:** In this study, itaconate production by *U. maydis* was further increased by integrated strain- and process engineering. Next-generation itaconate hyper-producing strains were generated using CRISPR/Cas9 and FLP/FRT genome editing tools for gene deletion, promoter replacement, and overexpression of genes. The handling and morphology of this engineered strain were improved by deletion of *fuz7*, which is part of a regulatory cascade that governs morphology and pathogenicity. These strain modifications enabled the development of an efficient fermentation process with in situ product crystallization with CaCO$_3$. This integrated approach resulted in a maximum itaconate titer of 220 g L$^{-1}$, with a total acid titer of 248 g L$^{-1}$, which is a significant improvement compared to best published itaconate titers reached with *U. maydis* and with *A. terreus*.

**Conclusion:** In this study, itaconic acid production could be enhanced significantly by morphological- and metabolic engineering in combination with process development, yielding the highest titer reported with any microorganism.

**Keywords:** *Ustilago maydis*, Itaconic acid, Metabolic engineering, Morphological engineering, Biochemical engineering, In situ precipitation

**Background**

More than 300 potential bio-based building blocks were selected from the U.S. Department of Energy according to criteria such as estimated processing costs, estimated selling price, and the technical complexity, to determine the most important chemicals that can be produced from biomass. In the top selection, nine belong to the group of organic acids [72], underlining the importance of this class of chemicals. One of these compounds is the unsaturated dicarboxylate itaconic acid. It was first described in 1837 [4] and primary reports about microbial production with *Aspergillus itaconicus* date back to 1931 [42]. Due to its two functional groups, radical polymerization of the methylene group and/or esterification of the carboxylic acid with different co-monomers is possible [59, 63, 67]. This leads to a wide range of applications in the paper, architectural, pharmaceutical, paint, lacquer, and medical industries [5, 6, 40, 43, 50, 55, 61, 71]. It can also be used as an intermediate for the production of 3-methyltetrahydrofuran, a potential biofuel with advantageous combustion properties [16]. Further, itaconate...
production by mammalian macrophages is reported, where it plays a key role in the human immune response [11, 57, 69], with possible applications as therapeutic agent for autoimmune diseases [2].

In spite of this wide variety of potential applications, the market size of itaconic acid in 2011 was relatively small, with 41,400 tons and a market value of $74.5 million [71]. This is caused by the relatively high price of approximately two dollars per kg and the availability of cheaper petro-based alternatives such as acrylic acid. Reduction of this price is, therefore, a major criterion for access to further markets. To be competitive against petro-based products, costs need to reduce to around $0.5 per kg [1]. Assuming that the price would decrease, itaconic acid has the possibility to replace acrylic acid in the production of poly(methyl methacrylate), the production of which is petroleum based with a market worth of $11 billion [39, 43, 59]. Since 1950, Aspergillus terreus is used for the industrial production of itaconate [59]. Charles Pfizer Co. was granted the first patent for the production of itaconate with the filamentous fungus A. terreus by submerged cultivation [37]. During the last decades, the responsible metabolic pathways and regulatory mechanisms of itaconate production in A. terreus were studied in detail [67]. Major advances were achieved through process development. This long history of optimization has enabled titers above 100 g L⁻¹ and yields near the theoretical maximum at low pH, making A. terreus the current best production host for itaconate production [7, 29, 34, 49, 50, 53, 66]. However, despite the long history and experience, itaconate production in A. terreus remains challenging. A specific pellet growth form is required for high productivity [25, 39] and therefore, morphology has to be strictly controlled. A. terreus reacts very sensitively to certain medium impurities, which can induce mycelium formation and stop itaconate production [15, 48, 50]. Thus, medium must be pretreated to remove impurities from production medium, especially when using less pure industrial substrates such as molasses [38, 59]. Consequently, morphological control influences the manufacturing process tremendously, leading to increased operational costs and failed batches.

Besides A. terreus, numerous itaconate producers have been engineered in recent years, such as E. coli [26], A. niger [31], and C. glutamicum [60]. Besides these heterologous hosts, Ustilaginaeaceae like the pH-tolerant Ustilago cynodontis or the yeast-like Ustilago maydis are natural itaconate producers which have recently been engineered to higher efficiency [21, 24, 33, 75]. Among the Ustilaginaeaceae, U. maydis is the most studied species in the fields of plant pathogenicity, cell biology, and biotechnology [17, 18, 52, 68, 70]. The Ustilaginaeaceae produce a broad spectrum of interesting products such as organic acids [21, 24, 76], glycolipids [14, 58], polyols [21, 35], and enzymes [14]. This, along with their yeast-like growth, makes them attractive for biotechnological applications [21].

That said, certain stresses can induce filamentous growth in U. maydis [45, 54] but efficient itaconate production with this species is, at least at small scale, not coupled to a specific morphology. In wild-type U. maydis, itaconate production is induced by nitrogen limitation [74] and requires pH values above five [21]. Like in A. terreus, the genes encoding the itaconate production pathway in U. maydis are clustered and co-regulated [19, 53]. Considerable progress has been made in increasing the yield, titer, and rate of itaconate production in U. maydis and related species by metabolic engineering and process development. Geiser et al. [19] characterized the itaconate production pathway and identified an itaconate oxidase Cyp3, which produces the downstream product (S)-2-hydroxypyraracionate. The disruption of this oxidase, and overexpression of the cluster-associated regulator Ria1, led to 4.5-fold increase in ITA production in U. maydis [18]. In U. vetiveriae, itaconate production from glycerol could be increased 2.5-fold by overexpression of ria1 or 1.5-fold by overexpression of the mitochondrial transporter mtt1 [75].

In another study, we could show that heterologous expression of the mitochondrial transporter MttA from A. terreus in U. maydis enables more efficient itaconate production than the native mitochondrial transporter [32]. Further, by deletion of fuzz in U. cynodontis, a stable yeast-like growth could be established for several relevant itaconic acid production conditions [33]. This is especially favorable for large-scale fermentation [62]. Furthermore, with optimization of growth media and the fermentation process, such as pulsed fed-batch strategies, product titers can be significantly increased [20, 21]. This is especially effective when combined with in situ product removal approaches such as reactive extraction or calcium precipitation [23, 43, 46, 75].

These optimizations have individually made a significant impact on the efficiency of itaconate production in Ustilago. In this study, we consolidate several of these metabolic and bioprocess engineering strategies to achieve itaconate titers that surpass those currently achieved by any other host.

Results and discussion

Engineering of a marker-free U. maydis MB215 for enhanced itaconate production

Previously, Geiser et al. [18] reached titers up to 63.2 ± 0.7 g L⁻¹ with production rates up to 0.38 ± 0.00 g L⁻¹ h⁻¹ and a yield up to 0.48 ± 0.02 g ITA g⁻¹ GLC in bioreactor experiments by
deletion of the itaconate oxidase encoding cyp3 gene and overexpression of the gene encoding transcriptional regulator Ria1 (∆cyp3 P_{etef} ria1). Unfortunately, two out of five possible antibiotic resistance markers available for U. maydis were genomically incorporated in this design, which limited further modification steps. Recently, Schuster et al. [65] established a CRISPR/Cas9 system for U. maydis enabling scarless and marker-free genome editing [12]. This technology, along with the FLP/FRT system for marker recycling already used in U. maydis [41] removes previous limitations of available antibiotic markers. We re-engineered the modifications described by Geiser et al. [18] using the CRISPR/Cas9 system from Schuster et al. [65]. To delete cyp3 and thereby abolish production of (S)-2-hydroxyxyparacatonate, a repair template was used to delete the whole gene. It consisted of 1000 bp flanks homologous to sequences up- and downstream of cyp3. The overexpression of ria1 was not achieved by the in trans insertion of an expression cassette at the cbx locus, but rather by a direct in cis replacement of the native P_{ria1} promoter by the strong and constitutive P_{etef} promoter. Here, the same strategy was chosen as for cyp3, including P_{etef} between the flanks of the repair templates. Promoter exchanges were previously shown to effectively upregulate native genes [20]. Two chosen transformants of the resulting strain (∆cyp3 ΔP_{ria1}::P_{etef} #1 and ∆cyp3 ΔP_{ria1}::P_{etef} #2) were compared to the control strain from Geiser et al. [18] in System Duetz® 24-well plates [13], in screening medium with 50 g L\(^{-1}\) glucose, buffered either with 30 mM MES or 33 g L\(^{-1}\) CaCO\(_3\) (Fig. 1). As expected, itaconate production was lower using 30 mM MES compared to 33 g L\(^{-1}\) CaCO\(_3\), since U. maydis prefers pH values above 5 [21]. In both tested conditions, the transformants showed no difference to the control except for one notable exception. Itaconate concentrations in the cultures with the ∆cyp3 P_{etef}ria1 strain decreased markedly at 96 h with 30 mM MES (Fig. 1a). This rapid decrease shows that U. maydis can degrade itaconate, likely through a similar pathway as that described for A. terreus [10, 18]. Possibly, the expression of the genes encoding this degradation pathway is affected by the promoter replacement which removed the native P_{ria1} promoter. For further investigations, we selected the strain U. maydis ∆cyp3 ΔP_{ria1}::P_{etef}.

In the cultures of these overproducing strains, we also observed a degree of filamentous growth. Although this is by far not as prominent as described for U. cynodontis [33], elongated cells and filaments were formed in all tested U. maydis strains for all conditions shown in Fig. 1, especially upon addition of CaCO\(_3\). Morphological engineering in U. maydis ∆cyp3 ΔP_{ria1}::P_{etef} Usually, filamentous growth in U. maydis is investigated in terms of pathogenicity. In its natural habitat, filamentous growth is indispensable to U. maydis for infection of Zea mays. This is strongly coupled with sexual development including a complex regulatory system [36, 51, 52]. Filamentous growth can also occur in haploid cells when they encounter stresses such as low pH, nitrogen limitation, or the presence of sunflower oil [45, 56]. This ability to grow filamentously is an obstacle in a biotechnological context, as it strongly influences bioprocess

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**Fig. 1** Itaconate production and growth of engineered U. maydis strains. Itaconate concentration (a), growth (OD\(_{600}\)) (b), pH (c) and DIC images at an magnification of 630× (d-l) with ∆cyp3 ΔP_{etef}ria1 (green, filled inverted triangle) and ∆cyp3 ΔP_{etef}ria1 (black, filled circle) in comparison to ∆cyp3 P_{etef}ria1 (red, filled diamond) during shake flask cultivation in screening medium with 30 mM MES (open symbols) or 33 g L\(^{-1}\) CaCO\(_3\) (filled symbols) with 50 g L\(^{-1}\) glucose. Error bars indicate the standard error of the mean (n = 3)
parameters such as oxygen transfer, viscosity, and clogging, and it increases the sensitivity to hydro-mechanical stress [44]. To solve this problem and to restore robust yeast-like growth, the fuz7 gene was deleted in the marker-free Δcyp3 ΔPria1::Petef #2 strain by replacement with a hygromycin marker through homologous recombination, followed by FLP/FRT-mediated marker excision [41]. Fuz7 is part of the Ras/mitogen-activated protein kinase (MAPK) pathway, which plays an important role in conjugation tube formation and filamentous growth [3]. By deletion of fuz7 in the strongly filamentous U. cynodontis, filamentous growth was repressed without influencing itaconate production and cell fitness under biotechnologically relevant conditions [33]. Deletion of fuz7 in U. maydis is known to abolish filamentous growth, and it also renders the strain completely apathogenic [3,45]. This inability to colonize the maize plant is an additional advantage in a biotechnological context, as it may alleviate possible regulatory hurdles for industrial application.

To assess the effect of fuz7 deletion, cultivation studies in screening medium with 30 mM and 100 mM MES, and 33 g L⁻¹ CaCO₃, were performed (Fig. 2). As expected, U. maydis Δcyp3 ΔPria1::Petef Δfuz7 grew completely yeast-like in all tested conditions. In contrast, U. maydis Δcyp3 ΔPria1::Petef grew filamentously (Fig. 2c), resulting in extensive adherence to the walls of the culture plates (Fig. 2d). This striking difference in morphology in the fuz7 mutant greatly improves handling of these cultures, while it did not negatively affecting itaconate production. Rather, production was significantly better at the end of cultivation for 30 mM and 100 mM MES.

**Mitochondrial transporter engineering in U. maydis Δcyp3 ΔPria1::Petef Δfuz7**

Recently, we could show by complementation experiments that overexpression of the mitochondrial transporter encoded by mttA from A. terreus enables higher itaconate production in U. maydis than overexpressing the native mtt1 [32]. Thus, to further increase itaconate production, we expressed mttA of A. terreus in U. maydis Δcyp3 ΔPria1::Petef Δfuz7 using plasmid pETEF_CbxR_At_mttA [32]. The best of three individual transformants was selected for further study. Upon cultivation
of this transformant with CaCO₃, a white precipitate was observed in samples of these cultures, indicating that the solubility limit of calcium itaconate was reached. As described for malic acid production with *U. trichophora*, and itaconate production with *U. vetricaria*, calcium salts of these organic acids have a lower solubility, leading to in situ precipitation in cultures where high titers are reached, usually preceded by a transient supersaturation of the product [75, 77]. To assess the effect of in situ itaconate precipitation in the engineered *U. maydis* strains, they were cultivated in System Duetz plates in screening medium with 100 g L⁻¹ glucose and 66 g L⁻¹ CaCO₃. Samples were analyzed with and without HCl treatment to re-solubilize the precipitated Ca-itaconate (Fig. 3).

Samples were analyzed with and without HCl treatment to re-solubilize the precipitated Ca-itaconate (Fig. 3). This value is below the measured aqueous concentrations of some samples from the abovementioned cultures (Fig. 2), indicating that these samples were in the supersaturation state and that some product may have been overseen due to precipitation.

An even more pronounced effect was observed with similar cultures using glycerol as C-source (Additional file 1: Fig. S1). Glycerol is a very poor substrate for wild-type *U. maydis* MB215 [77], and it invokes a high degree of filamentation and pigmentation in *U. cynodontis* [33]. The *fuz7* deletion had a very positive effect on the glycerol uptake rate and itaconate production, with the ∆cyp3 ∆Pria1::Petef ∆fuz7 strain producing 13.1 ± 0.04 g L⁻¹, compared to 4.3 ± 0.4 g L⁻¹ produced by the ∆cyp3 ∆Pria1::Petef control strain. Titors could be further increased with *U. maydis* MB215 ∆cyp3 ∆Pria1::Petef ∆fuz7 PetefmttA to 16.1 ± 0.4 g L⁻¹ itaconate.

**Optimized itaconate production in a stirred bioreactor**

In principle, the alleviation of product inhibition provided by the in situ precipitation of calcium itaconate enables much more extended cultures. In such cultures, productivity is only limited by the availability of the substrate and the stability of the biocatalyst. Therefore, to achieve high itaconate production, *U. maydis* MB215 ∆cyp3 ∆Pria1::Petef ∆fuz7 PetefmttA was cultivated in pulsed fed-batch fermentations with CaCO₃ in controlled 2-L bioreactors. The batch phase was started in screening medium containing 50 g L⁻¹ glucose and 1.6 g L⁻¹ NH₄Cl (Fig. 4). The CaCO₃ was added manually.
whenever pH dropped below 6.2, in the first 313 h as liquid suspension and after 313.5 h as a powder. Glucose was also pulsed into the fermenter to keep the concentration above 20 g L\(^{-1}\). The feeding schedule of CaCO\(_3\) and glucose is given in Additional file 1: Table S1. The resulting titer of 140 g L\(^{-1}\) itaconate was reached after 437 h. This is 2.2-fold more than the best published result of 0.2 ± 0.01 g\(_{\text{ITA}}\) g\(_{\text{GLC}}\) \(^{-1}\) from Geiser et al. [18] with \textit{U. maydis}. Biomass formation mainly occurred in the first 72 h and reached \textit{OD}_{600} values around 90, varying between 80 and 110 for the rest of the fermentation. These variations are likely due to analytical errors caused by CaCO\(_3\) precipitation upon sampling and OD measurement. An overall yield of 0.39 g\(_{\text{ITA}}\) g\(_{\text{GLC}}\) \(^{-1}\) was reached and the overall productivity was 0.32 g L\(^{-1}\) h\(^{-1}\), with a maximum productivity between 24 and 120 h of 0.65 g L\(^{-1}\) h\(^{-1}\), after which it stayed relatively linear at 0.23 g L\(^{-1}\) h\(^{-1}\). This decrease in productivity might be caused by the high solids load of 10–15% CaCO\(_3\) and Ca-itaconate in the fermentation broth, which could result in inhomogeneous mixing with pockets of low oxygen tension. For itaconate production, sufficient supply of oxygen is very important, with even transient oxygen limitations leading to a decrease of production [27, 44]. Future process development should, thus, focus on better mixing with these high solids loads, i.e., by changing stirrer geometry which can promote better oxygen distribution in viscous media [9]. In addition to itaconate, production of 31 g L\(^{-1}\) malate was also observed thereby increasing the total acid production to 170 g L\(^{-1}\) and the total acid yield to 0.48 g\(_{\text{ACID}}\) g\(_{\text{GLC}}\) \(^{-1}\). This increased by-product formation could be the result of the additional supply of CO\(_2\) by CaCO\(_3\). The efficient microbial production of malate via pyruvate relies on CO\(_2\) as co-substrate [77], and the additional CO\(_2\) provided by the CaCO\(_3\) might imbalance the precursor supply of itaconate.

In a similar approach where pH was controlled by titration with NaOH, a much lower level of itaconate production was observed (Additional file 1: Fig. S2), reaching a maximum titer of only 35.9 ± 1.5 g L\(^{-1}\) with a yield of 0.12 ± 0.004 g\(_{\text{ITA}}\) g\(_{\text{GLC}}\) \(^{-1}\) and an overall productivity of 0.12 ± 0.004 g L\(^{-1}\) h\(^{-1}\). In this titrated fermenter less than 1 g L\(^{-1}\) malate was produced, supporting the hypothesis that the additional CO\(_2\) from CaCO\(_3\) increases malate production. The overall decrease of productivity in the titrated culture is likely caused by the overexpression of \textit{mtnA}, which significantly stresses the cells leading to reduced growth and productivity as described previously [32]. The application of in situ itaconate crystallization with CaCO\(_3\), greatly reduced product inhibition, which is especially relevant with this deeply engineered strain, leading to almost threefold higher production rates.

To further improve the production rate, the cell density was increased by increasing the NH\(_4\)Cl concentration to 4 g L\(^{-1}\) and the starting glucose concentration to 200 g L\(^{-1}\) (Fig. 5). A similar feeding strategy of glucose and CaCO\(_3\) as above was applied (Additional file 1: Table S2). As expected, higher biomass formation was observed with the higher ammonium concentration, although the 2.5-fold higher nitrogen concentration only led to a moderate increase of the \textit{OD}_{600} to around 110. A similar trend was observed with \textit{A. terreus}, where a fourfold increase in phosphate as the growth-limiting nutrient only led to a twofold increase in biomass [48]. In spite of this, the overall production rate was increased significantly to 0.45 g L\(^{-1}\) h\(^{-1}\). The higher overall production rate was also reflected in a higher maximum rate of 0.74 g L\(^{-1}\) h\(^{-1}\) between 24 and 189 h followed by a fairly
linear rate of 0.35 g L$^{-1}$ h$^{-1}$ for the rest of the fermentation. This higher rate enabled the production of 220 g L$^{-1}$ itaconate and 28 g L$^{-1}$ malate resulting in a total acid titer of 248 g L$^{-1}$ in the same timeframe as the lower density culture. Of this total titer, approximately 14 g L$^{-1}$ of 248 g L$^{-1}$ acid will be dissolved in the aqueous phase [77], with a total acid yield of 0.37 gACID g$^{-1}$ itaconate prior to purification [47, 62]. In all, this study demonstrates the power of an integrated approach of strain and process engineering by greatly enhancing Ustilago-based itaconate production.

### Materials and methods

**Media and culture conditions**

All strains used in this thesis are listed in Table 1. E. coli strains were grown in medium containing 10 g L$^{-1}$ peptone, 5 g L$^{-1}$ sodium chloride, 5 g L$^{-1}$ yeast extract, and 5 g L$^{-1}$ glucose. U. maydis strains were grown in YEPS medium containing, 10 g L$^{-1}$ yeast extract, 10 g L$^{-1}$ peptone, and 10 g L$^{-1}$ sucrose. Growth and production experiments were performed using screening medium according to Geiser et al. [21] with varying glucose concentrations, C-sources (glycerol/glucose), and various buffer concentrations of 2-(N-morpholino)ethanesulfonic acid (MES) and 33 g L$^{-1}$ CaCO$_3$. This

### Conclusion

In this study, the combination of metabolic and morphological engineering together with in situ crystallization of itaconate yielded a titer of 220 g L$^{-1}$ itaconate, which corresponds to 284 g L$^{-1}$ calcium itaconate. This titer exceeds the 160 g L$^{-1}$ achieved with A. terreus [48], although the yield and production rate achieved with A. terreus are still higher [50]. Especially, the yield achieved with U. maydis could be further increased by the reduction of byproduct formation, as illustrated by the relatively high levels of malate production under these conditions. The strategy of in situ crystallization has not been reported in a biotechnological context with A. terreus, likely because the used pH values and the presence of solids strongly affect its morphology [48]. The use of in situ crystallization greatly enhanced itaconate production, but it will also pose new bioprocessing challenges such as solid/solid separation of biomass, CaCO$_3$ and Ca-itaconate, or pH shifts for resolubilization of itaconate prior to purification [47, 62].

### Table 1 U. maydis MB215 strains used in this study

| Strain designation | Resistance | References |
|--------------------|------------|------------|
| Ustilago maydis MB215 |             | [30]       |
| Ustilago maydis Δcyp3 P$_{etf}$nia1 | hyg$^R$, cbx$^R$ | (18)       |
| Ustilago maydis Δcyp3 |             | This study  |
| Ustilago maydis Δcyp3 ΔP$_{nia}$-P$_{etf}$ #1 |             | This study  |
| Ustilago maydis Δcyp3 ΔP$_{nia}$-P$_{etf}$ #2 |             | This study  |
| Ustilago maydis Δfuz7 ΔP$_{nia}$-P$_{etf}$ | hyg$^R$ | This study  |
| Ustilago maydis Δcyp3 ΔP$_{nia}$-P$_{etf}$ Δfuz7 P$_{etf}$mttA | hyg$^R$, cbx$^R$ | This study  |
medium further contained 0.8 g L⁻¹ NH₄Cl, 0.2 g L⁻¹ MgSO₄·7H₂O, 0.01 g L⁻¹ FeSO₄·7H₂O, 0.5 g L⁻¹ KH₂PO₄, 1 mL L⁻¹ vitamin solution, and 1 mL L⁻¹ trace element solution. The vitamin solution contained (per liter) 0.05 g d-biotin, 1 g d-calcium pantothenate, 1 g nicotinic acid, 25 g myo-inositol, 1 g thiamine hydrochloride, 1 g pyridoxol hydrochloride, and 0.2 g para-aminobenzoic acid. The trace element solution contained (per liter) 15 g EDTA, 0.45 g of ZnSO₄·7H₂O, 0.10 g of MnCl₂·4H₂O, 0.03 g of CoCl₂·6H₂O, 0.03 g of CuSO₄·5H₂O, 0.04 g of Na₂MoO₄·2H₂O, 0.45 g of CaCl₂·2H₂O, 0.3 g of FeSO₄·7H₂O, 0.10 g of H₂BO₃ and 0.01 g of KI. Shaking cultures of U. maydis and mutants strains were performed in System Duetz® (24 well plates) with a filling volume of 1.5 mL (d = 50 mm, n = 300 rpm, T = 30 °C and φ = 80%) or in 500 mL shaking flasks with a filling volume of 50 mL (d = 25 mm, n = 200 rpm, T = 30 °C and φ = 80%) [13]. If System Duetz® was used, cultures were parallelly inoculated into multiple plates and for each sample point, and a complete plate was taken as sacrificial sample to ensure continuous oxygenation.

Controlled batch cultivations were performed in a New Brunswick BioFlo® 115 bioreactor (Eppendorf, Germany) with a total volume of 1.3 L and a working volume of 0.5 L or a total volume of 2.0 L and a starting volume of 1.0 L if CaCO₃ was used. All cultivations were performed in batch medium containing 0.2 g L⁻¹ MgSO₄·7H₂O, 0.01 g L⁻¹ FeSO₄·7H₂O, 0.5 g L⁻¹ KH₂PO₄, 1 g L⁻¹ yeast extract (Merck Millipore, Germany) 1 mL L⁻¹ vitamin solution, and 1 mL L⁻¹ trace element solution and varying concentrations of glucose and NH₄Cl as indicated. During cultivation, pH 6.0 was maintained by automatic addition of 10 M NaOH or pH was kept above 6.2 by manual addition of CaCO₃. The stirring rate was kept constant at 1000 rpm with 2 Rushton impeller. The bioreactor was aerated with an aeration rate of 1 L min⁻¹ (2vvm) for working volume of 0.5 L or 2 L min⁻¹ (1vvm) for total volume of 2 L, while evaporation was limited by sparging the air through a water bottle. The temperature was set at 30 °C. The bioreactor was inoculated to a final OD₆₀₀ of 0.75 with cells from an overnight culture in 50 mL screening medium containing 50 g L⁻¹ glucose and 100 mM MES buffer.

**Analytical methods**

When using CaCO₃ as buffer, 1 mL of culture broth was taken for OD₆₀₀ determination and HPLC analysis. The CaCO₃ was dissolved with HCl prior to further measurements, basically as described by Zambanini et al. [77].

Cell densities were measured by determining the absorption at 600 nm with an Ultrospec 10 Cell Density Meter (Amersham Biosciences, Chalfont St Giles, UK).

For CDW determination of controlled high-density pulsed fed-batch fermentation of U. maydis MB215 Δcyp3 ΔPₐra1::Pₐra1 Δfuz7 Δpetef ∆hetb 10β from New England Biolab and confirmed by PCR, restriction or sequencing. Standard

**Plasmid cloning and strain engineering**

Plasmids were assembled by Gibson assembly [22] using the NEBuilder HiFi DNA Assembly kit (New England Biolabs, Ipswich, MA, USA). Primers were ordered as unmodified DNA oligonucleotides from Eurofins Genomics (Ebersberg, Germany). As polymerase, Q5 High-Fidelity Polymerase was used. Detailed information about utilized primers and plasmid are listed in Additional file 1: Table S3 and S4. All assembled plasmids were subcloned into E. coli 10β from New England Biolab and confirmed by PCR, restriction or sequencing. Standard
cloning techniques for E. coli were performed according to Sambrook et al. [64]. For transformation, preparation of protoplasts and isolation of genomic DNA of U. maydis protocols according to Brachmann et al. [8] were used. For deletion of fuz7 in U. maydis, homologous recombination with 1000 bp flanking regions (F1, F2) including FRT sites and a hygromycin resistance cassette were used. For integration of pETEF_CbxR_At_mttA [32], the plasmid was linearized with SspI and integrated into the genome. For exchange of the promoter of ria1, CRISPR/Cas9 system was used according to Schuster et al. [65] and sgRNA has been selected online with http://www.e-crisp.org/E-CRISPR/ [28]. A donor template was used to exchange the native promoter with the strong and constitutive P<sub>aroS</sub>. Successful integration and deletion were verified by PCR and sequencing.

**Supplementary information**

Supplementary information accompanies this paper at https://doi.org/10.1186/s13068-019-1605-6.

Additional file 1. Figure S1: Iaconate production from glycerol; Figure S2: NaOH-titrated fed-batch fermentation; Tables S1 and S2: Feeding procedures during high-density pulsed fed-batch fermentations; Table S3: Primers used in this work. Table S4: Plasmids used in this work.

Abbreviations

CRISPR, clustered regularly interspaced short palindromic repeats; FLP/FRT: flippase-mediated recombination; pH: potential hydrogen; HPLC: high performance liquid chromatography; CDW: cell dry weight; HCl: hydrochloric acid; GLC: glucose; HClO: hydrochloric acid; ITA: itaconic acid; C-source: carbon source; h: hour; FRT: flippase recognition target; PCR: polymerase chain reaction; A. terreus: Aspergillus terreus; E. coli: Escherichia coli; A. niger: Aspergillus niger; C. glutamicum: Corynebacterium glutamicum; U. maydis: Ustilago maydis; U. cynodontis: Ustilago cynodontis; U. vetiveriae: Ustilago vetiveriae; ria1: regulator of itaconic acid biosynthesis; mttA: mitochondrial transporter from Ustilago maydis; mttA: mitochondrial transporter from Aspergillus terreus; fuz7: dual specificity protein kinase; cyp3: oxidase encoding gene; P<sub>mitA</sub>: native promoter of ria1; P<sub>aroS</sub>: strong and constitutive promoter; MES: 2-(N-morpholino)ethanesulfonic acid; OD: optical density.

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Authors’ contributions

All authors contributed significantly to the work. NW conceived and supervised the study. HHT and NW performed experiments and analyzed results with the help of NW and LMB. HHT wrote the manuscript with help of NW and LMB. IB and AL engineered the strains and SM and KAS performed fermentation experiments. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

All authors have seen and approved the manuscript. All authors have contributed significantly to the work. The manuscript has not been published and is not being considered for publication elsewhere. The authors declare that they have no competing interests.

Author details

1. IAMF-Institute of Applied Microbiology, ABBt-Aachen Biology and Biotechnology, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany.
2. Institute of Bio- and Geosciences IBG-1, Biotechnology, Forschungszentrum Jülich, Wilhelm-Johanns-Str., 52425 Jülich, Germany.

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